eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Doctoral Thesis

Metagenomics of Idiazabal cheese: elucidating the microbiota, its impact on cheese quality and safety, and factors affecting its composition

Gorka Santamarina García

Vitoria-Gasteiz, 2024

Cover image generated for free with Microsoft Copilot using Bing Image Creator1 and edited for free with Photoroom.

Universidad del País Vasco Euskal Herriko Unibertsitatea

FARMAZIA FAKULTATEA
FACULTAD DE FARMACIA

Grupo de Investigación Lactiker Ikerketa Taldea

FACULTY OF PHARMACY
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
INTERNATIONAL DOCTORAL THESIS

FARMAZIA FAKULTATEA
BIOKIMIKA ETA BIOLOGIA MOLEKULARRA SAILA
NAZIOARTEKO DOKTOREGO-TESIA

Metagenomics of Idiazabal cheese: elucidating the microbiota, its impact on cheese quality and safety, and factors affecting its composition.

Idiazabal gaztaren metagenomika: mikrobiota, gaztaren kalitatean eta segurtasunean duen eragina, eta haren osaeran eragiten duten faktoreak argitzen.

Report to apply for the Degree of Doctor in Food Quality and Safety

Elikagaien Kalitatean eta Segurtasunean Doktore Gradua lortzeko memoria

Gorka Santamarina García

Supervisors/Zuzendariak:
Prof. Dr. G. Amores Olazagirre
Prof. Dr. M. Virto Lekuona

Vitoria-Gasteiz, July, 2024

Gasteiz, 2024ko uztaila

A Ama, Aita y Judit,

A mis abuelos,

A Laura.

¡Gracias por tanto!

"Success is not achieved only with special qualities. It is above all a work of constancy, method and organization."

Victor Hugo

"Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great."

Mark Twain

ESKER ONAK / AGRADECIMIENTOS / ACKNOWLEDGEMENTS

Una vez escuché una frase que dice "**Lo difícil se consigue, lo imposible se intenta**". Ese ha sido mi lema desde entonces. Aquellas personas que conocen lo que supone llevar a cabo una tesis son conscientes de su complejidad, mientras que quienes lo desconocen, solo necesitan escuchar la palabra "**doctorado**" para hacerse una idea. Por medio de las siguientes líneas quiero agradecer a todas las personas que, de una manera u otra, han contribuido a la consecución de esta tesis doctoral.

Lehenik eta behin, eskerrak eman nahi dizkiot **Euskal Herriko Unibertsitateari (UPV/EHU)** doktorego-tesi hau garatzeko emandako Ikertzaileak Prestatzeko Kontratuagatik. Nire **tesi-zuzendariei** ere eskerrak eman nahi dizkiet, urte hauetan zehar emandako aholkuengatik eta laguntzagatik. **Eskerrik asko Mailori** tesi hau garatzeko aukera emateagatik eta emandako laguntzagatik. **Esker berezienak Gustavorentzat** dira, nire ikerketa-proiektuari etengabeko arreta emateagatik, helburu guztiak nekaezinki lortzen laguntzeagatik, bikaintasuna lortzeko emandako aholkuengatik, eta datuen tratamendu estatistikoaren munduan sakontzen irakasteagatik.

Era berean, **Lactiker ikerketa taldeko lankideak** eskertu nahi ditut, batez ere **Igor**, ikerketa proiektuan zehar laguntzeagatik eta aholkuak emateko prest egoteagatik, **eta Luisja**, batez ere kudeketan, laguntza emategatik. Era berean, eskerrak eman nahi dizkiet **Diegori, Loreari, Emmari, Larari** eta ahazten ditudanei ikerketa prozesu honetan, era batean edo bestean, laborategiko lanetan lagundu didazuelako.

Nola ez, **Biokimika eta Biologia Molekularra saileko lankideak** eskertu nahi ditut. Besteak beste, eskerrik asko, **Nere, Olatz, Ainho…** doktoretza aurreko eta ondoko hainbeste aholkuengatik, gutxik kontatzen baitituzte; momentu onetan egoteagatik, baina, batez ere, txarretan aholku hoberenak eskaintzeagatik; eta nola ez, lanaz gain, bazkari, kafe eta bestelako une onengatik eta barreengatik. Ez dut **Nagore** ahaztu nahi, bai laguntza tekniko ezinhobea eskaintzeagatik, bai pertsonalki, hainbeste elkarrizketengatik eta bulego-kide hoberena izateagatik.

Ez ditut ahaztu nahi ikerketa taldetik edo sailetik kanpo laguntza teknikoa eta giza babesa eskaini duten gainerakoak. **SGIkerreko teknikariak** eskertu nahi ditut**, Irati, Irantzu**, **Feranando, Maite** eta ahazten ditudanak, DNA erauzketetan, sekuentziazioetan, qPCRetan eta abarretan laguntza tekniko eta giza babesagasa ezin hobea eskaini didazuelako. Igualmente, agradecer el apoyo técnico y humano del **Instituto Láctologico de Lekunberri**, en especial de **Marisa**, en el analsis de antibióticos. **MikroIker ikerketa taldeko kideak eta mikrobiologia saileko lankideak** eskertu nahi ditut baita ere, **Ilargi, Irati, Aitor, Ainhoa** etab., ekipo edo bioinfomatikako dudekin joan garenean laguntzeko prest egon zaretelako.

I would like to thank all the people from the **Moorepark Food Research Center** of the **Teagasc - Irish Agriculture and Food Development Authority** and specially, from the **Food Bioscience** division, who made my stay and my work easy. Many thanks to **Paul D. Cotter** and his great group **Fiona**, **Min, Cathy, John** and so on for their excellent instruction, advices and help, and for being such nice people. To all those people from the **Vision 1 lab**, **Alba, Enriqueta, Deyan, Ana, Gaston, Katie**..., who were always willing to help me and with whom I shared nice moments, thank you so much!

Nutrizio eta bromatologiako lakideak, espresuki, **Viki eta Marian** ere eskertu nahi ditut, tesi hau amaitzen nuen bitartean, irakasle lanetan hastea lagundu eta erraztu didazuelako, aholku on ugari emanez, bai eta **Gesala**, laborategi praktiketan laguntzeagatik. Eskerrik asko!

Igualmente, quisiera agradecer a **Ana Hurtado**, y a todos los compañeros del Departamento de Sanidad Animal de Neiker-Tecnalia, **Bea, Maitane** etc., haberme dado la oportunidad de introducirme en este mundo de la investigación y, especialmente, en el mundo de las resistencias, con la mejor instrucción y trato personal posible. Agradecer también al programa "Mentoring" de EHUALUMNI la oportunidad de conocer a **Javier Aranceta Bartrina**, que me transmitió su experiencia y conocimientos, facilitándome continuar adentrándome en este mundo de la investigación. También, mencionar a **Chema** y a su grupo de investigación BALAT de la Universidad de León, **Maria Eugenia, Daniel** etc., por haberme acogido de la mejor forma posible durante unos días enseñándome y compartiendo conmigo todo su trabajo y conocimientos.

Por último, y no menos importante, agradecer a toda mi **familia y amigos** que de una manera u otra siempre me han acompañado y animado. Especialmente gracias a mi núcleo, **Aita, Ama y Judit,** por todos estos años a mi lado y por todo el apoyo en los momentos buenos y malos, ya que sin él no habría podido llegar hasta aquí. Esker oso bereziak zuretzat **Laura**, lan honek suposatzen duena ezagutzen eta pairatu duzulako. Eskerrik asko zure uneoroko animoengatik eta nire helburuak lortzen nekaezinki laguntzagatik! Este trabajo va dedicado a todos ellos y en especial, a mis **abuelos**, por haberme transmitido desde pequeño tantos valores como tenacidad, constancia, valentía y responsabilidad, que me han permitido llegar hasta donde estoy. Gracias a todos ellos, y especialmente a mi abuela **Helio**, por enseñarme que la sabiduría más importante de la vida no se encuentra en los libros.

A todos los que he mencionado, y a los que me olvido…

Eskerrik asko! **¡Muchas gracias!** **Thank you!**

Gorka Santamarina

ACKNOWLEDGEMENTS FOR THE FINANCIAL SUPPORT

The predoctoral contract was provided by the **University of the Basque Country (UPV/EHU)**. The international stay at the Moorepark Food Research Center - Teagasc was also founded by the **University of the Basque Country (UPV/EHU)** (MOV22/23: IRLANDA. Teagasc Food Research Center). This research project has received financial support from the **Basque Government**, through the financial support of the Lactiker-Quality and Safety of Foods from Animal Origin Research Group (IT944-16 and IT1568-22), and from the **University of the Basque Country** (COLAB20/14) and the **Ministry of Science and Innovation** of the **Spanish Government** (PID2020-113395RB-C21).

ACKNOWLEDGEMENTS FOR THE TECHNICAL SUPPORT

The author thanks the **Idiazabal cheese PDO and cheesemakers** for collaborating on this project. Additionally, he expresses gratitude for the technical and human support provided by the **SGIker** (UPV/EHU/FEDER, EU), including Sequencing and Genotyping Unit, and Gene-Expression Unit-Genomics Service; and by the **Instituto Lactológico de Lekunberri**.

ACKNOWLEDGEMENTS TO THE EDITORIALS

The author extends his gratitude to the **editorials** for generously granting permission to reuse the published articles included in this PhD dissertation.

ESKER ONAK LAGUNTZA EKONOMIKOAGATIK

Doktoratu aurreko kontratua **Euskal Herriko Unibertsitatea**k **(UPV/EHU)** eman zuen. **Moorepark Food Research Center - Teagasc**-eko nazioarteko egonaldia ere **Euskal Herriko Unibertsitatea**k **(UPV/EHU)** finantzatu zuen (MOV22/23: IRLANDA. Teagasc Food Research Center). Ikerketa-proiektu honek laguntza ekonomikoa jaso du **Eusko Jaurlaritza**tik, Lactiker-Animalia Jatorriko Elikagaien Kalitatea eta Segurtasuna Ikerketa Taldeari emandako laguntza ekonomikoaren bidez (IT944-16 eta IT1568-22), **Euskal Herriko Unibertsitate**tik **(UPV/EHU)** (COLAB20/14) eta **Espainiako Gobernuko Zientzia eta Berrikuntza Ministeriotik** (PID2020-113395RB-C21).

ESKER ONAK LAGUNTZA TEKNIKOAGATIK

Egileak eskerrak eman nahi dizkie **Idiazabal gazta JDBri** eta **gaztagileei** proiektu honetan laguntzeagatik. Gainera, **SGIkerrek** (UPV/EHU/FEDER, EB) emandako laguntza teknikoa eta giza laguntza eskertzen ditu, Sekuentziazio eta Genotipo Azterketen Unitatea eta Gene-Adierazpen Unitatea-Genomika Zerbitzua barne; eta **Lekunberriko Esnekari Erakunde**ak emandakoa.

ESKERRAK ARGITALETXEEI

Egileak eskerrak eman nahi dizkie **argitaletxeei** doktore-tesi honetan argitaratutako artikuluak berrerabiltzeko baimen eskuzabala emateagatik.

FOREWORD

This document you are reading is an **international doctoral thesis**, developed at the **University of the Basque Country** (Lactiker Research Group, Faculty of Pharmacy, UPV/EHU), together with an stay at the **Teagasc - Irish Agriculture and Food Development Authority** (Moorepark Food Research Center). This thesis has been developed based on the different publications derived from the research project, therefore, it is a **thesis by published papers**. This dissertation is structured into three parts: (I) introduction, objectives, materials and methods, results and discussion, unpublished results and other contributions (II) conclusions, and (III) published articles. This thesis has been written in Basque and English. Anyway, it should be noted that all the information written in Basque is in the published manuscripts.

HITZAURREA

Irakurtzen ari zaren dokumentu hau **nazioarteko doktoretza tesi** bat da, **Euskal Herriko Unibertsitatea**n garatua (Lactiker Ikerketa Taldea, Farmazia Fakultatea), **Teagasc - Irish Agriculture and Food Development Authority**-n (Moorepark Food Research Center) egindako egonaldiarekin batera. Tesi hau ikerketa proiektutik eratorritako **argitalpenetan oinarrituta** garatu da, beraz, **artikuluen bildumaren bidezko tesia** da. Disertazio hau hiru zatitan egituratuta dago: (I) sarrera, helburuak, materialak eta metodoak, emaitzak eta eztabaida, argitaratu gabeko emaitzak eta bestelako ekarpenak; (II) ondorioak, eta (III) argitaratutako artikuluak. Tesi hau euskaraz eta ingelesez idatzi da. Nolanahi ere, aipatu behar da euskaraz idatzitako informazio guztia argitaratutako eskuizkribuetan dagoela.

INDEX / AURKIBIDEA

ABSTRACT

The present doctoral thesis has been developed within the **Lactiker Research Group** of the University of the Basque Country (UPV/EHU), which is focused, from a multidisciplinary perspective, on the quality and safety of animal-origin foods. Therefore, this thesis is contextualised within small artisanal cheese dairies affiliated with the Protected Designation of Origin for Idiazabal cheese. This semi-hard or hard cheese is produced in the Basque Country from the raw milk of Latxa or Carranzana sheep, with a minimum ripening period of 60 days.

It is well known that raw milk cheeses exhibit a richer and more intense aromatic profile than cheeses made from pasteurised milk, primarily due to the dynamics of microbial composition that take place during cheese production. Beyond contributing to quality and, specifically aroma, the microbiota also concerns food safety aspects due to the production of toxic compounds or the presence of antibiotic resistance. This has been extensively studied in various types of cheeses; however, information on raw sheep milk cheeses is scarce. Moreover, the microbiota of Idiazabal cheese was only described 20 years ago using culture-dependent methods, with their inherent limitations, and its impact on quality and safety is not fully understood. Therefore, the main **objective** of this doctoral thesis was to characterise the microbiota, namely the bacterial communities, of Idiazabal cheese using metagenomic techniques, to study the impact of the microbiota on the quality and safety of cheese, and finally, to investigate the main production factors determining the microbiota. The results obtained would not only provide novel information for the scientific community given the limited information on raw sheep milk cheeses, but also enable the producer sector to better understand the implications of microbial communities to improve the quality and safety of Idiazabal cheese.

The **first part** of the doctoral thesis involved the optimization of amplicon (V3–V4 hypervariable regions of the 16S rRNA gene) sequencing to characterize the microbiota of Latxa raw ewe milk and how it changes during the production and ripening of Idiazabal cheese. The results showed that raw milk microbiota was composed of lactic acid bacteria (LAB), e.g. *Lactococcus* or *Leuconostoc*, environmental bacteria, e.g. *Obesumbacterium*, and undesirable ones such as pathogens or those related to spoilage, e.g. *Staphylococcus* or *Clostridium*. However, during cheese production and ripening, LAB dominated, significantly reducing the abundance of

environmental and undesirable bacteria. Additionally, for the first time, the dynamics of LAB were described, with the starter LAB (SLAB) *Lactococcus* dominating the microbiota up to 30 or 60 days of ripening, followed by a significant increase in the abundance of non-starter LAB (NSLAB), specifically *Lactobacillus, Leuconostoc, Enterococcus* and *Streptococcus,* up to 120 days of ripening. Significant differences were observed between producers, suggesting that practices adopted by producers during herd management or cheese production determine the microbiota and, consequently, the quality and safety of the cheese (**Manuscript I**).

Considering the above, the **second part** of the doctoral thesis focused on studying the relationship between the dynamics of bacterial communities and the evolution of key quality and safety parameters in each Idiazabal cheese during ripening. Analytical techniques, including near-infrared spectroscopy and liquid and gas chromatography, were used to analyse quality parameters such as gross composition (pH, dry matter, protein, fat, Ca, Mg, P and NaCl), free fatty acids (FFAs), identifying a total of 21 FFAs; or volatile compounds, identifying 81 volatile compounds; and safety parameters such as biogenic amines (BAs), identifying 7 BAs. Subsequently, the statistical relationship was analysed using a bidirectional orthogonal partial least squares approach (O2PLS) with Spearman correlations.

The evolution of gross composition, such as pH or NaCl, determined bacterial dynamics, favouring the predominance of LAB as previously described, especially NSLAB such as *Lactobacillus, Streptococcus* and *Enterococcus*, and inhibiting the proliferation of environmental and undesirable bacteria, such as pathogens. Regarding aroma-affecting compounds, it was first described that SLAB *Lactococcus* was related to the production of certain ketones, such as 3-hydroxy-2-butanone, while NSLAB, such as *Enterococcus*, *Streptococcus*, and *Leuconostoc*, were related to the release of FFAs and the production of acids, esters, or alcohols, such as 3-hexenoic acid, ethyl butanoate, or 1-butanol. Undesirable or environmental bacteria, for example, *Psychrobacter*, *Brevibacterium*, and *Chromohalobacter*, were instead related to the synthesis of ketones, sulphur compounds, or hydrocarbons, such as 2-propanone, dimethyl sulphone, and *t*-3-octene. Considering the impact of FFAs on the aroma of Idiazabal cheese and the odour impact ratio values of many of the mentioned volatile compounds, the influence of the microbiota on aroma was confirmed. Regarding safety, the NSLAB *Lactobacillus* was described as the main

genus related to BA production, including toxigenic BAs, such as cadaverine and putrescine, but also γ-aminobutyric acid (GABA), related to different health benefits (**Manuscripts II and III**).

The **third part** of the doctoral thesis analysed the influence of the microbiota on one of the most challenging aspects of food safety and public health, antimicrobial resistances (AMRs). Initially, a literature review was conducted on the presence of antibiotic residues and AMRs in dairy products, concluding the current poor understanding in this regard, especially regarding the relationship between residue occurrence and resistance development, as well as the lack of information on sheep milk and derived cheeses (**Manuscript IV**).

Subsequently, the knowledge and practices of producers regarding antibiotic use were analysed through a cross-sectional survey, revealing the lack of knowledge of producers about antibiotic use, for example, compounds used. Next, the presence of antibiotic residues throughout the production chain of Idiazabal cheese was studied using commercial tests and chromatography. Only tylosin was identified in raw milk (3.28 ± 7.44 µg/kg), below maximum residual limits, and whey (2.91 ± 6.55 µg/kg), while no compound was detected in fresh or ripened cheeses, unlike various compounds such as chlortetracycline (15.7 ± 34.5 µg/kg) and sulfamethazine (7.69 ± 16.5 µg/kg), detected in sheep faeces, as published previously. However, screening techniques yielded antibiotic levels close to the LOD in 12.5 % of the samples, mainly in raw milk and whey, and 10.0 % tested positive, specifically in fresh and ripened cheeses, indicating that antibiotic metabolites or chromatographically unanalysed compounds are concentrated during cheese production, which is of particular interest as they maintain antimicrobial activity (**Manuscript V**).

In parallel, resistances to the most commonly used antibiotics on farms (dihydrostreptomycin, benzylpenicillin, amoxicillin, and polymyxin B) of the most important bacteria in cheese, i.e. LAB, were analysed using phenotypic (broth microdilution) and genotypic methods (High-throughput quantitative PCR). More than 200 isolates were obtained, which showed that dominant LAB in faeces and raw milk, such as *Enterococcus* (e.g., *E. hirae* and *E. faecalis*) and *Bacillus* (e.g., *B. thuringiensis* and *B. cereus*), along with *Lactococcus* (*L. lactis*) for whey and fresh cheeses, had the highest resistance rates (on average for each genus, 56.8 %, 53.4 % and 78.2 %, respectively). In contrast, *Lactobacillus* and *Lacticaseibacillus* species (e.g., *Lactobacillus* sp. and *L. paracasei*) dominating in ripened cheeses had lower resistance rates (39.1 % and 31.4 %,

respectively). Genotypically, 31 antimicrobial resistance genes (ARGs) and 6 mobile genetic elements (MGEs) were detected, dominating the ARGs *Str* (average relative abundance of 387), *Str*B (39.3), and *aad*A-01 (19.3) conferring resistance to aminoglycosides, and the transposons *tnp*A-02 and *tnp*A-01 (71.3 and 26.5, respectively). The abundance of ARGs and MGEs decreased throughout the production chain, corroborating phenotypic results and confirming the influence of the production chain on the reduction of resistant LAB (**Manuscript VI**).

The **fourth** and final **part** of this doctoral thesis aimed to study the sources of microorganisms in dairy environments of Idiazabal cheese PDO, to examine how they affect raw milk, whey and Idiazabal cheese microbiota, and to identify their genetic potential in terms of cheese quality and safety. Dairy environment samples included herd feed, teat skin surfaces, food contact surfaces, such as materials or equipment, and non-food contact surfaces, such as floors or walls, and processing ingredients, such as rennet and brine. Hence, shotgun sequencing methodology was optimised for this purpose. The results showed that all the collected samples were microbial niches with an impact on the microbiota of raw milk, whey, and Idiazabal cheese. However, commercial feed and teat skin were the main contributors to the raw milk microbiota (45.6 % and 33.5 %, respectively), sourcing *Lactococcus* and *Pantoea*, for example; along with rennet for whey and cheese (17.4 % and 41.0 %, respectively), contributing taxa such as *Streptococcus*, *Pseudomonas_E*, or *Lactobacillus_H*.

Functionally, these niches were also related to cheese quality and safety. For example, food contact surfaces and brine, related to genera such as *Brevibacterium*, *Methylobacterium*, and *Halomonas*, were related to metabolic pathways relevant to cheese quality and safety. Likewise, commercial feed and grass were the main reservoirs of virulence factors, related to, for example, *Brevibacillus_B* or *CAG-196*; while grass, teat skin, or rennet were the main contributors of ARGs, related to *Bact-11* or *Bacteroides_B*. In terms of quality, such as texture and aroma, brine, grass, and food contact surfaces were key reservoirs for genes encoding hydrolases, originating from, for example, *Lactococcus*, *Lactobacillus*, *Listeria*, or *Chromohalobacter*. Therefore, the impact of environments and practices carried out by each producer on the microbiota and, consequently, on the quality and safety of Idiazabal cheese was confirmed, suggesting these production factors as the main reason for the observed differentiation between producers (**Manuscript VII**).

In **conclusion**, the research project of this doctoral thesis confirms that the microbiota of Idiazabal cheese differs between producers, determining the final characteristics of the product in terms of quality and safety, and depends on the dairy environment or production factors, such as herd feeding, surfaces, or ingredients used during cheese making. The information obtained provides a comprehensive characterisation of the microbiota of Idiazabal cheese, its impact on quality and safety, and the factors determining it. Thus, these results offer essential knowledge to the scientific community, given the limited information for raw ewe milk cheeses in this regard, and especially to the production sector, to contribute to the improvement of the quality and safety of the final product.

LABURPENA

Doktorego-tesi hau Euskal Herriko Unibertsitateko (UPV/EHU) **Lactiker Ikerketa Taldea**n garatu da, diziplina anitzeko ikuspegi batetik, animalia-jatorriko elikagaien kalitatean eta segurtasunean oinarrituta. Beraz, tesi hau Idiazabal gazta Jatorri Deitura Babestuari (JDB) atxikitako artisau-gaztandegi txikien testuinguruan kokatzen da. Gazta erdigogor edo gogor hau Latxa edo Karrantzako ardien esne gordinarekin egiten da Euskal Herrian, eta gutxienez 60 egunez heltzen da.

Jakina da esne gordineko gaztek aroma-profil aberatsagoa eta biziagoa dutela esne pasteurizatuarekin egindako gaztek baino, batez ere gazta ekoiztean gertatzen den mikrobio-konposizioaren dinamikari esker. Kalitatean eta, bereziki, aroman laguntzeaz gain, mikrobiotak elikagaien segurtasuneko alderdietan ere eragiten du, konposatu toxikoak sortzen direlako edo antibiotikoekiko erresistentziak daudelako. Hori asko aztertu da hainbat gazta motatan; hala ere, ardi-esne gordineko gaztei buruzko informazioa urria da. Gainera, Idiazabal gaztaren mikrobiota duela 20 urte bakarrik deskribatu zen hazkuntza-mendeko metodoak erabiliz, berezko mugekin, eta horrek kalitatean eta segurtasunean duen eragina ez da erabat ulertzen. Beraz, doktore-tesi honen **helburu** nagusia Idiazabal gaztaren mikrobiota, hau da, bakterio-komunitateak, karakterizatzea izan zen, teknika metagenomikoak erabiliz; mikrobiotak gaztaren kalitatean eta segurtasunean duen eragina aztertzea; eta, azkenik, mikrobiotan eragiten duten ekoizpen-faktore nagusiak ikertzea. Lortutako emaitzek informazio berritzailea emango liokete komunitate zientifikoari, ardi-esne gordineko gaztei buruzko informazio mugatua dagoelako, eta, horrez gain, Idiazabal gaztaren kalitatea eta segurtasuna hobetzeko mikrobio-komunitateek dituzten inplikazioak hobeto ulertzeko aukera emango liokete ekoizpen-sektoreari.

Doktorego-tesiaren **lehen zatian**, anplikoietan (16S RNAe genearen V3-V4 zonalde aldakorrak) oinarritutako sekuentziazioa optimizatu zen, Latxa ardi-esne gordinaren mikrobiota karakterizatzeko eta Idiazabal gazta egiteko prozesuan eta heltzean nola aldatzen den zehazteko. Emaitzek erakutsi zutenez, esne gordinaren mikrobiotak bakterio azido laktikoak (BAL), hala nola *Lactococcus* edo *Leuconostoc*; ingurumen-bakterioak, esaterako *Obesumbacterium*; eta bakterio ez-desiragarriak, adibidez patogenoak edo gaztaren hondatzearekin zerikusia dutenak (*Staphylococcus* edo *Clostridium*) zituen. Hala ere, gazta egiteko prozesuan eta heltzean, BALak

nagusi izan ziren, eta nabarmen murriztu zen ingurumen-bakterioen eta bakterio ez-desiragarrien ugaritasuna. Gainera, lehen aldiz, BALen dinamikak deskribatu ziren. *Lactococcus* BAL abiarazleak (BALA) mikrobiota menderatu zuen 30. edo 60. heltze-egunera arte. Ondoren, BAL ez-abiarazleen (BALEA) ugaritasunak nabarmen egin zuen gora 120. heltze-egunera arte, bereziki *Lactobacillus*, *Leuconostoc*, *Enterococcus* eta *Streptococcus* generoenak. Alde nabarmenak ikusi ziren ekoizleen artean, eta horrek iradokitzen du ekoizleek artaldearen kudeaketan edo gaztaren ekoizpenean aukeratutako praktikek mikrobiota baldintzatzen dutela eta, ondorioz, gaztaren kalitatea eta segurtasuna (**I. eskuizkribua**).

Aurrekoa kontuan hartuta, doktorego-tesiaren **bigarren zatian** Idiazabal gazta bakoitzaren heltze-prozesuan zehar bakterio-komunitateen dinamikaren eta kalitate- eta segurtasun-parametro garrantzitsuen bilakaeraren arteko erlazioa aztertu zen. Teknika analitikoak erabili ziren, infragorri hurbileko espektroskopia eta gas eta likido kromatografia barne, kalitate-parametroak aztertzeko, hala nola konposizio gordina (pH-a, materia lehorra, proteina, gantza, Ca, Mg, P eta NaCl), gantz-azido askeak (GAA), guztira 21 GAA identifikatuz; edo konposatu lurrunkorrak, 81 konposatu lurrunkor identifikatuz; eta segurtasun-parametroak, esaterako amina biogenoak (AB), 7 AB identifikatuz. Ondoren, erlazio estatistikoa aztertu zen, bi noranzkoko karratu partzial minimo ortogonalen (BN-KPMO) eta Spearmanen korrelazioen arteko analisi baten bidez.

Konposizio gordinaren bilakaerak, pH-a edo NaCl-a barne, bakterioen dinamikak zehaztu zituen, BALen nagusitasuna bultzatuz, lehen deskribatu den bezala, bereziki BALEAena (*Lactobacillus, Streptococcus* eta *Enterococcus*), eta ingurumen-bakterioen eta bakterio ez-desiragarrien (patogenoak, esaterako) ugaritzea saihestuz. Aromari eragiten dioten konposatuei dagokienez, lehen aldiz deskribatu zen *Lactococcus* BALAa zetona jakin batzuen ekoizpenarekin erlazionatuta zegoela (3-hidroxi-2-butanona, adibidez), eta BALEAak (*Enterococcus, Streptococcus* eta *Leuconostoc*) GAAen askapenarekin eta azidoen, esterren edo alkoholen (azido 3-hexenoikoa, etil butanoatoa edo 1-butanola) ekoizpenarekin erlazionatuta zeuden. Aldiz, bakterio ez-desiragarriak edo ingurumenekoak (adibidez, *Psychrobacter*, *Brevibacterium* eta *Chromohalobacter*) zetonen, sufredun konposatuen eta hidrokarburoen sintesiarekin lotuta zeuden, esaterako 2-propanona, dimetil sulfona eta *t*-3-oktenoa. GAAek Idiazabal gaztaren aroman duten eragina eta aipatutako konposatu lurrunkor askoren usain-inpaktuaren ratioen balioak kontuan hartuta,

mikrobiotak aroman duen eragina berretsi zen. Segurtasunari dagokionez, *Lactobacillus* BALEAa ABen ekoizpenarekin lotutako genero nagusia zen, AB toxigenikoak barne, hala nola kadaberina (KAD) eta putreszina (PUT), baina baita osasunerako hainbat onurarekin zerikusia duen azido γ-aminobutirikoa (AGAB) ere (**II. eta III. eskuizkribuak**).

Doktorego tesiaren **hirugarren zatia**n mikrobiotak elikadura segurtasunaren eta osasun publikoaren alderdi desafiatzaileenetako batean duen eragina aztertu zen: antimikrobianoekiko erresistentziak (AME). Hasiera batean, esnekietan antibiotikoen hondakinen eta AMEen presentziari buruzko literatura berrikusi zen, eta horri buruz gaur egun dagoen ezagutza eskasa dela ondorioztatu zen, bereziki hondakinen agerpenaren eta erresistentzien garapenaren arteko loturari dagokionez, baita ardi-esneari eta gazta eratorriei buruzko informazio ezari dagokionez ere (**IV. eskuizkribua**).

Ondoren, ekoizleek antibiotikoen erabilerari buruz dituzten ezagutzak eta praktikak aztertu ziren zeharkako inkesta baten bidez, ekoizleek antibiotikoen erabilerari buruz duten ezagutza falta azalduz, adibidez, erabilitako konposatuen inguruan. Jarraian, Idiazabal gaztaren ekoizpen-katean antibiotikoen hondakinak dauden aztertu zen, baheketa testak eta kromatografia erabiliz. Bakarrik tilosina identifikatu zen esne gordinean (3,28 ± 7,44 µg/kg), ezarritako gehienezko hondakin-mugen (GHM) azpitik, eta gazuran (2,91 ± 6,55 µg/kg), eta gazta freskoetan edo helduetan, berriz, ez zen inolako konposaturik detektatu, klortetraziklina (15,7 ± 34,5 µg/kg) eta sulfametazina (7,69 ± 16,5 µg/kg) bezalako konposatuak ez bezala, ardi-gorotzetan detektatu zirenak, lehen argitaratu den bezala. Hala ere, baheketa testen arabera, laginen % 12,5ek detekzio-mugatik hurbil zeuden antibiotiko-mailak zituzten, batez ere esne gordinak eta gazurak, eta % 10,0 positiboak izan ziren, gazta freskoak eta helduak bereziki. Horrek esan nahi du antibiotikoen metabolitoak edo kromatografikoki aztertu gabeko konposatuak gaztaren ekoizpenean kontzentratu egiten direla, eta hori bereziki interesgarria da, jarduera antimikrobianoa mantentzen baitute (**V. eskuizkribua**).

Aldi berean, baserrietan gehien erabiltzen diren antibiotikoekiko (amoxizilina, dihidroestreptomizina, G penizilina eta B polimixina) erresistentziak aztertu ziren gaztako bakterio garrantzitsuenentzat, hau da, BALentzat, metodo fenotipikoak (salda-mikrodiluzioa) eta genotipikoak (Errendimendu handiko polimerasaren kate-erreakzio (PKE) kuantitatiboa) erabiliz. 200 isolatu baino gehiago lortu ziren, eta horiek erakutsi zuten gorotzetan eta esne

gordinean nagusi diren BALek, hala nola *Enterococcus* (adibidez, *E. hirae* eta *E. faecalis*) eta *Bacillus* (adibidez, *B. thuringiensis* eta *B. cereus*), *Lactococcus*ekin (*L. lactis*) batera gazuran eta gazta freskoetan, erresistentzia-tasa handienak zituztela (batez beste, % 56,8, % 53,4 eta % 78,2 genero bakoitzerako, hurrenez hurren). Aitzitik, gazta helduetan nagusi diren *Lactobacillus* eta *Lacticaseibacillus* espezieek (adibidez, *Lactobacillus* sp. eta *L. paracasei*) erresistentzia-tasa baxuagoak zituzten (% 39,1 eta % 31,4, hurrenez hurren). Genotipikoki, 31 antimikrobianoekiko erresistentzia gene (AEG) eta 6 elementu genetiko mugikor (EGM) detektatu ziren, *Str* (387ko batez besteko ugaritasun erlatiboa), *Str*B (39,3) eta *aad*A-01 (19,3) AEGak, aminoglukosidoekiko erresistentzia ematen dutenak, eta *tnp*A-02 eta *tnp*A-01 transposoiak (71,3 eta 26,5, hurrenez hurren), gailenduz. AEGen eta EGMen ugaritasuna murriztu egin zen produkzio-katean zehar, emaitza fenotipikoak berretsiz eta produkzio-kateak erresistenteak diren BALen murrizketan duen eragina berretsiz (**VI. eskuizkribua**).

Doktorego tesi honen **laugarren** eta azken **zatia**n, Idiazabal gazta JDBren gaztandegien inguruneetako mikroorganismo-iturriak aztertzea izan zen helburua, esne gordinaren, gazuraren eta Idiazabal gaztaren mikrobiotan nola eragiten duten aztertzeko, eta gaztaren kalitateari eta segurtasunari dagokionez duten potentzial genetikoa identifikatzeko. Gaztandegien inguruneetako laginen artean honako hauek zeuden: artaldearen bazka, titiburuaren gainazala, elikagaiak ukitzen dituzten gainazalak, hala nola materialak edo ekipoak, eta elikagaiak ukitzen ez dituzten gainazalak, hala nola zoruak edo hormak, eta prozesamenduaren osagaiak, hala nola gatzagia eta gatzuna. Beraz, helburu horretarako Shotgun sekuentziazio-metodologia optimizatu zen. Emaitzek erakutsi zuten bildutako lagin guztiak bakterio-iturriak zirela, eta eragina zutela esne gordinaren, gazuraren eta Idiazabal gaztaren mikrobiotan. Hala ere, pentsu komertziala eta titiburuaren gainazala izan ziren esne gordinaren mikrobiotari ekarpen handiena egiten ziotenak (% 45,6 eta % 33,5, hurrenez hurren), *Lactococcus* eta *Pantoea* generoak gehitzen zizkiotelarik, adibidez; gatzagiarekin batera gazurarako eta gaztarako (% 17,4 eta % 41,0, hurrenez hurren), *Streptococcus*, *Pseudomonas_E* edo *Lactobacillus_H* taxonak gehitzen zizkielarik.

Funtzionalki, gaztaren kalitatearekin eta segurtasunarekin ere lotuta zeuden bakterio-iturri horiek. Adibidez, elikagaiak ukitzen dituzten gainazalak eta gatzuna, *Brevibacterium*, *Methylobacterium* eta *Halomonas* generoekin lotutakoak, gaztaren kalitaterako eta segurtasunerako garrantzitsuak ziren bide metabolikoekin lotuta zeuden. Era berean, pentsu komertziala eta

belarra birulentzia-faktoreen gordailu nagusiak ziren, *Brevibacillus_B* edo *CAG-196* generoekin lotutakoak, adibidez. Belarra, titiburuaren gainazala edo gatzagia, berriz, AEGen gordailu nagusiak ziren, *Bact-11* edo *Bacteroides_B* generoekin lotuta zeudenak. Kalitateari dagokionez, esaterako testura eta aroma, gatzuna, belarra eta elikagaiak ukitzen dituzten gainazalak hidrolasak kodetzen dituzten geneen gordailuak ziren, adibidez, *Lactococcus*, *Lactobacillus*, *Listeria* edo *Chromohalobacter* generoenak. Horrenbestez, ekoizle bakoitzak mikrobiotan eta, ondorioz, Idiazabal gaztaren kalitatean eta segurtasunean izandako eragina berretsi zen, eta ekoizpen-faktore horiek ekoizleen artean ikusitako bereizketaren arrazoi nagusi gisa iradoki ziren (**VII. eskuizkribua**).

Funtsean, doktorego-tesi honen ikerketa-proiektuak berresten du Idiazabal gaztaren mikrobiota desberdina dela ekoizleen artean, produktuaren azken ezaugarriak zehaztuz kalitateari eta segurtasunari dagokienez, eta gaztandegien inguruneen edo ekoizpen-faktoreen mende dagoela, hala nola artaldearen elikadura, gainazalak edo gazta egiterakoan erabilitako osagaiak. Lortutako informazioak Idiazabal gaztaren mikrobiotaren karakterizazio integrala, kalitatean eta segurtasunean duen eragina eta berau zehazten duten faktoreak ematen ditu. Beraz, emaitza hauek funtsezko ezagutza eskaintzen dute komunitate zientifikoarentzat, ardi-esnez egindako gaztei buruzko informazio mugatua dela eta, eta, bereziki, ekoizpen-sektorearentzat, azken produktuaren kalitatea eta segurtasuna hobetzen laguntzeko.

ABBREVIATIONS

Abbreviation	Name
16S rRNA	16S Ribosomal Ribonucleic Acid
ADI	Acceptable Daily Intake
AMC	Antimicrobial Consumption
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AR	Antimicrobial-Resistant
ARB	Antimicrobial-Resistant Bacteria
ARG	Antimicrobial Resistance Gene
AST	Antimicrobial Susceptibility Testing
A_w	Water Activity
BA	Biogenic Amine
BD-FAE	Bifunctional Feruloyl and Acetyl Xylan Esterase
BPW	Buffered Peptone Water
C	Daily Intake
CCorA	Canonical Correlation Analysis
CT	Cycle Threshold
DAD	Diode Array Detector
DGGE/TGGE	Denaturing/Temperature Gradient Gel Electrophoresis
DMRM	Dynamic Multiple Reaction Monitoring
DNA	Deoxyribonucleic Acid
Duf_915	Hydrolase of Unknown Function
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological Cut-Off Value
EDI_i	Estimated Daily Intake
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
ESI+	Electrospray Ionization in Positive Ion Mode
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FA	Fatty Acid
FAA	Free Amino Acid
FDA	Food and Drug Administration of the United States of America
FEB	Feed Extraction Buffer
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FFA	Free Fatty Acid
FLD	Fluorescence Detector
FSM	Food Safety Margin

GC-MS	Gas Chromatography-Mass Spectrometry
GLC	Gas Liquid Chromatography
GLC-FID	Gas Liquid Chromatography-Flame Ionization Detector
HCA	Hierarchical Clustering Analysis
H_i	Concentration of Detected Antibiotic
HPLC-FLD	High-Performance Liquid Chromatography-Fluorescence detector
HT-qPCR	High-Throughput-Quantitative Polymerase Chain Reaction
HTS	High-Throughput Sequencing
I.D.	Inner Diameter
IDF	International Dairy Federation
IFC	Integrated Fluidic Circuit
IS	Internal Standard
ISO	International Organization for Standardization
LAB	Lactic Acid Bacteria
LC	Liquid Chromatography
LC-QqQ-MS/MS	Liquid Chromatography-Triple Quadrupole-Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LPL	Lipoprotein Lipase
LRI	Linear Retention Index
mADI	Microbial Acceptable Daily Intake
MAG	Metagenome Assembled Genome
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MIC_{50}	Minimum Inhibitory Concentration Required for the Inhibition of the Growth of 50 % of Isolates
MIC_{90}	Minimum Inhibitory Concentration Required for the Inhibition of the Growth of 90 % of Isolates
MRL	Maximum Residual Limit
MS	Mass Spectrometer
MS/MS	Tandem Mass Spectrometer
NBLAST	Nucleotide Basic Local Alignment Search Tool
NGS	Next-Generation Sequencing
NIR	Near Infrared Spectroscopy
NIST	National Institute of Standards and Technology
NOAEC	No Observed Adverse Effect Concentration
NSLAB	Non-Starter Lactic Acid Bacteria
O2PLS	Bidirectional Orthogonal Partial Least Squares

OIR	Odour Impact Ratio
OPLS	Orthogonal Partial Least Squares
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
OT	Odour Threshold
OTU	Operational Taxonomic Unit
PERMANOVA	Permutational Multivariate Analysis of Variance
PC	Principal Component
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PDO	Protected Designation of Origin
PL	Pregastric Lipase
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RP-HPLC	Reversed Phase-High performance Liquid Chromatography
RP-HPLC-FLD	Reversed Phase-High performance Liquid Chromatography-Fluorescence Detector
RPKM	Reads Per Kilobase Per Million Reads
rRNA	Ribosomal Ribonucleic Acid
SDG	Sustainable Development Goal
SLAB	Starter Lactic Acid Bacteria
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
SSCP	Single-Strand-Conformation Polymorphism
tADI	Toxicological Acceptable Daily Intake
TCA	Trichloroacetic Acid
TG	Triglyceride
TIC	Total Ion Current
TSB	Tryptic Soy Broth
UDI	Unique Dual Indice
UV	Unit Variance / Ultraviolet
VBNC	Viable But Non-Cultivable State
VF	Virulence Factor
VIP	Variable Influence on Projection
W	Body Weight
WHO	World Health Organization

LABURDURAK

Laburdura	Izena
16S RNAe	16S azido erribonukleiko erribosomikoa
AABAP	Aldagai Anitzeko Bariantza-Analisi Permutazionala
AEP	Aldagaiaren Eragina Proiekzioan
AF-BHKL	Alderantzizko Faseko-Bereizmen Handiko Kromatografia Likidoa
AF-BHKL-FLD	Alderantzizko Faseko-Bereizmen Handiko Kromatografia Likidoa-Fluoreszentzia-Detektagailuarekin
ESA	Ameriketako Estatu Batuetako Elikagaien eta Sendagaien Administrazioa
AB	Amina Biogenikoa
AEGPS	Animalien Elikaduran erabiltzen diren Gehigarriei eta Produktuei edo Substantziei buruzko ESEA Panela
AUE	Antibiotiko Ugariekiko Erresistentzia
AMEr	Antimikrobianoekiko Erresistentea
ARB	Antimikrobianoekiko Erresistenteak Diren Bakterioak
AME	Antimikrobianoekiko Erresistentzia
AEG	Antimikrobianoekiko Erresistentzia Genea
ASP	Antimikrobianoekiko Suszeptibilitate-Proba
ASPEB	Antimikrobianoekiko Suszeptibilitate-Proben Europako Batzordea
AMK	Antimikrobianoen Kontsumoa
AIL	Atxikipen-Indize Lineala
DNA	Azido Desoxirribonukleikoa
RNAe	Azido Erribonukleiko Erribosomikoa
RNA	Azido Erribonukleikoa
ATK	Azido Trikloroazetikoa
BALA	Bakterio Azido Laktiko Abiarazlea
BALEA	Bakterio Azido Laktiko Ez-Abiarazleak
BAL	Bakterio Azido Laktikoa
BA	Bariantza-Analisia
B.D.	Barne-Diametroa
BP	Barne-Patroia
BN-KPMO	Bi Noranzkoko Karratu Partzial Minimo Ortogonalak
BF	Birulentzia-faktorea
Hi	Detektatutako Antibiotikoaren Kontzentrazioa
DM	Detekzio-Muga
EAO	Eguneroko Ahorakin Onargarria
K	Eguneroko Ahorakina
ESI+	Elektrospray-Ionizazioa Modu Ioniko Positiboan

EGM	Elementu Genetiko Mugikorra
EH-PKEk	Errendimendu handiko PKE kuantitatiboa
ESEA	Elikagaien Segurtasunerako Europako Agintaritza
EST	Elikagaien Segurtasun-Tartea
HT-qPCR	Errendimendu Handiko Polimerasaren Kate-Erreakzio Kuantitatiboa
EHS	Errendimendu Handiko Sekuentziazioa
ENF	Esnekien Nazioarteko Federazioa
FSE	Fase Solidoko Erauzketa
FSME	Fase Solidoko Mikroerauzketa
FAX-EB	Feruloil eta Azetil Xilano Esterasa Bifuntzionala
FLD	Fluoreszentzia-Detektagailua
FEH_915	Funtzio Ezezaguneko Hidrolasa
GAA	Gantz-azido askea
GA	Gantz-Azidoa
GK-ME	Gas-Kromatografia-Masa-Espektrometria
GLK	Gas-Likido Kromatografia
GLK-SID	Gas-Likido Kromatografia-Sugarraren Ionizazio-Detektagailuarekin
GHM	Gehienezko Hondakin-Muga
P	Gorputz-Pisua
GKI	Gutxieneko Kontzentrazio Inhibitzailea
IPU	Indargetutako Peptona-Ura
IBB	Indize Bikoitz Bakarra
IHS	Infragorri Hurbileko Espektrometroa
IKT	Ioien Korronte Totala
JDB	Jatorri Deitura Babestua
KEA	Kalkulatutako Eguneko Ahorakina
KPMO	Karratu Partzial Minimo Ortogonalak
KPMO-AD	Karratu Partzial Minimo Ortogonalen-Analisi Diskriminatzailea
IKMB	Kilobaseko Milioi Bat Irakurketa
KNA	Koordenatu Nagusien Analisia
KKA	Korrelazio Kanonikoaren Analisia
LDD	Lerrokatutako Diodoen Detektagailua
LK	Likido Kromatografia
LK-KkK-ME/ME	Likido Kromatografia-Koadrupolo Hirukoitza-Tandem Masa-Espektrometria
ME	Masa-Espektrometria
MMG	Metagenoman Mihiztatutako Genoma
NNE	Nazioarteko Normalizazio Erakundea
ON	Osagai Nagusia
ONA	Osagai Nagusien Analisia

PEI	Pentsua Erauzteko Disoluzio Indargetzailea
PKE	Polimerasaren Kate-Erreakzioa
SST	Soja-Salda Triptikoa
THA	Taldekatze Hierarkikoaren Analisia
ME/ME	Tandem Masa-Espektrometria
TG	Triglizeridoa
UTO	Unitate Taxonomiko Operatiboa
UB	Unitate-bariantza
Au	Ur-aktibitatea
UIR	Usainaren Inpaktu-Ratioa
UA	Usain-Atalasea
ZA	Zikloaren atalasea
ZFI	Zirkuitu Fluidiko Integratua

FREE FATTY ACIDS NOMENCLATURE

Abbreviation	Common name	Systematic name
C2	Acetic acid	Ethanoic acid
C4	Butyric acid	Butanoic acid
iC4	Isobutyric acid	2-Methylpropanoic acid
2-ethylC4	2-ethyl-butyric acid	2-Ethylbutanoic acid
C5	Valeric acid	Pentanoic acid
iC5	Isovaleric acid	3-Methylbutanoic acid
C6	Caproic acid	Hexanoic acid
iC6	Isocaproic acid	4-Methylpentanoic acid
C7	Enanthic acid	Heptanoic acid
C8	Caprylic acid	Octanoic acid
4-methylC8	4-methyl-caprylic acid	4-Methyloctanoic acid
C9	Pelargonic acid	Nonanoic acid
C10	Capric acid	Decanoic acid
C11	Undecylic acid	Undecanoic acid
C12	Lauric acid	Dodecanoic acid
C13	Tridecylic acid	Tridecanoic acid
C14	Myristic acid	Tetradecanoic acid
C15	Pentadecylic acid	Pentadecanoic acid
C16	Palmitic acid	Hexadecanoic acid
C16:1	Palmitoleic acid	Hexadec-9-enoic acid
C17	Margaric acid	Heptadecanoic acid
C18	Stearic acid	Octadecanoic acid
C18:1	Oleic acid	Octadec-9-enoic acid
C18:2	Linoleic acid	Octadeca-9,12-dienoic acid
α-C18:3	α-Linolenic acid	Octadeca-9,12,15-trienoic acid

GANTZ-AZIDO ASKEEN NOMENKLATURA

Laburdura	Izen arrunta	Izen sistematikoa
C2	Azido azetikoa	Azido etanoikoa
C4	Azido butirikoa	Azido butanoikoa
iC4	Azido isobutirikoa	Azido 2-metilpropanoikoa
2-etilC4	Azido 2-etil-butirikoa	Azido 2-etilbutanoikoa
C5	Azido balerikoa	Azido pentanoikoa
iC5	Azido isobalerikoa	Azido 3-metilbutanoikoa
C6	Azido kaproikoa	Azido hexanoikoa
iC6	Azido isokaproikoa	Azido 4-metilpentanoikoa
C7	Azido enantikoa	Azido heptanoikoa
C8	Azido kaprilikoa	Azido oktanoikoa
4-metilC8	Azido 4-metil-kaprilikoa	Azido 4-metiloktanoikoa
C9	Azido pelargonikoa	Azido nonanoikoa
C10	Azido kaprikoa	Azido dekanoikoa
C11	Azido undezilikoa	Azido undekanoikoa
C12	Azido laurikoa	Azido dodekanoikoa
C13	Azido tridezilikoa	Azido tridekanoikoa
C14	Azido miristikoa	Azido tetradekanoikoa
C15	Azido pentadezilikoa	Azido pentadekanoikoa
C16	Azido palmitikoa	Azido hexadekanoikoa
C16:1	Azido palmitoleikoa	Azido 9-hexadezenoikoa
C17	Azido margarikoa	Azido heptadekanoikoa
C18	Azido estearikoa	Azido oktadekanoikoa
C18:1	Azido oleikoa	Azido 9-oktadezenoikoa
C18:2	Azido linoleikoa	Azido oktadeka-9,12-dienoikoa
α-C18:3	Azido α-linolenikoa	Azido oktadeka-9,12,15-trienoikoa

BIOGENIC AMINES NOMENCLATURE

Abbreviation	Name
AABA	α-Aminobutyric Acid
CAD	Cadaverine
ETY	Ethylamine
GABA	γ-Aminobutyric Acid
HIS	Histamine
MBA	Methylbutylamine
MEA	Ethanolamine
MMA	Methylamine
NBA	n-Butylamine
PHE	Phenylethylamine
PUT	Putrescine
SPD	Spermidine
SPM	Spermine
TYR	Tyramine

AMINA BIOGENIKOEN NOMENKLATURA

Laburdura	**Izena**
AAAB	Azido α-Aminobutirikoa
AGAB	Azido γ-Aminobutirikoa
EPD	Espermidina
EPM	Espermina
EAM	Etanolamina
ETI	Etilamina
FEA	Feniletilamina
HIS	Histamina
KAD	Kadaberina
MAM	Metilamina
MBA	Metilbutilamina
NBA	n-Butilamina
PUT	Putreszina
TIR	Tiramina

UNITS

Unit	Description
Å	Angstrom
bp	Base Pair
ºC	Celsius degree
cm	Centimetre
CFU	Colony-Forming Unit
cm^3	Cubic centimetre
eV	Electronvolt
x *g*	G force
g	Gram
h	Hour
kg	Kilogram
kV	Kilovolt
L	Litre
m	Metre
µL	Microlitre
µm	Micrometre
µmol	Micromol
mg	Miligram
mL	Mililitre
mm	Milimetre
mM	Milimolar
min	Minute
M	Molar
nm	Nanometre
nM	Nanomolar
N	Normal
%	Percentage/Relative abundance
psi	Pounds per Square Inch
rpm	Revolution Per Minute
s	Second
km^2	Square Kilometre

UNITATEAK

Unitatea	Deskribapena
Å	Angstrom
bp	Base pare
ºC	Celsius gradu
UKE	Unitate kolonia eratzaile
cm^3	Zentimetro kubiko
eV	Elektronvoltio
x *g*	G indar
g	Gramo
h	Ordu
kg	Kilogramo
kV	Kiloboltio
µL	Mikrolitro
µm	Mikrometro
µmol	Mikromol
mg	Miligramo
mL	Mililitro
mm	Milimetro
mM	Milimolar
min	Minutu
M	Molar
nm	Nanometro
nM	Nanomolar
N	Normal
%	Ehuneko/ugaritasun erlatibo
psi	Libra/Hazbete Karratuko
rpm	Bira/min
s	Segundo

LIST OF FIGURES / IRUDIEN ZERRENDA

LIST OF TABLES / TAULEN ZERRENDA

LIST OF MANUSCRIPTS / ESKUIZKRIBUEN ZERRENDA

The current doctoral thesis is based on the seven research manuscripts listed below (**Manuscripts I-VII**).

Doktore-tesi hau ondoren aipatzen diren zazpi ikerketa-eskuizkribuetan oinarritzen da (**I.-VII. eskuizkribuak**).

I. **Gorka Santamarina-García**, Igor Hernández, Gustavo Amores & Mailo Virto (2022). Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing. ***Biology*, *11*(5), 769**. DOI: 10.3390/biology11050769

II. **Gorka Santamarina-García**, Gustavo Amores, Emma López de Armentia, Igor Hernández & Mailo Virto (2022). Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese. ***Animals*, *12*(22), 3224**. DOI: 10.3390/ani12223224

III. **Gorka Santamarina-García**, Gustavo Amores, Igor Hernández, Luis Javier R. Barrón, Lara Morán & Mailo Virto (2023). Relationship between dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese. ***Current Research in Food Science*, *6*, 100425.** DOI: 10.1016/j.crfs.2022.100425

IV. Mailo Virto, **Gorka Santamarina-García**, Gustavo Amores & Igor Hernández (2022). Antibiotics in Dairy Production: Where Is the Problem? ***Dairy*, *3*(3), 541-564.** DOI: 10.3390/dairy3030039

V. **Gorka Santamarina-García**, Gustavo Amores, Nagore Gandarias, Igor Hernández & Mailo Virto (2024). Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain. ***Food Chemistry*, *442*, *138445*.** DOI: 10.1016/j.foodchem.2024.138445

VI. **Gorka Santamarina-García**, Gustavo Amores, Diego Llamazares, Igor Hernández & Mailo Virto (2024). Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese. ***Food Research International*, *187*, *114308*.** DOI: 10.1016/j.foodres.2024.114308

VII. **Gorka Santamarina-García**, Min Yap, Fiona Crispie, Gustavo Amores, Cathy Lordan, Mailo Virto & Paul D. Cotter (2024). Shotgun metagenomic sequencing reveals the influence of artisanal dairy environments on the microbiomes, quality and safety of a raw milk PDO cheese. **Under review in *Microbiome*.**

Section I
Summary

1. INTRODUCTION

1.1. Cheese: general framework

Cheese is defined as a "ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by: coagulating wholly or partly the protein of milk (…), through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation (...)" [1]. Cheese is manufactured worldwide using a wide range of production systems and technologies [2]. This leads to a myriad of cheeses in terms of aroma and texture, making it a versatile and enjoyable product that enriches cultures and, consequently, consumers' gastronomic experiences [3].

Cheese plays a considerable role in society beyond its cultural aspect due to its broad-reaching implications, particularly in terms of nutrition [4]. It provides substantial benefits, as it is a source of high-quality proteins, essential fatty acids, minerals, such as calcium, magnesium or potassium; and vitamins, such as A, B6, B12, D, and K vitamins, which favour proper bone health, muscle function or various metabolic processes [5,6]. Although it is noteworthy that excessive intake could affect cardiovascular health due to the amount of calories and saturated fatty acids [5], recent studies have also highlighted their protective effects against various health conditions, including metabolic syndrome and cardiovascular diseases [7]. Additionally, cheese contributes to overall well-being due to bioactive compounds, such as peptides, and probiotics, which influence metabolic health and the gut microbiota [8]. Thus, a balanced and varied diet containing cheese can contribute to overall nutritional well-being [4,5].

Likewise, cheese also has other implications for society beyond nutrition. In terms of social and economic importance, the cheese industry has been described as beneficial for the economic stimulation of rural areas, in addition to contributing to cultural heritage through traditional and artisanal practices [9]. Global cheese production has been increasing for years, at an average rate of 2.2 % over the last 10 years, reaching 22.35 million tons in 2023, indicating substantial economic importance worldwide and promoting international trade and cooperation [10,11].

Currently, there are more than a thousand varieties of cheese available [11]. Various attempts have been made to classify existing cheese varieties; however, the number of variables that must be taken into account makes it difficult to establish a complete scheme. The most notable variables are moisture (extra-hard, hard, semi-hard/semisoft or soft), the source of the milk (pasteurized or raw milk and animal origin, namely, cow, sheep, goat, etc.), the coagulant (rennet, acid or heat/acid), principal ripening microorganisms (internal bacteria, internal mould, surface mould, or surface smear (bacteria), for instance), or the manufacturing technology (for example, cooking temperature or salt concentration) [11].

1.2. Idiazabal Protected Designation of Origin cheese

By definition, Idiazabal cheese is a traditional semi-hard or hard cheese from the Basque Country made exclusively from the raw milk of Latxa and/or Carranzana autochthonous breed sheep (*Ovis aries*) (Figure 1A-B). Since 1996, its production has been regulated by the Idiazabal cheese Protected Designation of Origin (PDO) [12]. The majority of the producers affiliated with the PDO are small family dairies that oversee the entire process, from herd management to cheese manufacturing and finally to sales. Indeed, according to the PDO, there are currently a total of 108 dairies, of which only two are large producers. The overall annual amount of Idiazabal cheese produced is approximately 1,300 tonnes [13].

The geographical area of herd management and milk production for the subsequent manufacturing of Idiazabal cheese corresponds to the natural diffusion areas of the Latxa and Carranzana breeds, namely, Alava, Biscay, Gipuzkoa and Navarre, covering an area of 17,213.06 km^2 in southwestern Europe (43° 27' - 41° 54' N and 1° 5 ' - 3° 37' W). The municipalities in the Roncal Valley are excluded because they have their own designation of origin [14].

Herd management encompasses indoor foraging from October to March and semi-extensive or extensive grazing from March to October. Milk collection and cheese production primarily occur between January and June, following the traditional seasonal approach dictated by the biological rhythms of the sheep. Milking is primarily automated, being subsequently refrigerated (3–4 °C) until cheese making. For cheese manufacturing (Figure 1C), the milk is initially tempered to 25 °C, and a commercial mesophilic lyophilized starter culture is added, commonly composed of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and

Lactococcus lactis subsp. *lactis* biovar. *diacetylactis*. Milk coagulation occurs at 28–32 °C for 20–45 min, employing artisanal rennet (extracted from the stomachs of Latxa or Carranzana lambs, obtained during the first month of lactation, cleaned, dried, salted, and ground, as described by Bustamante et al. [15]) or commercial liquid rennet. Cheeses are ripened in chambers maintained at 80–95 % relative humidity and 8–14 °C for, at least, 60 days [14].

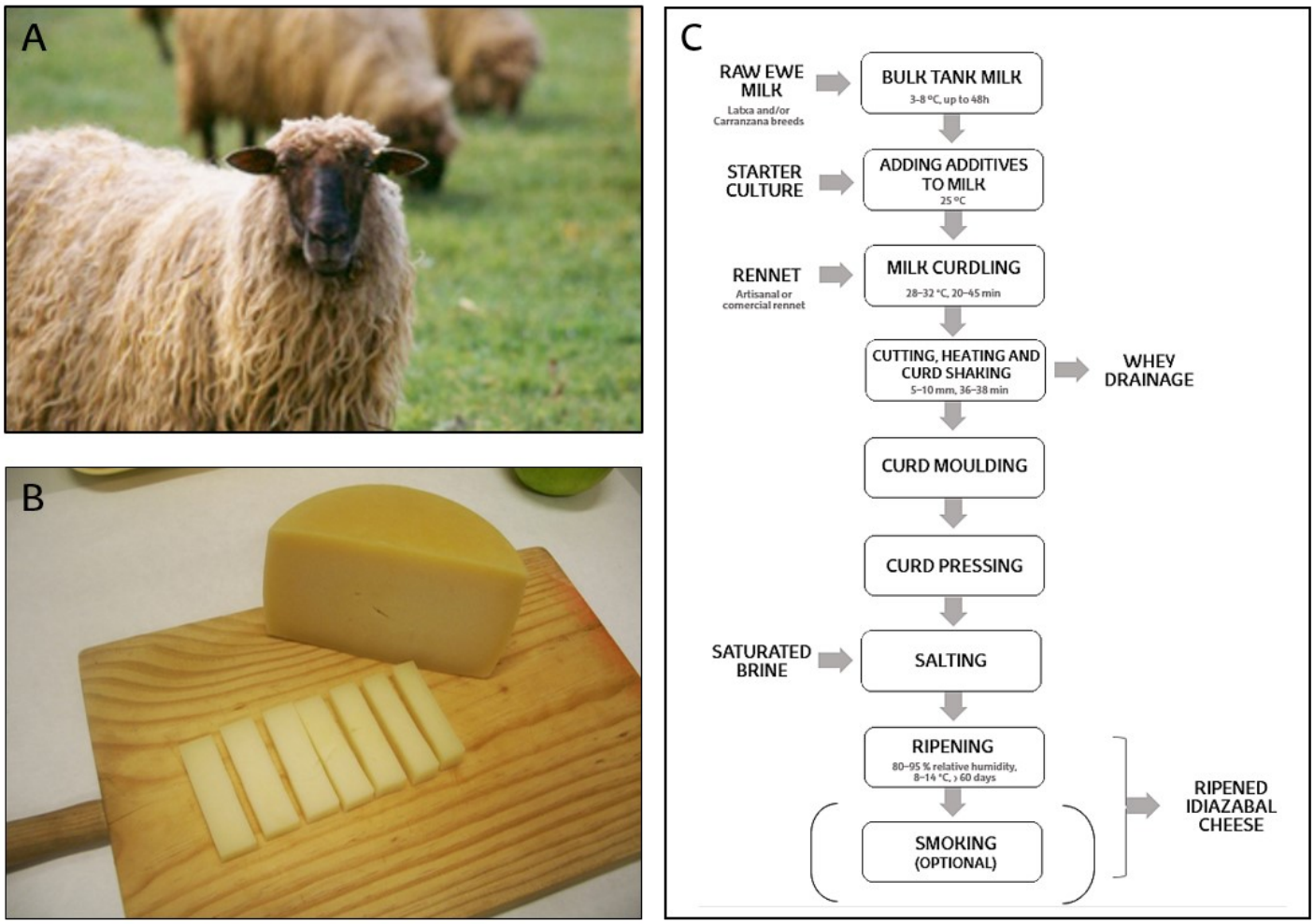


Figure 1. Latxa sheep breed (A), Idiazabal cheese (B) and flux diagram summarizing the Idiazabal cheese making process (C).

At the end of the ripening period, the typical Idiazabal cheese would present, approximately, a height between 8 and 12 cm, with a diameter of 10-30 cm, a minimum weight of 1 kg and a maximum of 3.5 kg (Figure 1B). In addition, it would have a minimum of 55 % dry extract, with a fat percentage of not less than 45 % on dry extract, as well as a total protein content not less than 25 % and a pH between 4.9 and 5.5. The typical form of an Idiazabal cheese consists of "a cylindrical shape, with flat faces, slightly convex heels and with uniform, rounded or sharp

edges". The rind is hard and smooth, with slight signs of the cloths used and with absence or slight tray marks on the faces. The cheese has a homogeneous rind that ranges from pale yellow or whitish grey to a dark brown colour, in the case of smoked cheeses. Paste, on the other hand, also has a homogeneous but variable colour, from ivory white to straw yellow. Likewise, paste may have a few, randomly distributed eyes, mostly irregular in shape, smaller than a short grain of rice, and without cracks. The texture of the paste has a variable elasticity from weak to medium, with variable firmness from medium to high and variable granulosity between weak and medium [14].

Regarding the olfactory-taste set, Idiazabal cheese has an intense and penetrating smell that integrates dairy, natural rennet and roasted smells. The taste consists of a sweet taste of zero to weak intensity, acid of weak to medium intensity, a salty taste of medium intensity and no bitterness. Finally, the main trigeminal sensation present is spicy and of weak to medium intensity. Its persistence is pronounced, with a prolonged global aroma and the absence of strange sensations [14].

1.3. Cheese microbiota

The microbiota of cheese is defined as a diverse group of prokaryotic, eukaryotic, and viral organisms [16]. This complex microbial ecosystem is primarily composed of bacteria, while smear-ripened and mold-ripened cheeses also includes significant populations of yeasts and moulds [17]. Therefore, for cheeses such as Idiazabal, the bacterial communities are the most important [16]. The complex dynamics of the microbial composition during cheese making and ripening have been linked to the interesting sensory properties of raw milk cheeses [18,19]. That is to say, the richer and more intense aromatic profile of raw milk cheeses, compared with those produced from pasteurized milk [18,20], has been primarily attributed to the microbiota [18,19].

The cheese microbiota has a diverse and complex composition, but it is mainly composed of lactic acid bacteria (LAB), environmental bacteria and undesirable bacteria [16,18,21–24]. LAB are essential during cheese making and ripening because of their contribution to various biochemical processes that affect cheese flavour and texture or pathogen inhibition [25–28]. Overall, LAB can be divided into starter LAB (SLAB) and non-starter LAB (NSLAB) [29,30]. SLAB are added to ensure one of the most important steps in cheese making, the production of lactic

acid through the metabolism of lactose [22,29]. Lactic acid leads to a decrease in the pH of milk and fulfils three main functions: promoting the activity of rennet, promoting the expulsion of whey from the curd and preventing the growth of undesirable bacteria, such as pathogens [22,29,31,32]. Among the SLAB, the mesophilic genus *Lactococcus* is the most common and is used in the production of Idiazabal cheese [28]. NSLAB are not directly added but are present in raw milk or come from the dairy environment. In general, after curd production, the abundance of NSLAB is low, but throughout ripening, NSLAB abundance increase or may even become predominant depending on the mortality rate of the SLAB [33]. The development of these bacteria is due to their ability to obtain energy from alternative sources through the metabolism of lactic and citric acids, as well as ribose, fatty acids or amino acids [34]. In the Idiazabal cheese, the most common LAB reported are *Lactococcus*, *Lactobacillus* and *Leuconostoc* [35,36].

Although LAB are predominant, other low-abundance microorganisms are also part of the microbial ecosystem of cheese [21,37,38]. These include bacteria derived from the natural environment, such as soil, water, or animal and human gut [23,39–41], and undesirable bacteria, encompassing genera exhibiting pathogenic potential [24] or related to spoilage [42]. Pathogenic potential is related to health hazards or illnesses [21]. Raw milk and derived cheese pathogens primarily originate from the mammary glands or lymph nodes of sheep affected by systemic diseases or infections, as well as from the dairy environment, including equipment, materials and staff [21]. The most reported bacterial pathogens in sheep milk and derived cheeses are, among others, *E. coli, Salmonella* sp., *Staphylococcus aureus* and *Clostridium perfringens.* These microorganisms can result in illnesses of varying severity, with the most common symptoms encompassing fever, nausea, vomiting, diarrhoea, and abdominal pains; however, in severe instances, they can lead to death [21].

On the other hand, spoilage bacteria can be defined as those communities coming from the dairy environment that cause the formation of undesirable products [42]. The most common microbial defects are related to gas formation, both early and late gas. Early gas typically emerges within 1 or 2 days after manufacturing, presenting as numerous small gas holes in the cheese. This phenomenon is attributed to the presence of coliform bacteria and/or yeast. Coliforms primarily produce H_2, while yeasts generate CO_2, both of which originate from lactose. Late gas formation, also known as "late blowing," manifests later in the ripening process. It results from

the fermentation of lactate to butyrate, CO_2, and H_2 by *Clostridium tyrobutyricum* and *Clostridium butyricum*, related to silage use. Butyrate contributes to off-flavour development and the formation of large holes in cheese [17]. Nonetheless, other bacteria, such as *Pseudomonas* spp., *Klebsiella* spp. and *Citrobacter* spp., and in some cases LAB, have also been related to the production of various compounds related to off-flavours due to lactose metabolism, for example, propionic or acetic acids [42].

1.4. Microbiota characterization methods

The bacterial communities present in Idiazabal cheese have been described in several studies [35,36]. However, these studies were carried out 20 years ago using culture-dependent methods, defined as those techniques that involve the isolation of strains in culture media before their phenotypic or genotypic identification [43]. Phenotypic identification includes morphological and physiological characterization, carbohydrate fermentation patterns and protein profiles, for example, whereas genotypic identification relies on polymerase chain reaction (PCR), which allows the selective amplification of specific DNA fragments through the use of oligonucleotide primers under controlled reaction conditions [43,44]. However, to identify all the microbial communities of a given ecosystem, such as cheese, culture-dependent techniques have many limitations, such as the detection of bacteria in relatively small numbers [45–48] or in a viable but non-cultivable state (VBNC) [49].

Nowadays, culture-independent methods, specifically next-generation sequencing (NGS) technologies, such as amplicon (targeting 16S rRNA gene hypervariable regions) and shotgun sequencing (sequencing all the genetic material), have emerged as indispensable tools for characterizing the microbiota of raw milk cheeses [50–52]. Even if each method has each advantages and disadvantages, overall, NGS technologies, compared to traditional culture-independent methods, such as denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), single-strand-conformation polymorphism (SSCP) or restriction fragment length polymorphism (RFLP), NGS technologies provide ample data, enabling us to grasp the functional dynamics of microbial communities [53].

Among NGS technologies, amplicon sequencing offers cost-effective and rapid profiling of microbial community structure and taxonomy [54], including those present in relatively small

numbers [45–48], those present in a VBNC state [49] and those not detected by other culture-dependent or culture-independent methods [49,55–58]. However, this approach also has several limitations, such as the accuracy of taxonomic identification at the species level or the need for trained personnel [59]. Shotgun sequencing, instead, facilitates a more comprehensive assessment of microbiomes [52], not only yielding more accurate taxonomic profiling, including identification of putative new species [51,60] but also providing an exhaustive understanding of the functional potential of microbial communities (e.g., metabolic pathways or genes) [51]. However, this technology involves great data complexity, bioinformatics requirements, and associated greater costs [52].

Although NGS technologies have been widely applied to monitor microbial communities in fermented products [61–63], including cheese [64–66], the majority of cheese studies have focused predominantly on those derived from raw cow milk [65,67,68]. Consequently, only a few studies have been conducted on cheese produced from the raw milk of ewes [69–71]. Moreover, little is known about the bacterial composition of raw ewe milk [72–74] and how it changes during cheese making and ripening processes [45,75,76]. Additionally, NGS results for Latxa raw ewe milk or Idiazabal cheese have not been reported.

1.5. The impact of cheese microbiota on cheese quality and safety

1.5.1. Cheese quality

Cheese quality is defined as a combination of appearance, texture, functionality, nutritive value and flavour [77], which determines consumer acceptability [78]. Nevertheless, cheese flavour is considered one of the most important attributes [26]. The flavour of cheese encompasses odour, referring to volatile compounds detected in the nose via the olfactory epithelium in an orthonasal pathway; aroma, which denotes volatile compounds detected in a retronasal pathway; taste, referring to non-volatile compounds detected on the tongue; and compounds perceived as mouthfeel and texture [26].

During cheese making and ripening, various biochemical processes that are responsible for flavour development occur [79]. There are primarily three groups of metabolic pathways (Figure 2): (1) the metabolism of lactose, lactate and citrate (Figure 2A-B); (2) lipolysis, that is to say, the enzymatic hydrolysis of milk triglycerides (TG) with the subsequent release and accumulation of free fatty acids (FFAs) and their metabolism (Figure 2C); and (3) proteolysis, namely, the enzymatic hydrolysis of milk caseins and subsequent release and accumulation of peptides and free amino acids (FAAs) and their metabolism (Figure 2D) [26]. Thus, the resulting compounds, e.g., FFAs, directly impact flavour, although their metabolism also leads to the production of volatile compounds [26]

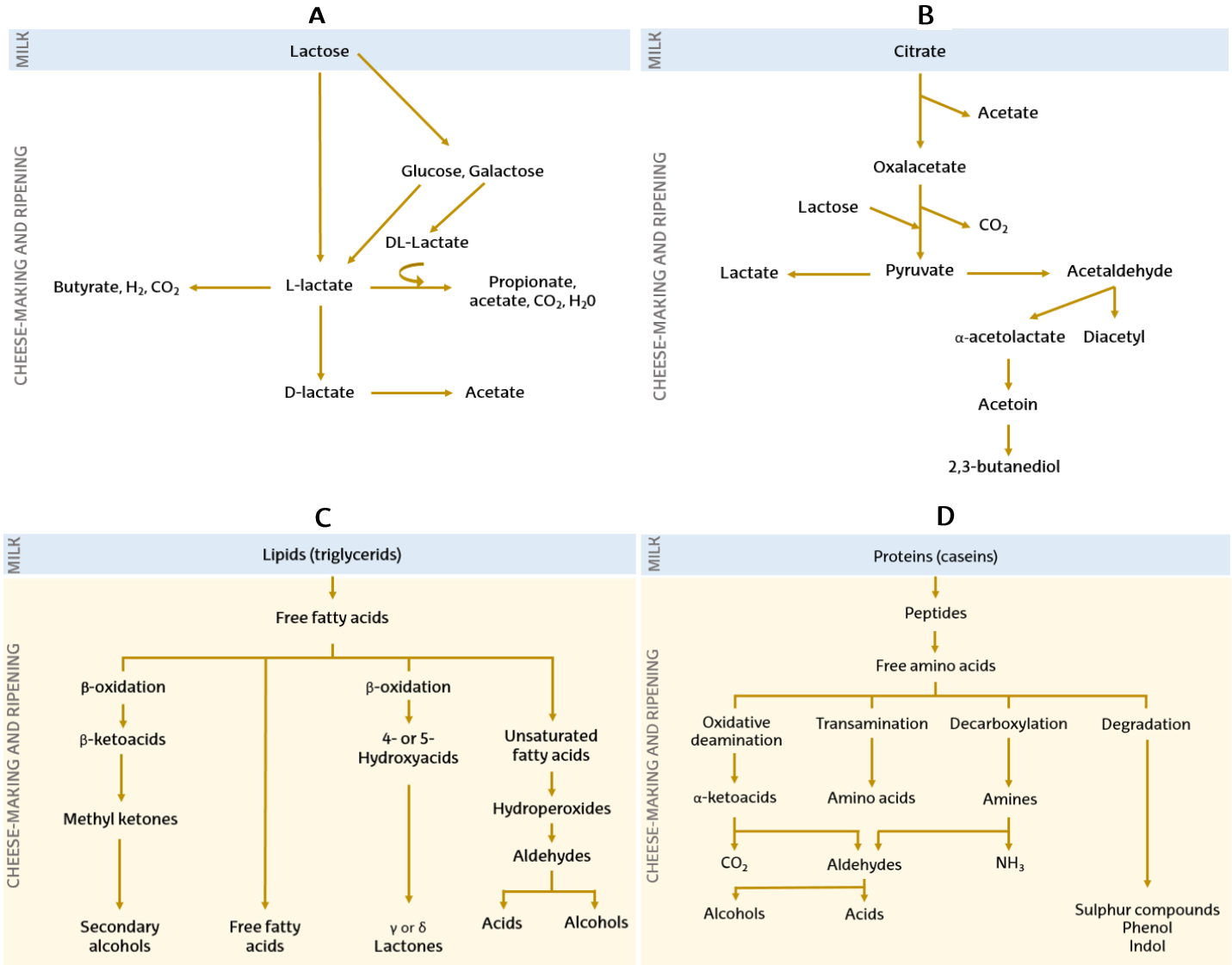


Figure 2. Summary of lactose metabolism (A) (adapted from McSweeney [79]), citrate metabolism (B) (adapted from Fox et al. [22]), lipolysis (C) and proteolysis (D) (adapted from Le Quéré & Buchin S [26]) during cheese making and ripening.

Lactose, lactate and citrate metabolism, along with related processes, is driven by living microorganisms (either starter or non-starter), whereas lipolysis and proteolysis are primarily catalysed by microbial enzymes, such as lipases or proteases [79]. However, there could also be a contribution of enzymes from other sources, such as raw milk, e.g., lipoprotein lipase (LPL), or rennet, e.g., pregastric lipase (PL) or proteases like chymosin and pepsin [79]. Nevertheless, in the case of LPL, its activity is very low in Idiazabal cheese [80]. Anyway, the contribution of microbial communities to the production of metabolites related to flavour, such as FAAs or volatile compounds, in different types of cheeses has been reported in several studies [81–84]. However, information on raw ewe milk-derived cheeses is scarce [45,69,85], and there is no information on the extent to which bacterial communities could affect the flavour of Idiazabal cheese.

1.5.2. Cheese safety

Cheese safety can be defined as the assurance that during preparation and/or eating process will not cause adverse health effects to the consumer, being the adverse health effects caused by physical, chemical, or biological hazards [86].

Apart from **pathogenic bacteria** themselves, as previously mentioned, there are also other **biological hazards** related to cheese safety, within which antimicrobial resistance is notable [87]. For decades, antibiotics have been overused in human medicine and animal production [88,89], including currently banned subtherapeutic growth promoters, resulting in bacteria developing resistance to antibiotics [2,90]. The proliferation of antimicrobial-resistant (AR) microorganisms has become one of the most important threats to human health [91] and is classified as one of the top 10 threats to global public health [87]. It causes approximately 700,000 deaths worldwide per year and is projected to increase to 10 million each year by 2050 [92]. The food and food production chain is classified as a possible vehicle for the dissemination of AR bacteria and genes [93], and fermented products are considered notable reservoirs for the transfer of antimicrobial resistance genes (ARGs) to other bacteria, including pathogenic bacteria [93–97]. However, information about dairy products, especially raw milk-derived cheeses, is limited [96,97].

Antibiotic residues, in addition to contributing to the development of antimicrobial resistances (AMRs) [98], are also **chemical hazards** *per se* since they pose a public health threat

due to various toxicological effects, such as allergies, dysfunction of the intestinal microbiota, immunopathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity, and anaphylactic shock [2]. Consequently, regulatory authorities worldwide have established maximum residual limits (MRLs) based on acceptable daily intake (ADI), representing the amount of substance that can be ingested daily throughout life without appreciable health risks [2,99]. However, MRLs have only been established for milk, and there are no limits for other dairy products, such as whey or cheese [2], in which these compounds can be concentrated [100]. There is no information in the literature on the prevalence of antibiotics throughout the entire dairy production chain because of livestock treatment, i.e., from animals to ripened cheese. The majority of published studies only analyse a single product, such as milk, or consist of intentionally spiked products to analyse the effect of different processes, such as sterilization [99–102]. Farmers' lack of knowledge about antibiotic use and awareness of its potential impacts have been linked to poor practices, resulting in the presence of residues in both foodstuffs and the environment [103,104]. Therefore, concerted efforts from all stakeholders are necessary to ensure the proper use of antibiotics and prevent the spread of residues [2,103,104].

Other **chemical hazards related to microbial communities** can also be found in cheese, within which **biogenic amines** (BAs) are notable. BAs are nitrogenous organic bases of low molecular weight with biological activity that are formed by microbial decarboxylation of FAAs [105–107]. At low concentrations, BAs are not harmful, but the ingestion of high concentrations can lead to toxicological effects [108,109]. In the context of dairy products, particularly cheese, histamine and tyramine are primarily related to intoxication. Histamine causes "histamine poisoning," characterized by symptoms such as low blood pressure and skin irritation resembling allergic reactions, while tyramine induces a "cheese reaction," with symptoms including migraines, headaches, or increased blood pressure. Putrescine and cadaverine are also significant BAs because they can enhance the toxic effects of other BAs by inhibiting detoxifying amine oxidases and are associated with the production of carcinogenic nitrosamines [108,110,111]. Among fermented foods, cheese is categorized as a potential source of BAs due to its high microbial activity [107,112]. However, legal limits for BAs in cheese and other fermented foods have not yet been established [113,114].

1.6. Source of microorganisms

Great **differences in the microbial composition** among types of cheeses have been reported [70,72,75,76–31], even among producers of the same type of cheese [28,69]. Thus, given their contribution to cheese quality and safety (section 1.5), the study of **microbial sources in dairies** is essential. In general, the sources of bacteria in milk and cheese are diverse and complex [21]. These include geographical location [115], flock management and feeding [116], microorganisms contaminating the teat surface [116], or practices, materials and ingredients employed during milking or in the dairy environment [27,117,118]. Although the impact of the aforementioned factors has been studied, in all cases, the impact of a single or few factors has been analysed [21,27,116–118], and none have comprehensively studied all the potential factors that could have affected. Furthermore, most studies have focused only on cow milk-derived cheeses, with little information on small ruminants, such as sheep [21,116].

This aspect is of special interest in the case of Idiazabal cheese since, although the Idiazabal cheese making process is strictly regulated, producers may employ different flock management and cheese making practices [119–122]. However, the impact of flock management and cheese making practices related to each artisanal dairy producing Idiazabal cheese on the microbiota of Latxa ewe raw milk or Idiazabal cheese has not been studied to date. This would greatly contribute to this expanding field of research, providing novel knowledge directly applicable to the sector, especially related to quality assurance.

2. AIMS AND OBJECTIVES

The present philosophiæ doctoral dissertation has been performed within **Lactiker-Quality and Safety of Foods from Animal Origin Research Group** of the **University of the Basque Country (UPV/EHU)**, whose main objective is to obtain scientific data from a multidisciplinary point of view (such as biochemistry, microbiology or food technology) to assess the quality and safety of traditional foods from animal origin.

The **hypothesis** raised in this research project is that **each Idiazabal cheese has its own microbiota**, namely bacterial communities, which has an **essential and unavoidable effect on the composition and characteristics** of the final product and which **depends on several factors**, such as the flock management or the cheese making process. Therefore, the **main objective** of the present PhD dissertation is to **characterize the microbiota**, namely the bacterial composition, **of Idiazabal PDO cheeses** by means of metagenomic techniques**, analyse to what extent** the microbiota **affects cheese quality and safety**, and **determine** the main **factors shaping the microbiota.**

In this way, so as to achieve this main objective, this PhD dissertation relies on four main aims that will be achieved throughout several specific objectives that are detailed below.

AIM 1. To characterize by metagenomic techniques the microbiota of raw ewe milk-derived Idiazabal cheese and how it evolves.

For this purpose, the following specific objective is formulated:

- **Objective 1.1.** To optimize the experimental techniques needed to carry out amplicon-based High-Throughput Sequencing (16S rRNA) (HTS) analysis, so as to characterize the microbiota of Latxa raw ewe milk and to determine how it changes during the production and ripening processes to make up the bacterial composition of Idiazabal cheese; and delve into the potential similarities or differences among PDO producers (**manuscript I**).

The **first aim** of **tuning up** the **methodology** for characterizing the microbiota of Idiazabal cheese by means of **amplicon-based HTS** is essential to allow **determining the bacterial communities** of Latxa raw ewe milk, including safety aspects such as pathogenic bacteria; and

studying how it changes during the cheese making process **to make up the final microbial composition of cheese**. Moreover, this methodology will also let knowing whether the bacterial composition of Idiazabal cheese **differs** according to the **manufacturer**, in relation to the hypothesis raised.

AIM 2. To elucidate to what extent the microbiota affects several parameters related to quality and safety of raw ewe milk-derived Idiazabal cheese.

For this purpose, the following specific objectives are set:

- **Objective 2.1.** To tackle, in general, by means of near-infrared spectrometry (NIR), gas-liquid chromatography–flame ionization detector (GLC-FID) and reversed phase-high-performance liquid chromatography-fluorescence detector (RP-HPLC-FLD) the evolution of gross composition, FFAs and BAs content, respectively, and its association with bacterial shifts, characterized by amplicon-based HTS, during the ripening of Idiazabal cheese (**manuscript II**).

- **Objective 2.2.** To examine by Solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) the evolution of volatile compounds, their odour impact (odour impact ratio (OIR) values), and their relationship with microbial dynamics, determined by amplicon-based HTS, during the ripening of Idiazabal cheese (**manuscript III**).

Reaching this **second aim** is of special interest, since it will allow knowing the extent **to what the bacterial communities** of a raw milk cheese **affect** the most important **cheese quality and safety** parameters. These results will also let unravelling whether differentiation in cheese microbiota between PDO Idiazabal producers **give rise to variations in terms of cheese quality**, mainly aroma; **and safety**, for example toxicity. These results will provide **novel information for the scientific community to help delving into the impact of bacterial metabolism involved in fermentation processes**, which has been little studied so far. Moreover, this topic is **extremely relevant to guide the dairy sector** and specifically Idiazabal PDO producers**, to understand the impact of bacterial communities on the final characteristics of the product.**

AIM 3. To study the extent to which the microbial communities inhabiting raw ewe milk-derived Idiazabal cheese can compromise cheese safety in terms of antimicrobial resistances.

For this purpose, the following specific objectives are formulated:

- **Objective 3.1.** To review the current knowledge on antibiotics' use and prevalence of residues and AMRs in milk and dairy products, specially cheese (**manuscript IV**).

- **Objective 3.2.** To survey Idiazabal PDO producers' knowledge, attitudes and practices on antibiotics use; and detection and quantification of antibiotic residues throughout Idiazabal cheese production by means of screening test (Charm KIS and Eclipse Farm^{3G}) and chromatographic techniques (Liquid Chromatography-Triple Quadrupole-Mass Spectrometry (LC-QqQ-MS/MS)), together with analysing the potential differences among PDO manufacturers (**manuscript V**).

- **Objective 3.3.** To study phenotypically, by broth microdilution, and genotypically, through HT-qPCR, the AMRs of the main microbial communities of Idiazabal cheese, namely LAB, and analyse trends throughout Idiazabal cheese production, together with the potential differences among PDO manufacturers (**manuscript VI**).

This **third aim** focuses on a **novel and challenging aspect of food safety**, that has nothing to do with the usual food safety parameters, such as the presence of toxic compounds in cheese (aim 2); namely AMRs. Determining the use of antibiotics, the presence of residues and **the phenotypic and genotypic prevalence of AMRs related to the microbiota** of Idiazabal cheese is **of great importance** to **provide knowledge that helps guarantee cheese safety** and, therefore, **protect consumers' health**. In this sense, it will be studied whether there is a **differentiation or similarities** in this regard **among PDO producers**, which will contribute to **promoting the knowledge and good practices** among producers to **ensure** the **safety related to microbiota inhabiting Idiazabal cheese**.

AIM 4. To determine the main production factors within artisanal dairies affecting the microbiota of raw ewe milk derived Idiazabal cheese.

For this purpose, the following specific objective is set:

- **Objective 4.1.** To tune up the experimental methodology required to carry out Shotgun Sequencing of Latxa raw ewe milk and derived Idiazabal cheeses, to elucidate potential microbial reservoirs within Idiazabal PDO dairies affecting cheese microbiota; and to delve into their functional potential and genetic diversity in relation to cheese quality and safety (**manuscript VII**).

The achievement of the **fourth aim** will make it easier to **identify those factors or sources of microorganisms within Idiazabal PDO dairies** that affect the **microbiomes of raw ewe milk and derived Idiazabal cheeses**, that is to say, the microbial composition and its genetic potential in relation to cheese quality and safety. This aspect has been little studied for raw ewe milk cheeses so far, which means **novel and important insights** for the scientific community to **confirm** the **associations** observed among **bacterial communities** and different compounds or parameters of **cheese quality and safety.** Moreover, the obtained knowledge will enable the **production sector** to **understand the impact of factors inherent to dairies on the microbiota**, from **herd management** to ingredients used for **cheese manufacturing**, and consequently, **select the most appropriate** ones in terms of cheese quality and safety. Additionally, these results will also help understanding the main factors that could **give rise** to **differentiation among PDO producers.** Up to now, no study has comprehensively analysed the genetic and functional diversity of bacterial communities of a raw ewe milk cheese, in relation to dairy environments.

3. MATERIALS AND METHODS

In this section, a concise overview of the **materials and methods** employed in the **manuscripts (I-VII)** constituting this thesis is provided. The objective is to prevent repetitions of the aspects described in the manuscripts. This **materials and methods** section is divided into four parts (Figure 3): **(3.1) area of study, (3.2) sampling, (3.3) methodology and (3.4) statistical analysis.** Additionally, these parts are categorised into **four phases (I-IV)** according to the four **aims (1-4)** of the thesis (grey horizontal stripes in Figure 3). The objective is to easily describe any differences among objectives.

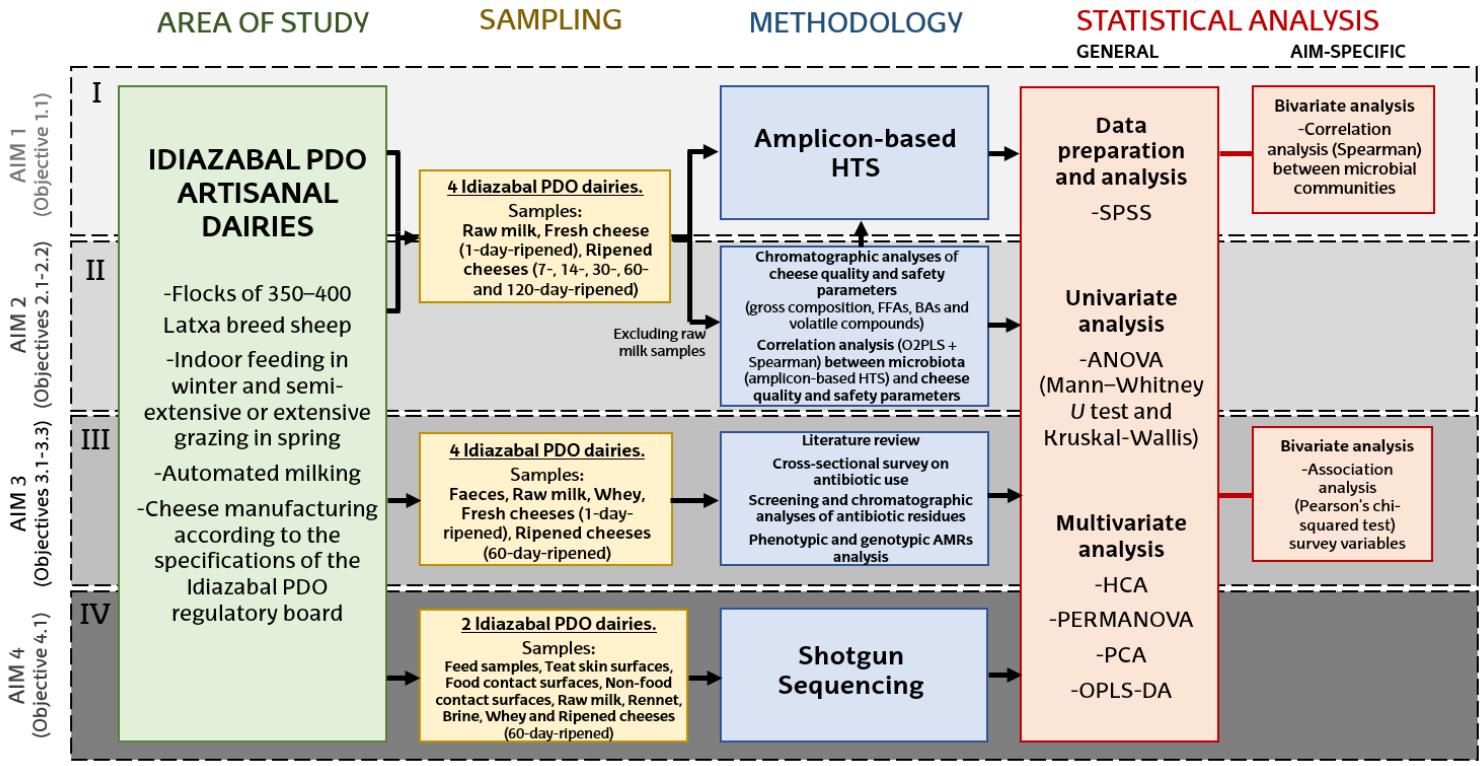


Figure 3. Schematic representation of the materials and methods used in this research project constituting the thesis.

3.1. Area of study

Several artisanal producers attached to the Idiazabal PDO, which were distributed along the Basque Country (Alava, Biscay, Gipuzkoa and Navarre), were selected for the project. These dairies managed the entire process, from herd management to cheese making and sales. All

producers adhered to uniform flock management and cheese making conditions in accordance with the specifications outlined by the Idiazabal PDO regulatory board [14]. In all cases, each flock comprised only the Latxa breed, approximately 350–400 heads, not considering sheep under antibiotic treatment, as indicated by producers. Flock management involved indoor feeding in winter and semi-extensive or extensive grazing in spring.

Milking was conducted automatically, and the milk was promptly refrigerated (3-4 °C) until cheese production. For cheese manufacturing, each producer employed the milk of its own flock. The manufacturing process began when the milk was warmed to 25 °C. Then, the commercial mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added. Milk coagulation occurred at 28–32 °C for 20–45 min, employing artisanal rennet (extracted from the stomachs of Latxa or Carranzana lambs, obtained during the first month of lactation, cleaned, dried, salted, and ground) or commercial NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). The resulting curds were cut into 5-10 mm diameter grains, heated to 36-38 °C, moulded, pressed and salted in saturated brine. Finally, cheeses were ripened in chambers maintained at 80-95 % relative humidity and 8-14 °C.

3.2. Sampling

Throughout the project, various **samplings** (**I-IV**) took place, each corresponding to the aims (**1-4**) described before. In all cases, aseptic collection was performed using appropriate personal protective equipment, disinfected gloves and sterile materials to avoid cross-contamination, and each set of samples corresponded to the same batch. All samples were collected by producers, except for **sampling IV** (**aim 4, objective 4.1**), which was conducted jointly by the producers and researchers. Overall, there was no need for approval from the Ethics Committee for Animal Experimentation. Informed verbal consent was obtained from the dairies prior to and during sample collection.

Sampling I-II: Characterization of the microbiota of Idiazabal cheese and how it evolves (I) and the impact of the microbiota on cheese quality and safety (II)
(aims 1-2, objectives 1.1, 2.1-2.2, manuscripts I-III)

To study the effect of the production and ripening processes on the microbiota, raw milk (~ 1.00 L), fresh cheeses (1-day-ripened) (~ 1.50 kg each) and cheese samples at different time points during ripening (namely, 7, 14, 30, 60 and 120 days) (~ 1.50 kg each) were taken from 4 producers in duplicate, except for raw milk, with each set of samples corresponding to the same batch (**aim 1, objective 1.1, manuscript I**). Excluding the raw milk samples, the collected fresh and ripened cheese samples from the aforementioned producers were also used to analyse the relationships among the microbiota and cheese quality and safety parameters (**aim 2, objectives 2.1-2.2, manuscripts II-III**). The samples were transported under refrigeration (3 ± 1 °C) to the laboratory. The raw milk samples were divided into aliquots (~ 0.2 L), and each cheese sample was divided into eights (~ 0.190 g). One fresh aliquot/eight of each sample type was directly used for metagenomic and gross composition analyses (except for NaCl), while the rest were stored in a freezer (−80 °C) for subsequent food quality- and safety-related parameter analyses (NaCl, FFAs, BAs and volatile compounds). The samples were allowed to thaw at 5 °C for 24 h and then kept at room temperature for 1 h prior to analysis.

Sampling III: The impact of the microbiota on cheese safety in terms of antimicrobial resistances
(aim 3, objectives 3.1-3.3, manuscripts IV-VI)

To analyse the presence of antibiotic residues and AMRs (**aim 3, objectives 3.2-3.3, manuscripts V-VI**), raw milk (~ 1.00 L), fresh (1-day-ripened) and ripened (60-day-ripened) cheese samples (~ 1.50 kg each) were collected from 4 producers. In addition, ovine faeces (~ 0.250 kg each) and whey samples (~ 1.00 L) were also collected. All samples were collected in quadruplicate, with each set of samples corresponding to the same batch, and transported under refrigeration (3 ± 1 °C) to the laboratory. The raw milk and whey samples were divided into aliquots (~ 0.2 L), and each cheese was divided into eighths (~ 0.190 g). For AMRs analyses, fresh samples were used, while for antibiotic residue analysis, samples were stored in a freezer (−80 °C) until analysis. The samples were allowed to thaw at 5 °C for 24 h and then kept at room temperature for 1 h prior to analysis.

Sampling IV: **Determination of the main production factors affecting the microbiota of Idiazabal cheese** (aim 4, objective 4.1, manuscript VII)

To identify the main factors or sources of microorganisms for Latxa raw ewe milk and Idiazabal cheese (**aim 4, objective 4.1, manuscript VI**), two Idiazabal PDO dairies located geographically close to each other were selected to avoid or minimize the impact of geographical factors (altitude, longitude, latitude, temperature, precipitation, humidity, etc.) on the microbiota. Several sample types were collected from each producer to identify all the potential microbial reservoirs within the Idiazabal PDO dairies. According to herd management and feeding, grass obtained from fresh pastures (~ 250 g) and commercial feed (~ 500 g) were collected from both producers. Additionally, from Producer A, straw obtained from their own fields (~ 150 g) and home-made feed (~ 500 g), comprising a blend of grass, corn, and beet used for feeding, were also collected. The day before milking, 30 swabs, each from 10 to 15 sheep, of teat skin surfaces were also collected with gauze swabs (7.5 cm × 7.5 cm) (Medrull, Dortmund, Deutschland) moistened in 0.9 % (w/v) sterile NaCl solution (Scharlab, Barcelona, Spain). After the morning milking, the raw milk was also collected (~ 1.00 L). From dairies, 30 swab samples from food contact surfaces (e.g., trays, shelves, equipment, materials, etc.) and 30 non-food contact surface swabs (e.g., floors, walls, etc.) were also taken with gauze swabs moistened in 0.9 % (w/v) sterile NaCl solution. During cheese manufacturing, artisanal rennet (~ 50.0 g) and brine (~ 1.00 L) samples were collected. After the cheese was made, the generated whey was collected (~ 1.00 L). The cheeses were ripened for 60 days prior to collecting 2 individual cheeses (~ 1.50 kg) from each producer. All samples were collected in duplicate, and each set of samples corresponded to the same batch. The samples were transported under refrigeration (3 ± 1 °C) to the laboratory, and all analyses were performed on fresh samples.

3.3. Metodologia

Metodologia lau zatitan banatu zen (**I-IV**), helburu bakoitzaren arabera (1-4).

I. metodologia: Idiazabal gaztaren mikrobiotaren karakterizazioa eta garapena (**1. xedea, 1.1. azpihelburua, I. eskuizkribua**)

3.3.1. Anplikoietan oinarritutako errendimendu handiko sekuentziazioa (EHS)

3.3.1.1. DNAren erauzketa

Lagin freskoetatik DNA bakterianoa erauzi zen Erkus et al.-en [123] protokoloan oinarrituta, aldaketa batzuekin. Gazta-laginetatik DNA erauzteko, 10 g gazta 90 mL sodio zitrato esterilean (% 2koa, m/b, pH 8.0, erreaktibo-maila, Scharlab) eseki ziren, eta sei aldiz homogeneizatu zen (20 s piztuta eta 10 s itzalita) Stomacher bat (Masticator Basic 400; IUL Instruments, Königswinter, Alemania) erabiliz. Ondoren, 1,5 mL zentrifugatu ziren (8000 × *g*-tan 10 minutuz 4 °C-tan) eta ur-gainazaleko gantza baztertu zen. Lortutako jalkina 600 µL sodio zitratorekin berreseki zen eta hiru aldiz zentrifugatu zen (8000 × *g*-tan 10 minutuz 4 °C-tan). DNA DNeasy Blood & Tissue Kit-a (Qiagen, Valentzia, Kalifornia, Estatu Batuak) erabilliz erauzi zen. Esne gordinaren laginak berdin prozesatu ziren, baina homogenizatu gabe.

3.3.1.2. Liburutegien prestaketa eta sekuentziazioa

EHS analisia Euskal Herriko Unibertsitateko SGIkerreko Sekuentziazio eta Genotipo Azterketen Unitatean-Genomika Zerbitzuan egin zen. 16S RNAe genearen liburutegia Nextera XT DNA Library Preparation Kit-a (Illumina, Inc., San Diego, Kalifornia, Estatu Batuak) erabiliz prestatu zen, Illuminako 16S RNAe genearen metagenomikako lan-fluxua jarraituz. 16S RNAe genearen V3-V4 zonaldeen anplifikazioa polimerasaren kate-erreakzioaren (PKE) bidez egin zen, Klindworth et al.-ek [124] deskribatutako abiarazleak erabilita (aurreranzko abiarazlea: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; alderantzizko abiarazlea: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). 16S RNAe genearen sekuentziazioa Illumina MiSeq plataforma batean egin zen, MiSeq Reagent Kit v3 (2 × 300 bp) kit-a (Illumina, Inc.) erabiliz.

3.3.1.3. Analisi bioinformatikoa

Irakurketa gordinak ebakitzeko eta horien kalitate-iragaztea egiteko, MiSeq Reporter softwarea (Illumina, Inc.) erabili zen. Sailkapen taxonomikoa MG-RAST erabiliz egin zen [125], Silva SSU datu-basean oinarrituta [126]. Bakterioen ugaritasuna ehuneko erlatibo gisa kuantifikatu zen (%), identifikatutako sekuentzien arabera kalkulatuta.

3.3.1.4. Dibertsitatearen azterketa

α- eta β-dibertsitatearen indizeak bakterio-genero guztien sekuentzien ugaritasuna kontuan hartuta kalkulatu ziren. α-dibertsitatea RStudio (1.3.959 bertsioa) eta R (3.6.3 bertsioa) softwareetan (R Core Team, Viena, Austria, 2020) kalkulatu zen. Shannon, Simpson, Simpson-en alderantzizkoa, Berger eta Shannon-en uniformetasuna (Jevenness eta Eevenness) indizeak BiodiversityR paketearen bidez kalkulatu ziren [127], eta Chao1 eta ACE dibertsitate-indizeak vegan paketearen bidez kalkulatu ziren [128]. β-dibertsitatea, hau da, Bray-Curtis-en eta Jaccard-en desberdintasun-indizeak, vegan paketearekin kalkulatu ziren, eta koordenatu nagusien analisietan (KNA) irudikatu ziren APE paketearekin [129].

II. metodologia: Mikrobiotak gaztaren kalitatean eta segurtasunean duen eragina (**2. xedea, 2.1.-2.2. azpihelburuak, II.-III. eskuizkribuak**)

3.3.2. Konposizio gordinaren analisia

pH-a pH-metro batekin neurtu zen (MicropH 2000; Crison Instruments S.A., Bartzelona, Espainia). Materia lehorraren, gantzaren, proteinaren, kaltzioaren, magnesioaren eta fosforoaren edukiak, SpectraAlyzer 2.0 FOOD infragorri hurbileko espektrometroarekin (IHS) neurtu ziren (ZEUTEC GmbH, Rendsburg, Alemania), aurretik deskribatu zen bezala [130]. NaCl-aren edukia Nazioarteko Normalizazio Erakundea (NNE) 5943 Esnekien Nazioarteko Federazioa (ENF) 88 metodo estandarrean oinarrituta zehaztu zen [131], baina determinazio kolorimetrikoa erabiliz (Mohr-en balorazio-metodoa). Analisi horiek bi aletan egin ziren.

3.3.3. Gantz-azido askeen (GAA) analisia

GAAak erauzi, eta gas-likido kromatografia (GLK) bidez bereizi, identifikatu eta kuantifikatu ziren deribatizaziorik gabe, aurretik deskribatu den bezala [132].

3.3.3.1. GAAen erauzketa

Laburbilduz, 0,5 g gazta 3,0 g sodio sulfato anhidrorekin (erreaktibo-maila, Scharlab) eho zen, eta 0,3 mL azido sulfuriko (2,5 M, erreaktibo-maila, Scharlab) eta barne-patroiaren (BP) disoluzioaren 100 µL gehitu ziren. BParen disoluzioak 1 mg/mL gantz-azido (azido pentanoikoa (C5), nonanoikoa (C9) eta heptadekanoikoa (C17)) dietil eter-heptanoan zituen (1:1, b/b, gas-kromatografia (GK) maila, ROMIL Ltd., Cambridge, Erresuma Batua). Lipidoak hiru aldiz erauzi ziren 3,0 mL dietil eter-heptanorekin eta zentrifugatu egin ziren gardentzeko. Ondoren, fase organikoak konbinatu ziren eta fase aminopropilatuko zutabe batean aplikatu ziren (Sep-Pak®, 3 cm^3, 500 mg, Waters, Bartzelona, Espainia), aldez aurretik 10,0 mL heptanorekin orekatuta. Triglizeridoak (TG) 10,0 mL kloroformo-isopropanol nahastearekin (2:1, b/b, GK kalitatea, ROMIL Ltd.) eluitu ziren eta GAAk, berriz, 5,0 mL azido formikoa zuen dietil eterrarekin (% 2an, erreaktibo-maila, Panreac Química, Bartzelona, Espainia) eluitu ziren.

3.3.3.2. Gas-likido kromatografia-sugarraren ionizazio-detektagailuarekin (GLK-SID)

GAAk FFAP zutabe kapilar batean banatu ziren (25 metroko luzera, 0,32 mm-ko barne-diametroa (B.D.), 0,50 µm-ko geruza-lodiera), 7890 Series II SIDdun gas-kromatografo batean instalatuta zegoena (Agilent Technologies, Inc., Madril, Espainia). Helioa (purutasuna % 99,999, Air Liquide, Madril, Espainia) gas eramaile gisa erabili zen (2 mL/min-ko fluxua). Tenperatura 10 °C/min-ko abiaduran igo zen 65 °C-tik 240 °C-ra, eta, ondoren, 240 °C-tan mantendu zen 30 minutuz. Banaketa/banaketa gabeko ratioa 1:5 balioan ezarri zen, eta injektorearen eta detektagailuaren tenperaturak 325 ºC-tan eta 275 ºC-tan mantendu ziren, hurrenez hurren. GAAak identifikatzeko, atxikitze-denborak purutasun handiko estandarrekin alderatu ziren (≥ % 90,0; Sigma-Aldrich, Madril, Espainia) eta barne-kalibrazioaren metodoaren bidez kuantifikatu ziren laginetan aurkitutako GAA bakoitzerako. GAAen kontzentrazioak, µmol/g gazta gisa adierazi ziren.

3.3.4. Konposizio lurrunkorraren analisia

3.3.4.1. Fase solidoko mikroerauzketa (FSME) metodologia

FSME prozedura aurretik deskribatu den bezala gauzatu zen [133]. Laburbilduz, 15 g gazta 20 g sodio sulfato anhidrorekin eho ziren, eta BParen soluzioa (ziklohexanona, 0,5 µg/L-ko,

≥ % 99,5, Sigma-Aldrich) gehitu zen, eta berriz homogeneizatu zen. Ondoren, 2,5 g 10 mL-ko anbar-ontzi batera transferitu ziren, eta PTFE septu batekin eta altzairuzko tapa magnetiko batekin zigilatu zen (18 mm PTFE/SIL, Agilent Technologies, Inc.). FSME prozedura PAL RSI 85 lagin-hargailu automatiko bat erabiliz egin zen, tenperatura kontrolatuko aire-inkubagailua zeukana (CTC CombiPAL, Zwingen, Suitza). Aurreorekaren (60 °C-tan 15 minutuz) ostean, konposatu lurrunkorrak 50/30 µm-ko DVB/Carboxen/PDMS zuntz batean (57298-U, Supelco, Madril, Espainia) harrapatu ziren 30 minutuz 60 °C-tan.

3.3.4.2. Gas-kromatografia-masa-espektrometria (GK-ME)

Konposatu lurrunkorrak aztertzeko, 7820A gas-kromatografiako sistema bat erabili zen, 5975 series masa-espektrometria (ME) detektagailu batekin (Agilent Technologies, Inc.), aurretik deskribatu den bezala [134]. Konposatu lurrunkorrak aurreko injekzio-atakan zuntzetik desorbatu ziren 10 minutuz 240 °C-tan, banaketa gabeko moduan. Ondoren, konposatu lurrunkorrak Supelcowax-10 silize urtuzko zutabe kapilar batean banandu ziren (0,25 µm-ko film-lodiera, 0,25 mm-ko B.D., 59,5 m-ko luzera, Supelco), helioa (% 99,999-ko purutasuna, Air Liquide) gas eramaile gisa erabiliz (16 psi-ko presio konstantea). Labearen hasierako tenperatura 40 °C-koa izan zen 10 minutuz; gero, 110 °C-ra igo zen 5 °C/min-ko abiaduran, eta, ondoren, 240 °C-ra igo zen 10 °C/min-ko abiaduran, eta 240 °C-tan mantendu zen 15 minutuz. Konposatu lurrunkorrak ME detektagailura transferitu ziren 280 °C-ko transferentzia-linea baten bidez, eta ME detektagailuak 150 °C-tan funtzionatu zuen eskaneatze osoko moduan, 70 eV-eko ioien korronte totalarekin (IKT).

Lortutako datu kromatografikoak MSD ChemStation Data Analysis softwarean (5.52 bertsioa, Agilent Technologies, Inc.) aztertu ziren. Konposatu lurrunkorren identifikazioa masa-espektroen konparazioan oinarritu zen (kointzidentzia-faktorea > 800), National Institute of Standards and Technology (NIST) espektro-liburutegia erabiliz (2.0 bertsioa, Gaithersburg, Estatu Batuak). Gailur-kromatografiko bakoitzerako batez besteko atxikipen-indize lineala (AIL) Idiazabal gaztaren laginetan (4 erreplika) eta alkano aseen nahaste estandar batean (49452-u, C7-C40, Sigma Aldrich) (3 × 2 aldiz esperimentu osoan) kalkulatu zen. Identifikazio positiboa egiteko, purutasun handiko estandarrekin (≥ % 90, Sigma-Aldrich eta Honeywell Fluka, Madril, Espainia) alderatu ziren AILak eta masa-espektroak. Detekzio-muga (DM) zarataren bikoitza izan zen (unitate arbitrarioak). Gailur-azalera IKTren bidez kuantifikatu zen, eta konposatu

lurrunkorren edukia ugaritasun erlatibo gisa adierazi zen (gailur-azalera unitate arbitrarioetan, BParekiko), hurrengo ekuazioak adierazten duen bezala:

$$\textbf{Konposatu lurrunkorren ugaritasun erlatiboa} = \frac{\textbf{Gailur} - \textbf{azalera}}{\textbf{BParen azalera}} \times \frac{\textbf{2,5 g}}{\textbf{laginaren pisua (g)}} \times \textbf{100}$$

Gazta-lagin bakoitzeko konposatu lurrunkorren batez besteko ugaritasuna konposatu bakoitzaren gailur-azaleretatik (> DM) abiatuta lortu zen, betiere laginaren lau errepliketatik gutxienez bitan detektatu baziren. Gazta-laginetan konposatu lurrunkorren ugaritasun erlatiboa batezbesteko ± desbideratze estandar gisa adierazi zen.

3.3.4.3. Usainaren inpaktu-ratioaren (UIR) balioen kalkulua

Konposatu lurrunkorren usain-inpaktua UIRen bidez kalkulatu zen, Abilleira et al.-ek [135] deskribatu duten bezala, aldaketa txikiekin, ekuazio honi jarraituz:

$$\textbf{UIR} = \frac{\textbf{Konposatu lurrunkorren ugaritasun erlatiboa}}{\textbf{usain} - \textbf{atalasea (UA)}(\mu\textbf{g/L} - \textbf{ko edo } \mu\textbf{g/kg} - \textbf{ko})}$$

UIRak 1etik gorakoak direnean, usain-konposatu aktiboak adierazten dituzte. Ahal zen neurrian, gaztan neurtutako UA balioak hartu ziren, matrizearen eragina saihesteko.

3.3.5. Amina biogenikoen (AB) analisia

ABak erauzi, bereizi, identifikatu eta kuantifikatu ziren alderantzizko faseko bereizmen handiko kromatografia likidoaren bidez (AF-BHKL), Busto et al.-ek [136] deskribatu duten bezala.

3.3.5.1. ABen erauzketa eta deribatizazioa

Laburbilduz, 1,0 g gazta 9,0 mL azido trikloroazetikorekin (ATK) (% 5ean, m/b, erreaktibo-maila, Scharlab) eho zen eta BP (1,7-diaminoheptanoa, 1,5 mM, ≥ % 98,0, Sigma-Aldrich) zuen 1,0 mL ATK gehitu zitzaion. Estraktu azidoa 25 minutuz sonikatu zen, eta 5000 rpm-ko abiaduran zentrifugatu zen 30 minutuz giro-tenperaturan. Ondoren, Durapore iragazkien (0,45 mm-ko poro-tamaina; Millipore, Madril, Espainia) bidez iragazi zen. Deribatizazio-erreaktiboa, 6-aminokinolil-N-hidroxisukzinimidil karbamatoa (erreaktibo-maila, Waters) 1,0 mL azetonitrilorekin (erreaktibo-maila, Waters) berrosatuz eratu zen, AccQ-Fluor

Reagent Kit-a (Waters) erabiliz. Ondoren, amina-estraktuaren 2,5 µL, borato indargetzailearen 75 µL-rekin (pH 8,8, erreaktibo-maila, Waters) eta AQC erreaktibo deribatzailearen 25 µL-rekin (erreaktibo-maila, Waters) nahastu ziren. Nahasketa astindu eta 55 °C-tan mantendu zen 10 minutuz.

3.3.5.2. Alderantzizko faseko bereizmen handiko kromatografia likidoa-fluoreszentzia-detektagailuarekin (AF-BHKL-FLD)

Deribatizatutako ABak XTerraTM MS C18 zutabe bat erabiliz bereizi ziren (125 Å-ko poro-tamaina, 5 µm-ko partikula-tamaina:, 4,6 mm-ko B.D. eta 250 mm-ko luzera), Alliance 2690 banaketa-modulu batean, Waters 474 FLD detektagailu bat zeukana (Waters). Zutabea eta aurrezutabea (XTerra MS C18 Sentry Guard Cartridge, 125 Å-ko poro-tamaina, 5 µm-ko partikula-tamaina:, 3,9 mm-ko B.D. eta 20 mm-ko luzera) 50 °C-tan mantendu ziren, eta bi disolbatzaile erabili ziren: (A) 0,05 M-eko sodio azetato disoluzioa % 1eko tetrahidrofuranoan (> % 99,9, ROMIL Ltd.), pH 7,0, eta (B) metanola (> % 99,9, ROMIL Ltd.), 1. taulan zehaztutako programari jarraituz. Detekzioa fluorimetria bidez egin zen, 250 nm-ko eta 395 nm-ko kitzikapen- eta igorpen-uhin-luzeratan, hurrenez hurren. Identifikazioa eta kuantifikazioa BPa eta ABen kontzentrazio ezagunak dituzten soluzio estandarrak erabiliz gauzatu zen. ABak honako hauek izan ziren: metilamina (MAM), azido α-aminobutirikoa (AAAB), azido γ-aminobutirikoa (AGAB), kadaberina (KAD), etanolamina (EAM), etilamina (ETI), histamina (HIS), metilbutilamina + espermidina (MBA + EPD), n-butilamina (NBA), feniletilamina (FEA), putreszina (PUT), espermina (EPM) eta tiramina (TIR) (erreaktibo-maila, Sigma-Aldrich).

1. taula. Esne gordinetik eratorritako Idiazabal gaztaren laginen ABak bereizteko programa. Santamarina-García et al.-en [137] argitaratuta.

Time (min)	Flow rate (mL/min)	Solvent A (%)[1]	Solvent B (%)[2]
-	1	75	25
10	1	75	25
35	1	20	80
45	1	0	100
55	1	75	25

[1] 0,05 M-eko sodio azetato disoluzioa % 1eko tetrahidrofuranoan (> % 99,9, ROMIL Ltd.), pH 7,0

[2] Metanola

3.3.6. Korrelazio-analisia

Mikrobiotaren eta gaztaren kalitate- eta segurtasun-parametroen (konposizio gordinaren, GAAen, konposatu lurrunkorren eta ABen) dinamiken arteko erlazioa bi noranzkoko karratu partzial minimo ortogonalen (BN-KPMO) analisi baten bidez aztertu zen. Analisi hori logaritmikoki eraldatutako datuei aplikatu zitzaien, beharrezkoa izan zenean, eta unitate-bariantzaren (UB) bidez eskalatutako datuei SIMCA softwarean (Umetrics AB, Umeå, Suedia). Bakterio-genero nagusiak X aldagai gisa aukeratu ziren, eta gaztaren kalitate eta segurtasun parametroak Y aldagai gisa aukeratu ziren. Eredua R2 eta Q2 balioen, permutazio-frogen edo barne-erlazioen grafikoen bidez balioztatu zen. Funtsezko bakterio-generoak identifikatzeko, aldagaiaren eragina proiekzioan (AEP) parametroaren balioak, karga-pisuak eta Spearmanen korrelazioak erabili ziren; azken horiek IBM SPSS statistical package softwarean (26.0 bertsioa, IBM SPSS, Inc., Chicago, IL, Estatu Batuak, 2019) kalkulatu ziren, eta bero-mapa batean interpretatu ziren taldekatze hierarkikoaren analisiarekin (THA), R-ko gplots paketea erabiliz [138]. Korrelazio kanonikoaren analisia (KKA) R-n egin zen, vegan paketearekin [128], korrelazioak baieztatzeko.

III. metodologia: Mikrobiotak gaztaren segurtasunean duen eragina antimikrobianoekiko erresistentziei dagokienez **(3. xedea, 3.1.-3.3. azpihelburuak, IV.-VI. eskuizkribuak)**

3.3.7. Literatura-berrikuspena

IV. eskuizkribua esnean eta esnekietan antibiotikoen hondakinak egoteari, esnekien elaborazioan duten eraginari, antibiotikoen erabileraren eta bakterio-erresistenteak agertzearen arteko erlazioari eta beste alderdi batzuei, hala nola antibiotikoen hondakinek giza osasunean eta ingurumenean duten eraginari, buruzko bibliografiaren berrikuspena da. Literaturaren berrikuspena egiteko, Web of Science datu-basea (Thomson Reuters, Toronto, Ontario, Kanada, 2022) erabili zen, 2022ko maiatzera arte argitaratutako lan guztiak barne hartuta, aipatutako gaiekin lotutako terminoak erabiliz eta bilaketa boolearra eginez. Lortutako lanak kritikoki aztertu ziren, berrikuspenean sartzeko egokiak ote ziren ebaluatzeko. Azkenik, informazio guztia laburbildu, antolatu eta idatzi zen.

3.3.8. Gaztandegien antibiotikoen erabilerari buruzko zeharkako inkesta

Artisau-ekoizleek antibiotikoen erabilerari buruz dituzten ezagutzak, praktikak eta jarrerak zeharkako inkesta baten bidez ebaluatu ziren. Galdetegia aurretik argitaratutako lanetan oinarrituta diseinatu zen [103,104,139,140], gaztelaniaz idatzita eta ezaugarri soziodemografikoak eta antibiotikoen erabilerarekin lotutako ezagutzak eta praktikak biltzen dituzten ataletan antolatuta. Idiazabal gazta JDBak eta albaitari espezialista batek egiaztatu zituzten egokitasun etikoa, ulermena eta alderdi teknikoak. Ekoizleek ikerketaren hainbat alderdiri buruzko informazioa jaso zuten, besteak beste, helburuak eta datuen babesa, JDBren bilera batean. Inkestak modu anonimoan egin ziren, aldez aurretik hitzezko baimena emanda, eta parte-hartzaileek edozein unetan erretiratzeko aukera izan zuten.

3.3.9. Antibiotikoen hondakinen analisia

3.3.9.1. Baheketa-analisia

Antibiotikoen hondakinen baheketa egiteko, Charm Kidney Inhibition Swab (KIS) (Charm Sciences, Inc., Lawrence, Massachusetts, Estatu Batuak) eta Eclipse Farm^{3G} (Zeulab S.L., Zaragoza, Espainia) testak erabili ziren gorotzen eta esnekien laginetarako, hurrenez hurren.

3.3.9.1.1. Charm KIS testa

Aldez aurretik ehotako 1 g gorotz, pentsua erauzteko disoluzio indargetzailearen (PEI) (Charm Sciences, Inc.) 30 mL-rekin nahastu ziren eta 5 minutuz pausatzen utzi zen. Minutu batez indarrez astindu ondoren, nahasketa minutu batez pausatu zen, solidoak jalkitzeko. Charm KIS testaren isipua (Charm Sciences, Inc.) 10 segundoz gorotz-estraktuan murgildu zen, xurgatzen laguntzeko. Lagin-estraktua zuen isipua 64 ± 2 °C-tan inkubatu zen (Biometra TB1 Thermoblock, Gottingen, Alemania), antibiotikorik gabeko lagin baten kolorea horira aldatzeko behar zen denboran (≈ 3 h). Emaitzak bisualki ebaluatu ziren kolore-aldaketaren arabera, hau da, urdina edo morea (positiboa), edo horia (negatiboa). Charm KIS testaren DMak 2. taulan azaltzen dira.

2. taula. Gorotz-laginei aplikatutako Charm KIS testaren DMak. Santamarina-García et al.-en [141] argitaratuta.

Antibiotic		LOD (µg/l)
Class	Agent	
β-lactams	Benzylpenicillin	900-1200
Tetracyclines	Oxytetracycline	90000
Sulfonamides	Sulfamethazine	15000
	Sulfadimethoxine	7500
Macrolides	Tulathromycin	30000-90000
	Tylosin	12000
Aminoglycoside	Neomycin	30000
	Gentamicin	22500

3.3.9.1.2. Eclipse Farm^{3G} testa

Esne gordinaren laginen kasuan, 100 µL saiakuntza-hodi batera (Zeulab S.L.) isuri ziren, eta ordu betez barreiatzen utzi zen giro-tenperaturan. Ondoren, 2–3 aldiz garbitu zen ur desionizatua erabiliz. Gazuraren kasuan, prozedura bera aplikatu zen, baina pH-a 6,5 eta 7,0 artean mantendu zen [142]. Gazta-laginen kasuan, ENFren 471/2014 buletinean adierazten den bezala [142], 30 g gazta eta 70 mL antibiotikorik gabeko esne, 45 °C-tan aurrez berotuta, nahastu ziren. Ondoren, nahasketa homogeneizatu zen 4 minutuz Stomacher batean (Masticator Basic 400) eta 3800 × *g*-tan zentrifugatu zen 10 minutuz, estraktu urtsua gordez. pH-a 6,5-7,0ra egokitu zen sodio hidroxidoarekin (2 N, erreaktibo-maila, PanReac AppliChem, Castellar del Vallés, Katalunia, Espainia). Ondoren, disoluzioaren 100 µL esnea eta gazura bezala prozesatu ziren. Test-hodiak 65 ± 1 ºC-tan inkubatu ziren (Biometra TB1 Thermoblock) antibiotikorik gabeko lagin baten kolorea horira aldatzeko behar zen denboran (2,25-3 h). Emaitzak bisualki ebaluatu ziren kolore-aldaketaren arabera, hau da, urdin-morea (positiboa), horia (negatiboa) edo berde-urdina (DMtik gertu). Eclipse Farm^{3G} testaren DMak 3. taulan adierazten dira.

3. taula. Eclipse Farm^{3G} testaren DMak ardi-esnerako.

Santamarina-García et al.-en [141] argitaratuta.

Antibiotic		LOD (µg/kg)
Class	Agent	
β-lactams	Benzylpenicillin	3
	Amoxicillin	4
	Cloxacillin	35
	Cephalexin	100
Tetracyclines	Tetracycline	150
	Oxytetracycline	150
Sulphonamides	Sulfathiazole	75
	Sulfamethazine	100
Macrolides	Tylosin	25
Aminoglycosides	Gentamicin	250
Lincosamides	Lincomycin	150

3.3.9.2. Analisi kromatografikoa

Esnekien laginetatik antibiotikoak erauzi eta analizatu ziren Lekunberriko Esnekari Erakundean (Lekunberri, Nafarroa), Quintanilla et al.-ek [100] deskribatutakoaren arabera, aldaketa txiki batzuekin. Labur-labur, 10 g edo mL 20 g trisodio zitratorekin (% 20 m/m, ≥ % 99,0, Sigma-Aldrich) homogeneizatu ziren 3 minutuz 40 °C-tan, bi aldiz, Stomacher batean (Masticator Basic 400). Zentrifugatu ostean (9000 × *g*-tan 10 minutuz giro-tenperaturan), ur-gainazalaren 2 g-rekin fase solidoko erauzketa (FSE) egin zen, Oasis HLB kartutxo bat (60 mg, 3 mL) erabiliz, aurrez egokituta 1 mL metanol (≥ 99,9, likido kromatografia (LK)-maila, Scharlab) eta 1 mL ur ultrapuru (Milli-Q sistema baten bidez lortuta, Millipore) erabiliz. Kartutxoa 2 mL ur erabiliz garbitu zen, 2 mL metanolarekin eluitu zen eta hutsean lehortu zen. Gero, 500 µL azido formikorekin (≥ % 98,0, LK-maila, Sigma-Aldrich) ultrasoinu-bainu batean homogeneizatu zen 5 minutuz, eta polibinilideno fluorurozko iragazkien bidez iragazi zen (0,45 µm, Sigma-Aldrich).

Antibiotikoak Alliance 2695 kromatografia likidoko sistema batean analizatu ziren, lerrokatutako diodoen detektagailu batekin (LDD) eta koadrupolo hirukoitzeko tandem ME (ME/ME) batekin (LK-KkK-ME/ME) (Micromass Quattro MicroTM, Waters). XBridge Cl8 zutabea erabili zen (100 mm-ko luzera, 34,6 mm, 2,1 mm-ko B.D., 3,5 µm-ko partikula-tamaina, Waters) antibiotikoak bereizteko, bi disolbatzaile erabiliz: (A) % 0,1 (b/b) azido formikoa uretan eta (B) % 0,1 azido formikoa azetonitrilotan (≥ % 99,9, LK-maila, Scharlab); eta 4. taulan zehaztutako programari jarraituz, oxitetraziklinarentzat izan ezik. Horretarako, gradiente-programa honako hau izan zen: 0-6 min, % 85 A eta % 15 B; 6-8 min,

% 82 A eta % 18 B; 8-15 min, % 50 A eta % 50 B; eta 15-20 min, % 85 A eta % 15 B. Injekzio-bolumena 20 µL izan zen.

4. taula. Antibiotikoen hondakinak ardi-esne gordinetik, esne-gazuretik eta Idiazabal gazta freskoetatik eta helduetatik bereizteko programa. Santamarina-García et al.-en [141] argitaratuta.

Time (min)	Flow rate (mL/min)	Solvent A (%)[1]	Solvent B (%)[2]
0-8	0.2	95	5
8-14	0.2	25	75
14-15	0.2	5	95
15-20	0.2	95	5

[1] % 0,1 (b/b) azido formikoa uretan

[2] % 0,1 (b/b) azido formikoa azetonitriloan

Datuak aztertzeko, Mass-Lynx softwarea (4.0 bertsioa; Waters) erabili zen. Antibiotikoak ME/ME bidez eta elektrospray-ionizazio bidez identifikatu ziren modu ioniko positiboan (ESI+). Iturriaren tenperatura 140 °C-tan mantendu zen, orratzaren tentsioa 3,0 kV-koa izan zen, leiarraren tentsioa 0,2 V-ekoa, eta desolbatazioaren eta kono-gasaren (nitrogenoaren) fluxuak 750 L/h eta 50 L/h izan ziren, hurrenez hurren, eta desolbatazio-tenperatura 450 °C-tan mantendu zen. Ohiko berreskuratzeak % 85 eta % 100 bitartekoak izan ziren β-laktamikoentzat eta tetraziklinentzat, % 80tik % 95era makrolidoentzat eta % 90-110 bitartekoak kinolonentzat. Antibiotiko bakoitzerako kalibrazio-kurbak sortu ziren.

3.3.9.3. Elikagaien segurtasun-tartearen (EST) kalkulua

Antibiotikoen hondakinak dituzten esnekiak (esnea, gazura edo gazta) kontsumitzeak kontsumitzailearen osasunerako duen arriskua EST adierazlearen bidez kalkulatu zen, Quintanilla et al.-ek [99] proposatzen duten bezala:

$$\mathrm{EST} = \begin{cases} 0 & \text{baldin eta } \mathrm{AA_i} > 1 \\ 1 - \mathrm{AA_i} & \text{baldin eta } \mathrm{AA_i} \leq 1 \end{cases} \qquad \text{non} \qquad \mathrm{AA_i} = \frac{(\mathrm{H_i} \times \mathrm{K})/\mathrm{P}}{\mathrm{EAO_i}}$$

non AA_i, arrisku-kotizazioa den, Hi detektatutako antibiotikoaren kontzentrazioa den (µg/kg-ko), K eguneroko ahorakina (kg/pertsona/eguneko), P batez besteko gorputz-pisua (kg-tan) adinaren arabera, eta EAOi eguneroko ahorakin onargarria. Kalkulatutako eguneko ahorakina (KEAi) zenbakitzaileari dagokio.

3.3.10. Antimikrobianoekiko erresistentzien (AME) analisia

3.3.10.1. AMEn karakterizazio fenotipikoa

3.3.10.1.1. BALen isolamendua eta zenbaketa

Gorotz- eta gazta-laginetarako, 10 g diluitu ziren (1:10) bikoiztuta indargetutako peptona-uretan (IPU), eta 30 segundoz homogeneizatu ziren, hiru aldiz, Stomacher batean (Masticator Basic 400). Serie-diluzioak IPUn egin ziren, eta, ondoren, Man-Rogosa-Sharpe (MRS) (Scharlab) agarrean erein ziren. Esne gordinaren eta gazuraren kasuan, 100 µL zuzenean hartu eta diluitu ziren. Plakak 37 ± 1 °C-tan inkubatu ziren 48 orduz. Kolonien aniztasun morfologikoaren arabera, ustezko BALen hiru isolatu ausaz aukeratu ziren lagin bakoitzeko, soja-salda triptikoan (SST) aberastu ziren (Condalab, Madril, Espainia), eta MRSn hazi ziren purutasuna bermatzeko. Isolatu guztiak –80 °C-tan kontserbatu ziren glizerolean (% 20, b/b, Honeywell Fluka).

3.3.10.1.2. BALen isolatuen identifikazioa Sanger sekuentziazioaren bidez

Isolatuen DNA bakterianoaren erauzketa Mag-Bind Bacterial DNA 96 kit-a (Omega Bio-Tek, Inc., Norcross, Estatu Batuak) erabiliz egin zen, eluzio-bolumena 60 µL-ra murriztuz DNA errendimenduak hobetzeko. NanoDrop ND-1000 espektrofotometro batekin lortutako DNAren kalitatea ebaluatu eta kuantifikatu ondoren, 16S RNAe genearen V1–V3 zonaldeak PKE bidez anplifikatu ziren KAPA HiFi HotStart ReadyMix kit-arekin eta abiarazle espezifikoekin (aurreranzko abiarazlea 16S–V1–8F: 5′-AGAGTTTGSTCCTGGCTCAG-3′ eta alderantzizko abiarazlea 16S–V3–534R: 5′-ATTACCGCGGCTGCTGG-3′). PKEaren produktuak CleanNGS kit (CleanNA, Waddinxveen, Herbehereak) batekin purifikatu ziren, eta kuantifikatu ziren NanoDrop ND-1000 espektrofotometro batekin, eta LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer, Inc., Waltham, Estatu Batuak) batekin, DNA 5K Reagent kit-a erabiliz (PerkinElmer). Purifikatutako produktua BigDye Terminator v3.1 Cycle Sequencing kit-arekin sekuentziatu zen (Thermo Scientific, Waltham, Estatu Batuak) eta ondoren perla magnetikoetan oinarritutako CleanDTR kit (CleanNA) batekin purifikatu zen. Sanger sekuentziazioa SeqStudio plataforma batean (Thermo Scientific) egin zen. DNAren erauzketa eta Sanger sekuentziazioa

Euskal Herriko Unibertsitateko (UPV/EHU) SGIkerreko Sekuentziazio eta Genotipo Azterketen Unitatean-Genomika Zerbitzuan egin zen.

Lortutako irakurketa gordinen kalitate-iragaztea eta ebaketa SeqStudio Reporter softwarearekin (Thermo Scientific) egin zen, eta BioEdit Sequence Alignment Editor 7.2.5 softwarearekin [143] editatu ziren. Sailkapen taxonomikoa Nucleotide Basic Local Alignment Search Tool (NBLAST) 2.14.0+ [144] erabiliz egin zen.

3.3.10.1.3. Antimikrobianoekiko suszeptibilitate-probak (ASP), salda-mikrodiluzioaren metodoaren bidez

BALen isolatuen gutxieneko kontzentrazio inhibitzailea (GKI) salda-mikrodiluzioaren metodoaren bidez zehaztu zen, ustiategietan erabiltzen diren antibiotiko ohikoenetarako, hau da, amoxizilina, dihidroestreptomizina, G penizilina eta B polimixinarako, NNEaren eta ENFaren metodo estandarrari jarraituz [145] eta Elikagaien Segurtasunerako Europako Agintaritzaren (ESEA) jarraibideei jarraituz [146]. Laburbilduz, 96 putzuko plaka bat (Deltalab, Bartzelona, Espainia), antibiotikoen kontzentrazio seriatuak (1:2) zituen MRS saldarekin (Scharlab) bete zen. Kontzentrazioak honako hauek izan ziren: amoxizilina: 0,0313-16 µg/mL (maila analitikoa, Sigma-Aldrich); dihidroestreptomizina: 1-512 µg/mL (maila analitikoa, Glentham Life Sciences, Corsham, Erresuma Batua); G penizilina: 0,0313-32 µg/mL (Tokyo Chemical Industry Co., Tokio, Japonia); eta B polimixina: 2-1024 µg/mL (maila analitikoa, Glentham Life Sciences). Inokuluak prestatzeko NaCl-aren soluzio esterila (% 0,85, m/b) erabili zen, kolonia bakarrak hartuz MacFarland eskalan 0,5eko dentsitate optikoa lortu arte. Ondoren, soluzioa diluitu egin zen (1:10) antibiotiko gabeko MRS saldan, eta 50 µL gehitu ziren putzu bakoitzean eta inkubatu egin ziren (37 ± 1 °C-tan 48 orduz). Kontrol positiboa antibiotikorik gabeko putzu bateko inokulu bat zen, eta kontrol negatiboa, berriz, antibiotikoak zituen inokulurik gabeko putzu bat. Sentikortasuna edo erresistentzia Animalien Elikaduran erabiltzen diren Gehigarriei eta Produktuei edo Substantziei buruzko ESEA Panelak (AEGPS) adierazitako balio mikrobiologikoei jarraituz interpretatu zen [146] eta Antimikrobianoekiko Suszeptibilitate-Proben Europako Batzordearen (ASPEB; http://www.eucast.org) balio epidemiologikoak erabiliz.

3.3.10.2. AMEn karakterizazio genotipikoa

3.3.10.2.1. DNAren erauzketa

Antimikrobianoekiko erresistentzia geneak (AEG) aztertzeko, DNA aurretik deskribatu den bezala erauzi zen (3.3.1.1 atala), aldaketa batzuekin. Gorotz- eta gazta-laginei dagokienez, 10 g 90 mL sodio zitrato esterilarekin (% 2, m/b) nahastu ziren, eta Stomacher batean (Masticator Basic 400) homogeneizatu ziren. Ondoriozko esekidura 6500 × *g*-tan zentrifugatu zen 8 minutuz 4 °C-tan, ur-gainazaleko gantza baztertu zen eta jalkina 50 mL sodio zitratorekin berreseki zen eta berriro zentrifugatu zen (6500 × *g*-tan 8 minutuz 4 °C-tan). Jalkina 800 µL sodio zitratorekin berreseki zen, eta hiru aldiz zentrifugatu zen (6500 × *g* 8 minutuz 4 °C-tan). Ondoren, DNA erauzi zen QIAamp® PowerFecal® Pro DNA kit-arekin (Qiagen) eta DNA erauzketaren errendimenduak hobetzeko, C6 disoluzioaren 25 µL-ko eluzio-urrats bikoitz bat gauzatu zen. Esne eta gazur-laginen kasuan, 10 mL deskribatu den bezala prozesatu ziren, baina homogeneizatu gabe.

3.3.10.2.2. Errendimendu handiko PKE kuantitatiboa (EH-PKEk)

AEGen analisia Euskal Herriko Unibertsitateko (UPV/EHU) SGIkerreko Gene-Adierazpen Unitatean-Genomika Zerbitzuan egin zen, EH-PKEkaren bidez, nanofluidic qPCR BioMarkTM HD sistema batean 96.96 matrize dinamikoko zirkuitu fluidiko integratuak (ZFI) (Fluidigm Corporation, San Frantzisko hegoaldea, Kalifornia, Estatu Batuak) erabiliz, aurretik deskribatu den bezala [147]. Zenbait abiarazle-sorta (48) erabili ziren ustiategietan erabiltzen diren antibiotiko ohikoenekiko erresistentzia ematen duten AEGak detektatzeko, hau da, dihidroestreptomizinarekiko erresistentzia ematen duten AEGak (12 gene), G penizilinarekiko eta amoxizilinarekiko erresistentzia ematen duten AEGak (24), B polimixinarekiko erresistentzia ematen duten AEGak (2) eta antibiotiko ugariekiko erresistentzia (AUE) ematen duten AEGak (2); bai eta transposoiak eta integroiak kodetzen dituzten elementu genetiko mugikorrak (EGM) (5 eta 2, hurrenez hurren) eta 16S RNAe erreferentzia-genea detektatzeko ere (1) (5. taula). Geneak BALetarako hautatu ziren CARD datu-basea kontuan hartuta [148], eta abiarazleak aldez aurretik balioztatu ziren PKEk egiteko [149,150].

5. **taula**. Aztertutako AEGak eta EGMak, horien sailkapena eta erresistentzia-mekanismoa, eta erabilitako abiarazleak**.** Santamarina-García et al.-en [151] argitaratuta.

Target gene	Forward Primer	Reverse Primer	Gene Classification	Resistance Mechanism
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA	-
*int*I	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	MGEs/Integrase	integrase
*int*I1	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	MGEs/Integrase	integrase
tnpA-03	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT	IS6 Group	transposase
*IS*613	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	IS613	transposase
*tnp*A-01	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT	IS21 Group	transposase
*tnp*A-02	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	IS4 Group	transposase
*tnp*A-07	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG	ISEcp1B	transposase
*Tp*614	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	Tp614	transposase
*aac*A/*aph*D	AGAGCCTTGGGAAGATGAAGTTT	TTGATCCATACCATAGACTATCTCATCA	Aminoglycoside	deactivate
*aad*A-01	GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA	Aminoglycoside	deactivate
*aad*A2-02	CTTGTCGTGCATGACGACATC	TCGAAGATACCCGCAAGAATG	Aminoglycoside	deactivate
*aad*A5-01	ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG	Aminoglycoside	deactivate
*aad*A9-01	CGCGGCAAGCCTATCTTG	CAAATCAGCGACCGCAGACT	Aminoglycoside	deactivate
*aad*D	CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA	Aminoglycoside	deactivate
*aad*E	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	Aminoglycoside	deactivate
aph	TTTCAGCAAGTGGATCATGTTAAAAT	CCAAGCTGTTTCCACTGTTTTTC	Aminoglycoside	deactivate
*aph*6ia	CCCATCCCATGTGTAAGGAAA	GCCACCGCTTCTGCTGTAC	Aminoglycoside	deactivate
str	AATGAGTTTTGGAGTGTCTCAACGTA	AATCAAAAACCCCTATTAAAGCCAAT	Aminoglycoside	deactivate
*str*A	CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG	Aminoglycoside	deactivate
*str*B	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside	deactivate
*amp*C-01	TGGCGTATCGGGTCAATGT	CTCCACGGGCCAGTTGAG	β-Lactamase	deactivate
*bla*1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	β-Lactamase	deactivate
bla-ACC-1	CACACAGCTGATGGCTTATCTAAAA	AATAAACGCGATGGGTTCCA	β-Lactamase	deactivate
*bla*CMY2-01	AAAGCCTCAT GGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA	β-Lactamase	deactivate
*bla*CTX-M-03	CGATACCACCACGCCGTTA	GCATTGCCCAACGTCAGATT	β-Lactamase	deactivate
*bla*GES	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG	β-Lactamase	deactivate
*bla*IMP-01	AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	β-Lactamase	deactivate
*bla*MOX/*bla*CMY	CTATGTCAATGTGCCGAAGCA	GGCTTGTCCTCTTTCGAATAGC	β-Lactamase	deactivate
*bla*OCH	GGCGACTTGCGCCGTAT	TTTTCTGCTCGGCCATGAG	β-Lactamase	deactivate
*bla*OKP	GCCGCCATCACCATGAG	GGTGACGTTGTCACCGATCTG	β-Lactamase	deactivate
*bla*OXA1/*bla*OXA30	CGGATGGTTTGAAGGGTTTATTAT	TCTTGGCTTTTATGCTTGATGTTAA	β-Lactamase	deactivate
*bla*OXY	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT	β-Lactamase	deactivate
*bla*ROB	GCAAAGGCATGACGATTGC	CGCGCTGTTGTCGCTAAA	β-Lactamase	deactivate
*bla*SHV-01	TCCCATGATGAGCACCTTTAAA	TTCGTCACCGGCATCCA	β-Lactamase	deactivate
*bla*TEM	AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	β-Lactamase	deactivate
*bla*VIM	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	β-Lactamase	deactivate
*bla*Z	GGAGATAAAGTAACAAATCCAGTTAGATATGA	TGCTTAATTTTCCATTTGCGATAAG	β-Lactamase	deactivate
*mec*A	GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA	β-Lactamase	protection
*NDM*1	ATTAGCCGCTGCATTGAT	CATGTCGAGATAGGAAGTG	β-Lactamase	deactivate
pbp	CCGGTGCCATTGGTTTAGA	AAAATAGCCGCCCCAAGATT	β-Lactamase	protection
*pbp*2x	TTTCATAAGTATCTGGACATGGAAGAA	CCAAAGGAAACTTGCTTGAGATTAG	β-Lactamase	protection
*pbp*5	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT	β-Lactamase	protection
*pen*A	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG	β-Lactamase	protection
mcr-1	GTCGGTATGCTCGTTGGCTTAG	CATAGGCATTGCTGTGCGTCTG	Tranferase	target modification
mcr-2	CCGCTACACCGATTTCGTGATTG	TCACCATGATCGGAG-ACGTAGAG	Tranferase	target modification
*mex*D	TTGCCACTGGCTTTCATGAG	CACTGCGGAGAACTGTCTGTAGA	Multidrug	efflux
*tol*C-01	GGCCGAGAACCTGATGCA	AGACTTACGCAATTCCGGGTTA	Multidrug	efflux

DNAren aurreanplifikazioa egiteko, QIAGEN ® Multiplex PCR kit-a (Qiagen) erabili zen, abiarazleen nahasketa batekin (abiarazle-pare bakoitzerako kontzentrazioa = 50 nM) eta anplifikazio-programari jarraituz (95 °C-tan 15 minutuz, eta 14 PKE ziklo 95 °C-tan 15 segundoz, 60 °C-tan 4 minutuz eta amaierako luzapen-urratsa 4 ºC-tan). Ondoren, laginak I exonukleasarekin (Thermo Scientific) tratatu ziren (37 ºC-tan 30 minutuz digestioa egiteko, 80 ºC-tan 15 minutuz I exonukleasa inaktibatzeko, eta amaitzeko 4 ºC-tan mantendu zen), eta itu espezifikoak anplifikatzeko erreakzioak ZFItan kargatu ziren 1:10-ko diluzioan, Fluidigm-en Fast Gene Expression Analysis-EvaGreen® Protocol (Fluidigm Corporation) jarraituz. Anplifikaziorako, Master Mix SsoFastTM EvaGreen® Supermix kit-a erabili zen Low ROXekin (Bio-Rad Laborategiak, Hercules, Estatu Batuak), eta abiarazleen amierako kontzentrazioa 500 nM-tan mantendu zen (aurreranzko zein alderantzizko abiarazleak). Anplifikazio-programak 95 ºC-ko desnaturalizazio urratsa izan zuen minutu batean zehar, ondoren 30 ziklo gauzatu ziren,

95 ºC-tan 5 segundoz eta 60 ºC-tan 20 segundoz. Horren ondorioz, fusio-kurba bat eman zen, 3 segundoz 60 °C-tan, eta arrapala-abiadura 1 °C/3 s-koa izan zen 95 °C arte. Lagin bakoitza lau aldiz erreplikatu zen.

3.3.10.2.3. Analisi bioinformatikoa

Datu gordinak Fluidigm Real-Time PCR Analysis Software-a (3.1.3 bertsioa, Fluidigm Corporation) erabiliz aztertu ziren, erreferentzia-puntu linealaren zuzenketarekin eta eskuzko atalase-doikuntzarekin. Zikloaren atalasearen (ZA) balioa 30ean ezarri zen, lortutako ZAren baliorik altuena kontuan hartuta (29). AEG edo EGM baten detekzio positiborako, lagin bakoitzeko 4 erreplika teknikoetatik gutxienez 3k DM gainditu behar zuten. AEGen ugaritasun erlatiboa ZAren metodo konparatiboaren bidez zehaztu zen [147], 16S RNAe genearen ugaritasunarekin normalizatua, eta aldaketaren zenbatekoa (AZ) bezala adierazia.

$$\Delta\text{ZA (Erreplika bakoitzeko)} = \text{ZA(Itu genea)} - \text{ZA(}\mathbf{16}\text{S RNAe genea)}$$

$$\Delta\Delta\text{ZA (Lagin bakoitzeko)} = \overline{\Delta\text{AZ}}$$

$$\text{AZ} = \mathbf{2}^{-\Delta\Delta\text{ZA}}$$

IV. metodologia: Idiazabal gaztaren mikrobiotari eragiten dioten ekoizpen-faktore nagusiak zehaztea **(4. xedea, 4.1. azpihelburua, VII. eskuizkribua)**

3.3.11. Shotgun sekuentziazioa

3.3.11.1. DNAren erauzketa

DNA genomikoa lagin freskoetatik erauzi zen aurretik deskribatutako metodoa jarraituz (3.3.1.1 atala), aldaketa batzuekin. Gazta- eta esne-laginak aurretik azaldu den bezala prozesatu ziren (3.3.10.2.1 atala), eta gazurak esne-laginak bezala prozesatu ziren. Gainerako ingurumen-laginen kasuan, metodoa egokitu zen hainbat lanetan oinarrituta [152–155]. Animalien elikagaietarako, 200 g pentsu komertzial eta 90 mL IPU nahastu ziren, 6 minutuz sonikatu ziren eta 100 x *g*-tan zentrifugatu ziren minutu batez 4 °C-tan. Gero, esekidura 15,000 x *g*-tan zentrifugatu zen 8 minutuz 4 ºC-tan, eta ur-gainazala baztertu ondoren, jalkina 50 mL IPUrekin berreseki zen eta berriz zentrifugatu egin zen (15,000 x *g*-tan 8 minutuz 4 ºC-tan). Ondoren, jalkina 800 µL IPUrekin berreseki zen eta hiru aldiz zentrigutau zen (15,000 x *g*-tan 8 minutuz 4 ºC-tan).

Prozedura bera aplikatu zitzaien etxeko pentsuari, belarrari eta lastoari, 200 g, 100 g eta 50 g izanda hasierako pisua, hurrenez hurren. Titiburuaren gainazalaren, elikagaiak ukitzen dituzten gainazalen eta elikagaiak ukitzen ez dituzten gainazalen kasuan, lagin mota bakoitzerako zazpi-zortzi gazako taldeak 90 mL IPUtan murgildu ziren, eta gogor astindu ziren minutu batez; prozesu hori hiru aldiz errepikatu zen mikrobio-komunitateak askatzeko. Esekidura 100 × *g*-tan zentrifugatu zen minutu batez 4 ºC-tan, ezpurutasun solidoak kentzeko, eta animalien elikagaien laginetarako erabilitako prozedurari jarraitu zitzaion. Gatzagi-laginen kasuan, 10 g gatzagi 90 mL IPUrekin nahastu zen, sei aldiz homogeneizatu zen (20 s piztuta eta 10 s itzalita) Stomacher batean (Masticator Basic 400), eta 100 x *g*-tan minutu batez 4 ºC-tan zentrifugatu zen. Ondoren, laginak animalien elikagaiak bezala prozesatu ziren. Gatzunen kasuan, 300 mL IPUrekin nahastu ziren eta gatzagi-laginak bezala prozesatu ziren, baina homogeneizatu gabe. Lagin guztietarako, DNA erauzketa QIAamp® PowerFecal® Pro DNA Kit-a erabiliz egin zen, fabrikatzailearen jarraibideei jarraituz eta DNA erauzketaren errendimenduak hobetzeko, C6 disoluzioaren 25 µL-ko eluzio-urrats bikoitz bat gauzatu zen. DNA –80 °C-tan mantendu zen sekuentziatu arte.

Erauzitako DNAren kantitatea eta kalitatea TryCell 2.0 (Hellma, Erresuma Batua) sistemaren bidez ebaluatu ziren. Sistema hori Cary 50 UV−Vis espektrofotometro batera konektatuta zegoen, Varian UV RNA−DNA estimation software-arekin (3.00 bertsioa (399), Agilent Technologies, Inc.,). DNAren osotasuna eta purutasuna egiaztatzeko, % 1eko agarosa gelean egindako elektroforesia gauzatu zen, GelRed koloratzailea (Biotium, Inc., Fremont, Kalifornia, Estatu Batuak), 10X BlueJuice gel-kargako indargetzailea (Roguen, Waltham Mbggeneted Markünggenp, Erresuma Batua), FastGene 100 bp DNA Marker DNA markatzailea (NIPPON Genetics EUROPE GmbH, Düren, Alemania), eta U:Genius 3 sistema (Synoptics, Cambridge, Erresuma Batua) erabiliz.

3.3.11.2. Liburutegien prestaketa eta sekuentziazioa

Liburutegiak prestatu aurretik, DNAren kuantifikazio zehatza egin zen Qubit® fluorimetro bat erabiliz Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) kit and broad-range (BR) assay kits-ekin (Bio-Sciences, Dublin, Irlanda). Illuminako DNA prep kit-a (Illumina, Inc.) erabili zen 150 bp-ko mutur-parekatuko sekuentziazio-liburutegiak eraikitzeko. Multiplexaziorako indize bikoitz bakarrak (IBB) (Integrated DNA Technologies, Coralville, Iowa, Estatu Batuak) gehitu ziren. Indexatu eta garbitu ostean, kuantifikatzeko eta kalitatea

egiaztatzeko, Qubit® fluorimetro bat eta Agilent 2100 BioAnalyzer sistema bat HS DNA kit batekin (Agilent Technologies, Inc.) erabili ziren, hurrenez hurren. Liburutegiak modu ekimolarrean batu ziren, eta garbiketa-etapa bat egin zen AMPure XP beads perlak (0,8X perla:produktua, Beckman Coulter, Inc., Pasadena, Kalifornia, Estatu Batuak) erabiliz. Sekuentziazioa Illumina NextSeq 2000 plataforma batean egin zen, P1 300 zikloko kartutxo batekin (Illumina, Inc.), eta Illuminaren sekuentziazio estandarreko protokoloak jarraituz Teagasc-eko DNA sekuentziazio-instalazioetan (Moorepark, Cork, Irlanda).

3.3.11.3. Analisi bioinformatikoak

Analisi bioinformatikoak Teagasc-eko goi errendimenduko konputazio klusterrean egin ziren. Mutur-parekatuko FASTQ fitxategi gordinak Cutadapt (1.18 bertsioa) [156] erabiliz ebaki ziren egokitzaileen sekuentziak ezabatzeko, eta kalitatea FastQC (0.11.8 bertsioa) [157] bidez ebaluatu zen, kalitate txikiko irakurketak baztertuz. Ardiaren (*Ovis aries*) genomarekin lerrokatzeko, Bowtie2 (2.4.4 bertsioa) erabili zen [158], mapatu gabeko ostalariarenak ez ziren irakurketa guztiak mikrobiar gisa hartuz. Mapatu gabeko irakurketak erauzteko, samtools (1.9 bertsioa) erabili zen [159], eta horren ondoriozko irakurketak mutur-parekatuko FASTQ artxiboetan banatu ziren, bedtools-eko bamtofastq (2.27.1 bertsioa) erabiliz [160]. Sailkapen taxonomikoa Kraken2 erabiliz egin zen [161], genome taxonomy database (GTDB) (89. argitalpena) erabiliz [162] eta bakterioen irakurketan arreta jarriz.

3.3.11.4. Dibertsitatearen azterketa

α- eta β-dibertsitatea Rstudio-n (2023.03.1 bertsioa) eta R-n (4.3.0 bertsioa) (R Core Team, Viena, Austria, 2023) kalkulatu ziren, 3.3.1.4 atalean aipatu den bezala.

3.3.11.5. SourceTracker analisia

Esne gordinaren, gazuraren eta gaztaren populazioen bakterio-jatorriak aztertzeko, SourceTracker2 (2.0.1 bertsioa) erabili zen [163]. Esne gordinaren mikrobiotan eragina izan zezaketen bakterio-iturriak honako hauek ziren: animalien elikagaiak, titiburuaren gainazala, elikagaiak ukitzen dituzten gainazalak eta elikagaiak ukitzen ez dituzten gainazalak. Horiez gain, gazurarentzat ere gatzagia zegoen, eta gaztarentzat gatzuna gehitu zen. Lehenetsitako konfigurazioa erabili zen.

3.3.11.6. Potentzial funtzionalaren azterketa

Potentzial funtzionala SUPERFOCUS (0.34 bertsioa) erabiliz ebaluatu zen [164], DIAMOND (2.1.7 bertsioa) sekuentzia-lerrokagailua [165] eta SEED datu-basea erabiliz (https://github.com/topics/seed-database). Horrela, gene-familia homologoei irakurketak esleitzen dizkie potentzial funtzionala zehazteko, gene-familia horiek antolaketa-maila altuagoetan gehituz, funtzio zabalagoa betetzeko.

3.3.11.7. AEGen eta birulentzia-faktoreen (BF) analisia

AEGak eta BFak ShortBRED (1.0 bertsioa) erabiliz identifikatu eta kuantifikatu ziren [166], Comprehensive Antibiotic Resistance Database (CARD) [148] eta virulence factor database (VFDB) [167] datu-baseetan oinarrituta, hurrenez hurren. AEGen eta BFen ugaritasun erlatiboa irakurketa normalizatu gisa adierazi zen kilobaseko milioi bat irakurketako (IKMB) [166].

3.3.11.8. Hidrolasak kodetzen dituzten geneen analisia

Hidrolasak kodetzen dituzten geneak identifikatzeko eta kuantifikatzeko DIAMOND [165] erabili zen, ESTerases and alpha/beta-Hydrolase Enzymes and Relatives (ESTHER) datu-basearekin [168]. Emaitza onenak hautatu ziren ondorengo analisirako, eta 10^5eko e-balioaren atalasea ezarri zen mapatutako kontigsentzat.

3.3.11.9. Correlation analysis

Mikrobio-komunitateen eta potentzial funtzionalaren, AEGen, BFen eta hidrolasak kodetzen dituzten geneen arteko korrelazioak 3.3.6 atalean deskribatu den bezala ebaluatu ziren, baina karratu partzial minimo ortogonalen (KPMO) analisia erabili zen.

3.3.11.10. Metagenoman mihiztatutako genomen (MMG) azterketa

MMGak metaSPAdes (3.13 bertsioa) erabiliz eraiki ziren [169], eta genomen binninga MetaBAT2 (2.12.1 bertsioa) erabiliz gauzatu zen [170], lehenetsitako konfigurazioarekin. MMGen kalitatea CheckM (1.0.18 bertsioa) erabiliz ebaluatu zen [171]. Kalitate txikiko MMGak (< % 50ko osotasuna edo/eta > % 5ko kutsadura) analisitik kanpo utzi ziren, kalitate ertaineko MMGak (% 50-90ko osotasuna, < % 5ko kutsadura) eta kalitate handiko MMGak (> % 90ko osotasuna,

< % 5ko kutsadura) mantendu ziren. MMGen sailkapen taxonomikoa GTDB datu-basearen bidez egin zen GTDB-tk 2.1.1 bertsioarekin [172].

3.4 Analisi estatistikoa

Analisi estatistikoak hiru urrats izan zituen: **(1) datuen prestaketa eta analisia, (2) aldagai bakarreko analisia eta (3) bi aldagaiko eta aldagai anitzeko analisiak**. Orokorrean, datuak estatistika deskriptiboa erabiliz laburbildu ziren (esaterako, batezbestekoa ± desbideratze estandarra edo tartea), eta hiru zifra esanguratsu erabili ziren emaitzak adierazteko. Datuen analisiak normaltasuna, homoskedastikotasuna eta independentzia egiaztatzea suposatu zuen. Datuen prestaketa eta azterketa IBM SPSS pakete estatistikoaren 26.0 bertsioan egin ziren. Oro har, **aldagai bakarreko** eta **bi aldagaiko analisiak** SPSS paketean egin ziren, eta **aldagai anitzeko analisiak** RStudio 1.3.959 bertsioan, R 3.6.3 bertsioan eta SIMCA softwarearen 15.0.0.4783 bertsioan gauzatu ziren. Oro har, esangura estatistikoa $P \leq 0{,}05$ean adierazi zen. A**ldagai bakarreko, bi aldagaiko eta aldagai anitzeko analisien** xehetasunak labur deskribatzen dira jarraian (**I-IV estatistikak**, 1-4 xede bakoitzaren arabera):

I. analisi estatistikoa: Idiazabal gaztaren mikrobiotaren karakterizazioa eta garapena (**1. xedea, 1.1. azpihelburua, I. eskuizkribua**)

Aldagai bakarreko analisiak, SPSS paketean kalkulatuta, Mann-Whitney *U* proba eta Kruskal-Wallis bariantza-analisia (BA) Bonferroni zuzenketarekin barne hartu zituen, esnearen eta gazta laginen arteko irakurketen eta unitate taxonomiko operatiboen (UTO) arteko desberdintasunak kalkulatzeko eta ekoizlearen, gazta egiteko prosezuaren eta heltze-prosezuaren eragina aztertzeko bakterioen ugaritasunean.

Bi aldagaiko analisiaren arabera, bakterio-genero nagusien arteko korrelazioak SPSS paketean kalkulatu ziren Spearmanen korrelazio-koefizienteen bidez eta R-n bero-mapa gisa irudikatu ziren gplots paketea erabiliz [138]. **Aldagai anitzeko analisiak** aldagai anitzeko bariantza-analisi permutazionala (AABAP) barne hartu zuen, R-n exekutatuta vegan paketearekin [128], ekoizlearen eta heltze denbora faktorearen eragin orokorra aztertzeko bakterio genero nagusien ugaritasunean. Osagai nagusien analisia (ONA) logaritmikoki eraldatutako datuetan, beharrezkoa zenean, eta UB bidez eskalatutako datuetan oinarrituta

gauzatu zen SIMCA softwarean, gaztetako bakterio-generoen dinamikak aztertzeko, aipatutako faktoreen arabera. Osagai nagusien (ON) kopurua balio propioen eta baliozkotze gurutzatuaren bidez zehaztu zen. Karratu partzial minimo ortogonalen-analisi diskriminatzailea (KPMO-AD) SIMCA softwarean egin zen, ekoizlearen arabera, laginen artean bakterio-genero nagusien arteko desberdintasunak baieztatzeko, eta ereduan aldagai bakoitzaren garrantzia AEP balioen eta karga-pisuen arabera estimatu zen.

II. analisi estatistikoa: Mikrobiotak gaztaren kalitatean eta segurtasunean duen eragina (**2. xedea, 2.1.-2.2. azpihelburuak, II.-III. eskuizkribuak**)

Aldagai bakarreko analisiak Kruskal-Wallis BA barne hartu zuen, heltze denborak eta ekoizleak gaztaren kalitate- eta segurtasun-parametro bakoitzean (konposizio gordinean, GAAetan, konposatu lurrunkorretan eta ABetan) duten eragina zehazteko.

Aldagai anitzeko analisiak AABAP barne hartu zuen heltze denborak eta ekoizleak gaztaren kalitate- eta segurtasun-parametroetan duten eragin orokorra neurtzeko, konposizio lurrunkorrean izan ezik. THA, bero-mapa gisa irudikatuta, taldekatzeak heltze denboran zehar aztertzeko egin zen. Heltzean zehar emandako joerak sakon aztertu ziren ONAren bidez, eta ekoizleen arteko bereizketa KPMO-ADren bidez aztertu zen.

III. analisi estatistikoa: Mikrobiotak gaztaren segurtasunean duen eragina antimikrobianoekiko erresistentziei dagokienez **(3. xedea, 3.1.-3.3. azpihelburuak, IV.-VI. eskuizkribuak)**

Aldagai bakarreko analisiak Kruskal-Wallis BA barne hartu zuen ekoizpen-katearen eta ekoizlearen eragina zehazteko baheketa-testen emaitzetan, antibiotikoen kontzentrazioetan, bakterioen zenbaketetan eta ugaritasunetan, eta emaitza fenotipikoetan eta genotipikoetan.

Bi aldagaiko analisiak Pearsonen chi-karratuaren proba barne hartzen zuen inkestaren aldagaien arteko loturak aztertzeko (gaztandegien eta ekoizleen ezaugarri soziodemografikoak eta antibiotikoen administrazioarekin lotutako ezagutzak eta praktikak), elkartze-maila Crameren V testaren bidez neurtuz SPSS paketean. **Aldagai anitzeko analisiak** AABAP barne hartu zuen ekoizpen-katearen eta ekoizlearen eragin orokorra ikertzeko baheketa-testen

emaitzetan, antibiotikoen kontzentrazioetan, bakterioen zenbaketetan eta ugaritasunetan, eta emaitza fenotipikoetan eta genotipikoetan. THA, bero-mapa gisa aurkeztua, taldekatzeak aztertzeko erabili zen. Emaitza fenotipikoen eta genotipikoen araberako laginen multzokatzea R-ko dendrograma baten bidez aztertu zen factoextra paketearekin [173]. Ekoizpen-kateak eta ekoizleak antibiotikoen kontzentrazioen, bakterioen zenbaketen eta ugaritasunen, eta emaitza fenotipikoen eta genotipikoen joeretan duten eragina ONAren bidez sakon aztertu zen, eta balizko desberdintasunak KPMO-AD bidez aztertu ziren.

IV. analisi estatistikoa: Idiazabal gaztaren mikrobiotari eragiten dioten ekoizpen-faktore nagusiak zehaztea **(4. xedea, 4.1. azpihelburua, VII. eskuizkribua)**

Aldagai bakarreko analisia, Mann-Whitney *U* proba eta Kruskal-Wallis BA barne, irakurketetan, bakterio-komunitateen ugaritasun erlatiboetan eta potentzial genetikoan (bide metabolikoekin lotutako geneetan, AEGetan, BFetan eta hidrolasak kodetzen dituzten geneetan) desberdintasunak aztertzeko egin zen, ekoizlearen eta lagin motaren arabera. Laginen arteko bakterio-genero komunen kopurua zehazteko, Venn diagramak sortu ziren R-n, ggvenn paketea erabiliz [174].

Aldagai anitzeko analisiak AABAP barne hartu zuen ekoizle eta lagin mota faktoreek irakurketetan eta bakterio-komunitateen ugaritasun erlatiboetan edo potentzial genetikoan duten eragin orokorra aztertzeko. ONA exekutatu zen mikrobio-komunitateen joerak aztertzeko ekoizlearen eta lagin motaren arabera, eta ondoren KPMO-ADren bidez baieztatu zen.

4. EMAITZAK ETA EZTABAIDA

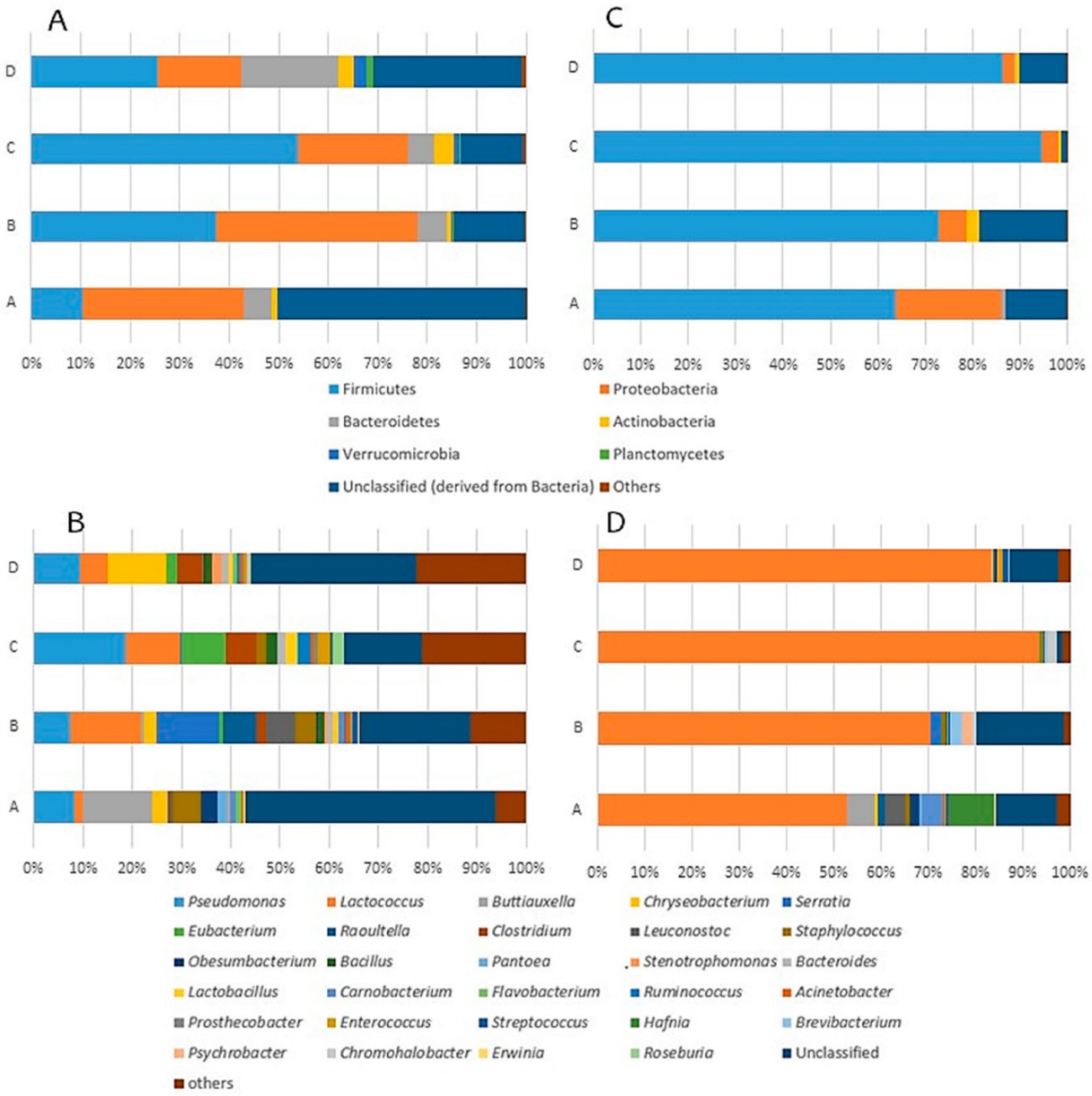
Atal honetan, **I-VII eskuizkribuetan** zehaztutako emaitza nagusiak azaltzen eta eztabaidatzen dira. Helburua da eskuizkribuetan deskribatutako alderdien errepikapena saihestea eta ikerketaren emaitzen ikuspegi bateratua eskaintzea. Atal hau lau sekziotan egituratu da, proiektuaren helburuen arabera: (4.1.) Idiazabal gaztaren mikrobiotaren karakterizazioa eta nola garatzen den (**1. helburua, 1.1. azpihelburua, I. eskuizkribua**), (4.2.) Idiazabal gaztaren mikrobiotaren eta kalitate- eta kaltegabetasun-parametro nagusien arteko erlazioa (**2. helburua, 2.1.-2.2. azpihelburuak, II.-III. eskuizkribuak**), (4.3.) mikrobiotaren eta antimikrobianoekiko erresistentzien (AME) arteko erlazioa (**3. helburua, 3.1.-3.3. azpihelburuak, IV.-VI. eskuizkribuak**), eta (4.4.) mikroorganismo-iturriak artisau-gaztandegietan eta bere eragina Idiazabal gaztaren mikrobiotan (**4. helburua, 4.1. azpihelburua, VII. eskuizkribua**).

4.1. Idiazabal gaztaren mikrobiotaren karakterizazioa eta garapena (1. helburua, 1.1. azpihelburua, I. eskuizkribua)

Anplikoien sekuentziazioaren bitartez, Latxa ardien esne gordinetik, Idiazabal gazta freskoetatik (heltze egun bateko gaztak) eta heldutako gaztetatik (7, 14, 30, 60 eta 120 egunetakoak) 10.798.992 16S RNAe gene-sekuentzia jaso ziren.

4.1.1. Latxa ardi-esne gordinaren mikrobiota

Guztira 21 bakterio-filum, 165 familia eta 455 genero identifikatu ziren esne gordinaren laginetan. Zehazki, 6 bakterio-filumek % 1etik gorako ugaritasuna zuten (4A. irudia). Genero mailan, 24k % 1etik gorako ugaritasuna zuten (4B. irudia) eta 10 genero nabarmendu ziren (> % 5), hain zuzen ere, *Pseudomonas* (% 7,36-18,5), *Lactococcus* (% 1,64-14,5), *Buttiauxella* (% 0-14,1), *Serratia* (% 0,0245–12,6), *Chryseobacterium* (% 0–11,7), *Eubacterium* (% 0,0766–9,18), *Raoultella* (% 0–6,86), *Clostridium* (% 0,183–6,09), *Leuconostoc* (% 0–6,10) eta *Staphylococcus* (% 0,291–5,48) (4B. irudia). Oro har, lagin guztien artean, *Pseudomonas* eta *Lactococcus* genero komun nagusiak ziren, eta gainerakoak ekoizle bakoitzaren araberakoak ziren.

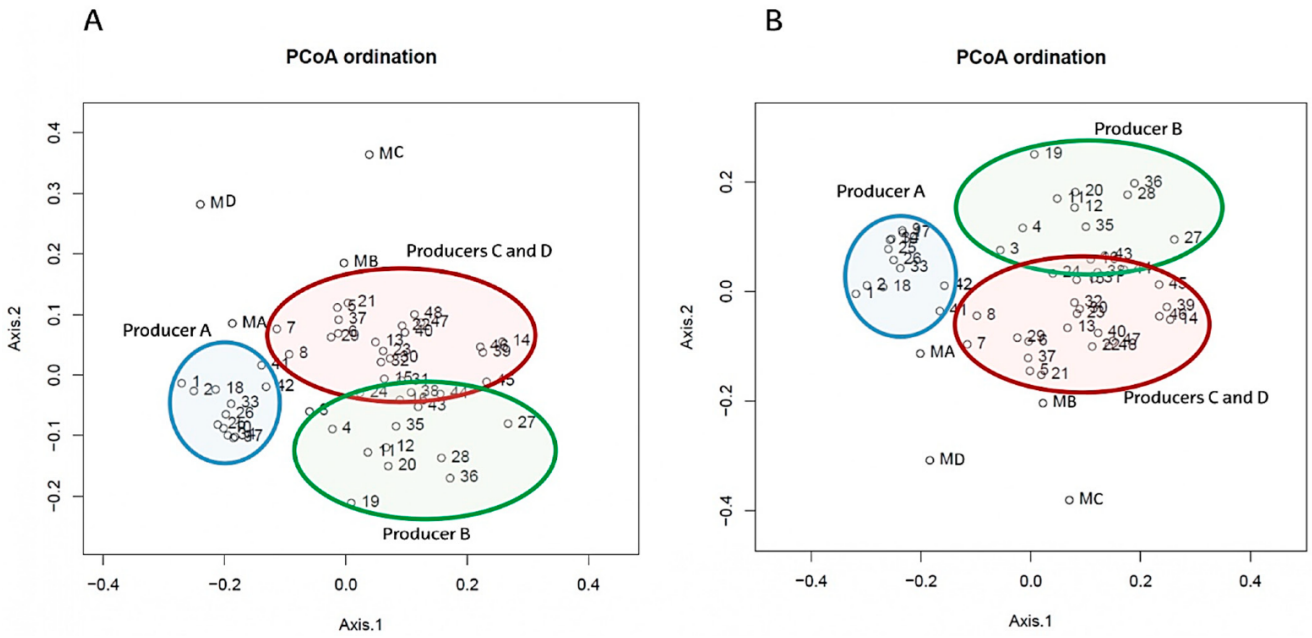


4. irudia. Bakterio-filumen eta generoen ugaritasun erlatiboa (%) Latxa ardiaren esne gordinaren (A, B) eta Idiazabal gazta freskoaren (C, D) laginetan, ekoizleen arabera (A, B, C, D). Santamarina-García et al.-en [28] argitaratuta.

Identifikatutako bakterio-generoen artean, hainbat bakterio azido-laktiko (BAL), ingurumen-bakterio eta ez-desiragarri zeuden. BALen artean, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Lactobacillus*, *Carnobacterium* eta *Streptococcus* zeuden, aurretik beste ardi-arraza batzuen esne gordinean identifikatu direnak, nahiz eta ugaritasun desberdinetan [74–76]. Ingurumen-bakterioen artean *Obesumbacterium*, *Roseburia* eta *Prosthecobacter* zeuden. Horiek hainbat iturri naturaletan (lurzoruan, ur gezan eta gazian, edo animalien eta gizakien hestean, besteak beste) topatu izan dira [39–41]. Hala ere, inongo ikerketak ez du oraindaino jakinarazi inongo arrazatako ardi esne gordinean topatu direnik. Bakterio ez-desiragarrien artean espezie patogeno oso ezagunak dituzten generoak zeuden, hala nola *Pseudomonas*, *Clostridium*,

Staphylococcus eta *Bacillus*. Gainera, beste genero batzuetako espezie batzuk patogeno emergente edo oportunista gisa ere deskribatu dira, bereziki *Buttiauxella*, *Serratia*, *Chryseobacterium*, *Eubacterium*, *Raoultella*, *Ruminococcus*, *Pantoea*, *Stenotrophomonas*, *Bacteroides*, *Flavobacterium* eta *Acinetobacter* [175,176,185,177–184]. Aipatzekoa da orain arte ez direla *Buttiauxella*, *Serratia*, *Eubacterium*, *Raoultella*, *Ruminococcus* eta *Bacteroides* identifikatu ardi esne gordinean. Era berean, gaztaren hondatzearekin lotutako generoak zeuden, *Serratia* eta *Clostridium* barne. Hauen espezieak zapore desatseginekin [186] eta CO_2-a ekoiztearen ondoriozko gaztaren puzte-akatsarekin erlazionatu dira [187].

Ekoizleen artean mikrobiotan hauteman ziren desberdintasunak, β-indizeek berretsi zituzten (5. irudia). Ezberdinatsun hauek zenbait faktore potentzialen ondorio zirela iradoki zen, besteak beste, artaldearen maneiua, mikroorganismo-iturriak (ugatz-guruineko gaixotasunak edo titiburuaren azala kutsatzen duten mikroorganismoak adibidez), edo ardiak jeztean erabilitako praktikak eta materialak [18,21,38,73]. Faktore horiek izan daitezke, halaber, Latxa ardiaren esne gordina bereizteko arrazoia, beste ardi-arraza batzuekin alderatuta [72–74].



5. irudia. β-dibertsitatearen KNA bakterio-genero mailan, Bray-Curtis (A) eta Jaccard (B) desberdintasunetan oinarrituta. Laginen identifikazioa 1etik 48ra doa. Horrela, 1etik 8ra bitarteko zenbakiak gaztagile bakoitzaren (A, B, C edo D) lehenengo heltze eguneko lagin bikoiztuei dagozkie. MA: A gaztagilearen esneari dagokio, MB: B gaztagilearen esneari dagokio; MC: C gaztagilearen esneari dagokio eta MD: D gaztagilearen esneari dagokio. Santamarina-García et al.-en [28] argitaratuta.

4.1.2. Bakterio-aldaketak gazta egiteko prozesuan

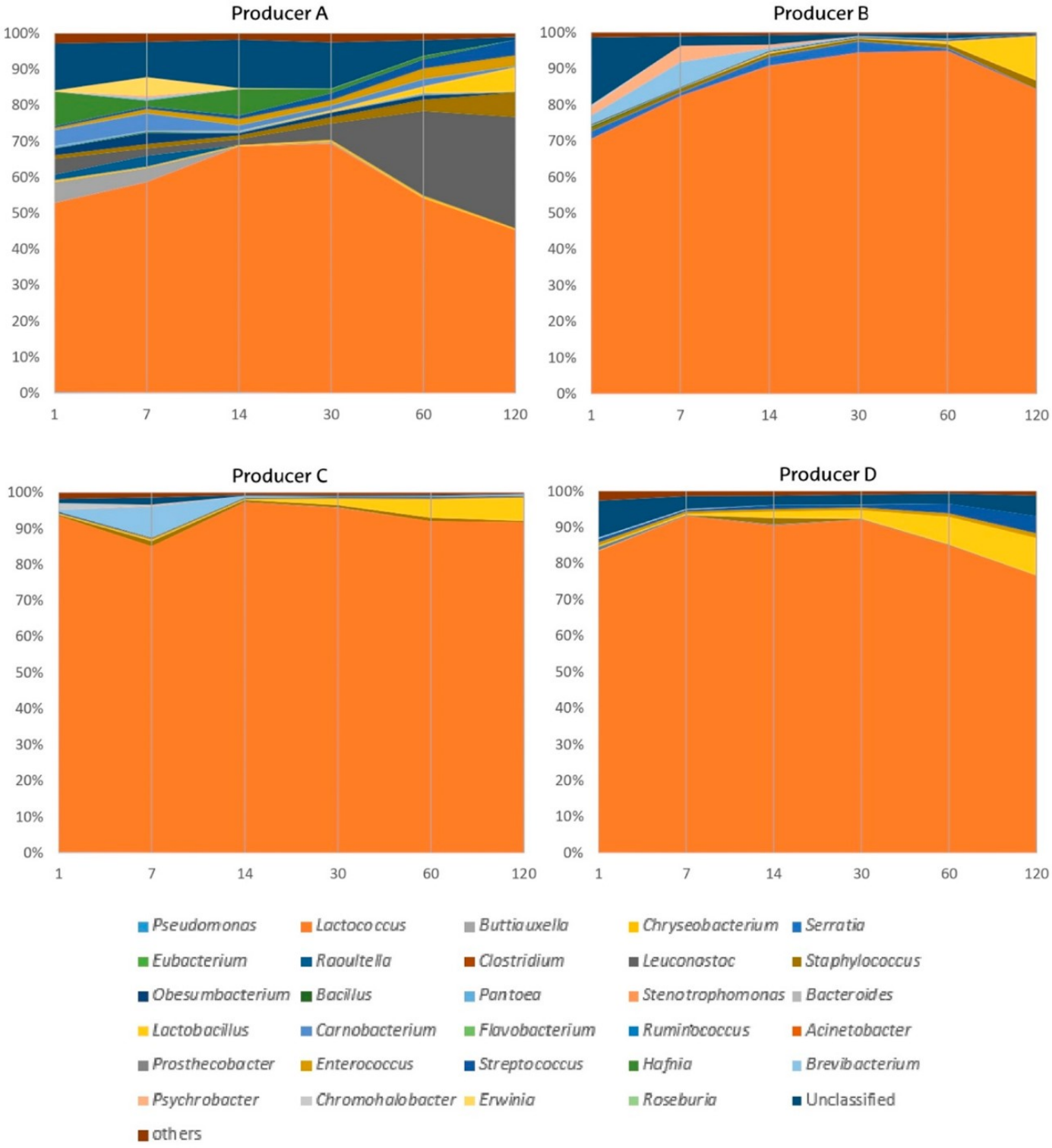
Gazta egiteko prozesuak, ekoizpen-etapa guztiak barne hartu zituenak, esne gordinetik hasita gazta freskoetaraino, mikrobiotari nabarmenki eragin zion (4C. eta 4D. irudiak). Horrela, 19 bakterio-filum, 160 familia eta 450 genero identifikatu ziren. BALen artean, *Lactococcus* generoak ugaritasun handiena izan zuen ekoizle guztien gazta freskoetan (% 52,5-93,2), BAL abiarazlea (BALA) zelako (4D. irudia), aurretik azaldu den bezala [71]. Gainerako BAL ez-abiarazleen (BALEA) kasuan, gaztaren elaborazio-prozesuaren eragina ezberdina izan zen ekoizlearen arabera. *Lactobacillus* generoaren ugaritasuna murriztu egin zen ekoizle gehienen laginetan (< % 0,200 ekoizle guztietan). Bestalde, *Leuconostoc* eta *Carnobacterium* generoen ugaritasuna handiagoa zen A ekoizlearen laginetan (% 4,48 eta % 4,40, hurrenez hurren), *Streptococcus* eta *Enterococcus* generoen antzera A eta D ekoizleen laginetan (% 0,507 eta % 0,458, eta % 0,993 eta % 0,892, hurrenez hurren) (4D. irudia). BALen agerpena eta ugaritzea aurretik ikusi da, baina osaera eta ugaritasun desberdinekin ardi-esne gordineko gaztaren arabera [45,75].

Bakterio ez-desiragarrien ugaritasuna, oro har, txikia izan zen (< % 1) (4D. irudia). Hala ere, zenbait generok ugaritasun nabarmena mantendu zuten, edo, are gehiago, ugaritu egin ziren ekoizle batzuen gaztetan; adibidez, *Buttiauxella* (% 0-5,79), *Serratia* (% 0,00179-2,16) eta *Raoultella* (% 0,0151-1,43) (4D. irudia). *Hafnia*, *Brevibacterium* eta *Psychrobacter* oportunistak ere [188–190], urriak zirenak esne gordinean (< % 1), gazta freskoetan ugaritu egin ziren, ekoizlearen arabera (% 0,00282-9,62, % 0,0210-2,43 eta % 0,00168-2,28, hurrenez hurren) (4D. irudia). Hau bereziki interesgarria da; izan ere, genero horiek lipasa- eta/edo proteasa-jarduerak dituzte [191–193] eta konposatu lurrunkor interesgarriak sortzen dituzte (hala nola 1-hexanola, 1-propanola, propilo-butanoatoa edo butilo-butanoatoa), gaztaren kalitateari eragiten diotenak [85,194,195]. Ingurumeneko bakterioen kasuan, gaztaren elaborazio-prozesuan haien ugaritasuna murriztu egin zen, baina *Obesumbacterium* generoak A ekoizlearen gaztetan antzeko ugaritasuna mantendu zuen (% 1,89) (4D. irudia). Era berean, inguruneko *Chromohalobacter* generoa [193], esne gordinean urria zena (< % 1), gazta freskoetan ugaritu zen, bereziki C ekoizlearen kasuan (% 1,78) (4D. irudia). Oro har, gazta egiteko prosezuan, ingurumeneko bakterioen eta bakterio ez-desiragarrien (adibidez, *Staphylococcus* edo *Pseudomonas*) proliferazioaren inhibizioa aurretik jakinarazi da [45,75].

Gaztaren elaborazio-prozesuan mikrobiotan eragina izan dezaketen beste faktore batzuk daude. Horrela, pH-a 4,5-5,3 tartera murrizteak bakterio gehienen hazkundea inhibitzen du, BALena izan ezik [22,196]. Gatzuneko NaCl-aren kontzentrazioak eta bakterio gehienek gatzarekiko duten tolerantzia txikiak, BALen [22] eta halofiloen hazkundea errazten dute, hala nola *Psychrobacter* eta *Chromohalobacter* generoena [197,198]. Hezetasunaren eta ur-aktibitatearen (A_u) jaitsierak, bakterio gehienen hazkundea inhibitzen du, BALena izan ezik [22,196]. Gazta egiterakoan erredox potentziala aldatzen da, bakterio anaerobiko fakultatiboen edo nahitaezkoen hazkuntza faboratuz [22]. Halaber, ardi-esne gordineko gazta batzuetan erabiltzen den artisau-gatzagia bakterio-iturri garrantzitsua izan liteke, BALak barne [117]. Gainera, gaztandegien inguruneetan dauden desberdintasunek tokian tokiko "etxeko mikrobiota" espezifikoa garatzea ekar dezakete, eta horrek gaztaren mikrobiotan eragin dezake [118].

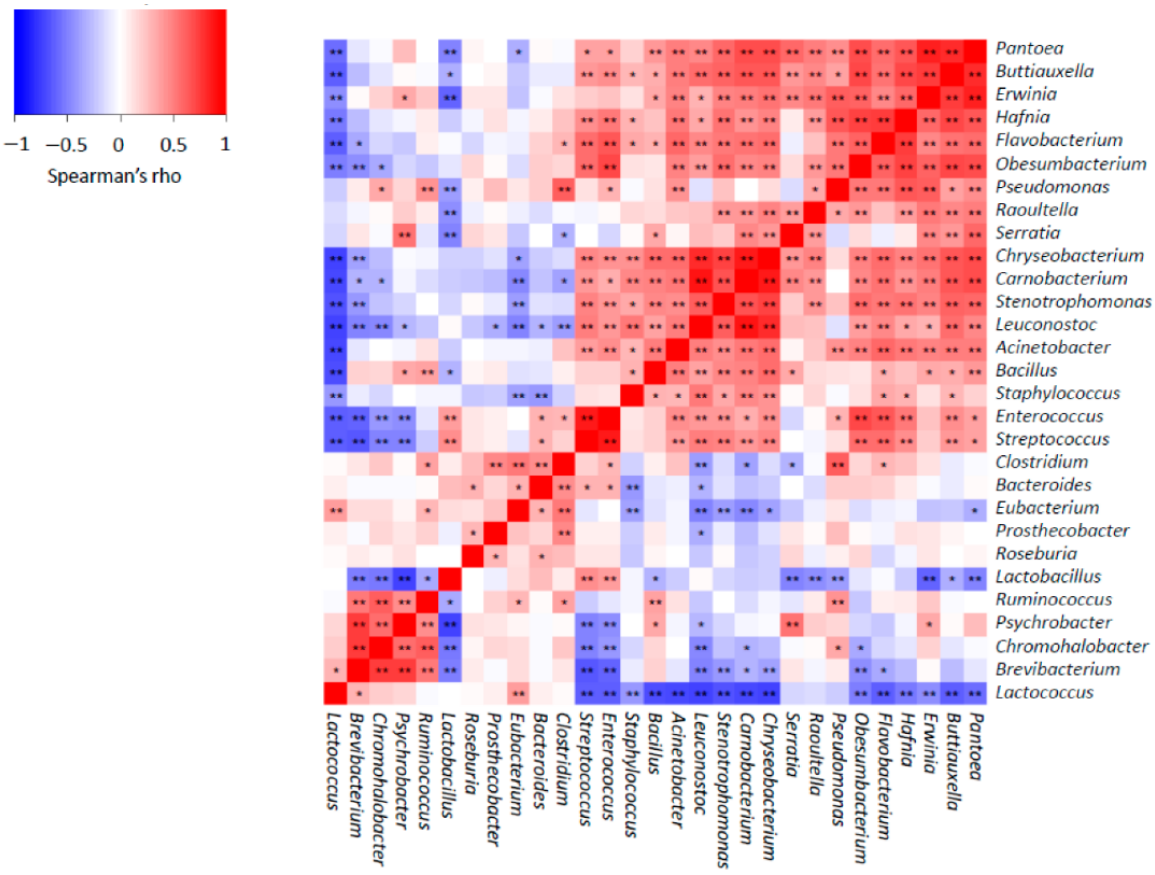
4.1.3. Bakterio-aldaketak gaztaren heltze-prozesuan

Gaztaren heltze-prozesuak ere eragin nabarmena izan zuen mikrobiotan (6. irudia). Heltzean zehar 23 bakterio-filum, 197 familia eta 583 genero identifikatu ziren. Oro har, BALen artean, *Lactococcus* izan zen generorik ugariena Idiazabal gaztaren laginetan (batez beste, % 74,9-74,5 bitartean 1. egunetik 120. egunera) (6. irudia). Estatistikoki, heltze-denborak eragin nabarmena izan zuen soilik *Lactobacillus* generoan, ugaritu egin zena ekoizle guztien laginetan (% 0,0949-tik % 8,96-ra) (6. irudia). Gainerako generoen ugaritasunaren dinamika aldatu egin zen ekoizlearen arabera. *Leuconostoc*en ugaritasuna nabarmen handitu zen heltzean zehar A ekoizlearen gaztetan (% 4,48-tik % 31,0-ra), eta *Carnobacterium*ena, berriz, murriztu egin zen (% 4,40-tik % 0,330-era). *Streptococcus* eta *Enterococcus* generoak ere ugaritu ziren A eta D ekoizleen gaztetan heltzean zehar (% 0,75-etik %4,52-ra eta % 0,675-etik % 2,12-ra, hurrenez hurren) (6. irudia). Oro har, BALen bilakaera argia ikusi zen heltzean zehar. *Lactococcus* BALAren ugaritasuna 30-60 egunetatik aurrera murriztu zen, *Leuconostoc*, *Lactobacillus*, *Streptococcus* eta *Enterococcus* BALEAak ugaritu zirenean. Heltzean zehar, laktosa gutxitzeak, pH-aren jaitsierak, NaCl-aren kontzentrazio handiak eta tenperatura baxuak BALAen bideragarritasuna txikitzen dute, eta, lisi-tasen arabera, BALEAek garrantzia hartzen dute [199]. BALAen heltzean zeharreko nagusitasuna aurretik jakinarazi da [70,71], bai eta BALAEen ugaritzea [45,70,71,200], baina desberdintasunak daude ardi-esne gordineko gazten artean [70,71,75,76].



6. irudia. Bakterio-generoen aldaketak Idiazabal gaztaren laginen heltzean zehar (1. egunetik 120. egunera), ekoizlearen arabera (A, B, C eta D). Santamarina-García et al.-en [28] argitaratuta.

Ingurumen-bakterioen ugaritasuna murriztu egin zen heltze-prozesuan zehar, *Obesumbacterium* generoaren kasuan izan ezik (6. irudia). Bakterio ez-desiragarrien artean, *Hafnia*, *Staphylococcus*, *Buttiauxella*, *Psychrobacter*, *Raoultella*, *Serratia* eta *Brevibacterium* ugari izan ziren gaztaren heltzean zehar (> % 1), ekoizlearen arabera. Hala ere, horien dinamikak ezberdinak izan ziren. *Buttiauxella*ren ugaritasuna gutxitu egin zen heltzean zehar; *Staphylococcus*aren ugaritasuna, berriz, handitu zen, eta gainerakoen ugaritasuna handitu egin zen tarteko puntuetan (7., 14. edo 30. egunetan) (6. irudia). Era berean, *Erwinia* patogenoa [201], urria zena esne gordinean (< % 1), ugariagoa izan zen heltzean zehar A ekoizlearen laginetan (% 5,15, heltzearen 7. egunean) (6. irudia). Oro har, identifikatu ziren ingurumeneko eta genero ez-desiragarri gehienak aurrez ikusi dira ardi-esne gordinetik eratorritako beste gazta batzuetan [45,69–71,75,202,203]. Hala ere, aipatutako bakterio gehienek heltze-prozesuan zehar duten prebalentziari buruzko informazioa urria da.



7. irudia. Spearmanen korrelazioak Idiazabal gaztaren laginetako bakterio-genero nagusien artean. Korrelazio esanguratsuak honela adierazten dira: ** $P \leq 0{,}01$ eta * $P \leq 0{,}05$. Santamarina-García et al.-en [28] argitaratuta.

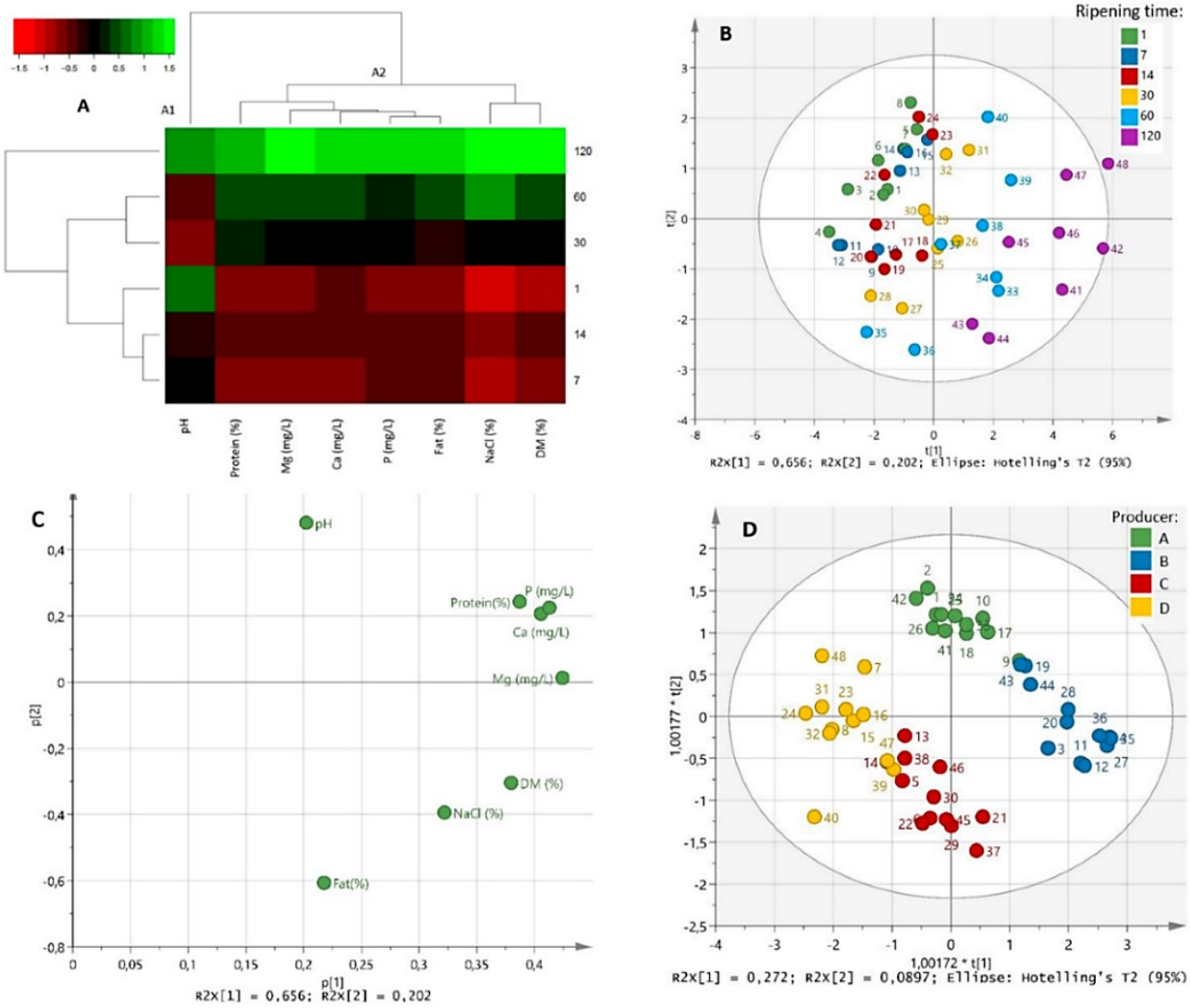
Korrelazio-azterketari erreparatuz (7. irudia), BALek bakterio ez-desiragarrien edo inguruneko bakterioen ugalketa menderatzeko edo murriztarazteko joera dutela ondorioztatu zen, aurretik ikusi den bezala [76]. Beraz, inguruneko bakterioek edo bakterio ez-desiragarriek gaztaren kalitateari egindako ekarpena heltzearen hasierako etapan izango zen [22]. Badaude ondo dokumentatutako elkarrekintza-mekanismo lehiakorrak bakterioen artean, azido organikoak edo bakteriozinak sortzen dituzten BALen kasuan, adibidez [22,204,205]. Era berean, heltzean zehar, zenbait faktorek bakterio gehienen ugalketan eragina izan dezakete, hala nola uraren jardueraren murrizketak, NaCl-aren kontzentrazio altuak, tenperatura baxuak, oxidazio erredukzio potentzialaren egoeraren aldaketak eta pH-aren murrizketak. Horrela, BALak dira ugaltzeko gai diren genero bakarrak [22]. Parametro horietan ekoizleen artean aurki daitezkeen desberdintasunek eragin dezakete mikrobiotan hautemandako ekoizleen arteko bereizketa.

4.2. Idiazabal gaztaren mikrobiotaren eta kalitate- eta kaltegabetasun-parametro nagusien arteko erlazioa (2. helburua, 2.1.-2.2. azpihelburuak, II.-III. eskuizkribuak)

Ondoren, heltzean zehar bakterio-dinamikak gaztaren kalitate eta kaltegabetasuneko parametro garrantzitsuenen bilakaeran (konposizio gordinean, gantz-azido askeetan (GAA) eta konposatu lurrunkorretan, eta amina biogenoetan (AB), hurrenez hurren) duen eragina aztertu zen, baita alderantzizko eragina ere.

4.2.1. Konposizio gordina

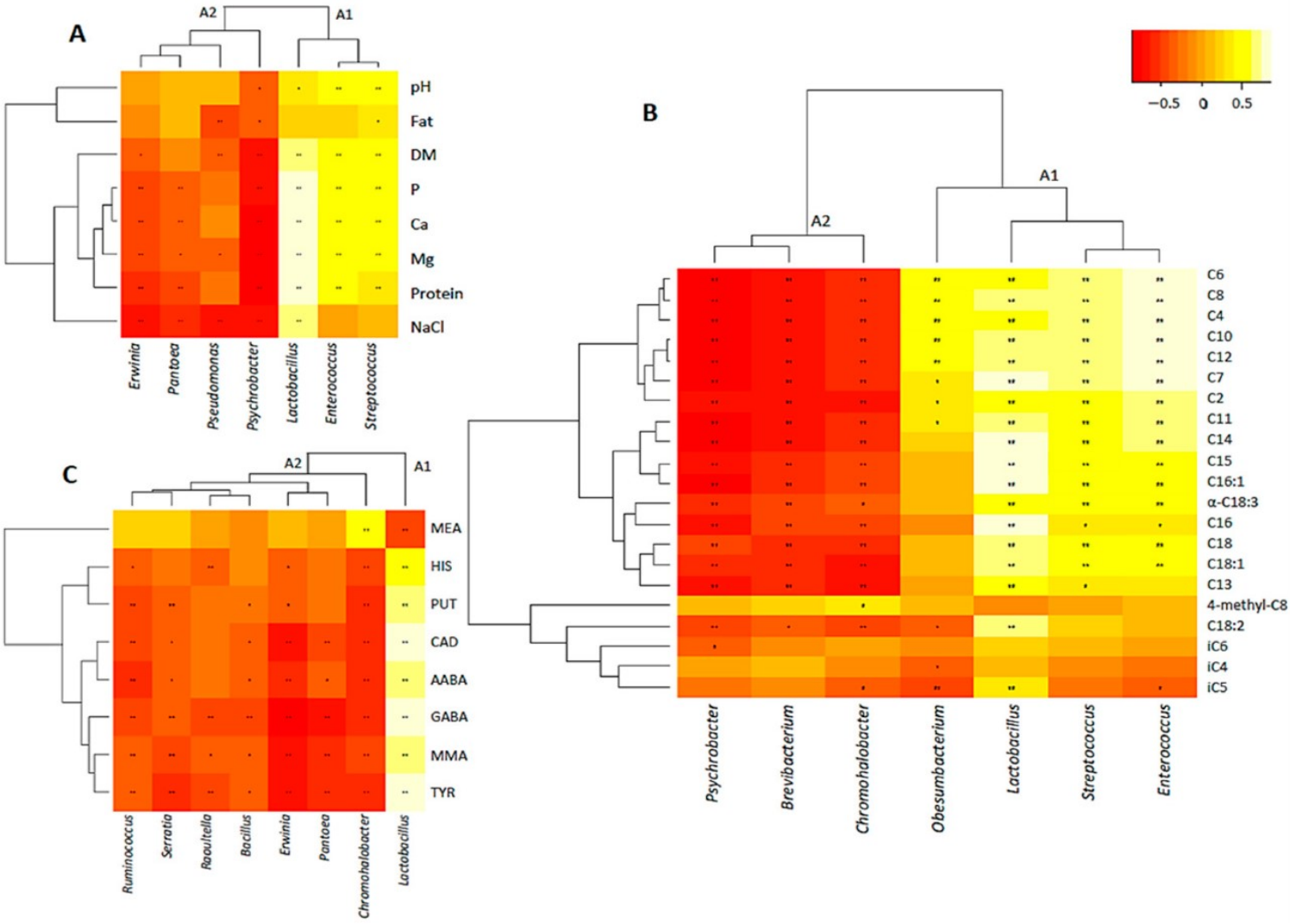
Konposizio gordinaren parametro guztiek goranzko joera izan zuten heltzean zehar ($P \leq 0,01$), pH-ak izan ezik; izan ere, 30 egunen buruan lortu zituen baliorik txikienak (4,91 ± 0,14), baina gero handitu egin zen hasierako balioetatik gertuko balioetaraino (5,11 ± 0,14) ($P \leq 0,05$) (8A-C. irudiak), aurreko emaitzekin bat datorrena [121,206–209]. Ardi-esne gordinarekin egindako beste gaztekin alderatuta, badira alde nabarmenak konposizio gordinean [210,211]. Hala ere, lortutako emaitzetan oinarrituta, aldaketak ere desberdinak izan ziren ekoizlearen arabera ($P \leq 0,05$) (8D. irudia), hainbat faktorerekin zerikusia izan lezaketenak, hala nola esne-konposizio desberdinekin [212,213], gazta egiteko eta heltzeko baldintzekin [122,130,209], edo erabilitako gatzagi motarekin eta entzima koagulatzailearen kontzentrazioarekin [214–216].



8. irudia. THAren bero-mapa (A), ONAren puntuazioen eta kargen grafikoak (B eta C, hurrenez hurren) eta ekoizlearen araberako KPMO-ADren puntuazioen grafikoa Idiazabal gaztaren laginen heltzean zehar (1, 7, 14, 30, 60 eta 120 egun) aztertutako konposizio gordinaren parametroetan oinarritua. Laginen identifikazioa 1etik 48ra doa. Horrela, 1etik 8ra bitarteko zenbakiak gaztagile bakoitzaren (A, B, C edo D) lehenengo heltze eguneko lagin bikoiztuei dagozkie. Santamarina-García et al.-en [137] argitaratuta.

Heltzean zehar, gaztaren konposizio gordinean ematen diren aldaketek bakterio-segidan eragiten dutela iradoki da [22,196]. Emaitzen arabera, zazpi bakterio-genero funtsezko bakterio gisa identifikatu ziren, konposizio gordinaren eboluzioarekin lotura estuena zutenak, hau da, *Lactobacillus*, *Psychrobacter*, *Erwinia*, *Enterococcus*, *Pseudomonas*, *Pantoea* eta *Streptococcus* generoak (9A. irudia). Hala ere, argi eta garbi bereizi ziren BALak, eboluzio horrekin korrelazio positiboa baitzuten (A1 klusterra); bereziki, *Lactobacillus* generoak, proteina, Ca edo P edukiarekin ($P \leq 0{,}01$), adibidez; eta ingurumeneko bakterioak edo bakterio ez-desiragarriak, negatiboki korrelazionatuta zeudenak (A2 klusterra), batez ere *Psychrobacter* generoa proteina, Ca eta Mg edukiarekin ($P \leq 0{,}01$), esaterako (9A. irudia). Emaitza hauek iradokitzen dutenez, konposizio

gordinaren bilakaerak BALen hazkundea bultzatu zuen, bereziki *Lactobacillus*ena, eta, neurri txikiagoan, *Streptococcus* eta *Enterococcus* generoena; eta, bitartean, eragotzi egiten zuen inguruneko bakterioen edo bakterio ez-desiragarrien hazkundea, bereziki *Psychrobacter*ena eta, neurri txikiagoan, *Erwinia*, *Pseudomonas* edo *Pantoea* generoena. Beraz, konposizio fisiko-kimikoaren eboluzioak mikrobiotan duen eragina egiaztatu zen [28]. Antzeko korrelazioak ere jakinarazi dira beste produktu hartzitu batzuetarako, nahiz eta alde nabarmenak dauden [217–219]. *Streptococcus* eta *Enterococcus* BALen eta pH-aren arteko korrelazio positibo handiek ere BALAaren eta BALEAen arteko ondorengotza baieztatu zuten (4.1.3. sekzioa) [28].

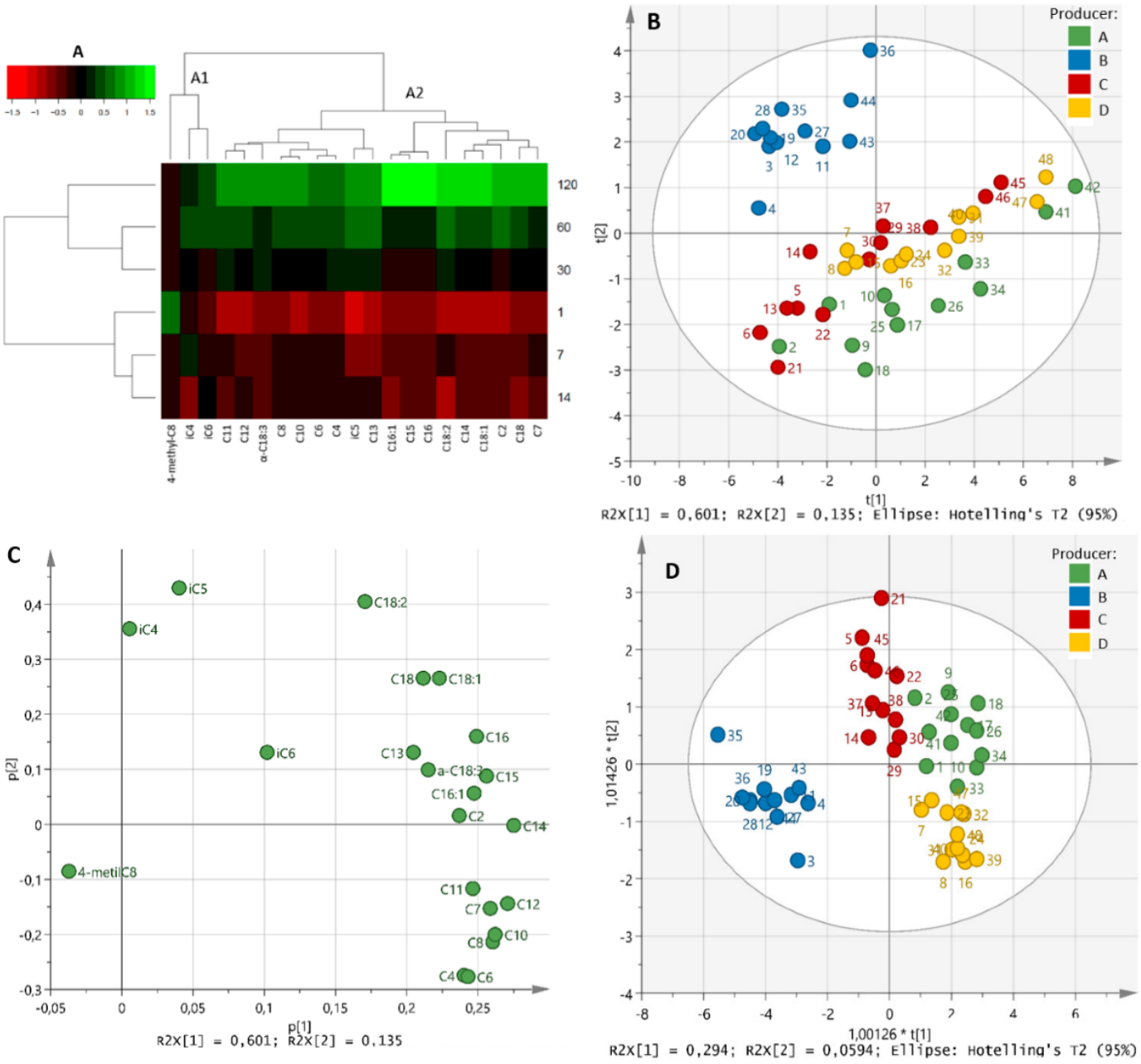


9. irudia. Spearmanen korrelazioak THArekin funtsezko bakterio-generoen eta konposizio gordinaren parametroen (A), GAAen (B) eta ABen (C) artean Idiazabal gaztaren laginen heltzean zehar (1, 7, 14, 30, 60 eta 120 egun). GAAen eta ABen nomenklatura XVII.-XX. orrialdetan azaltzen da. Korrelazio esanguratsuak honela adierazten dira: ** $P \leq 0{,}01$ eta * $P \leq 0{,}05$. Santamarina-García et al. [137] argitaratuta.

4.2.2. GAAak

Idiazabal gaztaren laginen GAA totalen, aseen, asegabeen, kate laburrekoen, ertainekoen eta luzeen kontzentrazioak handitu egin ziren heltze-prozesuan zehar ($P \leq 0,01$); GAA totalen kontzentrazioa 12,0 ± 5,95 µmol/g-tik (egun bateko gaztetan) 49,9 ± 24,5 µmol/g-ra (120 eguneko gazta helduetan), aseak eta kate laburrekoak nagusi zirelarik (10A-C. irudiak). Zehazkiago, 21 GAA identifikatu ziren, C2, C4, C6 eta Cl0 nagusi izanik. Heltzean, banakako GAA guztien kontzentrazioak ere nabarmen handitu ziren ($P \leq 0,05$), iC4, iC6 eta 4-metil-C8renak izan ezik ($P > 0,05$) (10A-C. irudiak). Emaitza horiek bat datoz aurreko ikerketekin [121,208,220–222], hala ere, 4-metil-C8, iC4 eta iC6 Idiazabal gaztan identifikatu diren lehen aldia da. Ardi-esne gordineko beste gaztekin alderatuta, badira desberdintasun nabarmen batzuk GAAen konposaketan [223–225]. Hala ere, ekoizleen artean ere alde nabarmenak hauteman ziren (10D. irudia), batez ere erabilitako gatzagi-motarekin lotuta [121,226,227].

GAAen bilakaerarekin lotura estuena izan zuten bakterio-generoak zazpi izan ziren, hau da, *Psychrobacter*, *Brevibacterium*, *Lactobacillus*, *Enterococcus*, *Chromohalobacter*, *Streptococcus* eta *Obesumbacterium* generoak (9B. irudia). BALek eta *Obesumbacterium* generoak harreman positiboa izan zuten GAAen bilakaerarekin (A1 klusterra); aldiz, bakterio ez-desiragarriak eta ingurumenekoak negatiboki erlazionatuta zeuden (A2 klusterra). BALen artean, *Lactobacillus* eta *Enterococcus* generoek korrelazio handienak izan zituzten (9B. irudia). *Lactobacillus*ek korrelazio estua izan zuen C7rekin eta kate ertain eta luzeko GAAekin, hala nola Cl4rekin, Cl5rekin eta C16rekin ($P \leq 0,01$); eta *Enterococcus*ek kate laburreko GAAekin lotura estua zuen, hala nola C4rekin, C6rekin eta C8rekin ($P \leq 0,01$). Bakterio ez-desiragarrien eta ingurunekoen artean, *Obesumbacterium*ek korrelazio positiboak erakutsi zituen kate laburreko GAAekin, hala nola C4rekin eta C6rekin ($P \leq 0,01$). Gainerakoek, berriz, korrelazio negatiboak izan zituzten ($P \leq 0,01$), *Psychrobacter* generoa nabarmenena izanda, bereziki C4rekin eta C6rekin ($P \leq 0,01$) (9B. irudia).



10. irudia. THAren bero-mapa (A), ONAren puntuazioen eta kargen grafikoak (B eta C, hurrenez hurren) eta ekoizlearen araberako KPMO-ADren puntuazioen grafikoa (D) Idiazabal gaztaren laginen heltzean zehar (1, 7, 14, 30, 60 eta 120 egun) identifikatutako GAAetan oinarritua. Laginen identifikazioa 1etik 48ra doa. Horrela, 1etik 8ra bitarteko zenbakiak gaztagile bakoitzaren (A, B, C edo D) lehenengo heltze eguneko lagin bikoiztuei dagozkie. GAAen nomenklatura XVII. eta XVIII. orrialdetan azaltzen da. Santamarina-García et al.-en [137] argitaratuta.

BALen eta ingurumeneko bakterioen edo bakterio ez-desiragarrien jarduera lipolitikoa, hala nola *Flavobacterium* edo *Pseudomonas* generoena, aurretik jakinarazi da [192,228–235], nahiz eta *Obesumbacterium* generoarentzat informaziorik ez dagoen. BALen artean aldea ikusi zen, *Lactobacillus*ek lipasa jarduera izango zuelako (kate ertain eta luzeko GAAen askapenagatik) eta

*Enterococcus*ek esterasa jarduera (kate motzeko GAAen askapenagatik) [227,236]. Horrek, bakterio-dinamikak produktu hartzitu baten GAAen profilaren garapenean duen eragina eta, oro har, aromaren garapenean duen inpaktua baieztatuko luke. Korrelazio negatiboek pentsaraz lezakete *Psychrobacter*, *Brevibacterium* eta *Chromohalobacter* generoek GAAen erabilera metabolikoa egiten dutela, orain arte gutxi aztertu dena [237]; edo lehia-inhibizioko mekanismoei egingo liekete erreferentzia, GAAek espektro zabaleko mikrobioen aurkako agente gisa jardun dezaketelako [238–240]. Horrela, BALek, nagusiki, GAAak askaraziko lituzkete, eta efektu inhibitzailea izango lukete inguruneko bakterioen edo bakterio ez-desiragarrien aurka (4.1.3. sekzioa).

4.2.3. Konposatu lurrunkorrak

Idiazabal gaztaren heltze-prosezuan, zortzi familia kimikotako 81 konposatu lurrunkor identifikatu ziren (6. taula), hau da, azidoak (9 azido indibidual identifikatu ziren), alkoholak (21), aldehidoak (7), zetonak (12), esterrak (24), hidrokarburoak (5), -ak (2) eta terpenoak (1). Esterrak eta alkoholak konposatu indibidual gehien barne hartzen zituzten familiak ziren. Hala ere, ugaritasunari dagokionez, azidoak nagusitu ziren (guztizko ugaritasunaren % 79,5-88,9 bitartean), bereziki azido hexanoikoagatik, butanoikoagatik eta oktanoikoagatik. Gero, esterrak (% 0,859-11,3), bereziki etil esterrak, hala nola etil-dekanoatoa, etil-hexanoatoa eta etil-dodekanoatoa; zetonak (% 3,68-7,16), batez ere metil zetonak (adibidez, 2-butanona); eta alkoholak (% 3,40-5,46), batez ere alkohol primarioak eta sekundarioak (esaterako, etanola eta 2-butanola) garrantzitsuak ziren (6. taula). Oro har, emaitza hauek bat datoz Idiazabal gaztaren inguruko ikerketekin [20,120,135,241], nahiz eta 18 konposatu lehen aldiz identifikatu diren. Alde nabarmenak daude ardi-esne gordineko beste gaztekin konparatuta, identifikatutako konposatuei eta horien ugaritasunari dagokienez [69,242].

6. taula. Idiazabal gaztaren laginen heltzen zehar (1, 7, 14, 30, 60 eta 120 egun) identifikatutako konposatu lurrunkorren batez besteko ugaritasun erlatiboa ± desbideratze estandarra. Santamarina-García et al.-en [243] argitaratuta.

ID	LRI[1]	Volatile compounds	Ripening time (days)[2]						P-value[3]	
			1	7	14	30	60	120	RT	P
Acids										
C1	1647	Acetic acid[4]	8.06 ± 22.8	353 ± 542	ND	24.3 ± 36.4	61.6 ± 101	120 ± 243	NS	NS
C2	1698	*n*- Butanoic acid[4]	1681 ± 1594	6884 ± 5928	9289 ± 7950	10674 ± 6878	12053 ± 7633	6624 ± 8670	*	**
C3	1880	*n*- Pentanoic acid[4]	10.5 ± 14.4	55.1 ± 50.4	1135 ± 3050	4166 ± 11566	48.1 ± 57.5	428 ± 942	NS	*
C4	1921	*n*- Hexanoic acid[4]	3168 ± 2911	12617 ± 9968	16782 ± 13507	24546 ± 15744	25632 ± 19485	15567 ± 22362	NS	***
C5	2067	(E)-3-Hexenoic acid[4,6]	ND	ND	ND	682 ± 1692	196 ± 365	2682 ± 5030	NS	***
C6	2105	*n*- Heptanoic acid[4]	ND	102 ± 113	435 ± 817	183 ± 199	192 ± 210	151 ± 237	NS	**
C7	2192	*n*- Octanoic acid[4]	1174 ± 1231	3727 ± 2899	5186 ± 4099	7982 ± 5158	9623 ± 8291	6362 ± 7524	*	***
C8	2395	*n*- Nonanoic acid[4]	ND	ND	ND	ND	ND	307 ± 617		
C9	2396	*n*- Decanoic acid[4]	490 ± 502	1439 ± 1119	2185 ± 1695	4073 ± 2525	4441 ± 3872	2959 ± 3862	*	**
		Total straight-chain acids	6532 ± 6275	25177 ± 20619	35012 ± 31119	52331 ± 43801	52247 ± 40014	35200 ± 49486	*	***
Alcohols										
C10	936	Ethanol[4]	171 ± 58.5	696 ± 931	743 ± 1065	1288 ± 2102	443 ± 505	145 ± 154	NS	NS
C11	1040	1-Propanol[4]	ND	ND	ND	ND	ND	287 ± 723		
C12	1145	1-Butanol[5]	ND	4.76 ± 8.93	10.6 ± 19.8	10.6 ± 19.6	20.3 ± 31.4	22.4 ± 24.9	*	***
C13	1205	3-Methyl-1-butanol[5]	7.46 ± 15.1	34.1 ± 29.8	58.2 ± 87.8	52.3 ± 55.9	29.5 ± 16.7	6.51 ± 7.76	*	***
C14	1249	1-Pentanol[5]	7.76 ± 6.20	4.60 ± 8.55	ND	19.4 ± 52.6	ND	ND	**	NS
C15	1351	1-Hexanol[5]	ND	ND	8.12 ± 15.5	8.69 ± 16.1	17.4 ± 20.8	26.0 ± 20.6	***	NS
C16	1482	2-Ethyl-1-hexanol[4]	9.66 ± 7.22	18.2 ± 22.2	5.41 ± 5.99	62.9 ± 147	15.8 ± 8.09	6.71 ± 9.78	NS	*
C17	1552	1-Octanol[5]	ND	ND	ND	ND	ND	3.15 ± 4.24		
		Total primary alcohols	196 ± 87.1	757 ± 1001	825 ± 1194	1442 ± 2393	526 ± 582	497 ± 945	NS	NS
C18	1025	2-Butanol[5]	ND	ND	ND	88.5 ± 124	812 ± 1102	424 ± 334	***	NS
C19	1120	2-Pentanol[5]	ND	ND	ND	1.60 ± 2.97	3.41 ± 8.25	27.0 ± 18.2	***	NS
C20	1122	3-Methyl-2-butanol[4,6]	ND	ND	ND	ND	4.41 ± 8.43	7.14 ± 13.3	NS	NS
C21	1218	2-Hexanol[5]	ND	ND	ND	ND	ND	1.44 ± 2.75		
C22	1316	2-Heptanol[4]	ND	ND	ND	30.7 ± 82.2	3.73 ± 6.99	12.2 ± 19.4	*	NS
C23	1509	2-Nonanol[5]	ND	ND	ND	ND	ND	7.29 ± 11.4		
C24	1640	2,3-Butanediol[4]	ND	27.1 ± 76.7	ND	32.1 ± 59.6	ND	ND	NS	*
C25	1646	Menthol[4,6]	6.65 ± 6.63	13.0 ± 10.4	27.5 ± 41.2	11.2 ± 12.6	ND	ND	**	**
		Total secondary alcohols	6.65 ± 6.63	40.1 ± 87.1	27.5 ± 41.2	164 ± 281	823 ± 1126	479 ± 399	***	NS
C26	1352	2-Methyl-3-pentanol[4]	9.41 ± 17.4	ND	ND	ND	ND	ND		
		Total tertiary alcohols	9.41 ± 17.4	ND	ND	ND	ND	ND		
C27	1115	2-Propen-1-ol[4,6]	ND	ND	10.7 ± 20.9	26.8 ± 49.7	31.8 ± 81.4	1.79 ± 5.06	NS	***
C28	1396	2-Nonen-1-ol[4,6]	0.497 ± 0.921	ND	ND	ND	ND	ND		
C29	1407	(E)-4-Hexen-1-ol[4,6]	ND	ND	ND	ND	ND	2.09 ± 3.94		
C30	1320	6-Heptene-2,4-diol[4,6]	8.00 ± 10.0	8.23 ± 18.7	ND	ND	ND	ND	**	*
		Total allyl alcohols	8.50 ± 11.0	805 ± 18.7	10.7 ± 20.9	26.8 ± 49.7	31.8 ± 81.4	3.88 ± 9.01	NS	***
		Total alcohols	221 ± 122	1602 ± 1107	863 ± 1256	1633 ± 2724	1381 ± 1789	980 ± 1353	*	NS
Aldehydes										
C31	1083	Hexanal[5]	ND	ND	1.55 ± 2.88	ND	ND	ND		
C32	1187	Heptanal[4]	3.62 ± 6.94	ND	ND	ND	ND	ND		
C33	1287	Octanal[5]	0.0911 ± 0.258	ND	ND	ND	ND	ND		
C34	1399	Nonanal[5]	7.93 ± 5.03	14.2 ± 4.22	15.2 ± 4.82	24.2 ± 35.2	14.4 ± 8.11	9.73 ± 12.1	NS	NS
		Total straight-chain aldehydes	11.6 ± 12.2	14.2 ± 4.22	16.7 ± 7.70	24.2 ± 35.2	14.4 ± 8.11	9.73 ± 12.1	NS	NS
C35	919	3-Methyl-butanal[4]	2.99 ± 5.54	3.96 ± 4.83	1.29 ± 2.44	2.44 ± 4.63	8.40 ± 10.8	43.5 ± 31.5	***	NS
		Total branched-chain aldehydes	2.99 ± 5.54	3.96 ± 4.83	1.29 ± 2.44	2.44 ± 4.63	8.40 ± 10.8	43.5 ± 31.5	***	NS
C36	1541	Benzaldehyde[5]	ND	ND	ND	3.90 ± 11.0	12.4 ± 23.0	1.80 ± 2.24	*	**
C37	1667	Benzeneacetaldehyde[4,6]	ND	ND	7.98 ± 15.6	ND	ND	ND		
		Total aromatic aldehydes	ND	ND	7.98 ± 15.6	3.90 ± 11.0	12.4 ± 23.0	1.80 ± 2.24	NS	NS
		Total aldehydes	14.6 ± 17.7	18.2 ± 9.05	26.0 ± 25.7	30.5 ± 50.8	35.2 ± 41.9	55.0 ± 45.8	*	NS
Ketones										
C38	817	2-Propanone[4]	13.8 ± 16.0	13.1 ± 16.9	10.6 ± 12.6	56.9 ± 142	ND	ND	*	***
C39	904	2-Butanone[5]	2.84 ± 5.25	ND	25.2 ± 21.3	1962 ± 2305	1465 ± 1572	243 ± 335	***	NS
C40	978	2-Pentanone[5]	ND	ND	ND	107 ± 129	57.2 ± 77.8	98.9 ± 73.9	***	NS
C41	1184	2-Heptanone[5]	17.5 ± 9.45	34.6 ± 12.7	39.7 ± 17.2	163 ± 226	82.1 ± 60.3	170 ± 211	**	*
C42	1288	2-Octanone[5]	1.82 ± 2.83	28.4 ± 74.6	1.57 ± 2.90	22.5 ± 57.0	1.92 ± 3.82	3.28 ± 6.14	NS	***
C43	1392	2-Nonanone[5]	12.4 ± 8.40	28.7 ± 13.1	34.5 ± 20.8	91.0 ± 69.7	64.7 ± 56.1	99.2 ± 111	*	*
C44	1447	8-Nonen-2-one[4]	ND	ND	ND	ND	ND	8.91 ± 11.2		
C45	1604	2-Undecanone[5]	ND	ND	2.72 ± 5.07	7.09 ± 10.8	6.59 ± 7.77	0.716 ± 1.35	*	NS
		Total methyl ketones	48.4 ± 42.0	105 ± 117	114 ± 79.8	2409 ± 2940	1678 ± 1778	624 ± 749	***	NS
C46	918	(E,E)-6,10-Dimethyl-5,9-dodecadien-2-one[4,6]	ND	ND	ND	ND	29.2 ± 64.9	ND		
C47	981	2,3-Butanedione[5]	60.3 ± 72.1	76.9 ± 79.1	86.2 ± 76.7	24.9 ± 46.4	22.7 ± 46.6	ND	**	**

6. taula (Jarraipena).

C48	1292	3-Hydroxy-2-butanone[4]	349 ± 564	477 ± 401	533 ± 451	380 ± 377	179 ± 245	66.8 ± 110	*	***
C49	1367	2-Hydroxy-3-pentanone[4,6]	ND	ND	4.69 ± 8.77	1.45 ± 2.85	ND	ND	NS	**
		Total diketones and other ketones	409 ± 636	554 ± 480	624 ± 536	406 ± 427	230 ± 357	66.8 ± 110	*	***
		Total ketones	457 ± 678	659 ± 597	738 ± 616	2815 ± 3367	1908 ± 2135	691 ± 859	**	*
Esters										
C50	1186	Methyl hexanoate[5]	ND	3.81 ± 7.83	3.33 ± 6.17	ND	ND	ND	NS	**
C51	1187	Methyl 4-methyl pentanoate[4,6]	2.87 ± 5.34	7.40 ± 8.64	3.33 ± 6.17	ND	3.84 ± 7.20	4.53 ± 8.66	NS	**
		Total methyl esters	2.87 ± 5.34	11.2 ± 16.5	6.66 ± 12.3	ND	3.84 ± 7.20	4.53 ± 8.66	NS	**
C52	1038	Ethyl butanoate[4]	4.32 ± 3.12	27.0 ± 45.3	53.8 ± 97.8	78.3 ± 116	62.8 ± 81.3	288 ± 362	**	**
C53	1055	Ethyl 2-methyl butanoate[4]	ND	ND	ND	ND	1.77 ± 3.28	3.11 ± 5.75	NS	**
C54	1069	Ethyl 3-methyl butanoate[4]	ND	ND	ND	ND	44.2 ± 112	33.9 ± 65.4	NS	**
C55	1134	Ethyl pentanoate[5]	ND	ND	ND	ND	2.12 ± 4.92	2.42 ± 4.54	NS	**
C56	1234	Ethyl hexanoate[5]	18.7 ± 12.0	183 ± 267	343 ± 554	453 ± 584	480 ± 647	216 ± 270	*	*
C57	1292	Ethyl hex-4-enoate[4,6]	ND	ND	ND	7.24 ± 14.1	7.70 ± 15.1	0.292 ± 0.559	NS	***
C58	1335	Ethyl heptanoate[5]	ND	ND	4.78 ± 8.89	5.77 ± 10.7	3.86 ± 9.01	33.8 ± 53.2	NS	***
C59	1434	Ethyl octanoate[5]	5.27 ± 3.72	31.7 ± 45.9	71.5 ± 117	120 ± 139	101 ± 171	58.3 ± 95.9	*	**
C60	1537	Ethyl nonanoate[4,6]	ND	ND	ND	ND	ND	1.18 ± 2.20		
C61	1641	Ethyl decanoate[4]	4.47 ± 3.82	20.3 ± 24.1	458 ± 1275	1002 ± 2560	87.9 ± 140	320 ±559	**	NS
C62	1855	Ethyl dodecanoate[4]	ND	ND	ND	ND	0.640 ± 1.80	1291 ± 2894	NS	*
		Total ethyl esters	32.7 ± 22.7	262 ± 383	930 ± 2051	1667 ± 3424	791 ± 1185	2248 ± 4312	***	**
C63	1122	Propyl butanoate[5]	ND	ND	ND	ND	3.35 ± 8.57	6.74 ± 7.88	**	***
C64	1320	Propyl hexanoate[5]	ND	ND	ND	ND	14.6 ± 19.6	27.1 ± 28.0	***	NS
C65	1521	Propyl octanoate[5]	ND	ND	ND	ND	ND	5.68 ± 8.53		
		Total propyl esters	ND	ND	ND	ND	18.0 ± 28.2	39.5 ± 44.4	***	NS
C66	1218	Butyl butanoate[4]	ND	ND	ND	ND	2.20 ± 5.05	2.98 ± 3.31	**	*
C67	1412	Butyl hexanoate[4]	ND	ND	ND	ND	3.34 ± 9.45	7.81 ± 7.31	***	NS
		Total butyl esters	ND	ND	ND	ND	5.54 ± 14.5	10.8 ± 10.6	***	NS
C68	1267	Pentyl butanoate[4,6]	ND	ND	2.11 ± 3.90	3.62 ± 6.81	2.45 ± 5.45	127 ± 237	NS	***
		Total pentyl esters	ND	ND	2.11 ± 3.90	3.62 ± 6.81	2.45 ± 5.45	127 ± 237	NS	***
C69	1414	Hexyl hexanoate[4]	ND	ND	ND	ND	ND	3.08 ± 6.05		
		Total hexyl esters	ND	ND	ND	ND	ND	3.08 ± 6.05		
C70	1131	1-Methylpropyl butanoate[4,6]	ND	ND	ND	ND	ND	5.56 ± 7.07		
C71	1323	2-Methylpropyl hexanoate[4]	ND	ND	ND	ND	11.4 ± 22.1	35.5 ± 32.4	***	NS
C72	1372	2-Propenyl hexanoate[4]	ND	ND	ND	ND	2.54 ± 5.47	0.485 ± 0.900	NS	**
C73	1461	3-Methylbutyl hexanoate[4,6]	ND	ND	ND	ND	1.55 ± 3.49	40.2 ± 74.8	NS	**
		Total branched-alkyl esters	ND	ND	ND	ND	15.5 ± 31.0	81.7 ± 115	***	NS
		Total esters	35.6 ± 28.0	273 ± 400	939 ± 2067	1671 ± 3431	836 ± 1271	2515 ± 4734	***	**
Hydrocarbons										
C74	700	Heptane[5]	7.46 ± 17.2	13.0 ± 17.6	7.10 ± 17.2	11.9 ± 14.2	5.45 ± 15.4	ND	NS	NS
		Total saturated hydrocarbons	7.46 ± 17.2	13.0 ± 17.6	7.10 ± 17.2	11.9 ± 14.2	5.45 ± 15.4	ND	NS	NS
C75	688	1,3-Pentadiene[4]	ND	ND	ND	ND	ND	2.42 ± 4.55		
C76	837	*t*-3-Octene[4]	35.6 ± 54.4	35.7 ± 44.8	36.9 ± 47.0	239 ± 621	16.3 ± 33.0	17.5 ± 33.5	NS	***
C77	956	1,3-Octadiene[4,6]	5.93 ± 11.3	7.08 ± 13.9	7.50 ± 14.7	98.5 ± 268	6.71 ± 15.3	6.22 ± 11.7	NS	***
C78	1041	Toluene[5]	20.2 ± 12.2	42.9 ± 28.4	24.9 ± 19.4	112 ± 261	19.9 ± 23.5	68.0 ± 112	NS	NS
		Total unsaturated hydrocarbons	61.7 ± 77.9	85.7 ± 87.1	69.3 ± 81.2	449 ± 1150	42.9 ± 71.8	94.1 ± 162	NS	***
		Total hydrocarbons	69.2 ± 95.1	98.7 ± 105	76.4 ± 98.4	461 ± 1164	48.4 ± 87.2	94.1 ± 162	NS	***
Sulphur compounds										
C79	746	Dimethyl sulphide[5]	1.55 ± 2.86	ND	ND	17.2 ± 46.3	ND	ND	NS	**
C80	1934	Dimethyl sulphone[4]	25.0 ± 34.0	8.05 ± 15.7	7.41 ± 14.0	61.4 ± 163	7.76 ± 14.5	6.77 ± 12.5	NS	***
		Total sulphur compounds	26.6 ± 36.8	8.05 ± 15.7	7.41 ± 14.0	78.6 ± 209	7.76 ± 14.5	6.77 ± 12.5	NS	***
Terpenes										
C81	1195	D-Limonene[5]	0.151 ± 0.430	1.35 ± 3.83	ND	1.02 ± 2.89	ND	ND	NS	NS
		Total terpenes	0.151 ± 0.430	1.35 ± 3.83	ND	1.02 ± 2.89	ND	ND	NS	NS

[1] LRI: atxikipen linealaren indizea; [2] ND: ez da detektatu; [3] RT: heltze-denbora faktorearen eragina; P: ekoizlea faktorearen eragina; NS: $P > 0,05$, * $P \leq 0,05$, ** $P \leq 0,01$, *** $P \leq 0,001$; [4] Konposatu lurrunkorraren saiakuntza identifikazioa; [5] Konposatu lurrunkorraren identifikazio positiboa; [6] Idiazabal gaztan aurretik deskribatu gabeko konposatu lurrunkorra.

Konposatu lurrunkor guztiek gaztaren aromari eragiten ez diotenez, batez ere usain-atalaseengatik (UA) [244], konposatu aromatikoak identifikatu ziren usain-inpaktuaren ratioen (UIR) balioen bitartez (7. taula). Esterrek UIR balio altuenak erakutsi zituzten, handitu zirenak heltze-prozesuak aurrera egin ahala, bereziki etil esterrengatik; esate baterako, etil 3-metil butanoatoa, etil butanoatoa eta etil hexanoatoa (7. taula). Azidoek ere UIR balio nabarmenak erakutsi zituzten, batez ere heltzearen erdiko etapan, azido butanoikoarekin, hexanoikoarekin eta oktanoikoarekin erlazionatuta, adibidez (7. taula). Gainerako familietako konposatuek UIR balio baxuak (< 1) izan zituzten, zenbait konposatuk izan ezik, hala nola, 2-butanonak, 3-metil-butanalak, 2-butanolak, toluenoak edo dimetil sulfonak (7. taula). Esterrak eta azidoak gazta askoren konposatu aromatiko nabarmenak dira, esaterako, Grana Padano eta Cheddar gaztetan, hurrenez hurren [26,245–247]. Hala ere, gazta-moten artean aldeak daude [248–251]. 2-butanonari dagokionez, metil zetonak hainbat gaztaren aroman nabariak dira, hala nola Cheddar gaztan edo gazta urdinetan [245,250–252]. Era berean, kate adarkatuko aldehidoak eta, zehazki, 3-metil-butanala, gaztaren aromarako aldehidorik nabarmenenak dira, Parmigiano Reggianoren kasuan bezala [245,252,253]. Sufredun konposatuak ere gazta askoren aromarentzat funtsezkoak dira, UA baxuak dituztelako [26,245]. Informazio gutxi dago 2-butanolak edo toluenoak usainean duten eraginari buruz [135,254].

7. taula. UIRen balio zenbatetsiak Idiazabal gaztaren laginen heltzean zehar (1, 7, 14, 30, 60 eta 120 egun) eta konposatu lurrunkor bakoitzaren deskribapen sentsoriala. Santamarina-García et al.-en [243] argitaratuta.

Volatile compounds	OT[1]	OIR[2] values during ripening (days)						Described odour notes	References
		1	7	14	30	60	120		
Acids									
Acetic acid	22000				< 1			Sour, vinegar, pungent, acid	[135,241,255]
n- Butanoic acid	50	**33.6**	**138**	**186**	**213**	**241**	**132**	Rancid, cheesy, putrid, sharp, sour, sweat	[135,241,255]
n- Pentanoic acid	137	< 1	< 1	**8.28**	**30.4**	< 1	**3.13**	Sweat, putrid, sharp, sour, cheesy, burned	[241,255]
n- Hexanoic acid	290	**10.9**	**43.5**	**57.9**	**84.6**	**88.4**	**53.7**	Sweat, sour, pungent, goat, rancid, cheesy, foot, faecal	[135,241,255]
(E)-3-Hexenoic acid	-				-			Pungent, sweat, vinegar, cheesy, green	[256,257]
n- Heptanoic acid	3000				< 1			Sweat, rancid, faecal	[135,255]
n- Octanoic acid	450	**2.61**	**8.28**	**11.5**	**17.7**	**21.4**	**14.1**	Sweat, goat, soapy, waxy, musty, faecal, dust, cleaner	[135,241,255]
n- Nonanoic acid	4000				< 1			Faecal, burned, fruity	[255]
n- Decanoic acid	10000				< 1			Fatty, soapy, dust, waxy, burned	[135,255]
Alcohols									
Ethanol	8				< 1			Alcohol, winey, sweet, ethereal	[241]
1-Propanol	5700				< 1			Alcohol, winey, sweet	[241]
1-Butanol	800				< 1			Winey, sweet, fruity, fusel oil	[241]
3-Methyl-1-butanol	250				< 1			Alcohol, winey, fruity, burned, herbal	[241,255]
1-Pentanol	4000				< 1			Alcohol, sharp, harsh	[241]
1-Hexanol	50				< 1			Winey, oily, flower, fruity,	[135,241]
2-Ethyl-1-hexanol	830				< 1			Green, citrus, floral, oily, sweet	[257,258]
1-Octanol	42				< 1			Fatty, waxy, citrus, oily, walnut, moss, chemical, metal, burned	[254,259]
2-Butanol	59	< 1	< 1	< 1	**1.50**	**13.8**	**7.19**	Winey, alcohol, sweet, fruity, fusel oil	[135,241]
2-Pentanol	41				< 1			Alcohol, slightly green, winey, fruity	[135,241]
3-Methyl-2-butanol	420				< 1			Fruity	[257]
2-Hexanol	82				< 1			Herbal, green, chemical, winey, fruity, fatty, terpenic, cauliflower	[257,260]
2-Heptanol	70				< 1			Earthy, sweet, fruity, oily, green, herbal	[135,241,255]
2-Nonanol	75				< 1			Fatty, mild, green, melon, coconut	[245,254,260]
2,3-Butanediol	11000				< 1			Fruity	[255]
Menthol	920				< 1			Minty, cooling	[261]
2-Methyl-3-pentanol	420				< 1			Grilled, bread	[255]
2-Propen-1-ol	5000				< 1			Pungent, mustard	[257]
2-Nonen-1-ol	130				< 1			Green, fatty, melon	[257]
(E)-4-Hexen-1-ol	100				< 1			Green, herbal, musty, tomato	[257]
6-Heptene-2,4-diol	-				-			-	-
Aldehydes									
Hexanal	5				< 1			Green, herbal, sharp	[241,255]
Heptanal	3	**1.21**	< 1	< 1	< 1	< 1	< 1	Fatty, fruity, soapy, green, waxy, herbal	[245,250,252,262,263]
Octanal	1.5				< 1			Fruity, green, citrus, fatty, fatty-fruity, lemon	[245,250,252,262–264]
Nonanal	10	< 1	**1.42**	**1.52**	**2.42**	**1.44**	< 1	Sweet, fatty-floral, floral-waxy, rosy, citrus, peas, plastic	[135,241,255]
3-Methyl-butanal	0.200	**15.0**	**19.8**	**6.46**	**12.2**	**42.0**	**217**	Malt, chocolate, toffee, green	[241]
Benzaldehyde	325				< 1			Almond, cherry stone, burned sugar	[250,254,265]
Benzeneacetaldehyde	5.50	< 1	< 1	**1.45**	< 1	< 1	< 1	Floral, honey, daisy, green, violet-like, hyacinth, styrene, rosy, dry fruit, sweet	[22,245,250,258,264,266]
Ketones									
2-Propanone	840				< 1			Sweet, fruity, ethereal, nauseating	[241]
2-Butanone	30	< 1	< 1	< 1	**65.4**	**48.8**	**8.09**	Sweet, ethereal, slightly nauseating	[241]
2-Pentanone	70000				< 1			Sweet, fruity, ethereal	[135,241]
2-Heptanone	5	**3.51**	**6.92**	**7.93**	**32.5**	**16.4**	**34.0**	Musty, blue cheese, pungent, soapy, flower	[135,241,255]
2-Octanone	41				< 1			Fruity, musty, floral, green, herbal, mouldy, humidity, soapy, musty, blue-cheese	[245,258–260,266]
2-Nonanone	5	**2.48**	**5.75**	**6.91**	**18.2**	**12.9**	**19.8**	Musty, floral, fruity, soapy	[135,241]
8-Nonen-2-one	-				-			Blue cheese, fruity baked	[245,258,259]
2-Undecanone	6.2	< 1	< 1	< 1	**1.14**	**1.06**	< 1	Fruity, herbal	[135]
(E,E)-6,10-Dimethyl-5,9-dodecadien-2-one	-				-			-	

7. taula. (Jarraipena)

2,3-Butanedione	3	**20.1**	**25.6**	**28.7**	**8.29**	**7.56**	< 1	Buttery, sweet, cream, caramel	[245,250,252,254,262–264,267]
3-Hydroxy-2-butanone	850			< 1				Buttery, flower	[241,255]
2-Hydroxy-3-pentanone	2500			< 1				Fatty, truffle, earthy, nutty	[245,257,258]
Esters									
Methyl hexanoate	390			< 1				Citrus, pineapple, ethereal	[260,264]
Methyl 4-methyl pentanoate	-			-				Strawberry, roasted cocoa	[268,269]
Ethyl butanoate	1	**4.32**	**27.0**	**53.8**	**78.3**	**62.8**	**288**	Fruity, apple, pineapple, banana, sweet, flower	[135,241,255]
Ethyl 2-methyl butanoate	-			-				Sweet, fruity	[245,262]
Ethyl 3-methyl butanoate	0.1	< 1	< 1	< 1	< 1	**442**	**339**	Fruity, olive, sweet	[135,255]
Ethyl pentanoate	8.7			< 1				Fruity, sweet, acid, apple, pineapple, green, berry, tropical	[257,263,266]
Ethyl hexanoate	1	**18.7**	**183**	**343**	**453**	**480**	**216**	Fruity, apple, pineapple, banana, mouldy, flower	[135,241,255]
Ethyl hex-4-enoate	-			-				-	
Ethyl heptanoate	2.2	< 1	< 1	**2.17**	**2.62**	**1.75**	**15.4**	Fruity, pineapple, sweet, banana, berry, cognac and slightly green with a seedy nuance	[257,260,266]
Ethyl octanoate	65	< 1	< 1	**1.10**	**1.85**	**1.55**	< 1	Fruity, winey, pineapple, apricot, burned, earthy, flower	[135,241,255]
Ethyl nonanoate	377			< 1				Cheesy, fruity	[255]
Ethyl decanoate	23	< 1	< 1	**19.9**	**43.6**	**3.82**	**13.9**	Fruity, winey, fatty, flower, humidity	[135,255]
Ethyl dodecanoate	400	< 1	< 1	< 1	< 1	< 1	**3.23**	Flower, vanilla	[255]
Propyl butanoate	124			< 1				Fruity, sweet, pineapple, banana	[135,241]
Propyl hexanoate	-			-				Fruity, pineapple, blackberry, fatty	[135,241]
Propyl octanoate	-			-				Coconut	[257]
Butyl butanoate	100			< 1				Fruity, pineapple, banana, sweet, fatty	[135,241]
Butyl hexanoate	700			< 1				Flower, fruity, pineapple, mouldy	[135,255]
Pentyl butanoate	210			< 1				Sweet, fruity, banana, pineapple, cherry, tropical	[257]
Hexyl hexanoate	6400			< 1				Green, sweet, waxy, fruity with tropical and berry notes	[257]
1-Methylpropyl butanoate	-			-				Sweet, fruity, pineapple, rum, cherry, apple, overripe fruit	[257]
2-Methylpropyl hexanoate	-			-				Apple	[241]
2-Propenyl hexanoate	200			< 1				Pineapple, fatty-fruity	[241]
3-Methylbutyl hexanoate	320			< 1				Fruity, sweet, pineapple with a slightly pungent sour cheesy note	[257]
Hydrocarbons									
Heptane	950			< 1				Solvent, sweet-ethereal, diffusive	[241]
1,3-Pentadiene	2500			< 1				Plastic, paint, kerosene	[270]
t-3-Octene	-			-				Sharp, herbal, leather-like	[135]
1,3-Octadiene	5600			< 1				Woody-moss	[262]
Toluene	1	**20.2**	**42.9**	**24.9**	**112**	**19.9**	**68.0**	Fruity, sweet-gassy, hydrocarbon	[241]
Sulphur compounds									
Dimethyl sulphide	1.2	**1.29**	< 1	< 1	**14.3**	< 1	< 1	Unpleasant wild radish, cabbage, sulphurous, pomegranate, corn, earthy, rancid	[245,252,263,267]
Dimethyl sulphone	2.5	**10.0**	**3.22**	**2.96**	**24.5**	**3.10**	**2.71**	Sweet, flower, sulphurous, hot milk, burned	[245,250,260]
Terpenes									
D-Limonene	70			< 1				Grass	[255]

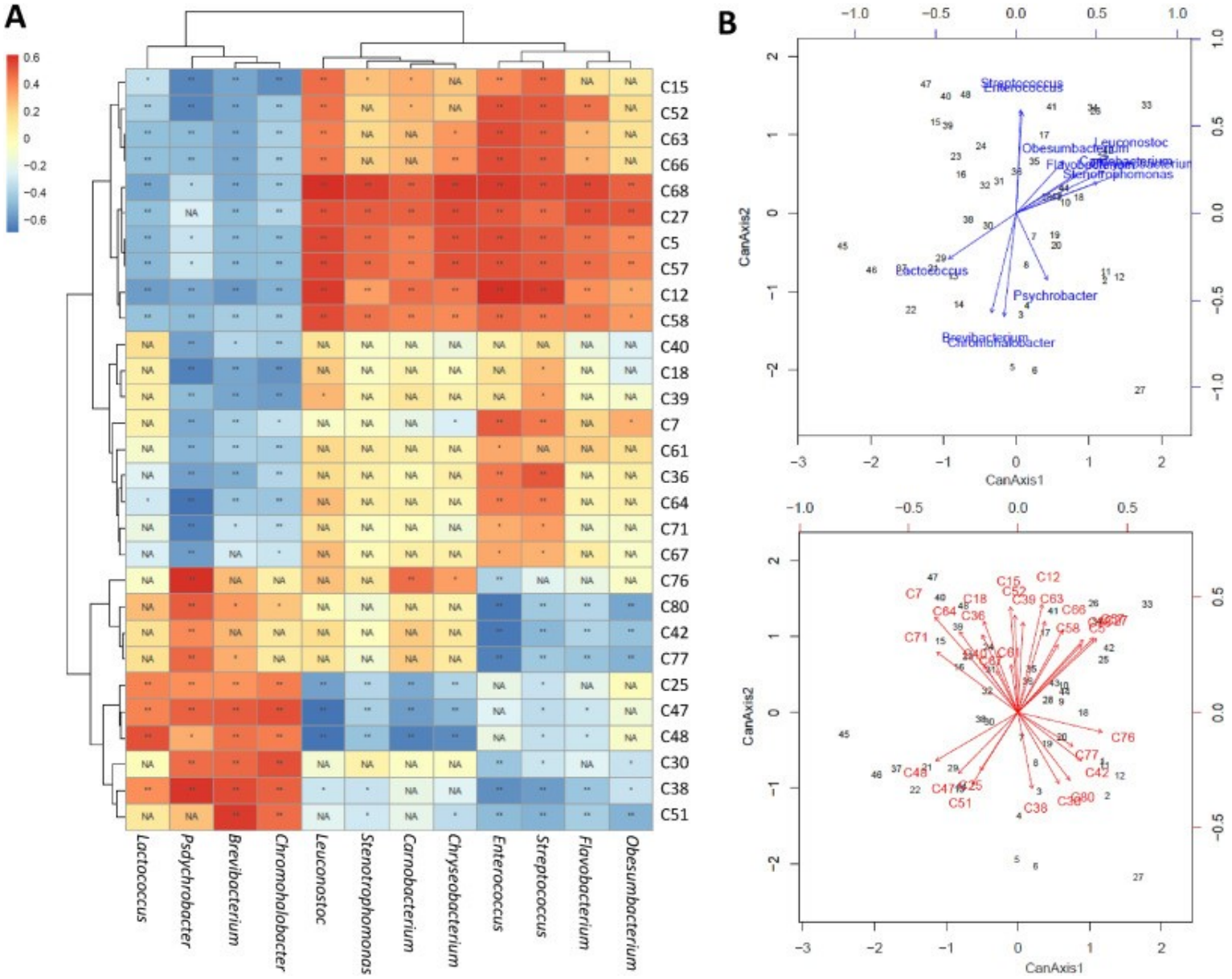
[1] UAk, µg/L-ko edo µg/kg-ko adierazita, honako hauetatik hartu dira: Abilleira et al. [135]; J. Wang et al. [251]; Natrella et al. [252]; Sarhir et al. [271]; Majcher and Jeleń [272]; Kubícková and Grosch [273]; Attaie [248]; van Gemert [274].

[2] UIRen balioak, 4 ekoizleen (A, B, C eta D) batez besteko ugaritasun erlatibo gisa kalkulatuak heltze-une bakoitzean (1, 7, 14, 30, 60, 120 egun)/UA. 1etik gorako UIRen balioak beltzez daude.

Konposatu lurrunkorren ekoizpena metabolismo mikrobiarrarekin erlazionatu da nagusiki [26,79]. Emaitzei erreparatuz, hamabi bakterio-genero funtsezkoak izan ziren Idiazabal gazten konposizio lurrunkorraren eboluziorako, hau da, *Psychrobacter*, *Enterococcus*, *Brevibacterium*, *Streptococcus*, *Leuconostoc*, *Chromohalobacter*, *Chryseobacterium*, *Carnobacterium*, *Lactococcus*, *Obesumbacterium*, *Stenotrophomonas* eta *Flavobacterium* generoak (11. irudia). Alde argiak ikusi ziren BALen eta ingurumeneko bakterioen edo bakterio ez-desiragarrien artean. BALen artean, *Enterococcus* eta *Streptococcus* generoek, eta neurri txikiagoan *Leuconostoc*ek, konposatu lurrunkorrekin korrelazio positibo handienak izan zituzten. Oro har, BAL hauek korrelazio positiboak izan zituzten azido lurrunkorrekin, batez ere azido 3-hexenoikoarekin eta oktanoikoarekin ($P \leq 0,01$); alkoholekin, batez ere 1-butanolarekin eta 2-propen-1-olarekin ($P \leq 0,01$); edo esterrekin, hala nola pentil butanoatoarekin, etil 4- hexenoatoarekin edo etil butanoatoarekin ($P \leq 0,01$) (11. irudia), horietako batzuk UIR balio altuak zituztelarik (7. taula). BALen eta konposatu horietako askoren arteko korrelazioak aurretik jakinarazi dira hartzitutako beste produktuetan [82,275,276], BALen metabolismoak konposatu lurrunkorren formazioan duen eragina baieztatuz [227,246,277]. Hala ere, aipatzekoa da kasu batzuetan ez dagoela informaziorik, adibidez, azido 3-hexenoikoaren kasuan edo esterrei dagokienez, pentil butanoatoaren edo etil 4-hexenoatoaren kasuan. *Lactococcus* BALAak korrelazio baxuagoak zituen, baina zetonekin korrelazio positiboak zitueneko BAL bakarra zen, zehazki 3-hidroxi-2-butanonarekin, zeinaren sintesia lehenago frogatu den genero horrentzat [26].

Ingurumen-bakterioek eta/edo bakterio ez-desiragarriek ere korrelazio positiboak izan zituzten konposatu lurrunkorrekin (11. irudia). *Psychrobacter*, *Brevibacterium* eta *Chromohalobacter* generoek, oro har, korrelazio positibo handienak erakutsi zituzten zetonekin, adibidez, 2-propanonarekin ($P \leq 0,01$), sufredun konposatuekin, adibidez, dimetil sulfonarekin ($P \leq 0,05$), edo *Psychrobacter*ren kasuan, hidrokarburoekin, adibidez, *t*-3-oktenoarekin ($P \leq 0,01$), besteak beste (11. irudia). UIR balioak kontuan hartuta (7. taula), ondoriozta daiteke ingurumen bakterio edo bakterio ez-desiragarri horiek ekarpen interesgarria egin ziotela Idiazabal gaztaren aromari. Oro har, orain arte ez da jakinarazi genero horiek konposatu horiek ekoizten dituztenik, baina beste konposatu lurrunkor batzuekin korrelazioak ikusi dira hartzitutako produktuetan [278,279]. Lipasa eta/edo proteasa jarduerak deskribatu dira aipatutako generoetako

batzuentzat, besteak beste, *Chryseobacterium*entzat eta *Stenotrophomonas*entzat [280,281], konposatu lurrunkorrak ekoizten laguntzen dutenak [26].



11. irudia. Funtsezko bakterio-generoen eta konposatu lurrunkorren arteko korrelazioen bero-mapa THArekin (0,500etik gorako korrelazio esanguratsuren bat duten konposatu lurrunkorrak soilik agertzen dira) (A) eta korrelazio kanonikoaren analisiaren (KKA) bidezko egiaztatzea (B). Konposatu lurrunkorrak IDaren arabera etiketatuta daude (6. taula). Korrelazio esanguratsuak honela adierazten dira: ** $P \leq 0{,}01$ eta * $P \leq 0{,}05$, eta korrelazio ez-esanguratsuak ($P > 0{,}05$) NA bidez.

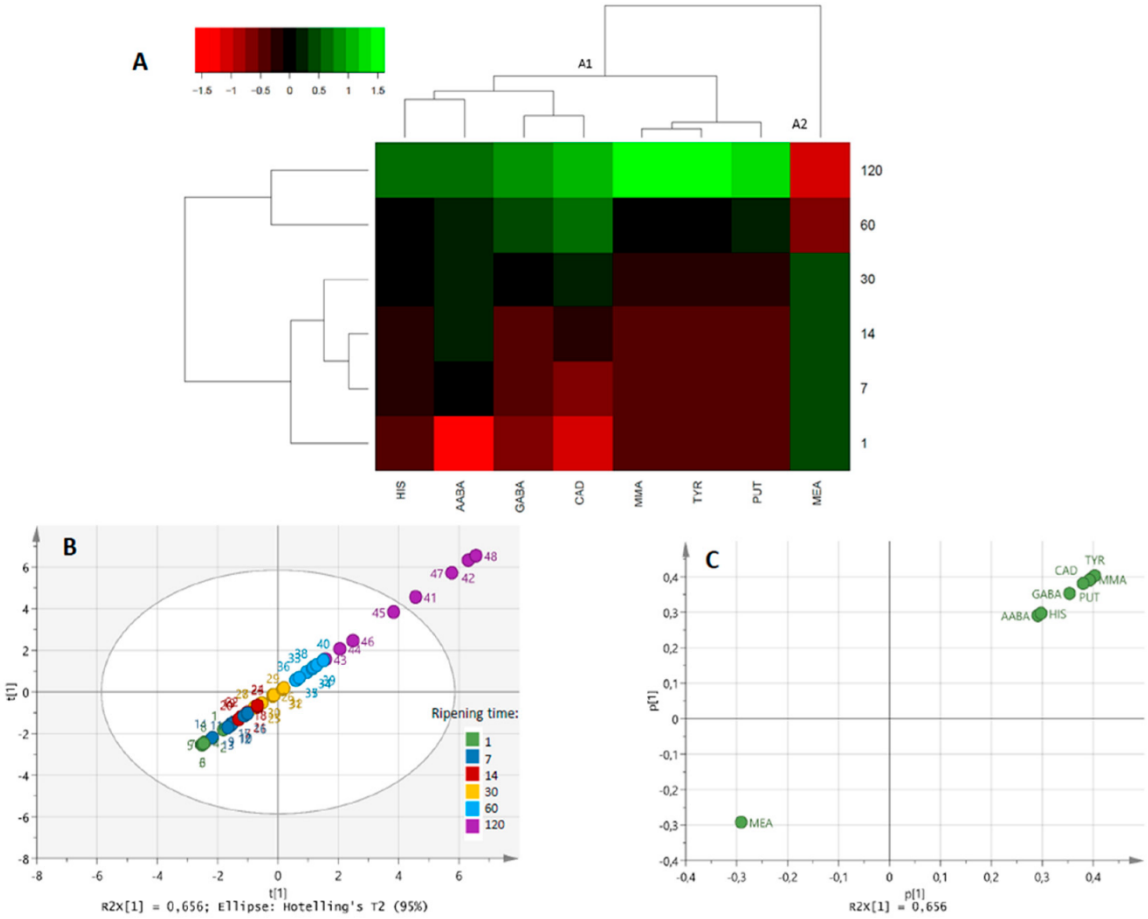
Santamarina-García et al.-en [243] argitaratuta.

Bestalde, zenbait korrelazio negatibo hauteman ziren bakterio-generoen eta konposatu lurrunkorren artean (11. irudia). Ingurumen-bakterioei dagokienez, *Psychrobacter*, *Brevibacterium* eta *Chromohalobacter* generoek korrelazio negatibo nabarienak zituzten, batez ere BALEAek sintetizatu ahal izan zituzketen konposatu lurrunkorrekin, adibidez alkoholekin eta esterrekin, eta alderantziz (BALEAek korrelazio negatiboak zituzten jatorria ingurumen-bakterioetan edota bakterio ez-desiragarrietan izan zezaketen konposatuekin, batez ere zetonekin eta, *Enterococcus*en kasuan, hidrokarburoekin) (11. irudia). Dagoeneko jakinarazi dira bakterio-generoen eta hartzitutako produktuen konposatu lurrunkorren arteko korrelazio negatiboak [276,282], bakterioek konposatu lurrunkorrak degradatzeko duten gaitasunekin erlazionatuta, baina orain arte gutxi deskribatu direnak [283].

4.2.4. ABak

Idiazabal gaztaren laginen heltzean zehar zortzi AB baino ez ziren identifikatu, hau da, AAAB, AGAB, KAD, HIS, EAM, MAM, PUT eta TIR (12. irudia). Oro har, ABen kontzentrazio totalak gora egin zuen heltzean, 4,29 ± 0,375 µmol/g-tik 14,9 ± 3,73 µmol/g-ra (120 egunetan). Indibidualki, AB gehienek goranzko joera erakutsi zuten. Hala ere, EMAk eredu ezberdin bat erakutsi zuen, konstante mantenduz 0,836 ± 0,0573 µmol/g-tan 30. egunera arte eta, oro har, gero behera eginez (12. irudia). Heltze-prozesuaren amaieran, MAM nagusi izan zen (3,16 ± 0,323 µmol/g-ko 120 egunetako gaztetan), eta ondoren PUT (2,72 ± 1,39 µmol/g-ko), KAD (2,58 ± 0,380 µmol/g-ko) eta AGAB (2,35 ± 1,12 µmol/g-ko). Ekoizleen arteko aldeak bakarrik HISen kasuan ikusi ziren ($P \leq 0.001$). Oro har, lortutako emaitzak bat datoz, partzialki, Idiazabal gaztari buruzko aurretiazko ikerketekin [284] eta ardi-esne gordinetik eratorritako beste gazta batzuekin [285–287], izan ere, aldeak daude identifikatutako ABetan eta/edo heltzean zehar duten bilakaeran [284–287]. SPD eta SPM poliamina endogenoak ez ziren detektatu, identifikatutako ABak batez ere deskarboxilazio bakterianoen ondorio direla adieraziz [108]. AB nagusiei dagokienez, MAMren inguruan informazio mugatua dago literaturan, baina maila altuetan abereentzat kaltegarritzat jotzen da [288]. PUT eta KAD, AB toxiko ezagunak direnak, toxikotasun mugen azpitik zeuden [111]. Nabarmentzekoa da AGABren prebalentzia bereziki interesgarria dela, osasunean dituen eragin positiboengatik, besteak beste, loaren nahasmenduen modulazioa, denbora eta espazioaren memoriaren hobekuntza [289], epilepsiaren tratamendua

[290], diabetesaren kudeaketa [291], depresioaren arintzea [292], eta minbiziaren tratamendua [293].



12. irudia. THAren bero-mapa (A) eta ONAren puntuazioen eta kargen grafikoak (B eta C, hurrenez hurren) Idiazabal gaztaren laginen heltzean zehar (1, 7, 14, 30, 60 eta 120 egun) identifikatutako ABetan oinarritua. Laginen identifikazioa 1etik 48ra doa. Horrela, 1etik 8ra bitarteko zenbakiak gaztagile bakoitzaren (A, B, C edo D) lehenengo heltze eguneko lagin bikoiztuei dagozkie. ABen nomenklatura XIX. eta XX. orrialdetan azaltzen da. Santamarina-García et al.-en argitaratuta [137].

ABen eboluzioarekin zerikusi estua zuten bakterio-generoak honako hauek izan ziren: *Lactobacillus*, *Erwinia*, *Chromohalobacter*, *Pantoea*, *Bacillus*, *Ruminococcus*, *Serratia* eta *Raoultella* (9C. irudia). Bakterio horien artean desberdintasun argi bat hauteman zen, *Lactobacillus* ABekin harreman positiboena zuen bakterio nagusia zen, zehazki TIRekin, AGABrekin eta KADrekin ($P \leq 0{,}01$); eta ingurumen-bakterioek eta bakterio ez-desiragarri gehienek korrelazio negatiboak zituzten, batez ere *Erwinia*k AGABrekin, MAMrekin edo KADrekin ($P \leq 0{,}01$). Hala ere, *Chromohalobacter*rek korrelazio positiboa zuen EAMrekin ($P \leq 0{,}01$) (9C. irudia). *Lactobacillus* generoko zenbait andui deskarboxilasak sintetizatzen dituzten bakterio gisa jakinarazi dira [294–

297], halotoleranteak barne [298,299]. Hori dela eta, hainbat korrelazio positiboren berri eman da *Lactobacillus*en eta AB ezberdinen artean, hala nola TIR, PUT edo HIS, beste elikagai hartzituetan [300,301]. Hala ere, emaitzak produktuaren araberakoak dira [302]. Era berean, *Chromohalobacter* generoaren eta ABen arteko korrelazio positiboak jakinarazi dira [303], baina ez dago informaziorik MEArentzat. Beste produktu hartzituetan korrelazio negatiboak ere jakinarazi dira [300,301], ABen degradazio-gaitasunekin erlazionatu direnak [303], nahiz eta *Lactobacillus* eta *Bacillus* espezieentzat bakarrik frogatu den gaitasun hau [304–307].

4.3. Mikrobiotaren eta antimikrobianoekiko erresistentzien (AME) arteko erlazioa (3. helburua, 3.1.-3.3. azpihelburuak, IV.-VI. eskuizkribuak)

Ondoren, mikrobiotak gaztaren kaltegabetasuna zenbateraino arriskuan jar dezakeen aztertu zen, AMEei dagokienez. Horrela, lehenik eta behin, esnekietan antibiotikoen hondakinen eta AMEen egungo ezagutza berrikusi zen (**IV. eskuizkribua**). Ondoren, Idiazabal gazta Jatorri Deitura Babestuko (JDB) gaztandegien antibiotikoen erabilerari buruzko ezagutzak eta praktikak ebaluatu ziren, baita Idiazabal gaztaren produkzioan zehar hondakinen agerpena ere (**V. eskuizkribua**). Azkenik, Idiazabal gaztaren ekoizpenean AMEen prebalentzia aztertu zen, Idiazabal gaztaren bakterio nagusietan arreta jarrita, hau da, BALetan (**VI. eskuizkribua**).

4.3.1. Berrikuspen bibliografikoa

4.3.1.1. Antibiotikoen erabilera esne-animalietan

Antibiotikoak asko erabiltzen dira abeltzaintzan, antibiotikoen erabilera globalaren % 73a baita [308]. Mastitisa esnekien produkzioan gehien agertzen den gaixotasuna da, eta galera ekonomikoak eragiten ditu kontrol-programak izanda ere [309]. Mastitisari eta, oro har, gaixotasun infekziosoei aurre egiteko, behietan erabiltzen diren antimikrobiano mota ohikoenak β-laktamikoak, tetraziklinak, sulfonamidak, makrolidoak eta linkosamidak dira [310,311]. Azken aldian, ardien eta ahuntzen esne-ekoizpenak gora egin duenez, antimikrobioanoak gehiago erabiltzen dira mastitisa eta beste gaixotasun batzuk tratatzeko, baina aukerak nahiko mugatuak dira kasu hauetan [309,312–314]. Oro har, antibiotikoen erabilerari buruzko datu gehienak behiei buruzkoak dira [310,311], hausnarkari txikiei (ardiak, adibidez) buruzko informazio mugatuarekin [309].

4.3.1.2. Esneko antibiotiko hondarren jatorria

Esneko antibiotiko hondarrak esne-animaliei antibiotikoak ematean ager daitezke, bai modu parenteralean, bai errape-barnekoan [315,316]. Errape-barneko tratamenduak kontzentrazio altuagoak eta iraunkorragoak eragiten ditu esnean bide parenteralarekin alderatuta, ugatz-guruinera zuzenean sartzearen ondorioz [316,317]. Zenbait faktorek eragina dute esnearen hondakin-mailetan, animalien osasunak eta antibiotikoaren ezaugarriek (mota, dosia, emateko metodoa, etab.) barne [318]. Agintari arautzaileek gehienezko hondakin-mugak (GHM) ezartzen dituzte esnean topa daitezkeneko antibiotikoentzat, ikerketa zientifikoen bidez, kontsumitzailearen segurtasuna bermatzeko [99,315]. Era berean, erretiro-aldiak ezartzen dira, hots, azken antibiotikoak ematen direnetik esnea ekoizten den arte behar den gutxieneko denbora, osasun publikoan eraginik ez duela bermatzeko [314]. Epe horiek aldatu egiten dira antibiotikoaren eta animaliaren arabera, zientifikoki zehaztutakoaren eta hornitzaileek adierazitakoaren arabera [314].

4.3.1.3. Antibiotiko hondarrak munduko esne komertzialetan

Zenbait ikerketak frogatu dute behi-esne komertzialean antibiotikoen hondarrak daudela mundu osoan [308,319–321]. Hala ere, oso ikerketa gutxik aztertzen dute hausnarkari txikien esnea, hala nola ahuntzena [322,323] eta ardiena [324,325]. Ardi eta ahuntz esnea Mediterraneoko herrialdeetan, hala nola Espainian, Frantzian, Italian eta Grezian, esnekiak egiteko erabiltzen da nagusiki, adibidez gazta eta jogurta [314]. Horregatik, esne hori ez dago ESEAaren zaintza-txostenetan, nahiz eta Espainiak antibiotikoen hondakinen testak egiten dituen esnea erabili aurretik [314]. Azterketa gutxi batzuek baino ez dituzte aztertu ardi- edo ahuntz-esne laginen kopuru handiak, adibidez, Espainiako ikerketa batek % 1,7-ko emaitza positiboak aurkitu zituen Mantxako artaldeen ardi-esne gordineko 2.686 laginetan [324]. Espainiako beste ikerketa batek erakutsi zuen ezarritako legezko mugekin bat ez zetozen laginak % 1,36tik % 0,30era jaitsi zirela 2004-2008 aldian, ziurrenik hezkuntza-programen ondorioz [325]. Ahuntz-esne komertzialari buruzko datuak Afrikatik eta Asiatik datoz batez ere eta ezarritako GHMen gainetik dauden hondakinak jakinarazi dituzte [322,323], lehen aipatutako ardi-esneari buruzko azterlanek baino proportzio handiagoetan [324,325].

4.3.1.4. Esnearen antibiotiko hondarrak esnekietara transferitzea

Hainbat ikerketak aztertu dute antibiotiko hondarren joera esnekiak ekoiztean, hala nola jogurta edo gazta [100,326,327]. Hala ere, gehienek ikuspegi esperimentaletan jartzen dute arreta, nahita kutsatutako laginekin, eta ez benetako ekoizpen-sistemetan [100,326,327]. Gaztagintzari dagokionez, oro har, antibiotiko β-laktamikoak gazurara transferitzen dira nagusiki, uretan disolbagarriak direlako, gaztan kontzentrazio txikia suposatuz [328–330]. Aminoglukosidoek, tetraziklinek eta kinolonek, berriz, gaztarekiko atxikipen handiagoa dute [100,330–332]. Bestalde, gaztaren heltze-prozesuan zehar antibiotikoen edukia murrizten doa, molekulak denborarekin degradatzen direlako. Hala ere, murrizketa-maila aldatu egiten da gazta moten eta azterlanen artean, heltze-baldintzek eragina izan dezaketelako [100,101,333,334].

4.3.1.5. Antibiotiko hondarrak esneki komertzialetan

Argitaratutako ikerketa gutxik aztertzen dituzte esneki komertzialetan dauden hondakinak, batez ere Afrikatik eta Asiatik datozenak, non bertako gazta, jogurta eta beste produktu batzuk aztertzen diren [335–339]. Oro har, hondakinen detekzio-tasa altuagoak jakinarazi dira eskualde horietako behi-esnearekin alderatuta, baina ez da GHMrik ezarri produktu hauetarako [335–339]. Gainera, produktu hauen azterketa ez da sartzen zaintza-txostenetan [340,341].

4.3.1.6. Antibiotiko hondarren eragina esnekietan

Esnean antibiotikoen hondarrak egoteak eragina izan dezake esneki hartzituen ekoizpenean, hala nola gaztarenean [342]. Antibiotikoek BALen hazkuntza inhibitu dezakete partzialki edo erabat [343], eta, beraz, azidotze zinetika eta koagulazio denbora atzeratu daiteke, eta horrek prozesatze denbora gehigarria suposatzen du nahi den pH-a lortzeko [100,343]. Azidotzea eskasa izanez gero, hartzidura goiztiarra gerta daiteke mikroorganismo ez-desiragarrien bidez (klostridioak edo legamiak, adibidez), gaztaren ezaugarri sentsorialetan akatsak eraginez [343]. Inpaktu txikiagoak ere deskribatu dira, hala nola GAAen, kolorearen edo testuraren aldaketak, eritromizina eta oxitetraziklina konposatu kaltegarrienak direlarik [100,101,333]. Bestetik, emaitza kontrajarriak argitaratu dira β-laktamikoentzat [100,344] eta kinolonek eraginik ez dutela iradoki da [334].

4.3.1.7. Antibiotikoen erabilera eta antibiotikoekiko erresistenteak diren bakterioen agerpena

Abeltzaintzan antimikrobianoak erabiltzea edo kontsumoa (AMK) zeharka lotuta egon da elikagaietan antibiotikoekiko erresistenteak diren bakterioak (AEB) egotearekin [345–347], nahiz eta erlazioa ezin den oraindik zehaztu zenbait azterlanen arabera [348,349]. Horren arrazoia izan daiteke AMEen sorreran eta hedapenean inplikatutako prozesu konplexuak ulertzeko eredu egokirik ez izatea [348]. Frogatu da antibiotikoen dosi subterapeutikoek aldakortasun genetikoa sustatzen dutela eta elikagaietan AEBen zenbaketa altuagoak eragiten dituztela, esnean barne [348,350]. Azterketa esperimentalek frogatu dute antibiotikoekin tratatutako behien esnetik isolatutako bakterioek erresistentzia handiagoa dutela tratatu gabeko behi-esnearen aldean [351,352]. Animalietan AMKren eta *Escherichia coli* eta *Campylobacter jejuni* erresistenteak topatzearen artean ere erlazio estatistikoak eman dira [353]. Hala ere, beste ikerketa batzuek ez dute erlazio esanguratsurik aurkitu [354–356]. Oro har, esnekiekin lotutako bakterio erresistente nagusien artean *Staphylococcus* sp. eta *Enterococcus* sp. daude, esaterako. Bakterio horiek antimikrobianoekiko erresistentzia geneak (AEG) dituzte, eta AME adierazten dute [94,357–359]. Hala ere, informazio mugatua dago esnekietatik isolatutako BALen AMEei buruz [346,360–362], eta horrek garrantzi berezia du, gaztaren mikrobiotaren zati handi bat suposatzen dutelako (4.1.3. sekzioa).

4.3.1.8. Beste alderdi batzuk

Elikagaietan eta, bereziki, esne eta esnekietan antibiotikoen hondakinak egoteak mehatxu larriak ekar ditzake gizakien osasunerako [363]. GHMk gainditzeak ondorio toxikoak eragin ditzake, hala nola alergiak, efektu immunopatologikoak, kartzinogenizitatea, mutagenizitatea, zenbait organoren asaldurak, ugalketa-desordenak eta baita shock anafilaktikoak ere [315,364]. GHM balioak eguneroko kontsumo onargarrian (EKO) oinarritzen dira, hau da, bizitza osoan zehar osasunarentzat arrisku hautemangarririk gabe egunero kontsumitu daitekeen substantzia baten kantitatean [363]. Antibiotikoen hondakinek hesteko mikrobiotaren osaeran eta funtzionaltasunean ere eragina izan dezakete [365]. Ikerketa bakar batek ere ez du aztertu esnearen eta esnekien antibiotiko hondarrek giza mikrobiotan duten eragina, baina ikerketa batek egiaztatu du sagu gazteei antibiotikoen dosi azpiterapeutikoak emateak haien mikrobiomaren garapena eten eta metabolismo hepatikoa aldatzen duela [366].

Esne-abeltzaintzako antibiotikoen hondakinek eragina dute ingurumenean ere [367]. Elikadurarako animalietan erabiltzen diren antibiotikoen % 75-90a gorotz-, gernu- eta gazur-hondakinetan iraizten dira, eta horrek eragina izan dezake lurzoruko mikrobiotan eta lurpeko uren kalitatean [368]. Gorotzen eta gazuraren azpiprodukTuak animalientzako ongarri edo pentsu gisa ere erabiltzen dira batzuetan, eta horrek bide gehiago ematen ditu hondakinak [369], AEBak eta AEGak ingurumenera transferitzeko [370].

4.3.2. Zeharkako inkesta

Inkestaren emaitzen arabera (8. taula), Idiazabal gaztaren JDBko fabrikatzaileen artean, ekoizle gehienek (% 93,3k) antibiotikoak ematen zizkieten haien artaldeei, normalean hilean behin baino gutxiagotan (% 64,3k), β-laktamikoak (adibidez, G penizilina eta amoxizilina) eta tetraziklinak (adibidez, tetraziklina eta oxitraziklina) nagusienak izanda, aurretik aipatu den bezala [139,140]. Hala ere, erabilitako konposatuak eta dosiak aldatu egiten dira baserrien eta lurraldeen artean [104,139,371]. Proportzio handi batek (% 92,9k) antibiotikoak erabiltzen zituen bakarrik infekzio baten ziurtasuna zegoenean, baina batzuk (% 7,14k) erabateko ziurtasunik gabe. Gainera, % 14,3k esan zuen antibiotikoak prebenitzeko erabiltzen zituztela, tratamendurako erabiltzeaz gain. Ekoizle guztiek albaitaritza zerbitzuen bitartez lortzen zituzten antibiotikoak, beste ikerketa batzuetan ez bezala, non hornikuntza errezetarik gabeko eskuratzea behatu den [103,140,372]. Ekoizle gehienek ez zuten antibiotikoei buruzko prestakuntza formalik edo informalik (% 86,7k), baina gehienek zehatz definitu zituzten antibiotikoak (% 60,0k), eta AMEekiko kezka erakutsi zuten (% 93,3k), bat datorrena aurreko ikerketekin [103,104,139,372,373]. Era berean, ekoizleek animalien osasunerako albaitaritza-zerbitzuekiko duten mendekotasuna oso urria zen, urtean behin edo bitan erabiliz (% 46,7k). Antibiotikoen administrazioan albaitarien zeregina mugatua izan zen (% 13,3an), gehienak jabeek edo enplegatuek eman baitzituzten (% 35,7an eta % 28,6an), guztiek dosiak eta administratzeko moduak errespetatuz. Gainera, ekoizleen % 53,3k bakarrik aztertu zuten antibiotikoen presentzia eta kontzentrazioa esnean eta gaztan. Beste ikerketa batzuekin alderatuta, emaitzak nabarmen aldatzen dira lurraldeen artean, baina, oro har, bat datoz aurretik jakinarazitako ezjakintasunarekin [103,140,371,373]. Ondorioz, ekoizleen hezkuntza funtsezkoa da antibiotikoak zuhurtziaz ematen laguntzeko [371].

8. taula. Ekoizleen antibiotikoen erabilera eta ezagutza. Santamarina-García et al.-en [141] argitaratuta.

Item	Percentage (%)
Training (formal or informal) on antibiotics	
Yes	6.67
No	86.7
No answer/don't know	6.67
Correct description of what antibiotics are	
Yes	53.3
No	40.0
No answer/do not know	6.67
In your experience, antibiotics are now less effective than in the past:	
I strongly disagree	0.00
I do not agree	13.3
Not sure	66.7
I agree	0.00
I strongly agree	6.67
No answer/don't know	13.3
Are you worried about antibiotic resistance?	
Yes	93.3
No	0.00
I do not know what antibiotic resistance is	0.00
No answer/don't know	6.67
Ever given antibiotics to animals	
Yes	93.3
No	6.7
Ease or difficulty of obtaining antibiotics	
Easy	53.3
Difficult	33.3
According to the veterinary staff	6.7
No answer/do not know	6.7
Antibiotics used	
Amoxicillin	5.26
Penicillin G	36.8
Dihydrostreptomycin	26.3
Oxytetracycline	15.8
Polymyxin b	10.5
Tetracycline	5.3
Antibiotics adquisition	
Veterinary staff	80.0
Veterinary staff of external company	13.3
Association	6.7
Availability of veterinary staff	
Always	33.3
Sometimes	53.3
Never	0.0
No answer/do not know	13.3
Veterinary services's use for the treatment of livestock diseases	
Never	6.67
Once a month	13.3
1 or 2 times a year	46.7
More than 2 times a year	40.0
Person administering antibiotics	
Veterinary staff	14.3
Employee	28.6
Owner	35.7
Veterinary staff and/or owner	21.4
Administered dose	
Dosage recommended by the veterinarian	100
Dose given based on your experience	0.0
Dose administered based on the recommendation of another farmer	0.0
When antibiotics are given to animal?	
When I am sure that the animal has a bacterial infection	92.9
When I am sure that the animal has a bacterial infection, whenever the animal is sick and/or whenever the animal appears ill	7.14
More than once a month	7.14
Once a month	0.0
Less than once a month	64.3
According to the need	21.4
No answer/do not know	7.14
Method of antibiotics administration	
Along with the feed	0.0
Along with the drinking water	0.0
Parenterally (injected)	100
Herd management during antibiotic administration	
Antibiotics are given always or often along with feed or drinking water to keep animals healthy and prevent disease.	0.0
Antibiotics are given to all animals in the herd, when some of the animals are sick.	0.0
Antibiotics are given only to sick animals	100
How long does it take for the antibiotic to disappear from the milk?	
Same day of treatment	0.00
3 days after treatment	0.00
One week after treatment	0.00
According to the antibiotic	92.9
I do not know.	7.14
Has milk or cheese ever been tested for the presence and/or concentration of antibiotics?	
Yes, routine within dairy control	53.3
Yes, once within dairy control	6.67
Yes, once within dairy control and sometime of my own free will	6.67
Yes, routine within dairy control and sometime of my own free will	6.67
Never	26.7
Method used to examine the presence and/or concentration of antibiotics in milk or cheese	
Inhibitor analysis	13.3
No answer/don't know	86.7

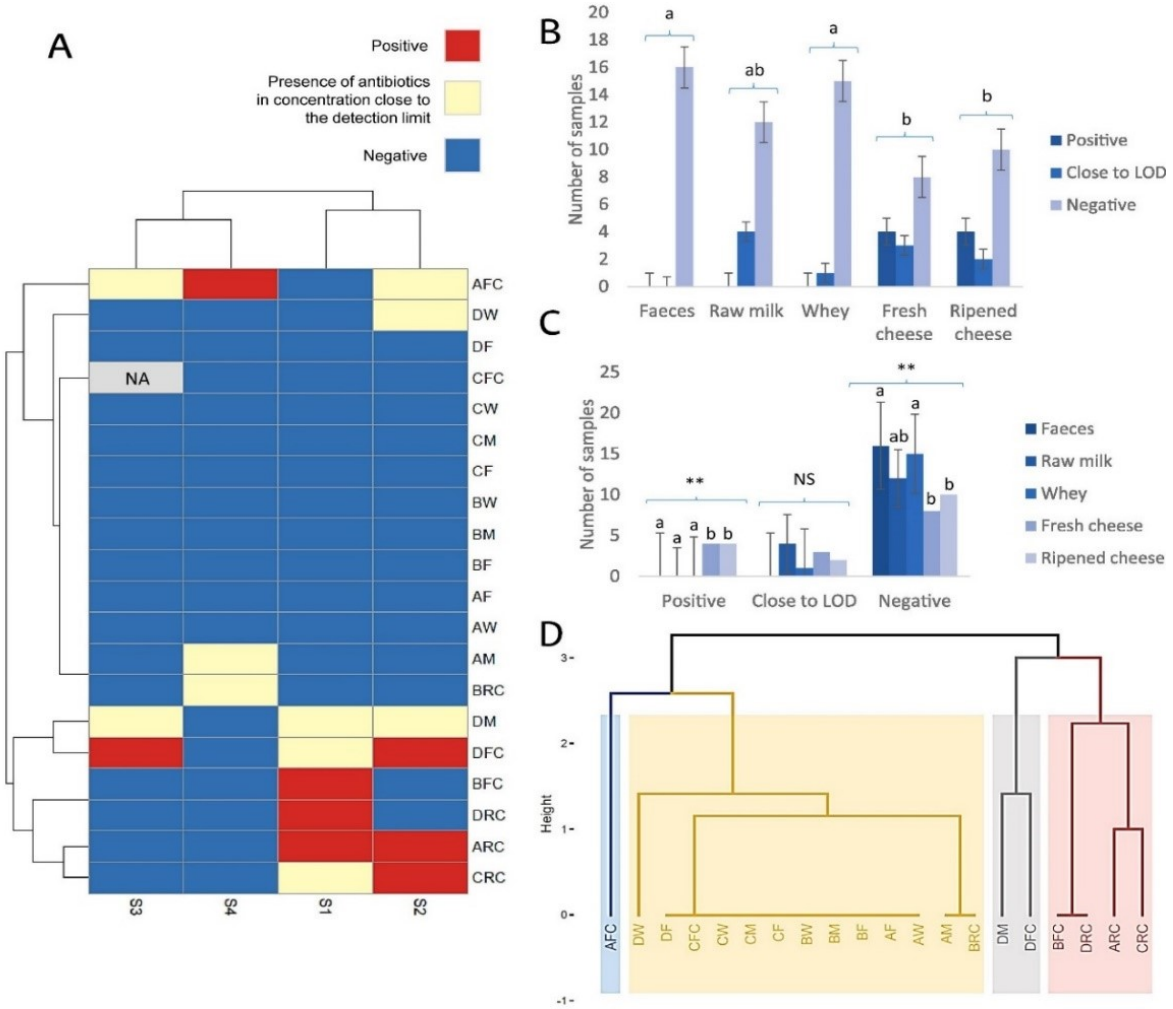
Faktore soziodemografikoen eta antibiotikoen erabilerari buruzko ezagutzen eta praktiken arteko erlazioak urriak ziren. Ikasketa-maila baxueneko (lehen hezkuntza) ekoizleak ziren antibiotikoetan prestakuntza zuten bakarrak ($P < 0,01$), eta eremu profesionalari dagokionez,

prestatutakoak izan ziren antibiotikoak modu sendagarrian eta prebentiboan erabili zituzten bakarrak ($P < 0,05$). Oro har, lotura gutxiago hauteman ziren generoaren, hezkuntzaren eta kokapenaren, eta antibiotikoen erabilera okerraren artean beste azterlan batzuekin alderatuta [103,371]. Hala ere, ekoizleek antibiotikoen erabilerari buruz dituzten ezagutzen eta praktiken arteko loturak ikusi ziren. Besteak beste, antibiotikoei buruzko trebakuntza jaso zuten ekoizleek, antibiotikoen hondakinak aztertzeko joera handiagoa izan zuten ($P < 0,01$); edo antibiotikoak erabili zituzten ekoizleek, infekzio bakterianoa zela ziur zeudenean, animalia sendatzeko baino ez zituzten erabili, eta infekzioaz ziur ez zeudenek modu prebentiboan erabili zituzten ($P < 0,01$). Eraginkortasunari buruzko iritziak administrazioarekin lotuta zeuden; izan ere, antibiotikoak inoiz administratu ez zituzten ekoizleek adierazi zuten antibiotikoak ez direla hain eraginkorrak orain iraganarekin alderatuta. Oro har, erlazio horiek berresten dute oso garrantzitsua dela ekoizleak antibiotikoen eta praktika egokien inguruan heztea, horiek modu egokian erabiltzen direla bermatzeko eta elikagaiak ekoizteko katean ez egoteko [140,371].

4.3.3. Antibiotiko hondarrak

Baheketa testen bidez, produkzio-katean bildutako laginen % 76,3k emaitza negatiboak eman zituen; % 12,5a (10/80), berriz, detekzio-mugara (DM) hurbildu zen, eta % 10,0k (8/80) emaitza positiboak eman zituen (13. irudia). Alde nabarmenak ikusi ziren lagin moten artean ($P \leq 0.01$) (13. irudia). Gorotz lagin guztiek negatibo eman zuten. Esne gordinaren laginak ere ez ziren positiboak izan, baina % 25,0a (4/16) DMtik gertu egon zen, gazur laginen antzera, horien artean, % 6,25a (1/16) DMtik gertu zegoen (13. irudia). Esnean antibiotikoen hondarrak detektatzeak eta gorotzetan ez detektatzeak ez du zentzurik, kontuan izanda konposatu horiek batez ere gernuaren eta gorotzen bitartez iraizten direla [2]. Antzemandako desadostasuna proben DMetan dauden desberdintasunen ondorio izan daiteke, Charm KIS testak Eclipse Farm^{3G} testak baino DM altuagoak (10^2–10^3 aldiz) baititu (3.3.9.1. sekzioa). Ezarritako GHMetan oinarrituta, esne gordinaren lagin guztiak mugen azpitik daude [374], azken hamarkadan ikusitako ez-betetze tasa baxuarekin bat etorriz, EFSAk (% 0,09 – 0,44) [374] eta Ameriketako Estatu Batuetako Elikagaien eta Sendagaien Administrazioak (ESA) (% 0,02 – 0,008) [340] adierazi duten bezala. Hala ere, emaitza horiek behi-esneari buruzkoak dira; ardien esneari buruzko

informazioa mugatua da [2], aurretik aipatu den bezala (4.3.1. sekzioa). Ardi-esneari buruz argitaratutako emaitzek, oro har, ez-betetze tasa handiagoak erakusten dituzte [325,375].



13. irudia. THAren bero-mapa (A), barra-diagramak (B eta C) eta THAren dendrograma (D) baheketa testen (Charm KIS eta Eclipse Farm^{3G}) emaitzetan oinarrituta (D). Letra xeheek ezberdintasun esanguratsuak adierazten dituzte. Laburdurak: NA: ezin da aztertu; S1: lehen laginketa astea; S2: bigarren laginketa astea; S3: hirugarren laginketa astea; S4: laugarren laginketa astea; AF, BF, CF, DF: A, B, C eta D ekoizleen gorotz-laginak, hurrenez hurren; AM, BM, CM, DM: A, B, C eta D ekoizleen esne-laginak, hurrenez hurren; AW, BW, CW, DW: A, B, C eta D ekoizleen gazur-laginak, hurrenez hurren; AFC, BFC, CFC, DFC: A, B, C eta D ekoizleen gazta fresko-laginak, hurrenez hurren; ARC, BRC, CRC, DRC: A, B, C eta D ekoizleen gazta heldu-laginak (60 egun), hurrenez hurren; **: $P \leq 0{,}01$; NS: ez-esanguratsua, $P > 0{,}05$. Santamarina-García et al.-en [141] argitaratuta.

Gazta freskoen kasuan, % 18,8k (3/16) DMtik gertuko emaitzak eman zituen, eta % 25,0k (4/16) emaitza positiboa eman zuen. Heldu ondoren, laginen % 25,0k (4/16) emaitza positiboa eman zuen, eta % 12,5ek (2/16) DMtik hurbil (13. irudia). Azpimarratzekoa da gazta freskoetan

eta gazta helduetan DMtik hurbil edo positibo gisa sailkatutako emaitza batzuk faltsuak zirela (3/16 eta 5/16, hurrenez hurren), gaztak egiteko erabilitako esne laginak negatibo eman zutelako (13. irudia). Detekzio-proben emaitza faltsu horiek bakterioen hazkundea inhibitzen duten substantziekin lotu ziren, hala nola lisozimarekin edo GAAekin [376]. Hori guztia kontuan hartuta, emaitzetako batzuen arabera, Latxa ardi-esne gordinean dauden antibiotikoak Idiazabal gaztan kontzentratu daitezke, eta horrek interes berezia du, kontuan hartuta elikagaien kaltegabetasunerako agintari nazionalek eta nazioartekoek ez dituztela esnekiak kontrolatzen [340,374]. Lortutako beste emaitza batzuek berriz, heltze-prozesuaren degradazio-eragina, aurretik aipatutakoa (4.3.1.4. sekzioa) berresten dutela dirudi. Orain arte ez da argitaratu gaztan antibiotikoak detektatzeko proben emaitzarik, eta hori interesgarria izan liteke elikagaien kaltegabetasuna sustatzeko eta, aldi berean, ekoizleei galera ekonomikoak saihesteko, antibiotikodun gisa gaizki sailkatutako laginak deuseztatu behar ez izateagatik, eta analisi berresleen beharra murrizteagatik [343].

Gorotzen antibiotikoen erauzketa eta analisi kromatografikoa Euskal Herriko Unibertsitateko Kimika Analitikoko Sailak eta Itsas Biologia eta Bioteknologia Esperimentaleko Ikerketa Zentroak (PiE) egin zuten, eta emaitzak aurretik argitaratu dira [377]. Tesi honetan emaitza horiek esnekiekin alderatzeko baino ez dira erabiltzen. Zenbait antibiotiko atzeman ziren gorotzetan, zehazki, kinolona bat (enrofloxazina), bi sulfonamida (sulfadiazina eta sulfametazina) eta bi tetraziklina (oxitraziklina eta klortetraziklina), klortetraziklina nagusi izanda (3,00 ± 4,24 - 52,0 ± 58,4 µg/kg), eta sulfametazina jarraian (2,10 ± 0,28 - 45,0 ± 2,20 µg/kg). Aurkitutako kontzentrazio horiek baheketa testaren DMen azpitik daude (3.3.9.1.1. sekzioa), baheketa metodoaren eta metodo analitikoren artean hautemandako desadostasuna azalduz. Gorotz-laginetarako detekzio-proba espezifikoak garatzeak alde interesdunen gaitasuna areagotu lezake, animalia batek konposatu jakin bat organismoan noiz ez duen zehazteko, eta, horrela, ingurumenera eta elikadura-katera lekualdatzea eragotziz [2,378]. Identifikatutako konposatuak aurretik identifikatu dira abere-gorotzetan [2,379], ardiak barne [380], gorotzen bidezko antibiotikoen iraizketa baieztatuz [2]. Horrela, emandako antibiotikoen % 70-90 inguru, metabolizatu gabe edo metabolito aktibo gisa, animalien gorotzetan eta gernuan daudela jakinarazi da [103]. Tetraziklinak, kinolonak, sulfonamidak eta makrolidoak maiz antzeman dira simaurretan [2,381]. Hala ere, emaitza hauek bereziki interesgarriak dira, gorotz-laginak, printzipioz, antibiotikoak jaso ez zituzten artalde osasuntsuetatik lortu baitziren.

9. taula. Lau ekoizleen (A, B, C eta D) Idiazabal gaztaren ekoizpen-katean zehar identifikatutako antibiotikoen batez besteko kontzentrazioa ± desbiderapen estandarra. Santamarina-García et al.-en [141] argitaratuta.

Antibiotics		Antibiotic concentration (μg/kg)[1]																				P-value[3]	
Class	Compound	Faeces[2]				Raw milk				Whey				Fresh cheese				Ripened cheese					
		Producer				Producer				Producer				Producer				Producer					
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	C	P
Ansamycin	Rifaximin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (penicillin)	Amoxicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ampicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Penicillin G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Dicloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Nafcillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephapirin + Desacetylcephapirin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefoperazone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalexin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (cephalosporin)	Cefquinome	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalonium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ceftiofur + Desfuroylceftiofur	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefazolin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefacetril	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefuroxime	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Lincosamide	Lincomycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Tylosin	n.d.	n.d.	n.d.	n.d.	**6.15 ± 12.3**	n.d.	**6.98 ± 8.24**	n.d.	**5.35 ± 10.7**	n.d.	**6.28 ± 7.39**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	0.052
Macrolide	Erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Spiramycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Tilmicosin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Quinolone	Enrofloxacin + Ciprofloxacin	n.d.	**2.05 ± 2.90**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.092	0.392
	Flumequine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sarafloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Danofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Marbofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxolinic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Sulfonamide	Sulfadiazine	n.d.	**9.10 ± 5.52**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**< 0.001**	0.108
	Sulfathiazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfapyridine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamerazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamethazine	**45.0 ± 2.20**	n.d.	**2.30 ± 0.850**	**2.10 ± 0.28**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**< 0.001**	0.540
	Sulfachloropyridazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfaquinoxaline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfametizole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Tetracycline	Oxytetracycline	**9.30 ± 0.60**	n.d.	**1.75 ± 2.47**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**< 0.001**	0.278
	Tetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Chlortetracycline	n.d.	**3.00 ± 4.24**	n.d.	**52.0 ± 58.4**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**< 0.001**	0.278
	Doxycycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000

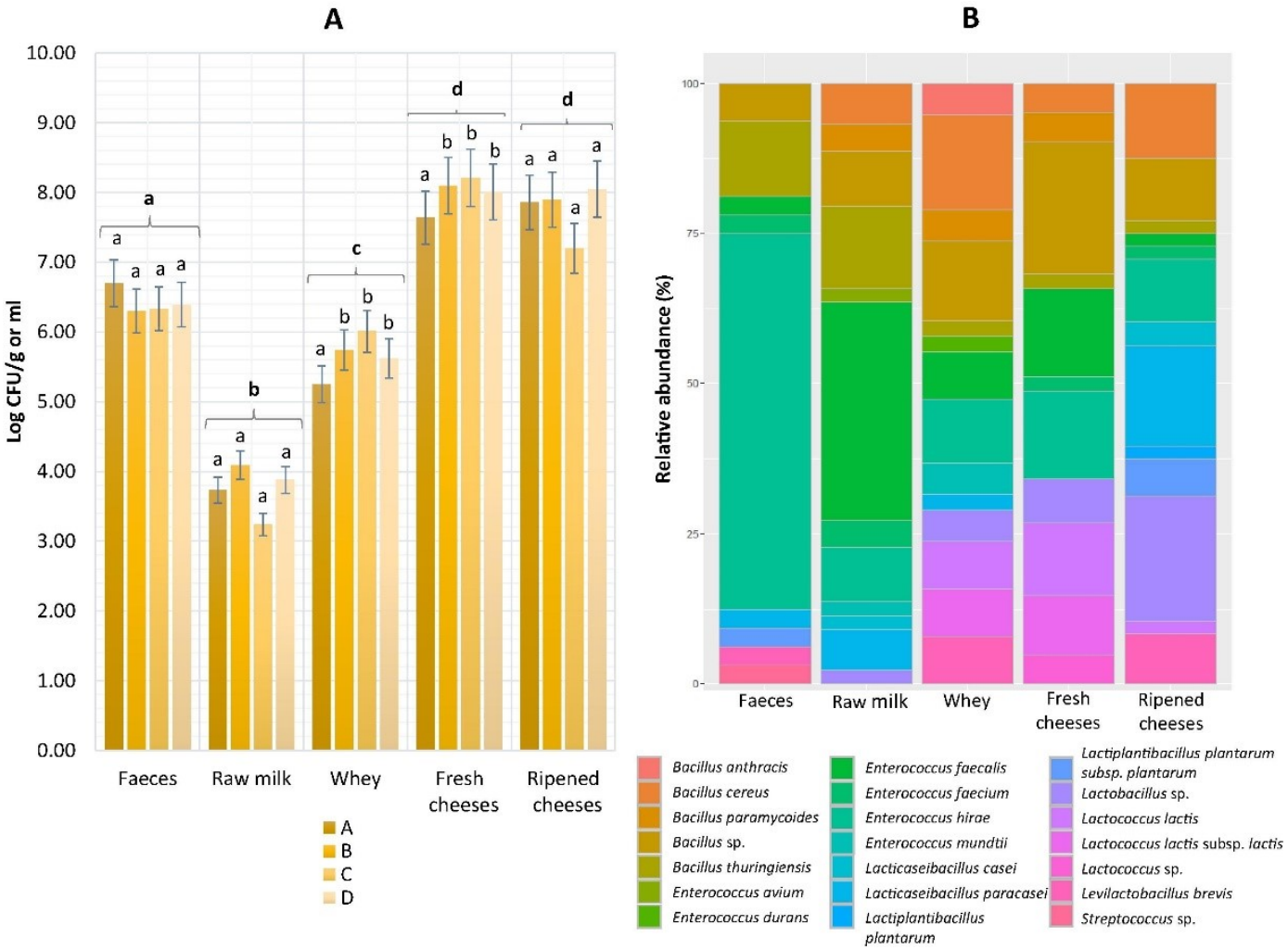
[1] Konposatu bakoitzaren kontzentrazioa batezbestekoa ± desbiderapen estandarra bezala adierazten da. Kuantifikazio-mugetatik (KM) gorako balioak letra lodiz daude. n.d.: ez da detektatu.
[2] Vergara-Luis et al.-ek [377] metodoaren garapenean eta baliozkotzean aurrez argitaratutako datuak.
[3] C: ekoizpen-katea faktorearen eragina; P: ekoizlea faktorearen eragina

Esne- eta esneki-laginetan, makrolido bat (tilosina) baino ez zen identifikatu, hain zuzen ere esne gordinean (6,15 ± 12,3 µg/kg-tik, 6,98 ± 8,24 µg/kg-ra) eta gazuran (5,35 ± 10,7 µg/kg-tik, 6,28 ± 7,39 µg/kg-ra). Gazta fresko edo helduetan, berriz, ez zen antibiotikorik identifikatu (9. taula). Esneko osagaiak gaztara edo gazurara pasatzea, disolbagarritasunaren araberakoa ez ezik, proteina eta/edo gantz frakzioarekin elkarreragiteko duten gaitasunaren araberakoa ere bada [99], baita esploratu gabeko beste faktore batzuen araberakoa ere [102]. Lortutako emaitzen arabera, tilosina gazta egiterako prozesuan zehar gazuraren bidez kentzen zen batez ere, aurretik aipatu den bezala [102]. Esneko antibiotikoak gazurara pasatzea gaztaren kaltegabetasunerako positiboa bada ere [102], funtsezkoa da gazuraren kaltegabetasunaz arduratzea, hainbat aplikazio baititu, hala nola ongarria nekazaritzan edo elikadura iturria gizakientzat eta animalientzat [2]. Kontzentrazioak esneko GHMen azpitik zeuden, eta horrek iradokitzen du kontsumitzaileen osasunerako arriskurik ez zegoela [374], elikagaien segurtasun-tartearen (EST) balioek (1,00 ± 0,00143) baieztatu zuten bezala [99]. Azpimarratzekoa da ekoizleen artean ere alde nabarmenak ikusi zirela, gazta egiterakoan erabiltzen diren baldintzetan dauden ezberdintasunekin erlazionatuta egon daitekeena. Izan ere, tratamendu termikoez gain, bestelako parametroek antibiotikoen kontzentrazioetan duten eragina gutxi aztertu da orain arte [99,332].

4.3.4. AMEk

Guztira BALen 203 isolatu lortu ziren Idiazabal gaztaren ekoizpen katean. Guztira, BALen 8 genero (*Bacillus*, *Enterococcus*, *Lacticaseibacillus*, *Levilactobacillus*, *Lactiplantibacillus*, *Lactobacillus*, *Lactococcus* eta *Streptococcus*) eta 21 espezie idenfikatu ziren, *E. hirae*, *E. faecalis*, *Bacillus* sp., *B. cereus* eta *Lactobacillus* sp. nagusiak zirelarik (14. irudia). BALen konposizioan alde nabarmenak ikusi ziren produkzio-katean bildutako laginen artean ($P \leq 0{,}001$) (14. irudia). Oro har, *Enterococcus*eko (*E. hirae* eta *E. faecalis*) eta *Bacillus*eko (*B. thuringiensis*, *B. cereus* edo *Bacillus* sp.) isolatuak esne- eta gorotz-laginetan nagusi ziren, *Lactococcus*eko isolatuekin batera (*L. lactis* eta *L. lactis* subsp. *lactis*) gazuretan eta gazta freskoetan. Heldu ostean, *Lactobacillus* eta *Lacticaseibacillus* (*L. paracasei*) generoetako isolatuak nagusi ziren (14. irudia). Oro har, ez zen alderik ikusi ekoizleen artean ($P > 0{,}05$). Lortutako emaitzak partzialki bat datoz Idiazabal gaztari [35,36,382] eta ardi-esne gordineko beste gazta batzuei buruzko ikerketekin [383,384], identifikatutako BALetan eta horien ugaritasunean aldaketak baitaude. Nabarmendu behar da emaitza hauek ez datozela bat sekuentziazio bidez lortutako emaitzekin (4.1. sekzioa) [28].

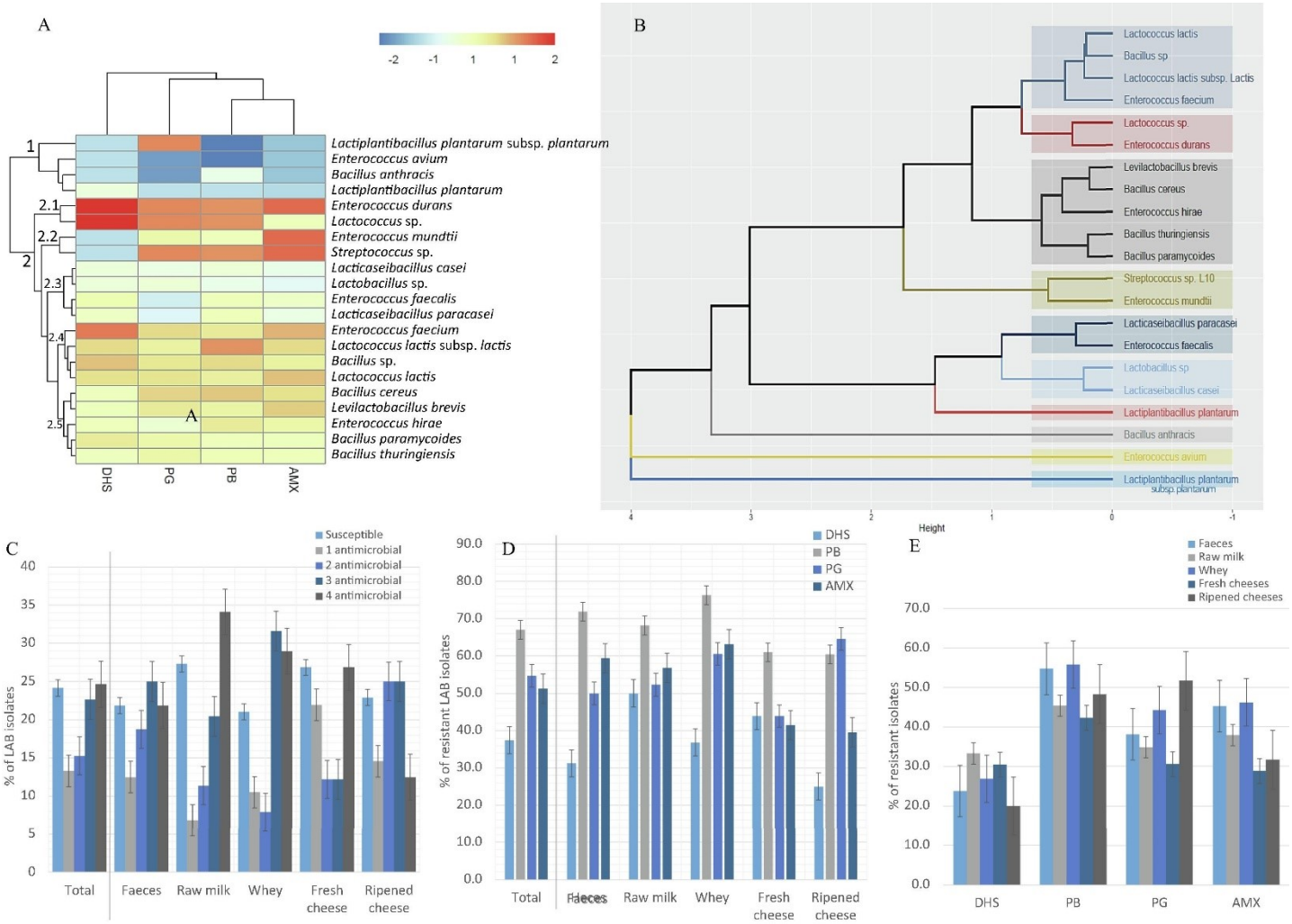
Sekuentziazio-teknikek bakterio sorta zabala identifika dezaketen bitartean, urriak direnak barne [28,47,55], ezin dute organismoen bideragarritasuna zehaztu [385].



14. irudia. Idiazabal gaztaren ekoizpen-katean zehar dauden bakterio azido laktikoen batez besteko zenbaketak (log UKE/g-ko edo mL-ko) eta ugaritasun erlatiboak (%). Letra xeheek ezberdintasun esanguratsuak adierazten dituzte ($P \leq 0.05$). Santamarina-García et al.-en [151] argitaratuta.

Erresistentzia fenotipikoari dagokionez, oro har, BALen isolatuen % 24,1ek frogatutako agente antimikrobiano guztiekiko sentikortasuna erakutsi zuten, eta % 75,9k gutxienez antimikrobiar batekiko erresistentzia erakutsi zuten, 3 edo 4 agente antimikrobianorekiko erresistentzia nagusiena izanik (% 22,7 eta % 24,6, hurrenez hurren) (15. irudia). Hala ere, ezberdintasunak ikusi ziren gaztaren ekoizpen-katean zehar (15. irudia). BAL erresistenteen proportzioa murriztu egin zen 3 edo 4 agente antimikrobianorekiko, eta handitu egin zen 1 edo 2 agente antimikrobianorekiko. Dinamika hori batez ere BALen konposizio-aldaketekin

erlazionatu zen ($P \leq 0{,}001$). Izan ere, gorotzetan, esne gordinean, gazuran, edo gazta freskoetan nagusi ziren bakterioak, hala nola *E. faecalis*, *B. thuringiensis*, *E. hirae*, *B. cereus* edo *L. lactis*, batez ere 3 edo 4 antimikrobianorekiko erresistenteak (AMEr) ziren. *Lactobacillus* sp. eta *L. paracasei*, aldiz, gazta helduak menderatu zituztenak, 2 AMEr izan ziren. Ondorioz, gazta egiterakoan ematen diren dinamika bakterianoak gaztaren kalitatea eta kaltegabetasuna baldintzatzen dituela egiaztatu zen. Orain arte, azterlan bakar batek ere ez du aztertu ardietatik isolatutako BALen antibiotikoekiko sentikortasuna, eta ardi-esne gordinaren edo eratorritako gazten inguruko informazioa urria da [386–390].

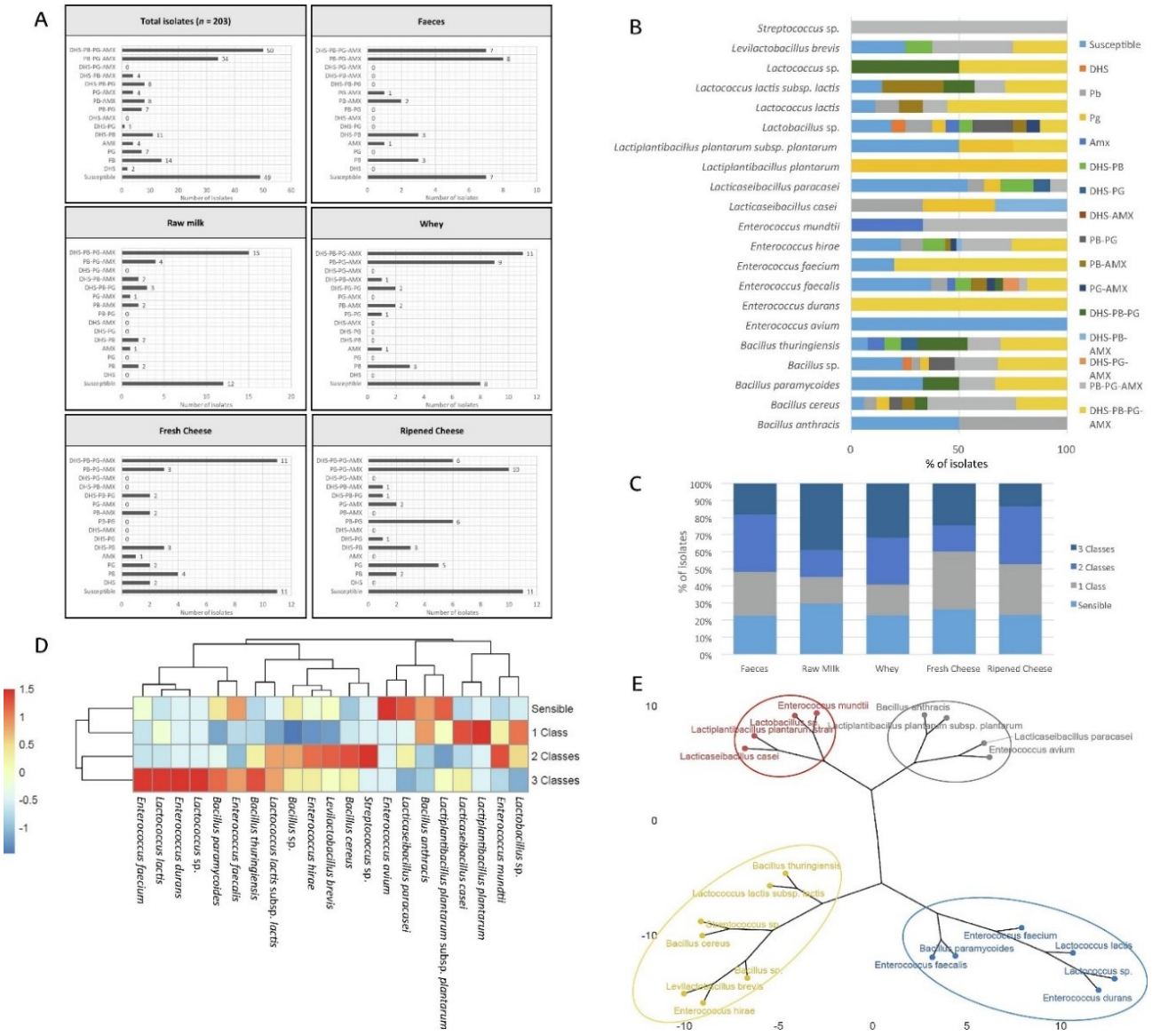


15. irudia. THAren bero-mapa (A), THAren dendrograma (B) eta barra-diagramak (C, D eta E) antimikrobianoekiko sentikortasun-proben emaitzetan oinarrituta, bakterio-espeziearen (A eta B), antimikrobiano-kopuruaren (C) eta lagin motaren arabera (D eta E). Laburdurak: DHS: dihidroestreptomizina; PB: B polimixina; PG: G penizilina; AMX: amoxizilina. Santamarina-García et al.-en [151] argitaratuta.

Antiobiotikoei dagokienez, B polimixinarekiko erresistentzia nagusiena izan zen (% 67,0), eta lagin guztietan ikusi zen, gazta helduetan izan ezik; dihidroestreptomizinarekiko erresistentzia, berriz, txikiena izan zen (% 37,4), eta batez ere esne gordinetan eta gazta freskoetan eman zen. G penizilinarekiko erresistentzia, nagusiki gazta helduetan behatu zena, eta amoxizilinarekiko erresistentzia, gorotzetan eta gazuran ikusitakoa, tartekoak izan ziren (% 54,7 eta % 51,2, hurrenez hurren). Kasu honetan ere, produkzio-katean zehar ikusitako aldakuntza lagin-mota bakoitzeko BALen konposaketarekin lotuta zegoen ($P \leq 0.05$). Adibidez, *E. durans*, *Lactococcus* sp. eta *E. faecium*, dihidroestreptomizinarekiko isolatu erresistenteenen artean zeuden; isolatu sentikorrenak, berriz, *B. anthracis*, *E. avium*, *E. mundtii*, *L. plantarum* edo *Streptococcus* sp. ziren (15. irudia).

BALen AME-profilei dagokienez (16A-B. irudia), frogatutako antibiotikoen konbinazio posible guztiak hartzen dituztenak, antibiotiko guztiekiko eta B polimixina-G penizilina-amoxizilina konbinaziorekiko erresistentziak nagusitu ziren (% 24,6 eta % 16,7, hurrenez hurren), ondoren B polimixina (% 6,90) eta dihidroestreptomizina-B polimixina (% 5,42) profilak egonda. Profil horiek batez ere *E. hirae* eta, neurri txikiagoan, *Bacillus* sp., *B. cereus* eta *E. faecalis* espezieetan ikusi ziren. Ondorioz, oro har, AME-profil nagusiak murriztu egin ziren ekoizpen-katean zehar, BALen dinamikarekin lotuta ($P \leq 0.01$). Era berean, antibiotiko ugariekiko erresistentzia (AUE), 3 antibiotiko klase edo gehiagorekiko erresistentzia bezala definitua, BALen isolatuen % 30,5ean ikusi zen, bereziki *E. hirae* (% 17,2), *E. faecalis* (% 15,6), *B. thuringiensis* (% 15,6) eta *Bacillus* sp. (% 15,6) espezieetan (16C-E. irudia). Horrenbestez, AUEren prebalentzia ere aldatu egin zen ekoizpen-katean zehar, eta gaztaren elaborazio- eta heltze-prozesuetan behera eginez (% 12,9ra), BALen dinamikaren ondorioz ($P \leq 0.01$). Oro har, ez da topatu beste gazta batzuen ekoizpen katean zehar erresistentzia eta AUE ereduei buruzko emaitzarik.

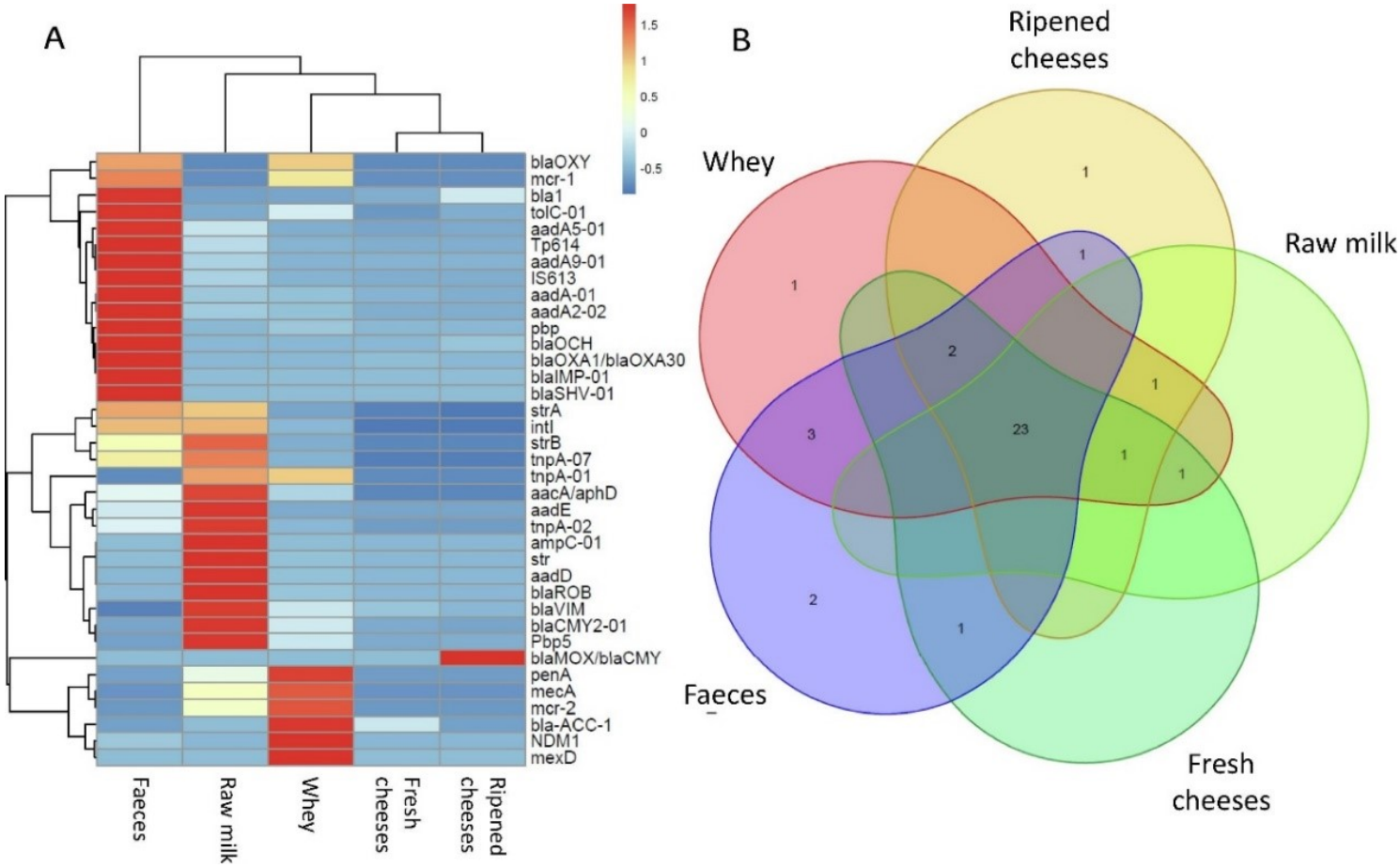
Oro har, *Lactococcus* eta *Streptococcus* espezieek izan zituzten erresistentzia-tasa handienak (batez beste, % 78,2ko eta % 75,0ko erresistentzia zuten isolatu guztiek antibiotiko guztiekiko, hurrenez hurren), eta, ondoren, *Levilactobacillus*, *Enterococcus* eta *Bacillus* espezieek (% 65,6, % 56,8 eta % 53,4, hurrenez hurren). *Lactiplantibacillus*, *Lacticaseibacillus* eta *Lactobacillus* espezieak izan ziren, aldiz, bakterio sentikorrenak (% 31,3, % 31,4 eta % 39,1, hurrenez hurren). Oro har, lortutako emaitzak bat datoz, hein batean, azkeneko urteetan argitaratutako emaitzekin; izan ere, kasu askotan, BALen AMEei buruzko informazioa urria da [386–389,391,392].



16. irudia. AME-profiletan oinarritutako barra-diagramak, ekoizpen-kateko lagin motaren eta bakterio-espezieen arabera (A eta B, hurrenez hurren), eta barra-diagramak (C), THAren bero-mapa (D) eta THAren dendrograma (E) frogatutako antimikrobiano-klaseen aurkako erresistentzietan oinarrituta, lagin motaren (C) eta bakterio-espezieen arabera (D eta E). Laburdurak: DHS: dihidroestreptomizina; PB: B polimixina; PG: G penizilina; AMX: amoxizilina. Santamarina-García et al.-en [151] argitaratuta.

Genotipikoki, ikertutako 47 AEG eta elementu genetiko mugikorretatik (EGM), 37 identifikatu ziren lagin guztien artean (17A-B. irudiak). Lagin guztien artean, AEG nagusiak aminoglukosidoekiko erresistentziari lotutakoak ziren, bereziki *Str* (387ko batez besteko ugaritasun erlatiboa), eta ondoren *Str*B (39,3) eta *aad*A-01 (19,3), literaturan esnekien BALetarako jakinarazten den bezala, nahiz eta ezberdintasunak egon detektatutako AEGetan [96,391,393]. β-laktamikoen AEGei dagokienez, *bla*-ACC-1, *pbp*, eta *bla*CMY2-01 nagusiak ziren, zeinentzat ez baitago informaziorik literaturan. Nagusi gisa deskribatu direnak beste batzuk dira, *bla*Z, *amp*C edo *mec*A besteak beste [391,394], eta gehienak detektatu egin ziren. Polimixinei dagokienez, *mcr*-

2 nagusi izan zen, *mcr*-1 ez bezala, polimixinetarako identifikaturiko lehen AEGa, aurretik abereetako gorotzetan eta esnekietan jakinarazia [395–397]. Nolanahi ere, *mcr* geneak enterobakterioentzako bakarrik deskribatu dira, eta ez dago BALekin erlazionaturiko emaitzarik [395–397]. Antibiotiko ugariekiko AEGen artean, *tol*C-01 eta *mex*D geneak identifikatu ziren. BALetan AUE ematen duten gene gutxi deskribatu dira, nahiz eta profil fenotipikoa, batez ere *Enterococcus*en behatutakoa, aurretik jakinarazi den [96,393,398], ikerketa honetan bezala. EGMen artean, transposoiak nagusienak ziren, bereziki *tnp*A-02 eta *tnp*A-01 (71,3 eta 26,5eko batez besteko ugaritasun erlatiboak), AEGen ugaritasuna gaindituz, *Str* genearena izan ezik. BALen EGMei buruzko informazioa mugatua da, nahiz eta aurretik hainbat integroi eta transposoi jakinarazi diren [391].



17. irudia. THAren bero mapa (A) eta Venn diagrama (B), AEGak eta EGMak Idiazabal gaztaren ekoizpen-katean zehar nola banatzen diren erakutsiz. Santamarina-García et al.-en [151] argitaratuta.

Bereizte genotipiko handia zegoen ekoizpen-katean zehar lagin-moten artean ($P \leq 0.001$), emaitza fenotipikoekin bat datorrena. Esne gordineko laginek AEGen eta EGMen ugaritasun handiena izan zuten, eta, ondoren, gorotzek eta gazurek; gazta freskoek eta helduek, berriz,

ugaritasun txikiena izan zuten ($P \leq 0.001$) (17A. irudia). Oro har, emaitzek iradokitzen dute dinamika mikrobiarrek, gazta egitean, erraztu egiten dutela AEGen eta EGMen murrizketa. Hori bat dator bakterioen dinamikak elikagaien kalitate eta kaltegabetasunean duen inplikazioarekin [28,137], 4.2. sekzioan adierazten den bezala.

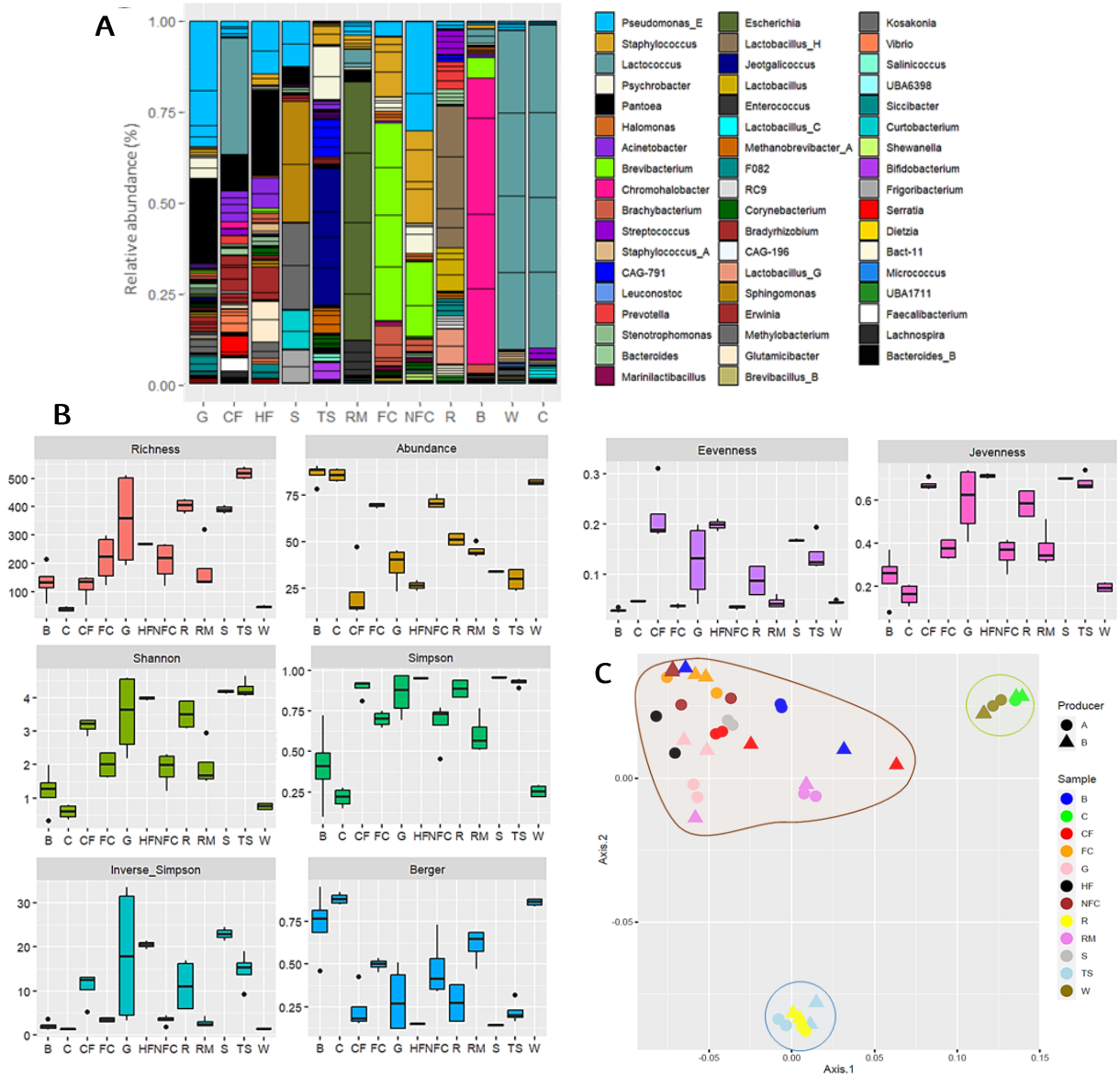
4.4. Mikroorganismo-iturriak artisau-gaztandegietan eta bere eragina Idiazabal gaztaren mikrobiotan (4. helburua, 4.1. azpihelburua, VII. eskuizkribua)

Azkenik, ekoizleen artean mikrobiotan hauteman diren alde handiak eta gaztaren kalitatean eta kaltegabetasunean duten eragina direla eta, Idiazabal gazta JDBko gaztandegietako mikroorganismo-iturri nagusiak identifikatu ziren eta gaztaren mikrobiotan eta, ondorioz, haren kalitatean eta kaltegabetasunean duten eragina ebaluatu zen (**VII. eskuizkribua**).

4.4.1. Mikrobio-komunitateak artisau-gaztandegietan

Shotgun sekuentziazio metagenomikoak 414.367.387 irakurketa sortu zituen. Taxonomikoki, 56 bakterio-filum, 370 familia, 1312 genero eta 3467 espezie identifikatu ziren lagin guztien artean. Oro har, 53 generok % 1etik gorako batez besteko ugaritasun erlatiboak zituzten, eta 14 generok % 5etik gorakoa. Horien artean, *Lactococcus*, *Staphylococcus*, *Brevibacterium*, *Pseudomonas_E*, *Chromohalobacter*, *Escherichia*, *Lactobacillus_H*, *Psychrobacter*, *Brachybacterium*, *Pantoea*, *Jeotgalicoccus*, *Lactobacillus_G* eta *Streptococcus* zueden (18A. irudia). Alde esanguratsuak ikusi ziren lagin moten artean ($P \leq 0.01$), α- eta β-dibertsitatearen indizeek berretsi zituztenak (18B-C. irudia). *Lactococcus* pentsu komertzialean nagusitu zen, *Sphingomonas* eta *Methylobacterium* lastoan, eta *Pantoea* eta *Pseudomonas_E* belarretan eta etxeko pentsuan. Titiburuaren gainazalean *Jeotgalicoccus* eta *Psychrobacter* nagusi ziren, eta elikagaiak ukitzen dituzten gainazaletan, aldiz, *Brevibacterium*, *Staphylococcus* edo *Brachybacterium*. Elikagaiak ukitzen ez dituzten gainazaletan, *Pseudomonas_E*, *Staphylococcus*, *Brevibacterium* edo *Psychrobacter* nagusi ziren. Esne gordinaren mikrobiota, batez ere, *Escherichia*k osatzen zuen, eta, ondoren, *Enterococcus*ek eta *Lactococcus*ek. Gatzagian, batez ere, *Lactobacillus_H*, *Lactobacillus*, *Lactobacillus_G*, *Prevotella*, eta *Streptococcus* topatzen ziren. Gatzun-laginetan *Chromohalobacter* nagusitu zen, ondoren *Brevibacterium* eta *Lactococcus* zeudelarik. Azkenik, *Lactococcus* eta *Staphylococcus_A* nagusi ziren gazuran, eta *Lactococcus*, *Streptococcus* eta *Lactobacillus_C*, berriz, gaztan. Oro har, esnearen eta gaztaren mikrobiotaren emaitzak bat datoz 4.1. sekzioan adierazitakoarekin, baina desberdintasunak daude identifikatutako bakterioetan eta horien

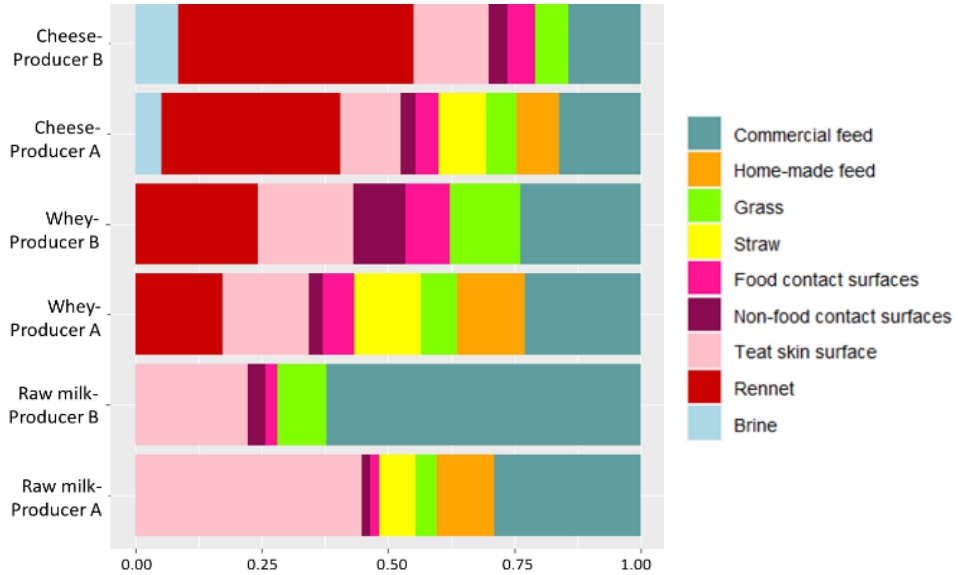
ugaritasunean [28]. Gazurari dagokionez, bakterio ez-desiragarriak ugariagoak izan ziren gaztarekin konparatuta, orain arte jakinarazi ez dena. Gazuran edo gaztan ugariak ziren bakterio asko ez zeuden esne gordinean, adibidez, *Lactobacillus_C*, *Leuconostoc* edo *Staphylococcus_A*. Beraz, horrek adierazten du artisau-gaztandegietan beste mikrobio-iturri batzuk zeudela [18,38,73], lehenago espekulatu zen bezala [28] (4.1. sekzioa).



18. irudia. Esne gordinaren, gazuraren, Idiazabal gaztaren eta gaztandegiaren inguruko laginen mikrobiotaren barra-diagrama (bakterio-genero mailan, % 1,00tik gorako ugaritasun erlatiboarekin gutxienez) (A); α-dibertsitatearen indizeen barra-diagramak (B) eta β-dibertsitatearen KNA (C). Laburdurak: B: gatzuna, C: gazta, CF: pentsu komertziala, FC: elikagaiak ukitzen dituzten gainazalak, G: belarra, HF: etxeko pentsua, NFC: elikagaiak ukitzen ez dituzten gainazalak, R: gatzagia, RM: esne gordina, S: lastoa, TS: titiburuaren gainazala, W: gazura.

4.4.2. Gaztandegi-inguruneek Latxa ardiaren esne gordinaren eta Idiazabal gaztaren mikrobiotan duten eragina

SourceTracker analisiari erreparatuz, pentsu komertziala eta titiburuaren gainazala izan ziren esne gordinaren bakterio-iturri nagusiak (% 45,6 ± 21,6 eta % 33,5 ± 14,2, hurrenez hurren) (19. irudia). Pentsu komertziala *Lactococcus*en eta *Pantoea*ren iturria zen, *Bradyrhizobium*ekin edo *Acinetobacter*rekin batera, besteak beste. Titiburuaren gainazala, aldiz, *Staphylococcus*, *Jeotgalicoccus*, *Psychrobacter*, *CAG-791*, *Methanobrevibacter_A* edo *Bifidobacterium* generoen iturria zen, adibidez. Gazta egin ondoren, gatzagia zen gaztaren bakterio-iturri nagusia (% 41,0 ± 7,58), pentsu komertzialak (% 15,3 ± 2,47) eta titiburuaren gainazalak (% 13,4 ± 2,74) ere ekarpen handia egin zutelarik. Gazurarentzat antzeko emaitzak jaso ziren, pentsu komertziala bakterio-iturri nagusiena izanda (% 20,2 ± 4,18), eta ondoren, gatzagia (% 17,4 ± 2,59) eta titiburuaren gainazala (% 15,4 ± 2,80) (19. irudia). Gatzagia, besteak beste, *Streptococcus*, *Pseudomonas_E*, *Lactobacillus_H*, *Lactobacillus* edo *Lactobacillus_G* generoen iturria zen. Gainerako laginak ere (artaldeentzako gainerako elikagaiak, elikagaiak ukitzen dituzten eta ez dituzten gainazalak, eta gatzuna) esne gordinaren eta gaztaren mikrobiotaren bakterio-iturri ziren, nahiz eta neurri txikiagoan (19. irudia). Adibidez, elikagaiak ukitzen dituzten eta ez dituzten gainazalak *Brevibacterium*, *Staphylococcus* eta *Pseudomonas_E* generoen iturri esanguratsuak ziren, eta gatzuna, berriz, *Chromohalobacter* generoaren iturria. Beraz, baieztatu zen ingurumen lagin guztiek bakterio gordailu nabarmenak suposatzen dutela [18,28,38,73].



19. irudia. SourceTracker analisiaren emaitzen barra-diagrama (ez dira proportzio ezezagunak erakusten).

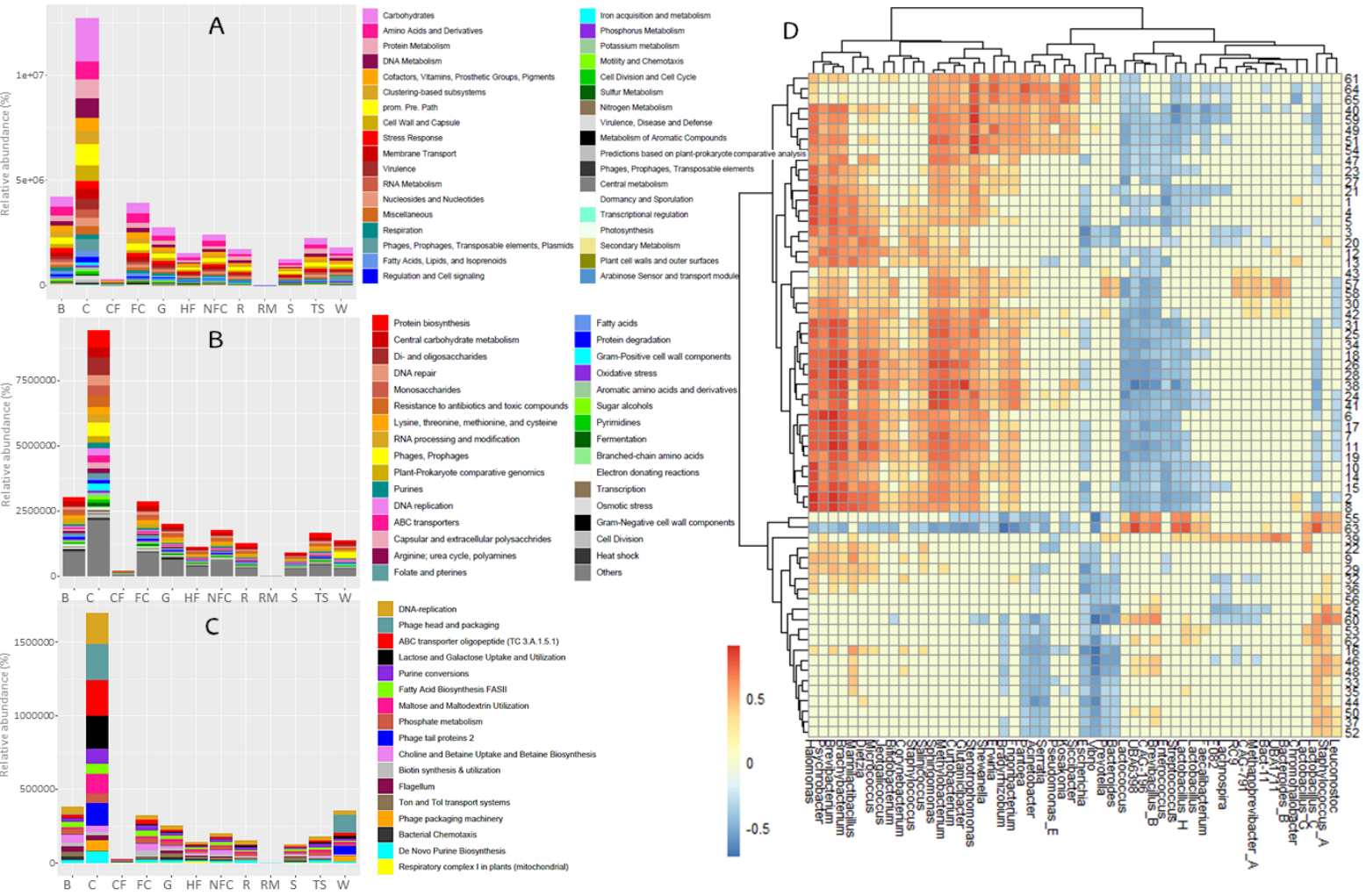
Jakina da artaldearekin erabilitako elikagaiek esne gordinaren eta gazta eratorrien konposizioan eta kalitatean eragiten dutela [399,400]. Hala ere, horien inpaktua esne gordinaren mikrobiotan ez da asko ezagutzen [400], eta ez dago gaztari buruzko ikerketarik. Titiburuaren gainazalaren kasuan, esne gordinaren mikroorganismoen iturri garrantzitsutzat hartzen da [116,401–403], eta hori animalien elikaduraren eta estabulazio-baldintzen araberakoa da [116], *Corynebacterium*, *Staphylococcus*, *Moraxella*, *Mannheimia*, *Jeotgalicoccus* edo *Methanobrevibacter* nagusi direlarik [116,403–405]. Hala ere, azterketa horiek behiekin egin dira [116,403–405] eta ardien titiburuaren gainazalei buruzko informazioa urria da, *Bacteroides* eta *Prevotella* nagusi direlarik [406]. Artisau-gatzagiak gaztaren mikrobiotan duen eraginari buruzko informazioa urria da [208,407–409]. Besteak beste, BALen iturri garrantzitsua izan daitekeela jakinarazi da, hala nola *Streptococcus* eta *Lactobacillus*ena [407]; bai eta beste bakterioena, esaterako, *E. coli* edo *S. aureus*ena [208,408,409]. Hala ere, ikerketa bakar batek ere ez du aztertu bakterio-komunitateen transferentzia gatzagietatik esne gordineko gaztetara.

Gainerako laginei dagokienez, elikagaiak ukitzen dituzten eta ez dituzten gainazaletako mikrobiota oso aztertua izan da [410–412], mikroorganismoen nitxo potentzialak baitira, biofilmak sortzeko duten gaitasunari esker [410]. Gainera, gaztandegi bakoitzeko espezifikotzat deskribatzen dira [118]. Besteak beste, *Pseudomonas* eta *Psychrobacter* deskribatu dira [410], *Brevibacterium*ekin eta *Halomonas*ekin batera [118]. Hala ere, ez dago informaziorik ardi-esne gordinarekin gaztak ekoizten dituzten gaztandegiei buruz. Era berean, gatzunen mikrobiota ere prozesatze-instalazioetarako espezifikotzat jotzen da [413], BALen eta bakterio halofiloen (adibidez, *Lactococcus*, *Chromohalobacter*, *Halomonas* edo *Tetragenococcus*) nagusitasunarekin gaztagintzarako erabiltzen diren gatzunetan [413–415]. Hala ere, ez dago informaziorik ardi-esne gordineko gaztak egiteko erabiltzen diren gatzunei buruz.

4.4.3. Gaztandegi-inguruneek gaztaren kalitatean eta kaltegabetasunean duten eragin funtzionala

Potentzial funtzionalari dagokionez, bide metaboliko ugari identifikatu ziren (20A-C. irudiak). Besteak beste, biofilmen eraketarekin lotutako bide metabolikoak identifikatu ziren, gaztandegien gainazaletako hainbat bakteriorekin erlazionatuta, adibidez, *Brevibacterium*ekin edo *Brachybacterium*ekin (20D. irudia). Horrek baieztatuko luke biofilmen eraketa gaztandegietako komunitate espezifikoen arrazoi potentzialetako bat dela [410] (4.4.2. sekzioa).

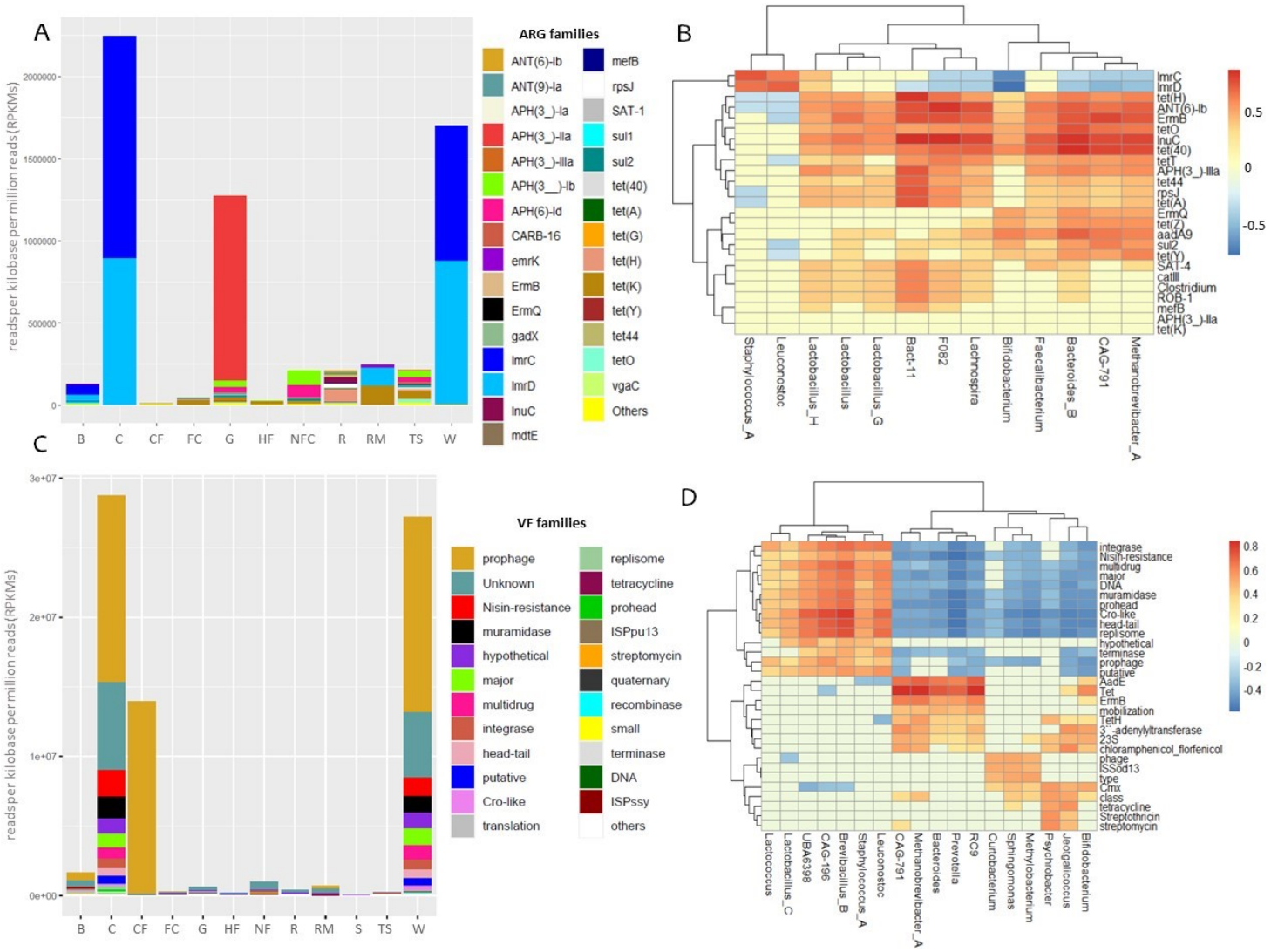
Gainera, lehia-inhibizioko mekanismoekin zerikusia duten bideak identifikatu ziren, hala nola bakteriozinen ekoizpena [16], *Sphingomonas*, *Methylobacterium* edo *Lactobacillus_H* bezalako bakterioekin erlazionatuta (20D. irudia), genero horiek gazta helduetan edo ingurumen-laginetan ugariak izatearen arrazoia izan litekeena [28,204,205] (4.1.3. eta 4.4.1. sekzioak).



20. irudia. 1., 2. eta 3. azpisistema-mailetako bide metabolikoen barra-diagrama (A, B eta C, hurrenez hurren), eta elikagaien kalitatearekin eta kaltegabetasunarekin erlazionatutako bide metabolikoen eta funtsezko bakterio-generoen arteko korrelazioen bero-mapa (D). Laburdurak: B: gatzuna, C: gazta, CF: pentsu komertziala, FC: elikagaiak ukitzen dituzten gainazalak, G: belarra, HF: etxeko pentsua, NFC: elikagaiak ukitzen ez dituzten gainazalak, R: gatzagia, RM: esne gordina, S: lastoa, TS titiburuaren gainazala, W: gazura. Bero-mapan azaltzen diren bide metabolikoen laburdurak VII. eskuizkribuko S6 taula osagarrian zehazten dira.

Gaztaren kaltegabetasunarekin lotutako hainbat bide metaboliko identifikatu ziren, hala nola patogenotasun-uharteekin edo birulomekin lotutakoak, gaztan, titiburuaren gainazalean eta gatzunan identifikatu zirenak nagusiki (20A-C. irudiak). Beraz, inguruneek esne gordineko gazten kaltegabetasunean duten inplikazioa berretsi zen [17,28,416]. Bestalde, gaztaren kalitatearekin lotutako bideak ere aurkitu ziren, adibidez, aroma- eta testura-konposatuen metabolismoarekin lotutakoak, esaterako, aminoazidoen metabolismoa, elikagaiak ukitzen dituzten gainzalaletan eta gatzunan; edo konposatu lurrunkorren sorrerarekin erlazionatutakoak, gaztan, elikagaiak ukitzen dituzten gainzalaletan eta gatzunan, esaterako (20A-C. irudiak). Bide metaboliko horietarako, *Halomonas* eta *Brevibacterium* ziren lotura handienak zituzten bakterioetako batzuk (20D. irudia). Emaitza horiek berretsi egingo lukete ingurumen bakterioek eta bakterio ez-desiragarriek gaztaren aroman duten eragina (4.2. sekzioa) [26,243,275].

Espresuki, AEGei dagokienez (21A. irudia), 86 AEG familia identifikatu ziren, peptido antimikrobianoekiko eta 12 antibiotiko-klaseekiko erresistentzia emanez. AEG nagusiak linkosamida eta aminoglikosido antibiotiko-klaseekin erlazionatuta zeuden, indibidualki, *lmr*C (2.245.572,17 IKMB), *lmr*D (1.910.857,10 IKMB) eta *APH*(3_)-IIa (1.125.938,83 IKMB) AEGak gailenduz. Lagin moten artean ezberdintasunak zeuden ($P \leq 0.001$), gaztak eta gazurak AEGen ugaritasun altuena izanik, *lmr*C eta *lmr*D geneekin erlazionatuta; eta ondoren, belarrak, *APH*(3_)-IIa AEGaren ugaritasunaren ondorioz (21A. irudia). Esnekietan edo gaztandegi-inguruneetan AEGak daudela jakinarazi da [417–419], baina ez dago informaziorik ardi-esne gordinari eta eratorritako gaztei buruz. Gainera, gaztandegi-inguruneak AEGen iturri gisa egiten duen ekarpenari buruzko informazioa urria da, baina tetraziklina, aminoglikosido, β-laktamiko eta antibiotiko ugariekiko AEGak ekoizpen-azaleretatik jakinarazi dira batez ere [419]. Belarrari dagokionez, aminoglikosido, β-laktamiko, kinolona, tetraziklina edo bankomizinarekiko AEGak ugarienak dira [420,421], nahiz eta ardiei ematen zaien belarrari buruzko informaziorik ez izan. Era berean, 13 bakterio-generok (adibidez, *Staphylococcus_A* edo *Leuconostoc*) AEGekin lotura zuten (21B. irudia). BALak eta enterobakterioak (adibidez, *Staphylococcus* edo *Escherichia*) AEGen gordailu nagusi gisa aipatzen badira ere [2], esnekietan eta horiekin lotutako inguruneetan dauden AEGak eta horien gordailuak deskribatzen dituen ikerketa lanik ez da topatu.

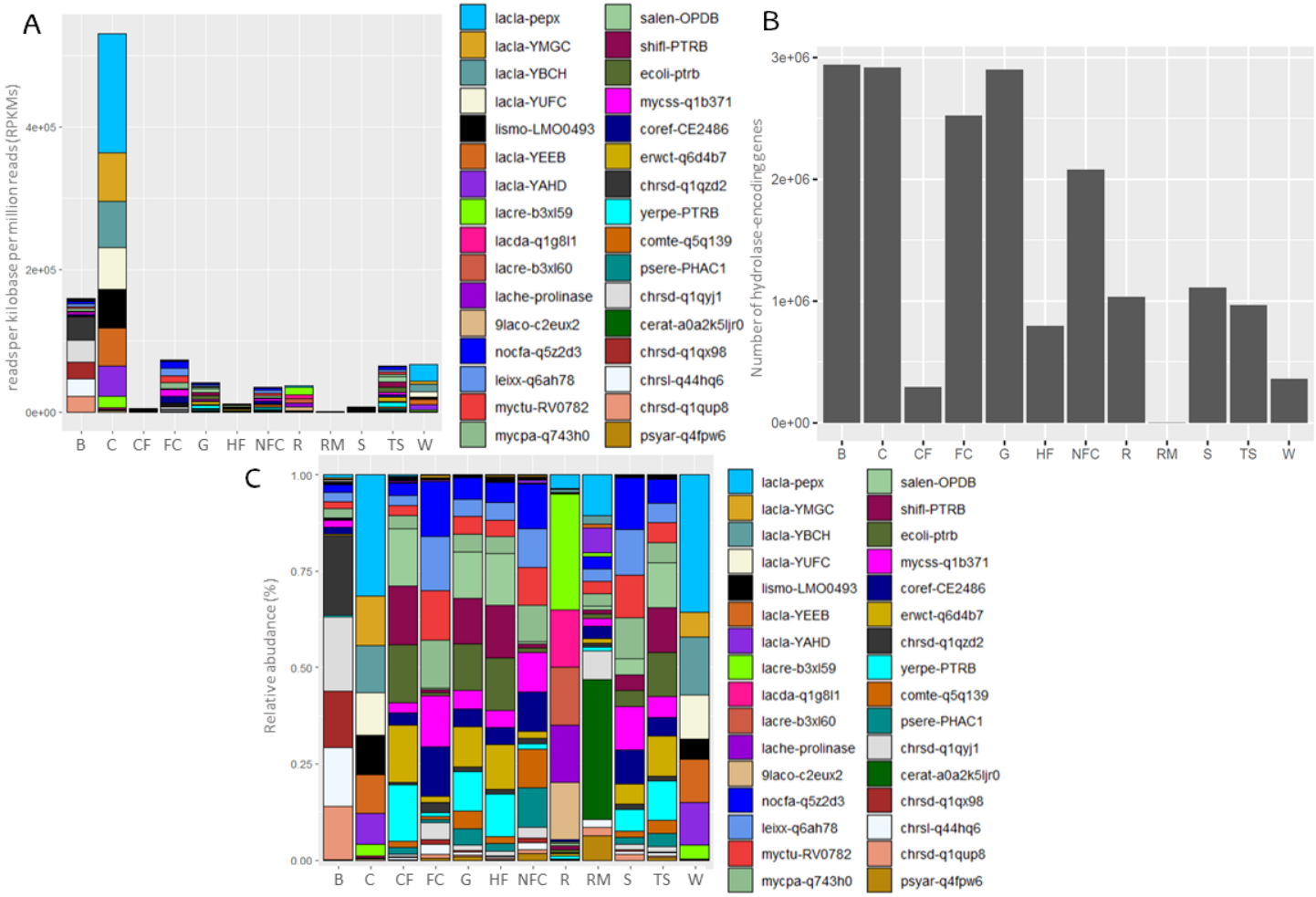


21. irudia. AEG eta BF nagusien barra-diagrama lagin-motaren arabera (A eta C) eta AEGen eta BFen, eta funtsezko bakterio-generoen arteko korrelazioen arteko bero-mapa (B eta D). Laburdurak: B: gatzuna, C: gazta, CF: pentsu komertziala, FC: elikagaiak ukitzen dituzten gainazalak, G: belarra, HF: etxeko pentsua, NFC: elikagaiak ukitzen ez dituzten gainazalak, R: gatzagia, RM: esne gordina, S: lastoa, TS: titiburuaren gainazala, W: gazura.

Birulentzia-faktoreei (BF) dagokienez (21C. irudia), 159 BF familia ezberdin identifikatu ziren, batez ere entzimekin eta egiturazko faktoreekin (% 19,5), antibiotikoekiko erresistentziarekin (% 13,8), elementu erregulatzaileekin eta genetikoekin (% 13,2) eta metabolismoarekin eta azido nukleikoekin (% 13,2) erlazionatuta. Indibidualki, profagoak (42.213.014,74 IKMB) eta nisinarekiko erresistentzia BFak (3.337.734,67 IKMB) ugarienak ziren (21C. irudia). Profagoek zeregin garrantzitsua dute bakterioen arteko lehia-mekanismoetan, eta, ondorioz, bakterio-komunitateen eraketan, patogeno kritiko askoren birulentziarekin eta eboluzioarekin oso lotuta daudelako [422,423]. AMEekin zerikusia duten proteinak kodetzen dituzten geneen presentzia partzialki bat dator 4.3.4 sekzioko emaitzekin. Lagin-motaren arabera ($P \leq 0.001$), gaztak eta gazurak, eta, ondoren, pentsu komertzialak, BFen ugaritasun handiena izan zuten (21C. irudia). BFak asko aztertu dira esne gordinaren edo gaztaren isolatuetan

[424,425]. Hala ere, oraindik ez da jakinarazi sekuentziazioan oinarritutako identifikazio sakonik esnekien eta gaztandegien inguruneetako mikrobiotan dauden BFei buruz. Pentsu komertzialari dagokionez, ez dago informaziorik literaturan. Hala ere, emaitza horiek bat datoz belar-mikrobiotari buruzko aurreko ikerketetan aurkitutakoekin, non gizakientzako hainbat patogeno detektatu diren, adibidez, *Pseudomonas* espezieak [421,426]. Gainera, 18 bakterio-generok BFekin lotura zuten, adibidez, *Psychrobacter*, *Lactococcus*, *CAG-791* edo *Staphylococcus_A* generoek (21D. irudia). Izan ere, genero horien barruan espezie patogeno asko, patogeno oportunistak edo emergenteak barne, aurretik jakinarazi dira [28].

Azkenik, gaztaren kalitateari dagokionez, konposatu aromatikoen metabolismoarekin lotutako entzima aipagarrienak hidrolasak dira, hala nola lipasak, proteasak edo esterasak [79]. Lortutako emaitzen arabera, hidrolasak kodetzen dituzten 58.593 gene-familia identifikatu ziren eta nagusienak honako hauek izan ziren: alfa/beta hidrolasa abh_upf00227, abhidrolasa_5, eta abhidrolasa_6; feruloil eta azetil xilano esterasa bifuntzionala (FAX-EB), funtzio ezezaguneko hidrolasa (FEH_915), lisofosfolipasa/karboxilesterasa, lipasa pankreatikoa, peptidasa_S9, peptidasa_S15 eta prolina_iminopeptidasa. Geneak ugarienak *Lactococcus* (*L. lactis*) eta *Lactobacillus* (*L. delbrueckii* subsp. *lactis*) espezieetakoak ziren, eta atzetik *Listeria*renak (*L. monocytogenes*) (22. irudia). Gatzunak gene kopuru handiena aurkeztu zuen, kokaina-esterasa eta B oligopeptidasa kodetzen dituzten *Chromohalobacter*en geneekin erlazionatuta. Gero gazta zegoen, Xaa-Pro dipeptidil aminopeptidasak kodetzen dituzten *Lactococcus*en geneekin lotuta. Azkenik, belarra nagusitzen zen, non B oligopeptidasa kodetzen duten *Salmonella*ren geneak eta II proteasa kodetzen duten *Escherichia*ren eta *Shigella*ren geneak nabarmenak ziren (22. irudia). Oro har, emaitzek BALek aromaren garapenean duten inplikazioa baieztatuko lukete [26,79,137,243]. Hala ere, beste ingurumen-bakterio edo bakterio ez-desiragarri batzuek ere (adibidez, *Chromohalobacter*ek eta *Listeria*k) aroman duten eragina baieztatu daiteke [137,243] (4.2. sekzioa). Orain arte, mikrobio-komunitateen eta gaztaren kalitate-konposatuen arteko korrelazioez gain [45,69,85,137,243], ez da argitaratu mikrobiotaren potentzial genetikoari buruzko lanik.



22. irudia. Hidrolasak kodetzen dituzten gene familia nagusien barra-grafikoak lagin motaren arabera (A eta C) eta hidrolasak kodetzen dituzten geneen kopuru totalaren barra grafikoa lagin motaren arabera (B). Laburdurak: B: gatzuna, C: gazta, CF: pentsu komertziala, FC: elikagaiak ukitzen dituzten gainazalak, G: belarra, HF: etxeko pentsua, NFC: elikagaiak ukitzen ez dituzten gainazalak, R: gatzagia, RM: esne gordina, S: lastoa, TS: titiburuaren gainazala, W: gazura. Hidrolasak kodetzen dituzten geneen laburdurak VII. eskuizkribuko S9 taula osagarrian zehazten dira.

5. REFERENCES

1. Codex Alimentarius Commission. General Standard for Cheese CXS 283-1978. In: Codex Alimentarius: International Food Standards. Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO). 2022. https://www.fao.org/fao-who-codexalimentarius/sh-proxy/fr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B283-1978%252FCXS_283e.pdf. Accessed 03 Mar 2024.

2. Virto M, Santamarina-García G, Amores G, Hernández I. Antibiotics in dairy production: Where is the problem? Dairy. 2022;3:541-64.

3. Kilcawley KN. Cheese Flavour. In: Fox PF, Guinee TP, Cogan TM, McSweeney PLH, editors. Fundamentals of cheese science. 2nd ed. New York: Springer US; 2017. p. 443–74.

4. Dekker LH, Vinke PC, Riphagen IJ, Minović I, Eggersdorfer ML, van den Heuvel EGHM, et al. Cheese and Healthy Diet: Associations With Incident Cardio-Metabolic Diseases and All-Cause Mortality in the General Population. Front Nutr. 2019;6:185.

5. Thorning TK, Raben A, Tholstrup T, Soedamah-Muthu SS, Givens I, Astrup A. Milk and dairy products: good or bad for human health? An assessment of the totality of scientific evidence. Food Nutr Res. 2016;60:32527.

6. Lock AL, Bauman DE. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids. 2004;39:1197–206.

7. Mazidi M, Mikhailidis DP, Sattar N, Howard G, Graham I, Banach M. Consumption of dairy product and its association with total and cause specific mortality - A population-based cohort study and meta-analysis. Clin Nutr. 2019;38:2833–45.

8. Dahiya D, Nigam PS. The Gut Microbiota Influenced by the Intake of Probiotics and Functional Foods with Prebiotics Can Sustain Wellness and Alleviate Certain Ailments like Gut-Inflammation and Colon-Cancer. Microorganisms. 2022;10:665.

9. Fusté-Forné F. Developing cheese tourism: A local-based perspective from Valle de Roncal (Navarra, Spain). J Ethn Foods. 2020;7:26.

10. Statista. Ströer Media, Köln. 2024. https://www.statista.com. Accessed 03 Mar 2024.

11. Fox PF, Guinee TP. Overview of Cheese. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. 3rd ed. Cambridge, United States: Academic Press; 2022. Vol. 3. p. 250–61.

12. Official Journal of the European Communities (OJEC). Commission Regulation (EC) No 1107/96 of 12 June 1996 on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) No 2081/92. OJEC. 1996;148:1-10.

13. Denominación de Origen Protegida Queso Idiazabal. https://www.quesoidiazabal.eus (2024). Accessed 10 Feb 2024.

14. Boletín Oficial del Estado (BOE). Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen "Idiazabal" y su Consejo Regulador. BOE.

1993;289:34591–6.

15. Bustamante MA, Virto M, Aramburu M, Barron LJR, Pérez-Elortondo FJ, Albisu M, et al. Lamb rennet paste in ovine cheese (Idiazabal) manufacture. Proteolysis and relationship between analytical and sensory parameters. Int Dairy J. 2003;13:547–57.

16. Mayo B, Rodríguez J, Vázquez L, Flórez AB. Microbial interactions within the cheese ecosystem and their application to improve quality and safety. Foods. 2021;10:602.

17. Hayaloglu AA. Microbiology of Cheese. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. 3rd ed. Cambridge, United States: Academic Press; 2022. Vol. 3. p. 225–37.

18. O'Sullivan O, Cotter PD. Microbiota of Raw Milk and Raw Milk Cheeses. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Cheese. 4th ed. Cambridge, United States: Academic Press; 2017. Vol. 1. p. 301–16.

19. Peláez C, Requena T. Exploiting the potential of bacteria in the cheese ecosystem. Int Dairy J. 2005;15:831–44.

20. Barron LJR, Redondo Y, Aramburu M, Gil P, Pérez-Elortondo FJ, Albisu M, et al. Volatile composition and sensory properties of industrially produced Idiazabal cheese. Int Dairy J. 2007;17:1401–14.

21. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, et al. The complex microbiota of raw milk. FEMS Microbiol Rev. 2013;37:664–98.

22. Fox PF, Guinee TP, Cogan TM, McSweeney PLH. Fundamentals of cheese science. 2nd ed. New York: Springer US; 2017.

23. Quintana ÁR, Seseña S, Garzón A, Arias R. Factors affecting levels of airborne bacteria in dairy farms: A review. Animals. 2020;10:526.

24. van den Brom R, de Jong A, van Engelen E, Heuvelink A, Vellema P. Zoonotic risks of pathogens from sheep and their milk borne transmission. Small Rumin Res. 2020;189:106123.

25. McSweeney PLH. Biochemistry of cheese ripening. Int J Dairy Technol. 2004;57:127–44.

26. Le Quéré J-L, Buchin S. Cheese Flavor. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. 3rd ed. Cambridge, United States: Academic Press; 2022. Vol. 3. p. 79–90.

27. Coelho MC, Malcata FX, Silva CCG. Lactic Acid Bacteria in Raw-Milk Cheeses: From Starter Cultures to Probiotic Functions. Foods. 2022;11:2276.

28. Santamarina-García G, Hernández I, Amores G, Virto M. Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing. Biology-Basel. 2022;11:769.

29. Beresford T, Williams A. The Microbiology of Cheese Ripening. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP, editors. Cheese: Chemistry, Physics and Microbiology. 3rd ed. Cambridge, United States: Academic Press; 2004. Vol. 1. p. 287–317.

30. Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM. Recent advances in cheese microbiology. Int Dairy J. 2001;11:259–74.

31. Blaya J, Barzideh Z, LaPointe G. Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. J Dairy Sci. 2018;101:3611–29.

32. Crow V, Curry B, Christison M, Hellier K, Holland R, Liu SQ. Raw milk flora and NSLAB as adjuncts. Aust J Dairy Technol. 2002;57:99–105.

33. Irlinger F, Helinck S, Jany JL. Secondary and Adjunct Cultures. In: Fox PF, McSweeney PLH, Fox PF, Cotter PD, Everett, DW. Cheese: Chemistry, Physics and Microbiology. 4th ed. Cambridge, United States: Academic Press; 2017. Vol. 1. p. 273–300.

34. Broadbent JR, Houck K, Johnson ME, Oberg CJ. Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. J Dairy Sci. 2003;86:2773–82.

35. Pérez-Elortondo FJ, Albisu M, Barcina Y. Changes in the microflora of Idiazabal cheese with the addition of commercial lactic starters. Aust J Dairy Technol. 1993;48:10–4.

36. Pérez-Elortondo FJ, Aldámiz Echobarria P, Albisu M, Barcina Y. Indigenous lactic acid bacteria in Idiazabal ewes' milk cheese. Int Dairy J. 1998;8:725–32.

37. Kamilari E, Anagnostopoulos DA, Papademas P, Kamilaris A, Tsaltas D. Characterizing Halloumi cheese's bacterial communities through metagenomic analysis. LWT-Food Sci Technol. 2020;126:109298.

38. Zhao Z, Ning C, Chen L, Zhao Y, Yang G, Wang C, et al. Impacts of manufacture processes and geographical regions on the microbial profile of traditional Chinese cheeses. Food Res Int. 2021;148:110600.

39. Paradh AD. Gram-negative spoilage bacteria in brewing. In: Hill AE, editor. Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste. Cambridge, United States: Academic Press; 2015. p. 175–94.

40. Staley JT. The Genus *Prosthecobacter*. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG, editors. The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria. New York: Springer US; 1981. p. 477–9.

41. Suchodolski JS. Gastrointestinal Microbiota. In: Washabau RJ, Day MJ, editors. Canine and Feline Gastroenterology. St. Louis, United States: W.B. Saunders; 2012. p. 32–41.

42. Lu M, Wang NS. Spoilage of Milk and Dairy Products. In: Bevilacqua A, Corbo MR, Sinigaglia M, editors. The Microbiological Quality of Food: Foodborne Spoilers. Sawston: Woodhead Publishing; 2017. p. 151–78.

43. Ndoye B, Rasolofo EA, LaPointe G, Roy D. A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. Dairy Sci Technol. 2011;91:495–524.

44. Temmerman R, Huys G, Swings J. Identification of lactic acid bacteria: culture-dependent and culture-independent methods. Trends Food Sci Technol. 2004;15:348–59.

45. De Pasquale I, Calasso M, Mancini L, Ercolini D, La Storia A, De Angelis M, et al. Causal relationship between microbial ecology dynamics and proteolysis during manufacture and ripening of Protected Designation of Origin (PDO) cheese Canestrato Pugliese. Appl Environ Microbiol. 2014;80:4085–94.

46. Ercolini D, De Filippis F, La Storia A, Iacono M. "Remake" by high-throughput sequencing of

the microbiota involved in the production of water Buffalo mozzarella cheese. Appl Environ Microbiol. 2012;78:8142–5.

47. Michailidou S, Pavlou E, Pasentsis K, Rhoades J, Likotrafiti E, Argiriou A. Microbial profiles of Greek PDO cheeses assessed with amplicon metabarcoding. Food Microbiol. 2021;99:103836.

48. Jiang Y, Yu P, Liu X, Zhao J, Zhang H, Chen W. Shifts in diversity and function of bacterial community during manufacture of rushan. J Dairy Sci. 2021;104:12375–93.

49. Zhang F, Wang Z, Lei F, Wang B, Jiang S, Peng Q, et al. Bacterial diversity in goat milk from the Guanzhong area of China. J Dairy Sci. 2017;100:7812–24.

50. Papadimitriou K, Anastasiou R, Georgalaki M, Bounenni R, Paximadaki A, Charmpi C, et al. Comparison of the Microbiome of Artisanal Homemade and Industrial Feta Cheese through Amplicon Sequencing and Shotgun Metagenomics. Microorganisms. 2022;10:1073.

51. Anastasiou R, Kazou M, Georgalaki M, Aktypis A, Zoumpopoulou G, Tsakalidou E. Omics Approaches to Assess Flavor Development in Cheese. Foods. 2022;11:188.

52. Zotta T, Ricciardi A, Condelli N, Parente E. Metataxonomic and metagenomic approaches for the study of undefined strain starters for cheese manufacture. Crit Rev Food Sci Nutr. 2022;62:3898–912.

53. Su C, Lei L, Duan Y, Zhang KQ, Yang J. Culture-independent methods for studying environmental microorganisms: Methods, application, and perspective. Appl Microbiol Biotechnol. 2012;93:993–1003.

54. Notario E, Visci G, Fosso B, Gissi C, Tanaskovic N, Rescigno M, et al. Amplicon-Based Microbiome Profiling: From Second- to Third-Generation Sequencing for Higher Taxonomic Resolution. Genes. 2023;14:1567.

55. Abriouel H, Martín-Platero A, Maqueda M, Valdivia E, Martínez-Bueno M. Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. Int J Food Microbiol. 2008;127:200–8.

56. Alegría Á, Szczesny P, Mayo B, Bardowski J, Kowalczyk M. Biodiversity in Oscypek, a traditional Polish Cheese, determined by culture-dependent and -independent approaches. Appl Environ Microbiol. 2012;78:1890–8.

57. Masoud W, Vogensen FK, Lillevang S, Abu Al-Soud W, Sørensen SJ, Jakobsen M. The fate of indigenous microbiota, starter cultures, *Escherichia coli*, *Listeria innocua* and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. Int J Food Microbiol. 2012;153:192–202.

58. Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol. 2014;12:635–45.

59. Rizal NSM, Neoh HM, Ramli R, Periyasamy PRALK, Hanafiah A, Samat MNA, et al. Advantages and Limitations of 16S rRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: Perspectives from a Middle-Income Country. Diagnostics. 2020;10:816.

60. Yap M, Ercolini D, Álvarez-Ordóñez A, O'Toole PW, O'Sullivan O, Cotter PD. Next-

Generation Food Research: Use of Meta-Omic Approaches for Characterizing Microbial Communities Along the Food Chain. Annu Rev Food Sci Technol. 2022;13:361–84.

61. Hu Y, Huang X, Yang B, Zhang X, Han Y, Chen X-X, et al. Contrasting the microbial community and metabolic profile of three types of light-flavor Daqu. Food Biosci. 2021;144:101395.

62. Liang T, Xie X, Zhang J, Ding Y, Wu Q. Bacterial community and composition of different traditional fermented dairy products in China, South Africa, and Sri Lanka by high-throughput sequencing of 16S rRNA genes. LWT-Food Sci Technol. 2021;144:111209.

63. Zhang QQ, Li D, Zhang W, Jiang M, Chen XH, Dong MS. Comparative analysis of the bacterial diversity of Chinese fermented sausages using high-throughput sequencing. LWT-Food Sci Technol. 2021;150:111975.

64. Biolcati F, Ferrocino I, Bottero MT, Dalmasso A. Short communication: High-throughput sequencing approach to investigate Italian artisanal cheese production. J Dairy Sci. 2020;103:10015–21.

65. Nero LA, Andretta M, Almeida TT, Ferreira LR, Camargo AC, Yamatogi RS, et al. Lactic microbiota of the minas artisanal cheese produced in the serro region, Minas Gerais, Brazil. LWT-Food Sci Technol. 2021;148:111698.

66. Salazar JK, Carstens CK, Ramachandran P, Shazer AG, Narula SS, Reed E, et al. Metagenomics of pasteurized and unpasteurized gouda cheese using targeted 16S rDNA sequencing. BMC Microbiol. 2018;18:189.

67. Bertani G, Levante A, Lazzi C, Bottari B, Gatti M, Neviani E. Dynamics of a natural bacterial community under technological and environmental pressures: The case of natural whey starter for Parmigiano Reggiano cheese. Food Res Int. 2020;129:108860.

68. de Paula ACL, Medeiros JD, Fernandes GR, da Silva VL, Diniz CG. Microbiome of industrialized Minas Frescal Cheese reveals high prevalence of putative bacteria: A concern in the One Health cotext. LWT-Food Sci Technol. 2021;139:110791.

69. Cardinali F, Ferrocino I, Milanović V, Belleggia L, Corvaglia MR, Garofalo C, et al. Microbial communities and volatile profile of Queijo de Azeitão PDO cheese, a traditional Mediterranean thistle-curdled cheese from Portugal. Food Res Int. 2021;147:110537.

70. Dimov SG, Gyurova A, Zagorchev L, Dimitrov T, Georgieva-Miteva D, Peykov S. NGS-Based Metagenomic Study of Four Traditional Bulgarian Green Cheeses from Tcherni Vit. LWT-Food Sci Technol. 2021;152:112278.

71. Gaglio R, Franciosi E, Todaro A, Guarcello R, Alfeo V, Randazzo CL, et al. Addition of selected starter/non-starter lactic acid bacterial inoculums to stabilise PDO Pecorino Siciliano cheese production. Food Res Int. 2020;136:109335.

72. Endres CM, Castro ÍMS, Trevisol LD, Severo JM, Mann MB, Varela APM, et al. Molecular characterization of the bacterial communities present in sheep's milk and cheese produced in South Brazilian Region via 16S rRNA gene metabarcoding sequencing. LWT-Food Sci Technol. 2021;147:111579.

73. Esteban-Blanco C, Gutiérrez-Gil B, Puente-Sánchez F, Marina H, Tamames J, Acedo A, et al. Microbiota characterization of sheep milk and its association with somatic cell count using 16s

rRNA gene sequencing. J Anim Breed Genet. 2020;137:73–83.

74. Biçer Y, Telli AE, Sönmez G, Telli N, Uçar G. Comparison of microbiota and volatile organic compounds in milk from different sheep breeds. J Dairy Sci. 2021;104:12303–11.

75. Cardinali F, Osimani A, Taccari M, Milanović V, Garofalo C, Clementi F, et al. Impact of thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia* on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese. Int J Food Microbiol. 2017;255:7–16.

76. Ramezani M, Hosseini SM, Ferrocino I, Amoozegar MA, Cocolin L. Molecular investigation of bacterial communities during the manufacturing and ripening of semi-hard Iranian Liqvan cheese. Food Microbiol. 2017;66:64–71.

77. Guinee TP. Cheese Rheology and Texture. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. 3rd ed. Cambridge, United States: Academic Press; 2022. Vol. 3.;112–30.

78. Drake MA, Delahunty CM. Sensory Character of Cheese and Its Evaluation. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP, editors. Cheese: Chemistry, Physics and Microbiology. 3rd ed. Cambridge, United States: Academic Press; 2004. Vol. 1. 517–45.

79. McSweeney PLH. Biochemistry of Cheese Ripening. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. 3rd ed. Cambridge, United States: Academic Press; 2022. Vol. 3. p. 22–9.

80. Chávarri F, Santisteban A, Virto M, de Renobales M. Alkaline Phosphatase, Acid Phosphatase, Lactoperoxidase, and Lipoprotein Lipase Activities in Industrial Ewe's Milk and Cheese. J Agric Food Chem. 1998;46:2926–32.

81. Afshari R, Pillidge CJ, Read E, Rochfort S, Dias DA, Osborn AM, et al. New insights into cheddar cheese microbiota-metabolome relationships revealed by integrative analysis of multi-omics data. Sci Reports. 2020;10:3164.

82. Zheng X, Liu F, Shi X, Wang B, Li K, Li B, et al. Dynamic correlations between microbiota succession and flavor development involved in the ripening of Kazak artisanal cheese. Food Res Int. 2018;105:733–42.

83. Unno R, Suzuki T, Matsutani M, Ishikawa M. Evaluation of the Relationships Between Microbiota and Metabolites in Soft-Type Ripened Cheese Using an Integrated Omics Approach. Front Microbiol. 2021;12:1464.

84. Bertuzzi AS, Walsh AM, Sheehan JJ, Cotter PD, Crispie F, McSweeney PLH, et al. Omics-Based Insights into Flavor Development and Microbial Succession within Surface-Ripened Cheese. mSystems. 2018;3:e00211-17.

85. De Pasquale I, Di Cagno R, Buchin S, De Angelis M, Gobbetti M. Spatial distribution of the metabolically active microbiota within Italian PDO ewes' milk cheeses. PLoS One. 2016;11:e0153213.

86. Codex Alimentarius Commission. General Principles of Food Hygiene CXC 1-1969. Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO). 2022. https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B1-1969%252FCXC_001e.pdf. Accessed 22 Feb 2024.

87. World Health Organization (WHO). Antimicrobial resistance. 2022. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed 22 Feb 2024.

88. Patel SJ, Wellington M, Shah RM, Ferreira MJ. Antibiotic Stewardship in Food-producing Animals: Challenges, Progress, and Opportunities. Clin Ther. 2020;42:1649–58.

89. Sobierajski T, Mazińska B, Chajęcka-Wierzchowska W, Śmiałek M, Hryniewicz W. Antimicrobial and Antibiotic Resistance from the Perspective of Polish Veterinary Students: An Inter-University Study. Antibiotics-Basel. 2022;11:115.

90. Konopka JK, Chatterjee P, LaMontagne C, Brown J. Environmental impacts of mass drug administration programs: exposures, risks, and mitigation of antimicrobial resistance. Infect Dis Poverty. 2022;11:78.

91. Wang Y, Xu Y, Guo X, Wang L, Zeng J, Qiu H, et al. Enhanced antimicrobial activity through the combination of antimicrobial photodynamic therapy and low-frequency ultrasonic irradiation. Adv Drug Deliv Rev. 2022;183:114168.

92. Interagency Coordination Group on Antimicrobial Resistance (IACG). No Time To Wait: Infections From Drug-Resistant Securing the Future From Drug-Resistant Infections. 2019. https://cdn.who.int/media/docs/default-source/antimicrobial-resistance/amr-gcp-tjs/iacg/summaries/iacg_final_summary_en.pdf?sfvrsn=f346e650_5. Accessed 28 Feb 2024.

93. Caniça M, Manageiro V, Abriouel H, Moran-Gilad J, Franz CMAP. Antibiotic resistance in foodborne bacteria. Trends Food Sci Technol. 2019;84:41–4.

94. Wang HH, Manuzon M, Lehman M, Wan K, Luo H, Wittum TE, et al. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiol Lett. 2006;254:226–31.

95. Yasir M, Al-Zahrani IA, Bibi F, Abd El Ghany M, Azhar EI. New insights of bacterial communities in fermented vegetables from shotgun metagenomics and identification of antibiotic resistance genes and probiotic bacteria. Food Res Int. 2022;157:111190.

96. Gaglio R, Couto N, Marques C, de Fatima Silva Lopes M, Moschetti G, Pomba C, et al. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int J Food Microbiol. 2016;236:107–14.

97. Slyvka I, Tsisaryk O, Musii L, Kushnir I, Koziorowski M, Koziorowska A. Identification and Investigation of properties of strains *Enterococcus* spp. Isolated from artisanal Carpathian cheese. Biocatal Agric Biotechnol. 2022;39:102259.

98. World Health Organization (WHO). Fact sheets on SDG: health targets and Antimicrobial Resistance. 2017. https://www.who.int/europe/publications/i/item/WHO-EURO-2017-2375-42130-58025. Accessed 28 Feb 2024.

99. Quintanilla P, Doménech E, Escriche I, Carmen Beltrán M, Pilar Molina M. Food Safety Margin Assessment of Antibiotics: Pasteurized Goat's Milk and Fresh Cheese. J Food Prot. 2019;82:1553–9.

100. Quintanilla P, Beltrán MC, Molina A, Escriche I, Molina MP. Characteristics of ripened Tronchón cheese from raw goat milk containing legally admissible amounts of antibiotics. J Dairy Sci. 2019;102:2941-53.

101. Cabizza R, Rubattu N, Salis S, Pes M, Comunian R, Paba A, et al. Transfer of oxytetracycline from ovine spiked milk to whey and cheese. Int Dairy J. 2017;70:12–7.

102. Giraldo J, Igualada C, Cabizza R, Althaus RL, Beltrán MC. Transfer of antibiotics from goat's milk to rennet curd and whey fractions during cheese-making. Food Chem. 2022;392:133218.

103. Phares CA, Danquah A, Atiah K, Agyei FK, Michael OT. Antibiotics utilization and farmers' knowledge of its effects on soil ecosystem in the coastal drylands of Ghana. PLoS One. 2020;15: e0228777.

104. Visschers VHM, Iten DM, Riklin A, Hartmann S, Sidler X, Siegrist M. Swiss pig farmers' perception and usage of antibiotics during the fattening period. Livest Sci. 2014;162:223-32.

105. Fernández M, Hudson JA, Korpela R, De Los Reyes-Gavilán CG. Impact on human health of microorganisms present in fermented dairy products: An overview. Biomed Res. Int. 2015; 2015:412714.

106. Şanli T, Şenel E. Formation of Biogenic Amines in Cheese. In: Preedy V, editor. Processing and Impact on Active Components in Food. Cambridge, United States: Academic Press; 2015. p. 223–30.

107. Spano G, Russo P, Lonvaud-Funel A, Lucas P, Alexandre H, Grandvalet C, et al. Biogenic amines in fermented foods. Eur J Clin Nutr. 2010;64:S95–100.

108. Ruiz-Capillas C, Herrero AM. Impact of Biogenic Amines on Food Quality and Safety. Foods. 2019;8:62.

109. Møller CO d. A, Ücok EF, Rattray FP. Histamine forming behaviour of bacterial isolates from aged cheese. Food Res Int. 2020;128:108719.

110. Linares DM, MartÍn MC, Ladero V, Alvarez MA, Fernández M. Biogenic Amines in Dairy Products. Crit. Rev. Food Sci. Nutr. 2011;51:691–703.

111. Özogul Y, Özogul F. Chapter 1: Biogenic Amines Formation, Toxicity, Regulations in Food. In: Saad B, Tofalo R, editors. Food Chemistry, Function and Analysis. London: Royal Society of Chemistry; 2020. pp. 1–17.

112. Barone C, Barbera M, Barone M, Parisi S, Zaccheo A. Biogenic Amines in Cheeses: Types and Typical Amounts. In: Barone C, Barbera M, Barone M, Parisi S, Zaccheo A, editors. Chemical Evolution of Nitrogen-based Compounds in Mozzarella Cheeses. New York: Springer US; 2018. p. 1–18.

113. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on risk based control of biogenic amine formation in fermented foods. EFSA J. 2011;9:2393.

114. Mah JH, Park YK, Jin YH, Lee JH, Hwang HJ. Bacterial Production and Control of Biogenic Amines in Asian Fermented Soybean Foods. Foods. 2019;8:85.

115. Zheng X, Liu F, Li K, Shi X, Ni Y, Li B, et al. Evaluating the microbial ecology and metabolite profile in Kazak artisanal cheeses from Xinjiang, China. Food Res Int. 2018;111:130–6.

116. Frétin M, Martin B, Rifa E, Isabelle VM, Pomiès D, Ferlay A, et al. Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci Reports. 2018;8:200.

117. Cruciata M, Sannino C, Ercolini D, Scatassa ML, De Filippis F, Mancuso I, et al. Animal rennets as sources of dairy lactic acid bacteria. Appl Environ Microbiol. 2014;80:2050–61.

118. Bokulich NA, Mills DA. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl Environ Microbiol. 2013;79:5214–23.

119. Abilleira E, Virto M, Nájera AI, Salmerón J, Albisu M, Pérez-Elortondo FJ, et al. Effects of seasonal changes in feeding management under part-time grazing on the evolution of the composition and coagulation properties of raw milk from ewes. J Dairy Sci. 2010;93:3902–9.

120. Valdivielso I, Albisu M, de Renobales M, Barron LJR. Changes in the volatile composition and sensory properties of cheeses made with milk from commercial sheep flocks managed indoors, part-time grazing in valley, and extensive mountain grazing. Int Dairy J. 2016;53:29–36.

121. Virto M, Chávarri F, Bustamante MA, Barron LJR, Aramburu M, Vicente MS, et al. Lamb rennet paste in ovine cheese manufacture. Lipolysis and flavour. Int Dairy J. 2003;13:391–9.

122. Aldalur A, Bustamante MÁ, Salmerón J, Barron LJR. Relationships between cheese-processing conditions and curd and cheese properties to improve the yield of Idiazabal cheese made in small artisan dairies: A multivariate approach. J Dairy Sci. 2021;104:253–69.

123. Erkus O, de Jager VCL, Geene RTCM, van Alen-Boerrigter I, Hazelwood L, van Hijum SAFT, et al. Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. Int J Food Microbiol. 2016;228:1–9.

124. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41:e1.

125. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, et al. The metagenomics RAST server - A public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 2008;9:386.

126. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 2007;35:7188–96.

127. Kindt R, Coe R. Tree diversity analysis. In: A manual and software for common statistical methods for ecological and biodiversity studies. World Agroforestry Centre (ICRAF). 2005. http://www.worldagroforestry.org/output/tree-diversity-analysis. Accessed 15 Jan 2021.

128. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package "vegan". 2020. https://cran.r-project.org/web/packages/vegan/vegan.pdf. Accessed 15 Jan 2021.

129. Paradis E, Claude J, Strimmer K. APE: Analyses of phylogenetics and evolution in R language. Bioinformatics. 2004;20:289–90.

130. Aldalur A. Technical factors affecting cheese properties, yield and whey generated from raw sheep milk during cheesemaking in small rural dairies. Ph.D. Thesis. University of the Basque Country; 2019.

131. International Standardisation Organisation (ISO)/International Dairy Federation (IDF). ISO 5943/IDF 88: Cheese and processed cheese products — Determination of chloride content — Potentiometric titration method. ISO/IDF. 2006. https://www.iso.org/standard/43922.html.

Accessed 15 May 2021.

132. Chavarri F, Virto M, Martin C, Nájera AI, Santisteban A, Barrón LJR, et al. Determination of free fatty acids in cheese: Comparison of two analytical methods. J Dairy Res. 1997;64:445–52.

133. Amores G, Valdivielso I, Virto M, Salazar A, Vera R, Aguilar C, et al. Lipid and volatile fractions of hard and semi-hard farmhouse sheep milk cheeses from Chile. Dairy Sci Technol. 2013;93:83–98.

134. Barron LJR, Redondo Y, Flanagan CE, Pérez-Elortondo FJ, Albisu M, Nájera AI, et al. Comparison of the volatile composition and sensory characteristics of Spanish PDO cheeses manufactured from ewes' raw milk and animal rennet. Int Dairy J. 2005;15:371–82.

135. Abilleira E, Schlichtherle-Cerny H, Virto M, de Renobales M, Barron LJR. Volatile composition and aroma-active compounds of farmhouse Idiazabal cheese made in winter and spring. Int Dairy J. 2010;20:537–44.

136. Busto O, Guasch J, Borrull F. Determination of biogenic amines in wine after precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. J Chromatogr A. 1996;737:205–13.

137. Santamarina-García G, Amores G, López de Armentia E, Hernández I, Virto M. Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese. Animals. 2022;12:3224.

138. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Huber W, Liaw A, et al. "gplots": Various R Programming Tools for Plotting Data. 2020. https://cran.r-project.org/package=gplots. Accessed 15 Jan 2021.

139. Casseri E, Bulut E, Llanos Soto S, Wemette M, Stout A, Greiner Safi A, et al. Understanding Antibiotic Resistance as a Perceived Threat towards Dairy Cattle through Beliefs and Practices: A Survey-Based Study of Dairy Farmers. Antibiotics-Basel. 2022;11:997.

140. Dyar OJ, Zhang T, Peng Y, Sun M, Sun C, Yin J, et al. Knowledge, attitudes and practices relating to antibiotic use and antibiotic resistance among backyard pig farmers in rural Shandong province, China. Prev Vet Med. 2020;175:104858.

141. Santamarina-García G, Amores G, Gandarias N, Hernández I, Virto M. Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain. Food Chem. 2024;442:138445.

142. Diserens J-M. Bulletin of the International Dairy Federation (IDF) N° 471/2014: Detection of inhibitors and antimicrobial residues in milk and dairy products by screening methods - Guidance on preparation of the test portion. IDF. 2014. https://shop.fil-idf.org/products/detection-of-inhibitors-and-antimicrobial-residues-in-milk-and-dairy-products-by-screening-methods-guidance-on-preparation-of-the-test-portion. Accessed 29 Jun 2023.

143. Hall T, Biosciences I, Carlsbad C. BioEdit: An important software for molecular biology. GERF Bull Biosci. 2011;2:60–61.

144. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol. 2000;7:203–14.

145. International Standardisation Organisation (ISO)/International Dairy Federation (IDF). ISO 10932/IDF 233 - Milk and milk products - Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). ISO/IDF. 2010. https://www.iso.org/standard/46434.html. Accessed 8 Sep 2022.

146. Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos M de L, Bories G, et al. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA J. 2018;16:5206.

147. Jauregi L, Epelde L, Alkorta I, Garbisu C. Antibiotic Resistance in Agricultural Soil and Crops Associated to the Application of Cow Manure-Derived Amendments From Conventional and Organic Livestock Farms. Front Vet Sci. 2021;8:633858.

148. Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, et al. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Res. 2023;51:D690–9.

149. Hu HW, Wang JT, Li J, Li JJ, Ma YB, Chen D, et al. Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils. Environ Microbiol. 2016;18:3896–909.

150. Gorecki A, Musialowski M, Wolacewicz M, Decewicz P, Ferreira C, Vejmelkova D, et al. Development and validation of novel PCR primers for identification of plasmid-mediated colistin resistance (*mcr*) genes in various environmental settings. J Hazard Mater. 2022;425:127936.

151. Santamarina-García G, Amores G, Llamazares D, Hernández I, Barron LJR, Virto M. Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese. Food Res Int. 2024;187:114308.

152. Allard SM, Walsh CS, Wallis AE, Ottesen AR, Brown EW, Micallef SA. *Solanum lycopersicum* (tomato) hosts robust phyllosphere and rhizosphere bacterial communities when grown in soil amended with various organic and synthetic fertilizers. Sci Total Environ. 2016;573:555–63.

153. Sharma R, Damgaard D, Alexander TW, Dugan MER, Aalhus JL, Stanford K, et al. Detection of transgenic and endogenous plant DNA in digesta and tissues of sheep and pigs fed roundup ready canola meal. J Agric Food Chem. 2006;54:1699–709.

154. Mormile MR, Hong BY, Benison KC. Molecular Analysis of the Microbial Communities of Mars Analog Lakes in Western Australia. Astrobiology. 2009;9:919–30.

155. Daniels S, Hepworth J, Moore-Colyer M. The haybiome: Characterising the viable bacterial community profile of four different hays for horses following different pre-feeding regimens. PLoS One. 2020;15:e0242373.

156. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011;17:10–2.

157. Andrews S. FastQC: A Quality Control Tool for High Throughput Sequence Data. 2010. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed 15 Jun 2023.

158. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.

159. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. Gigascience. 2021;10:giab008.

160. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 2010;26:841–2.

161. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. Genome Biol. 2019;20:257.

162. Parks DH, Chuvochina M, Rinke C, Mussig AJ, Chaumeil PA, Hugenholtz P. GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. Nucleic Acids Res. 2022;50:D785–94.

163. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian community-wide culture-independent microbial source tracking. Nat Methods. 2011;8:761–3.

164. Silva GGZ, Green KT, Dutilh BE, Edwards RA. SUPER-FOCUS: a tool for agile functional analysis of shotgun metagenomic data. Bioinformatics. 2016;32:354–61.

165. Buchfink B, Reuter K, Drost HG. Sensitive protein alignments at tree-of-life scale using DIAMOND. Nat Methods. 2021;18:366–8.

166. Kaminski J, Gibson MK, Franzosa EA, Segata N, Dantas G, Huttenhower C. High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. PLOS Comput Biol. 2015;11:e1004557.

167. Liu B, Zheng D, Zhou S, Chen L, Yang J. VFDB 2022: a general classification scheme for bacterial virulence factors. Nucleic Acids Res. 2022;50:D912–7.

168. Lenfant N, Hotelier T, Velluet E, Bourne Y, Marchot P, Chatonnet A. ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Res. 2013;41:D423–9.

169. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017;27:824–34.

170. Kang DD, Li F, Kirton E, Thomas A, Egan R, An H, et al. MetaBAT 2: An adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ. 2019;7:e7359.

171. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;25:1043–55.

172. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. Bioinformatics. 2020;36:1925–7.

173. Kassambara A, Mundt F. Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. 2020. Available from: https://cran.r-project.org/package=factoextra. Accessed 08 Sep 2023.

174. Yan L. ggvenn: Draw Venn Diagram by 'ggplot2'. 2023. https://cran.r-project.org/web/packages/ggvenn/index.html. Accessed 18 Jun 2023.

175. Actor JK. Clinical Bacteriology. In: Actor JK, editor. Elsevier's Integrated Review

Immunology and Microbiology. 2nd ed. Philadelphia: Saunders; 2012. p. 105–20.

176. Antonello VS, Dallé J, Domingues GC, Ferreira JAS, Fontoura M do CQ, Knapp FB. Post-cesarean surgical site infection due to *Buttiauxella agrestis*. Int J Infect Dis. 2014;22:65–6.

177. Appel TM, Quijano-Martínez N, De La Cadena E, Mojica MF, Villegas MV. Microbiological and Clinical Aspects of *Raoultella* spp. Front Public Heal. 2021;9:686789.

178. Bergogne-Bérézin E. Bacteria: *Acinetobacter*. In: Motarjemi Y, editor. Encyclopedia of Food Safety. San Diego: Academic Press; 2014. p. 337–41.

179. Fusco V, Abriouel H, Benomar N, Kabisch J, Chieffi D, Cho G-S, et al. Opportunistic Food-Borne Pathogens. In: Grumezescu AM, Holban AM, editors. Food Safety and Preservation. San Diego: Academic Press; 2018. p. 269–306.

180. Guiu A, Buendía B, Llorca L, Gómez Punter RM, Girón R. *Chryseobacterium* spp., a new opportunistic pathogen associated with cystic fibrosis? Enferm Infecc Microbiol Clin. 2014;32:497–501.

181. Li N, Wang Y, You C, Ren J, Chen W, Zheng H, et al. Variation in Raw Milk Microbiota Throughout 12 Months and the Impact of Weather Conditions. Sci Rep. 2018;8:2371.

182. Morin A. *Pantoea*. In: Batt CA, Tortorello ML, editors. Encyclopedia of Food Microbiology. 2nd ed. San Diego: Academic Press; 2014. p. 1028–32.

183. Murphy EC, Mörgelin M, Cooney JC, Frick IM. Interaction of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* with the kallikrein-kinin system. Microbiology. 2011;157:2094–105.

184. Titécat M, Wallet F, Vieillard MH, Courcol RJ, Loïez C. *Ruminococcus gnavus*: An unusual pathogen in septic arthritis. Anaerobe. 2014;30:159–60.

185. Waśkiewicz A, Irzykowska L. *Flavobacterium* spp. - Characteristics, Occurrence, and Toxicity. In: Batt CA, Tortorello ML, editors. Encyclopedia of Food Microbiology. 2nd ed. San Diego: Academic Press; 2014. p. 938–42.

186. Lo R, Xue T, Weeks M, Turner MS, Bansal N. Inhibition of bacterial growth in sweet cheese whey by carbon dioxide as determined by culture-independent community profiling. Int J Food Microbiol. 2016;217:20–8.

187. Morandi S, Battelli G, Silvetti T, Tringali S, Nunziata L, Villa A, et al. Impact of salting and ripening temperatures on late blowing defect in Valtellina Casera PDO cheese. Food Control. 2021;120:107508.

188. Bowman JP. The Genus *Psychrobacter*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E, editors. The Prokaryotes. New York: Springer; 2006. p. 920–30.

189. Shweta FNU, Gurram PR, O'Horo JC, Khalil S. *Brevibacterium* Species: An Emerging Opportunistic Cause of Bloodstream Infections. Mayo Clin Proc. 2021;96:1093–4.

190. Yin Z, Yuan C, Du Y, Yang P, Qian C, Wei Y, et al. Comparative genomic analysis of the *Hafnia* genus reveals an explicit evolutionary relationship between the species *alvei* and *paralvei* and provides insights into pathogenicity. BMC Genomics. 2019;20:768.

191. Anast JM, Dzieciol M, Schultz DL, Wagner M, Mann E, Schmitz-Esser S. *Brevibacterium* from Austrian hard cheese harbor a putative histamine catabolism pathway and a plasmid for

adaptation to the cheese environment. Sci Rep. 2019;9:6164.

192. Ozturkoglu-Budak S, Wiebenga A, Bron PA, de Vries RP. Protease and lipase activities of fungal and bacterial strains derived from an artisanal raw ewe's milk cheese. Int J Food Microbiol. 2016;237:17–27.

193. Zhang D, Palmer J, Teh KH, Flint S. Identification and selection of heat-stable protease and lipase-producing psychrotrophic bacteria from fresh and chilled raw milk during up to five days storage. LWT-Food Sci Technol. 2020;134:110165.

194. Deetae P, Bonnarme P, Spinnler HE, Helinck S. Production of volatile aroma compounds by bacterial strains isolated from different surface-ripened French cheeses. Appl Microbiol Biotechnol. 2007;76:1161–71.

195. Irlinger F, In Yung SAY, Sarthou AS, Delbès-Paus C, Montel MC, Coton E, et al. Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. Int J Food Microbiol. 2012;153:332–8.

196. Possas A, Bonilla-Luque OM, Valero A. From cheese-making to consumption: Exploring the microbial safety of cheeses through predictive microbiology models. Foods. 2021;10:355.

197. Quillaguamán J, Delgado O, Mattiasson B, Hatti-Kaul R. *Chromohalobacter sarecensis* sp. nov., a psychrotolerant moderate halophile isolated from the saline Andean region of Bolivia. Int J Syst Evol Microbiol. 2004;54:1921–6.

198. García-López ML, Santos JA, Otero A, Rodríguez-Calleja JM. *Psychrobacter*. In: Batt CA, Tortorello ML, editors. Encyclopedia of Food Microbiology. 2nd ed. San Diego: Academic Press; 2014. p. 261–8.

199. Blaya J, Barzideh Z, LaPointe G. Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. J Dairy Sci. 2018;101:3611–29.

200. Fuka MM, Wallisch S, Engel M, Welzl G, Havranek J, Schloter M. Dynamics of Bacterial Communities during the Ripening Process of Different Croatian Cheese Types Derived from Raw Ewe's Milk Cheeses. PLoS One. 2013;8:e80734.

201. Mohamaden WI, Zhen-fen Z, Hegab IM, Shang-li S. Experimental infection in mice with *Erwinia persicina*. Microb Pathog. 2019;130:38–43.

202. Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD. High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses. Appl Environ Microbiol. 2012;78:5717–23.

203. Sádecká J, Šaková N, Pangallo D, Koreňová J, Kolek E, Puškárová A, et al. Microbial diversity and volatile odour-active compounds of barrelled ewes' cheese as an intermediate product that determines the quality of winter bryndza cheese. LWT-Food Sci Technol. 2016;70:237–44.

204. Gontijo MTP, Silva J de S, Vidigal PMP, Martin JGP. Phylogenetic distribution of the bacteriocin repertoire of lactic acid bacteria species associated with artisanal cheese. Food Res Int. 2020;128:108783.

205. Kaya HI, Simsek O. Characterization of pathogen-specific bacteriocins from lactic acid bacteria and their application within cocktail against pathogens in milk. LWT-Food Sci Technol.

2019;115:108464.

206. Pérez-Elortondo FJ, Albisu M, Barcina Y. Brining time effect on physicochemical and microbiological parameters in Idiazabal cheese. Int J Food Microbiol. 1999;49:139–49.

207. Nájera AI, Barcina Y, De Renobales M, Barron LJR. Influence of salt content on the triglyceride composition of Idiazabal cheese during ripening. Lait. 1999;79:527–34

208. Etayo I, Pérez-Elortondo FJ, Gil PF, Albisu M, Virto M, Conde S, et al. Hygienic quality, lipolysis and sensory properties of Spanish Protected Designation of Origin ewe's milk cheeses manufactured with lamb rennet paste. Lait. 2006;86:415–34.

209. Aldalur A, Bustamante MÁ, Barron LJR. Effects of technological settings on yield, curd, whey, and cheese composition during the cheese-making process from raw sheep milk in small rural dairies: Emphasis on cutting and cooking conditions. J Dairy Sci. 2019;102:7813–25.

210. Pappa EC, Kondyli E, Samelis J. Microbiological and biochemical characteristics of Kashkaval cheese produced using pasteurised or raw milk. Int Dairy J. 2019;89:60–7.

211. De Pasquale I, Di Cagno R, Buchin S, De Angelis M, Gobbetti M. Use of autochthonous mesophilic lactic acid bacteria as starter cultures for making Pecorino Crotonese cheese: Effect on compositional, microbiological and biochemical attributes. Food Res Int. 2019;116:1344–56.

212. Valdivielso I, Bustamante MÁ, Buccioni A, Franci O, Gordoa JCR de, Renobales M de, et al. Commercial sheep flocks – fatty acid and fat-soluble antioxidant composition of milk and cheese related to changes in feeding management throughout lactation. J Dairy Res. 2015;82:334–43.

213. Valdivielso I, Bustamante MA, Aldezabal A, Amores G, Virto M, Ruiz De Gordoa JC, et al. Case study of a commercial sheep flock under extensive mountain grazing: Pasture derived lipid compounds in milk and cheese. Food Chem. 2016;197:622–33.

214. Hayaloglu AA, Karatekin B, Gurkan H. Thermal stability of chymosin or microbial coagulant in the manufacture of Malatya, a Halloumi type cheese: Proteolysis, microstructure and functional properties. Int Dairy J. 2014;38:136–44.

215. Soltani M, Sahingil D, Gokce Y, Hayaloglu AA. Effect of blends of camel chymosin and microbial rennet (*Rhizomucor miehei*) on chemical composition, proteolysis and residual coagulant activity in Iranian Ultrafiltered White cheese. J Food Sci Technol. 2019;56:589–98.

216. Sanjuán E, Saavedra P, Millán R, Castelo M, Fernández-Salguero J. Effect of ripening and type of rennet on the mineral content of Los Pedroches cheese. J Food Qual. 1998;21:187–200.

217. An F, Li M, Zhao Y, Zhang Y, Mu D, Hu X, et al. Metatranscriptome-based investigation of flavor-producing core microbiota in different fermentation stages of dajiang, a traditional fermented soybean paste of Northeast China. Food Chem. 2021;343:128509.

218. Wang H, Su W, Mu Y, Zhao C. Correlation Between Microbial Diversity and Volatile Flavor Compounds of Suan zuo rou, a Fermented Meat Product From Guizhou, China. Front Microbiol. 2021;12:3216.

219. Li C, Zhao Y, Wang Y, Li L, Yang X, Chen S, et al. Microbial community changes induced by *Pediococcus pentosaceus* improve the physicochemical properties and safety in fermented tilapia sausage. Food Res Int. 2021;147:110476.

220. Chávarri F, Bustamante MA, Santisteban A, Virto M, Barrón LJR, De Renobales M. Changes

in Free Fatty Acids During Ripening of Idiazabal Cheese Manufactured at Different Times of the Year. J Dairy Sci. 1999;82:885–90.

221. Hernández I, Barrón LJR, Virto M, Pérez-Elortondo FJ, Flanagan C, Rozas U, et al. Lipolysis, proteolysis and sensory properties of ewe's raw milk cheese (Idiazabal) made with lipase addition. Food Chem. 2009;116:158–66.

222. Barron LJR, Hernández I, Bilbao A, Flanagan CE, Nájera AI, Virto M, et al. Changes in lipid fractions and sensory properties of Idiazabal cheese induced by lipase addition. J Dairy Res. 2004;71:372–9.

223. Esmaeilzadeh P, Ehsani MR, Mizani M, Givianrad MH. Characterization of a traditional ripened cheese, Kurdish Kope: Lipolysis, lactate metabolism, the release profile of volatile compounds, and correlations with sensory characteristics. J Food Sci. 2021;86:3303–21.

224. Caboni P, Maxia D, Scano P, Addis M, Dedola A, Pes M, et al. A gas chromatography-mass spectrometry untargeted metabolomics approach to discriminate Fiore Sardo cheese produced from raw or thermized ovine milk. J Dairy Sci. 2019;102:5005–18.

225. Fernández D, Combarros-Fuertes P, Renes E, Abarquero D, Fresno JM, Tornadijo ME. Influence of the Breed of Sheep on the Characteristics of Zamorano Cheese. Dairy. 2021;2:242–55.

226. Amores G, Pérez-Elortondo FJ, Albisu M, Barron LJR. Short communication: To what extent do environmental or technological conditions affect the sensory differentiation of raw ewe milk cheeses produced in valley or mountain farms? J Dairy Sci. 2021;104:301–7.

227. Thierry A, Collins YF, Mukdsi, M. C. A. McSweeney PLH, Wilkinson, M. G. Spinnler HE. Lipolysis and metabolism of fatty acids in cheese. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Cheese: Chemistry, Physics and Microbiology. 4th ed. San Diego: Academic Press; 2017. p. 423– 444.

228. Collins YF, McSweeney PLH, Wilkinson MG. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int Dairy J. 2003;13:841–66.

229. Dellali A, Karam HZ, Karam N-E. Lipase and esterase activities of lactic acid bacteria isolated from different biotopes. African J Biotechnol. 2020;19:156–64.

230. Katz M, Medina R, Gonzalez S, Oliver G. Esterolytic and lipolytic activities of lactic acid bacteria isolated from ewe's milk and cheese. J Food Prot. 2002;65:1997–2001.

231. Esteban-Torres M, Mancheño JM, de las Rivas B, Muñoz R. Characterization of a halotolerant lipase from the lactic acid bacteria *Lactobacillus plantarum* useful in food fermentations. LWT-Food Sci Technol. 2015;60:246–52.

232. Esteban-Torres M, Mancheño JM, de las Rivas B, Muñoz R. Production and characterization of a tributyrin esterase from *Lactobacillus plantarum* suitable for cheese lipolysis. J Dairy Sci. 2014;97:6737–44.

233. Ramakrishnan V, Goveas LC, Suralikerimath N, Jampani C, Halami PM, Narayan B. Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization. Biocatal Agric Biotechnol. 2016;6:19–27.

234. Özer B, Yaman H. Milk and Milk Products: Microbiology of Liquid Milk. In: Batt CA,

Tortorello ML, editors. Encyclopedia of Food Microbiology. 2nd ed. San Diego: Academic Press; 2014. p. 721–7.

235. Schmitz-Esser S, Dzieciol M, Nischler E, Schornsteiner E, Bereuter O, Mann E, et al. Abundance and potential contribution of Gram-negative cheese rind bacteria from Austrian artisanal hard cheeses. Int J Food Microbiol. 2018;266:95–103.

236. Holland R, Liu SQ, Crow VL, Delabre ML, Lubbers M, Bennett M, et al. Esterases of lactic acid bacteria and cheese flavour: Milk fat hydrolysis, alcoholysis and esterification. Int Dairy J. 2005;15:711–8.

237. Forquin MP, Weimer BC. *Brevibacterium*. In: Batt CA, Tortorello ML, editors. Encyclopedia of Food Microbiology. 2nd ed. 2014. p. 324–30.

238. Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol. 2009;85:1629–42.

239. Nieman C. Influence of trace amounts of fatty acids on the growth of microorganisms. Bacteriol Rev. 1954;18:147.

240. Yoon BK, Jackman JA, Valle-González ER, Cho N-J. Antibacterial Free Fatty Acids and Monoglycerides: Biological Activities, Experimental Testing, and Therapeutic Applications. Int J Mol Sci. 2018;19:1114.

241. Barron LJR, Redondo Y, Aramburu M, Pérez-Elortondo FJ, Albisu M, Nájera AI, et al. Variations in volatile compounds and flavour in Idiazabal cheese manufactured from ewe's milk in farmhouse and factory. J Sci Food Agric. 2005;85:1660–71.

242. Gaglio R, Cruciata M, Scatassa ML, Tolone M, Mancuso I, Cardamone C, et al. Influence of the early bacterial biofilms developed on vats made with seven wood types on PDO Vastedda della valle del Belìce cheese characteristics. Int J Food Microbiol. 2019;291:91–103.

243. Santamarina-García G, Amores G, Hernández I, Morán L, Barrón LJR, Virto M. Relationship between the dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese. Curr Res Food Sci. 2023;2023:100425.

244. Starowicz M. Analysis of Volatiles in Food Products. Separations. 2021;8:157.

245. Curioni PMG, Bosset JO. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int Dairy J. 2002;12:959–84.

246. Liu SQ, Holland R, Crow VL. Esters and their biosynthesis in fermented dairy products: a review. Int Dairy J. 2004;14:923–45.

247. Bertuzzi AS, McSweeney PLH, Rea MC, Kilcawley KN. Detection of Volatile Compounds of Cheese and Their Contribution to the Flavor Profile of Surface-Ripened Cheese. Compr Rev Food Sci Food Saf. 2018;17:371–90.

248. Attaie R. Quantification of volatile compounds in goat milk Jack cheese using static headspace gas chromatography. J Dairy Sci. 2009;92:2435–43.

249. Taylor K, Wick C, Castada H, Kent K, Harper WJ. Discrimination of Swiss Cheese from 5 Different Factories by High Impact Volatile Organic Compound Profiles Determined by Odor Activity Value Using Selected Ion Flow Tube Mass Spectrometry and Odor Threshold. J Food Sci. 2013;78:C1509–15.

250. Wang B, Wang J, Xu LY, Zhang JH, Ai NS, Cao YP. Characterization of the key odorants in kurut with aroma recombination and omission studies. J Dairy Sci. 2020;103:4164–73.

251. Wang J, Yang ZJ, Xu LY, Wang B, Zhang JH, Li BZ, et al. Key aroma compounds identified in Cheddar cheese with different ripening times by aroma extract dilution analysis, odor activity value, aroma recombination, and omission. J Dairy Sci. 2021;104:1576–90.

252. Natrella G, Faccia M, Lorenzo JM, De Palo P, Gambacorta G. Short communication: Sensory characteristics and volatile organic compound profile of high-moisture mozzarella made by traditional and direct acidification technology. J Dairy Sci. 2020;103:2089–97.

253. Qian M, Reineccius GA. Quantification of Aroma Compounds in Parmigiano Reggiano Cheese by a Dynamic Headspace Gas Chromatography-Mass Spectrometry Technique and Calculation of Odor Activity Value. J Dairy Sci. 2003;86:770–6.

254. Juric M, Bertelsen G, Mortensen G, Petersen MA. Light-induced colour and aroma changes in sliced, modified atmosphere packaged semi-hard cheeses. Int Dairy J. 2003;13:239–49.

255. Zabaleta L, Gourrat K, Barron LJR, Albisu M, Guichard E. Identification of odour-active compounds in ewes' raw milk commercial cheeses with sensory defects. Int Dairy J. 2016;58:23–30.

256. Câmara JS, Lourenço S, Silva C, Lopes A, Andrade C, Perestrelo R. Exploring the potential of wine industry by-products as source of additives to improve the quality of aquafeed. Microchem J. 2020;155:104758.

257. The Good Scents Company Information System. Providing information for the flavor, fragrance, food and cosmetic industries. 2021. https://www.thegoodscentscompany.com/. Accessed 17 Nov 2021.

258. Poveda JM, Sánchez-Palomo E, Pérez-Coello MS, Cabezas L. Volatile composition, olfactometry profile and sensory evaluation of semi-hard Spanish goat cheeses. Dairy Sci Technol. 2008;88:355–367.

259. Jung HJ, Ganesan P, Lee SJ, Kwak HS. Comparative study of flavor in cholesterol-removed Gouda cheese and Gouda cheese during ripening. J Dairy Sci. 2013;96:1972–83.

260. Moio L, Piombino P, Addeo F. Odour-impact compounds of Gorgonzola cheese. J Dairy Res. 2000;67:273–85.

261. Zhang J, Li M, Zhang H, Pang X. Comparative investigation on aroma profiles of five different mint (*Mentha*) species using a combined sensory, spectroscopic and chemometric study. Food Chem. 2022;371:131104.

262. Sympoura F, Cornu A, Tournayre P, Massouras T, Berdagué JL, Martin B. Odor compounds in cheese made from the milk of cows supplemented with extruded linseed and α-tocopherol. J Dairy Sci. 2009;92:3040–8.

263. Karagul Yuceer Y, Tuncel B, Guneser O, Engin B, Isleten M, Yasar K, et al. Characterization of aroma-active compounds, sensory properties, and proteolysis in Ezine cheese. J Dairy Sci. 2009;92:4146–57.

264. Whetstine MEC, Cadwallader KR, Drake MA. Characterization of aroma compounds responsible for the rosy/floral flavor in Cheddar cheese. J Agric Food Chem. 2005;53:3126–32.

265. Chen C, Zhou W, Yu H, Yuan J, Tian H. Characterization of major odor-active compounds responsible for nutty flavor in Cheddar cheese according to Chinese taste. Flavour Fragr J. 2021;36:171–81.

266. Qian M, Reineccius G. Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J Dairy Sci. 2002;85:1362–9.

267. Boscaini E, Van Ruth S, Biasioli F, Gasperi F, Märk TD. Gas Chromatography−Olfactometry (GC-O) and Proton Transfer Reaction−Mass Spectrometry (PTR-MS) Analysis of the Flavor Profile of Grana Padano, Parmigiano Reggiano, and Grana Trentino Cheeses. J Agric Food Chem. 2003;51:1782–90.

268. Campo E, Ferreira V, López R, Escudero A, Cacho J. Identification of three novel compounds in wine by means of a laboratory-constructed multidimensional gas chromatographic system. J Chromatogr A. 2006;1122:202–8.

269. Takeoka GR, Buttery RG, Turnbaugh JG, Benson M. Odor thresholds of various branched esters. LWT-Food Sci Technol. 1995;28:153–6.

270. Horwood JF, Lloyd GT, Ramshaw H, Stark W. An off-flavour associated with the use of sorbic acid during Feta cheese maturation. Aust J Dairy Technol. 1981;36:38–40.

271. Sarhir ST, Amanpour A, Bouseta A, Selli S. Fingerprint of aroma-active compounds and odor activity values in a traditional Moroccan fermented butter "Smen" using GC–MS–Olfactometry. J Food Compos Anal. 2021;96:103761.

272. Majcher MA, Jeleń HH. Key odorants of Oscypek, a traditional Polish ewe's milk cheese. J Agric Food Chem. 2011;59:4932–7.

273. Kubícková J, Grosch W. Quantification of potent odorants in camembert cheese and calculation of their odour activity values. Int Dairy J. 1998;8:17–23.

274. van Gemert LJ. Odour thresholds : compilations of odour threshold values in air, water and other media. 2nd ed. Zeist: Oliemans Punter & Partners BV; 2011.

275. Meng Y, Chen X, Sun Z, Li Y, Chen D, Fang S, et al. Exploring core microbiota responsible for the production of volatile flavor compounds during the traditional fermentation of Koumiss. LWT-Food Sci Technol. 2021;135:110049.

276. Zhong A, Chen W, Duan Y, Li K, Tang X, Tian X, et al. The potential correlation between microbial communities and flavors in traditional fermented sour meat. LWT-Food Sci Technol. 2021;149:111873.

277. Zuljan FA, Mortera P, Alarcón SH, Blancato VS, Espariz M, Magni C. Lactic acid bacteria decarboxylation reactions in cheese. Int. Dairy J. 2016;62:53–62.

278. Yao Y, Zhou X, Hadiatullah H, Zhang J, Zhao G. Determination of microbial diversities and aroma characteristics of Beitang shrimp paste. Food Chem. 2021;344:128695.

279. Zhao D, Hu J, Chen W. Analysis of the relationship between microorganisms and flavour development in dry-cured grass carp by high-throughput sequencing, volatile flavour analysis and metabolomics. Food Chem. 2022;368:130889.

280. Mukhia S, Kumar A, Kumar R. Generation of antioxidant peptides from soy protein isolate through psychrotrophic *Chryseobacterium* sp. derived alkaline broad temperature active protease.

LWT-Food Sci Technol. 2021;143:111152.

281. Rathakrishnan D, Gopalan AK. Isolation and characterization of halophilic isolates from Indian salterns and their screening for production of hydrolytic enzymes. Environ Challenges. 2022;6:100426.

282. Li K, Tang J, Zhang Z, Wu Z, Zhong A, Li Z, et al. Correlation between flavor compounds and microorganisms of Chaling natural fermented red sufu. LWT-Food Sci Technol. 2022;154:112873.

283. Fonseca HC, Melo D de S, Ramos CL, Menezes AGT, Dias DR, Schwan RF. Sensory and flavor-aroma profiles of passion fruit juice fermented by potentially probiotic *Lactiplantibacillus plantarum* CCMA 0743 strain. Food Res Int. 2021;152:110710.

284. Ordóñez AI, Ibáñez FC, Torre P, Barcina Y. Formation of biogenic amines in Idiazabal ewe's-milk cheese: Effect of ripening, pasteurization, and starter. J Food Prot. 1997;60:1371–5.

285. Tofalo R, Perpetuini G, Battistelli N, Pepe A, Ianni A, Martino G, et al. Accumulation γ-aminobutyric acid and biogenic amines in a traditional raw milk ewe's cheese. Foods. 2019;8:401.

286. Torracca B, Pedonese F, López MB, Turchi B, Fratini F, Nuvoloni R. Effect of milk pasteurisation and of ripening in a cave on biogenic amine content and sensory properties of a pecorino cheese. Int Dairy J. 2016;61:189–95.

287. Schirone M, Tofalo R, Fasoli G, Perpetuini G, Corsetti A, Manetta AC, et al. High content of biogenic amines in Pecorino cheeses. Food Microbiol. 2013;34:137–44.

288. Mao S, Huo W, Liu J, Zhang R, Zhu W. In vitro effects of sodium bicarbonate buffer on rumen fermentation, levels of lipopolysaccharide and biogenic amine, and composition of rumen microbiota. J Sci Food Agric. 2017;97:1276–85.

289. Sigel E, Steinmann ME. Structure, Function, and Modulation of GABA(A) Receptors. J Biol Chem. 2012;287:40224–31.

290. Bagheri S, Heydari A, Alinaghipour A, Salami M. Effect of probiotic supplementation on seizure activity and cognitive performance in PTZ-induced chemical kindling. Epilepsy Behav. 2019;95:43–50.

291. Abdelazez A, Abdelmotaal H, Evivie SE, Melak S, Jia FF, Khoso MH, et al. Screening potential probiotic characteristics of *Lactobacillus brevis* strains in vitro and intervention effect on type I diabetes in vivo. Biomed Res Int. 2018;2018:7356173.

292. Boonstra E, de Kleijn R, Colzato LS, Alkemade A, Forstmann BU, Nieuwenhuis S. Neurotransmitters as food supplements: The effects of GABA on brain and behavior. Front Psychol. 2015;6:1520.

293. Wang C, Zhu CL, Huang ZJ, Wang G, Huang Q, Liu CH, et al. γ-aminobutyric acid inhibits the growth of cholangiocarcinoma via cAMP/PKA signal pathway. Int J Clin Exp Med. 2016;9: 9992–9998.

294. Barbieri F, Montanari C, Gardini F, Tabanelli G. Biogenic Amine Production by Lactic Acid Bacteria: A Review. Foods. 2019;8:17.

295. Espinosa-Pesqueira D, Roig-Sagués AX, Manuela Hernández-Herrero M. Screening Method to Evaluate Amino Acid-Decarboxylase Activity of Bacteria Present in Spanish Artisanal Ripened

Cheeses. Foods. 2018;7:182.

296. Diaz M, del Rio B, Ladero V, Redruello B, Fernández M, Martin MC, et al. Isolation and typification of histamine-producing *Lactobacillus vaginalis* strains from cheese. Int J Food Microbiol. 2015;215:117–23.

297. Rodríguez-Sánchez S, Ramos IM, Seseña S, Poveda JM, Palop ML. Potential of *Lactobacillus* strains for health-promotion and flavouring of fermented dairy foods. LWT-Food Sci Technol. 2021;143:111102.

298. Joosten HMLJ, Northolt MD. Conditions allowing the formation of biogenic amines in cheese, 2: decarboxylative properties of some non-starter bacteria. Netherlands milk dairy J. 1987;41:259–80.

299. Aliakbarlu J, Alizadeh M, Razavi-Rohani SM, Agh N. Biogenic amines in Iranian white brine cheese: modelling and optimisation of processing factors. Int J Dairy Technol. 2011;64:417–24.

300. Zhang C, Zhang J, Liu D. Biochemical changes and microbial community dynamics during spontaneous fermentation of Zhacai, a traditional pickled mustard tuber from China. Int J Food Microbiol. 2021;347:109199.

301. Zhao Y, Wang Y, Li C, Li L, Yang X, Wu Y, et al. Novel insight into physicochemical and flavor formation in naturally fermented tilapia sausage based on microbial metabolic network. Food Res Int. 2021;141:110122.

302. Santiyanont P, Chantarasakha K, Tepkasikul P, Srimarut Y, Mhuantong W, Tangphatsornruang S, et al. Dynamics of biogenic amines and bacterial communities in a Thai fermented pork product Nham. Food Res Int. 2019;119:110–8.

303. Jung JY, Chun BH, Jeon CO. *Chromohalobacter* is a causing agent for the production of organic acids and putrescine during fermentation of ganjang, a korean traditional soy sauce. J Food Sci. 2015;80:M2853–9.

304. Lee YC, Kung HF, Huang CY, Huang TC, Tsai YH. Reduction of histamine and biogenic amines during salted fish fermentation by *Bacillus polymyxa* as a starter culture. J Food Drug Anal. 2016;24:157–63.

305. Chong CY, Bakar FA, Russly AR, Jamilah B, Mahyudin NA. The effects of food processing on biogenic amines formation. Int. Food Res. J. 2011;18:867–876.

306. Butor I, Pištěková H, Purevdorj K, Jančová P, Buňka F, Buňková L. Biogenic amines degradation by microorganisms isolated from cheese. Potravin Slovak J Food Sci. 2017;11:302–308.

307. Herrero-Fresno A, Martínez N, Sánchez-Llana E, Díaz M, Fernández M, Martin MC, et al. *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental model. Int J Food Microbiol. 2012;157:297–304.

308. Treiber FM, Beranek-Knauer H. Antimicrobial residues in food from animal origin—a review of the literature focusing on products collected in stores and markets worldwide. Antibiotics-Basel. 2021;10:534.

309. Pengov A, Kirbis A. Risks of antibiotic residues in milk following intramammary and intramuscular treatments in dairy sheep. Anal Chim Acta. 2009;637:13–7.

310. McEwen SA, Fedorka-Cray PJ. Antimicrobial Use and Resistance in Animals. Clin Infect Dis. 2002;34:S93–106.

311. Pol M, Ruegg PL. Treatment Practices and Quantification of Antimicrobial Drug Usage in Conventional and Organic Dairy Farms in Wisconsin. J Dairy Sci. 2007;90:249–61.

312. Linage B, Gonzalo C, Carriedo JA, Asensio JA, Blanco MA, De La Fuente LF, et al. Performance of Blue-Yellow Screening Test for Antimicrobial Detection in Ovine Milk. J Dairy Sci. 2007;90:5374–9.

313. Molina A, Molina MP, Althaus RL, Gallego L. Residue Persistence in Sheep Milk Following Antibiotic Therapy. Vet J. 2003;165:84–9.

314. Beltrán MC, Althaus RL, Molina A, Berruga MI, Molina MP. Analytical strategy for the detection of antibiotic residues in sheep and goat's milk. Spanish J Agric Res. 2015;13:e0501–e0501.

315. Beyene T. Veterinary Drug Residues in Food-animal Products: Its Risk Factors and Potential Effects on Public Health. J Vet Sci Technol. 2016;7:1.

316. Fejzić N, Begagić M, Šerić-Haračić S, Smajlović M. Beta lactam antibiotics residues in cow's milk: comparison of efficacy of three screening tests used in Bosnia and Herzegovina. Bosn J Basic Med Sci. 2014;14:155–9.

317. Rossi R, Saluti G, Moretti S, Diamanti I, Giusepponi D, Galarini R. Multiclass methods for the analysis of antibiotic residues in milk by liquid chromatography coupled to mass spectrometry: A review. Food Addit Contam Part A-Chem. 2017;35:241–57.

318. Official Journal of the European Union (OJEU). Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on Veterinary Medicinal Products and Repealing Directive 2001/82/EC. OJEU. 2019;4:1–125.

319. Sachi S, Ferdous J, Sikder H, Azizul SM, Hussani K. Antibiotic residues in milk: Past, present, and future. J Adv Vet Anim Res. 2019;6:315–32.

320. Mitchell JM, Griffiths MW, McEwen SA, McNab WB, Yee AJ. Antimicrobial Drug Residues in Milk and Meat: Causes, Concerns, Prevalence, Regulations, Tests, and Test Performance. J Food Prot. 1998;61:742–56.

321. Katz SE, Brady MS. Antibiotic residues in food and their significance. Food Biotechnol. 2000;14:147–71.

322. Baazize-Ammi D, Dechicha AS, Tassist A, Gharbi I, Hezil N, Kebbal S, et al. Screening and quantification of antibiotic residues in broiler chicken meat and milk in the central region of Algeria. Rev Sci Tech. 2019;38:863–77.

323. Wanniatie V, Sudarwanto MB, Purnawarman T, Jayanegara A. Chemical compositions, contaminants, and residues of organic and conventional goat milk in Bogor District, Indonesia. Vet World. 2019;12:1218–24.

324. Yamaki M, Berruga MI, Althaus RL, Molina MP, Molina A. Occurrence of Antibiotic Residues in Milk from Manchega Ewe Dairy Farms. J Dairy Sci. 2004;87:3132–7.

325. Gonzalo C, Carriedo JA, García-Jimeno MC, Pérez-Bilbao M, de la Fuente LF. Factors influencing variation of bulk milk antibiotic residue occurrence, somatic cell count, and total

bacterial count in dairy sheep flocks. J Dairy Sci. 2010;93:1587–95.

326. Tian L, Khalil S, Bayen S. Effect of thermal treatments on the degradation of antibiotic residues in food. Crit Rev Food Sci Nutr. 2017;57:3760–70.

327. Grunwald L, Petz M. Food processing effects on residues: penicillins in milk and yoghurt. Anal Chim Acta. 2003;483:73–9.

328. Cayle T, Guth JH, Hynes JT. Penicillin Distribution During Cheese Manufacture and Membrane Treatment of Whey. J Food Prot. 1986;49:796–8.

329. Shappell NW, Shelver WL, Lupton SJ, Fanaselle W, Van Doren JM, Hakk H. Distribution of animal drugs among curd, whey, and milk protein fractions in spiked skim milk and whey. J Agric Food Chem. 2017;65:938–49.

330. Giraldo J, Althaus RL, Beltrán MC, Molina MP. Antimicrobial activity in cheese whey as an indicator of antibiotic drug transfer from goat milk. Int Dairy J. 2017;69:40–4.

331. Hassan HF, Saidy L, Haddad R, Hosri C, Asmar S, Jammoul A, et al. Investigation of the effects of some processing conditions on the fate of oxytetracycline and tylosin antibiotics in the making of commonly consumed cheeses from the East Mediterranean. Vet World. 2021;14:1644–9.

332. Gajda A, Nowacka-Kozak E, Gbylik-Sikorska M, Posyniak A. Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. Food Addit Contam Part A-Chem. 2018;35:66–76.

333. Cabizza R, Rubattu N, Salis S, Pes M, Comunian R, Paba A, et al. Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications. LWT-Food Sci Technol. 2018;96:236–43.

334. Quintanilla P, Beltrán MC, Molina MP, Escriche I. Enrofloxacin treatment on dairy goats: Presence of antibiotic in milk and impact of residue on technological process and characteristics of mature cheese. Food Control. 2021;123:107762.

335. Tona GO, Olusola AD. Determination of tetracycline antibiotic residue in dairy products sold in Ogbomoso, south-western Nigeria. Int J Food Agric Vet Sci. 2014;4:136–40.

336. Olatoye IO, Daniel OF, Ishola SA. Screening of antibiotics and chemical analysis of penicillin residue in fresh milk and traditional dairy products in Oyo state, Nigeria. Vet World. 2016;9:948.

337. Bagre T, Samandoulougou S, Traore M, Illy D, Bsadjo-Tchamba G, Bawa-Ibrahim H, et al. Détection biologique des résidus d'antibiotiques dans le lait et produits laitiers de vache consommés à Ouagadougou, Burkina Faso. J Appl Biosci. 2015;87:8105–12.

338. Srimulyati A, Purnawarman T, Latif H. Tetracycline Residue Detection on Cheese Imported Trough Tanjung Priok Seaport, Jakarta. Glob Vet. 2015;14:819–23.

339. Adetunji VO. Effects of Processing on Antibiotic Residues (Streptomycin, Penicillin-G and Tetracycline) in Soft Cheese and Yoghurt Processing Lines. Pakistan J Nutr. 2011;10:792–5.

340. National Milk Drug Residue Database Informational Web Page. United States Food and Drug Administration Agency (FDA), White Oak, Maryland, United States. 2023. https://www.nmdrd.com/. Accessed 29 Jun 2023.

341. Brocca D., Salvatore S. Report for 2019 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. EFSA Support Publ. 2023;18:7886.

342. Marth EH, Ellickson BE. Problems created by the presence of antibiotics in milk and milk products–a review. J Food Prot. 1959;22:266–72.

343. Chiesa LM, DeCastelli L, Nobile M, Martucci F, Mosconi G, Fontana M, et al. Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. LWT-Food Sci Technol. 2020;131:109783.

344. Berruga I, Battacone G, Molina MP, Román M, Molina A. Influence of β-lactams on Manchego cheese manufacture. In: Pirisi A, Ayerbe A, Piredda G, Psathas G, Soustre Y, editors. Proceedings of the 5th International Symposium on the Challenge to Sheep and Goats Milk Sectors. Alghero: Small Ruminant Res. 2007. p. 222–24.

345. Aarestrup FM. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int J Antimicrob Agents. 1999;12:279–85.

346. Mathur S, Singh R. Antibiotic resistance in food lactic acid bacteria—a review. Int J Food Microbiol. 2005;105:281–95.

347. Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, et al. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect. 2015;6:22–9.

348. Wall BA, Mateus A, Marshall L, Pfeiffer D, Lubroth J, Ormel HJ, et al. Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Rome: Food and Agriculture Organization of the United Nations (FAO); 2016.

349. Firth CL, Käsbohrer A, Pless P, Koeberl-Jelovcan S, Obritzhauser W. Analysis of Antimicrobial Use and the Presence of Antimicrobial-Resistant Bacteria on Austrian Dairy Farms— A Pilot Study. Antibiotics-Basel. 2022;11:124.

350. Manie T, Brözel VS, Veith WJ, Gouws PA. Antimicrobial Resistance of Bacterial Flora Associated with Bovine Products in South Africa. J Food Prot. 1999;62:615–8.

351. Aarestrup FM, Wegener HC, Collignon P. Resistance in bacteria of the food chain: epidemiology and control strategies. Expert Rev Anti Infect Ther. 2008;6:733–50.

352. Zeina K, Pamela AK, Fawwak S, Zeina K, Pamela AK, Fawwak S. Quantification of Antibiotic Residues and Determination of Antimicrobial Resistance Profiles of Microorganisms Isolated from Bovine Milk in Lebanon. Food Nutr Sci. 2013;4:1–9.

353. European Centre for Disease Prevention and Control (ECDC); European Food Safety Authority (EFSA); European Medicines Agency (EMA). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA. EFSA J. 2021;19: e06712.

354. Brown K, Mugoh M, Call DR, Omulo S. Antibiotic residues and antibiotic-resistant bacteria detected in milk marketed for human consumption in Kibera, Nairobi. PLoS One. 2020;15:e0233413.

355. Zanella GN, Mikcha JMG, Bando E, Siqueira VLD, MacHinski M. Occurrence and Antibiotic Resistance of Conform Bacteria and Antimicrobial Residues in Pasteurized Cow's Milk from Brazil. J Food Prot. 2010;73:1684–7.

356. El-Zubeir IE, El Owni O. Antimicrobial Resistance of Bacteria Associated with Raw Milk Contaminated by Chemical Preservatives Value chain and processing of camel milk functional food View project Risk Assessments in Food Safety and Health (RAFaH) View project. World J Dairy Food Sci. 2009;4:65–9.

357. Jamet E, Akary E, Poisson MA, Chamba JF, Bertrand X, Serror P. Prevalence and characterization of antibiotic resistant *Enterococcus faecalis* in French cheeses. Food Microbiol. 2012;31:191–8.

358. Hammad AM, Hassan HA, Shimamoto T. Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. in Egyptian fresh raw milk cheese. Food Control. 2015;50:815–20.

359. Výrostková J, Regecová I, Dudriková E, Marcinčák S, Vargová M, Kováčová M, et al. Antimicrobial resistance of *Enterococcus* sp. Isolated from sheep and goat cheeses. Foods. 2021;10:1844.

360. Devirgiliis C, Barile S, Perozzi G. Antibiotic resistance determinants in the interplay between food and gut microbiota. Genes Nutr. 2011;6:275–84.

361. Abriouel H, Knapp CW, Gálvez A, Benomar N. Antibiotic Resistance Profile of Microbes From Traditional Fermented Foods. In: Frias J, Martinez-Villaluenga C, Peña E, editors. Fermented Foods in Health and Disease Prevention. 2017. p. 675–704.

362. Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, et al. Characterization of Antibiotic Resistance Genes from *Lactobacillus* Isolated from Traditional Dairy Products. J Food Sci. 2017;82:724–30.

363. Cerniglia CE, Pineiro SA, Kotarski SF. An update discussion on the current assessment of the safety of veterinary antimicrobial drug residues in food with regard to their impact on the human intestinal microbiome. Drug Test Anal. 2016;8:539–48.

364. Bacanlı M, Başaran N. Importance of antibiotic residues in animal food. Food Chem. Toxicol. 2019;125:462–466.

365. Francino MP. Antibiotics and the human gut microbiome: Dysbioses and accumulation of resistances. Front Microbiol. 2016;6:164577.

366. Cho I, Yamanishi S, Cox L, Methé BA, Zavadil J, Li K, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 2012;488:621–6.

367. Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol. Indic. 2008;8:1–13.

368. Marshall BM, Levy SB. Food animals and antimicrobials: Impacts on human health. Clin Microbiol Rev. 2011;24:718–33.

369. Dolliver H, Kumar K, Gupta S. Sulfamethazine Uptake by Plants from Manure-Amended Soil. J Environ Qual. 2007;36:1224–30.

370. Oliver JP, Gooch CA, Lansing S, Schueler J, Hurst JJ, Sassoubre L, et al. Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. J Dairy Sci. 2020;103:1051–71.

371. Bedekelabou AP, Talaki E, Dzogbema KFX, Dolou M, Savadogo M, Seko MO, et al. Assessing farm biosecurity and farmers' knowledge and practices concerning antibiotics and antibiotic resistance in poultry and pig farms in Southern Togo. Vet world. 2022;15:1727–37.

372. Lee K, Pereira R V., Martínez-López B, Busch RC, Pires AFA. Assessment of the knowledge and behavior of backyard and small-scale producers in California regarding disease prevention, biosecurity practices and antibiotics use. PLoS One. 2022;17:e0277897.

373. Moreno MA. Opinions of Spanish pig producers on the role, the level and the risk to public health of antimicrobial use in pigs. Res Vet Sci. 2014;97:26–31.

374. Brocca D, Salvatore S. Report for 2021 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. EFSA Support Publ. 2023;EN-7886:1–111.

375. Yamaki M, Berruga MI, Althaus RL, Molina MP, Molina A. Screening of antibiotic residues in ewes' milk destined to cheese by a commercial microbiological inhibition assay. Food Addit Contam. 2006;23:660–667.

376. Richards ED, Pereira R V., Davis JL, Rowe JD, Clapham MO, Wetzlich SE, et al. Comparison of florfenicol depletion in dairy goat milk using ultra-performance liquid chromatography with tandem mass spectrometry and a commercial on-farm test. Front Vet Sci. 2022;9:991772.

377. Vergara-Luis I, Bocayá N, Irazola-Duñabeitia M, Zuloaga O, Lacuesta M, Olivares M, et al. Multitarget and suspect screening of antimicrobials in soil and manure by means of QuEChERS — liquid chromatography tandem mass spectrometry. Anal Bioanal Chem. 2023;415:6291–6310.

378. Zhang Y, Cheng D, Xie J, Zhang Y, Wan Y, Zhang Y, et al. Impacts of farmland application of antibiotic-contaminated manures on the occurrence of antibiotic residues and antibiotic resistance genes in soil: A meta-analysis study. Chemosphere. 2022;300:134529.

379. Berendsen BJA, Wegh RS, Memelink J, Zuidema T, Stolker LAM. The analysis of animal faeces as a tool to monitor antibiotic usage. Talanta. 2015;132:258–68.

380. Peng S, Zhang H, Song D, Chen H, Lin X, Wang Y, et al. Distribution of antibiotic, heavy metals and antibiotic resistance genes in livestock and poultry feces from different scale of farms in Ningxia, China. J Hazard Mater. 2022;440:129719.

381. Kuppusamy S, Kakarla D, Venkateswarlu K, Megharaj M, Yoon YE, Lee YB. Veterinary antibiotics (VAs) contamination as a global agro-ecological issue: A critical view. Agric Ecosyst Environ. 2018;257:47–59.

382. Pérez-Elortondo FJ, Albisu M, Barcina Y. Physicochemical properties and secondary microflora variability in the manufacture and ripening of Idiazabal cheese. Lait. 1999;79:281–90.

383. Blaiotta G, Marrone R, Aponte M, Peruzy MF, Smaldone G, Vollano L, et al. Characterisation of Conciato Romano: one of the oldest Italian cheeses. Int Dairy J. 2021;120:105077.

384. Rocha R, Couto N, Pinto RP, Vaz-Velho M, Fernandes P, Santos J. Microbiological Characterization of Protected Designation of Origin Serra da Estrela Cheese. Foods. 2023;12:2008.

385. Ferrocino I, Rantsiou K, Cocolin L. Investigating dairy microbiome: an opportunity to ensure quality, safety and typicity. Curr Opin Biotechnol. 2022;73:164–70.

386. Chen T, Wang L, Li Q, Long Y, Lin Y, Yin J, et al. Functional probiotics of lactic acid bacteria

from Hu sheep milk. BMC Microbiol. 2020;20:228.

387. Kmet' V, Drugdová Z. Antimicrobial susceptibility of microflora from ovine cheese. Folia Microbiol. 2012;57:291–3.

388. Nalepa B, Markiewicz LH. Microbiological Biodiversity of Regional Cow, Goat and Ewe Milk Cheeses Produced in Poland and Antibiotic Resistance of Lactic Acid Bacteria Isolated from Them. Animals. 2023;13:168.

389. Rajput K, Dubey RC, Kumar A. Probiotic potential and immunomodulatory properties in *Enterococcus faecium* GMB24 and *Enterococcus hirae* SMB16 isolated from goat and sheep milk. Arch Microbiol. 2022;204:619.

390. Tsigkrimani M, Panagiotarea K, Paramithiotis S, Bosnea L, Pappa E, Drosinos EH, et al. Microbial Ecology of Sheep Milk, Artisanal Feta, and Kefalograviera Cheeses. Part II: Technological, Safety, and Probiotic Attributes of Lactic Acid Bacteria Isolates. Foods. 2022;11:459.

391. Nunziata L, Brasca M, Morandi S, Silvetti T. Antibiotic resistance in wild and commercial non-enterococcal Lactic Acid Bacteria and Bifidobacteria strains of dairy origin: An update. Food Microbiol. 2022;104:103999.

392. Tsigkrimani M, Bakogianni M, Paramithiotis S, Bosnea L, Pappa E, Drosinos EH, et al. Microbial Ecology of Artisanal Feta and Kefalograviera Cheeses, Part I: Bacterial Community and Its Functional Characteristics with Focus on Lactic Acid Bacteria as Determined by Culture-Dependent Methods and Phenotype Microarrays. Microorganisms. 2022;10:161.

393. Obioha PI, Anyogu A, Awamaria B, Ghoddusi HB, Ouoba LII. Antimicrobial Resistance of Lactic Acid Bacteria from Nono, a Naturally Fermented Milk Product. Antibiotics-Basel. 2023;12:843.

394. Rosander A, Connolly E, Roos S. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Appl Environ Microbiol. 2008;74:6032–40.

395. Chen K, Chan EWC, Xie M, Ye L, Dong N, Chen S. Widespread distribution of *mcr*-1-bearing bacteria in the ecosystem, 2015 to 2016. Eurosurveillance. 2017;22:17–00206.

396. Nagy Á, Székelyhidi R, Hanczné Lakatos E, Kapcsándi V. Review on the occurrence of the *mcr*-1 gene causing colistin resistance in cow's milk and dairy products. Heliyon. 2021;7:e06800.

397. Zheng B, Feng C, Xu H, Yu X, Guo L, Jiang X, et al. Detection and characterization of ESBL-producing *Escherichia coli* expressing *mcr*-1 from dairy cows in China. J Antimicrob Chemother. 2019;74:321–325.

398. Oguntoyinbo FA, Okueso O. Prevalence, distribution and antibiotic resistance pattern among enterococci species in two traditional fermented dairy foods. Ann Microbiol. 2013;63:755–761.

399. Caprioli G, Kamgang Nzekoue F, Fiorini D, Scocco P, Trabalza-Marinucci M, Acuti G, et al. The effects of feeding supplementation on the nutritional quality of milk and cheese from sheep grazing on dry pasture. Int J Food Sci Nutr. 2020;71:50–62.

400. Tzora A, Nelli A, Kritikou AS, Katsarou D, Giannenas I, Lagkouvardos I, et al. The "Crosstalk" between Microbiota and Metabolomic Profile of Kefalograviera Cheese after the

Innovative Feeding Strategy of Dairy Sheep by Omega-3 Fatty Acids. Foods. 2022;11:3164.

401. Doyle CJ, Gleeson D, O'Toole PW, Cotter PD. Impacts of seasonal housing and teat preparation on raw milk microbiota: A high-throughput sequencing study. Appl Environ Microbiol. 2017;83.

402. Verdier-Metz I, Delbès C, Bouchon M, Rifa E, Theil S, Chaucheyras-Durand F, et al. Dietary Live Yeast Supplementation Influence on Cow's Milk, Teat and Bedding Microbiota in a Grass-Diet Dairy System. Microorganisms. 2023;11:673.

403. Ruegg PL. The bovine milk microbiome – an evolving science. Domest Anim Endocrinol. 2022;79:106708.

404. Dahlberg J, Williams JE, McGuire MA, Peterson HK, Östensson K, Agenäs S, et al. Microbiota of bovine milk, teat skin, and teat canal: Similarity and variation due to sampling technique and milk fraction. J Dairy Sci. 2020;103:7322–30.

405. Guo W, Bi SS, Wang WW, Zhou M, Neves ALA, Degen AA, et al. Maternal rumen and milk microbiota shape the establishment of early-life rumen microbiota in grazing yak calves. J Dairy Sci. 2023;106:2054–70.

406. Bi Y, Cox MS, Zhang F, Suen G, Zhang N, Tu Y, et al. Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. Environ Microbiol. 2019;21:2333–46.

407. Cruciata M, Sannino C, Ercolini D, Scatassa ML, De Filippis F, Mancuso I, et al. Animal Rennets as Sources of Dairy Lactic Acid Bacteria. Appl Environ Microbiol. 2014;80:2050.

408. Cosentino S, Palmas F. Hygienic conditions and microbial contamination in six ewe's-milk-processing plants in Sardinia, Italy. J Food Prot. 1997;60:283–7.

409. Gil PF, Conde S, Albisu M, Pérez-Elortondo FJ, Etayo I, Virto M, et al. Hygienic quality of ewes' milk cheeses manufactured with artisan-produced lamb rennet pastes. J Dairy Res. 2007;74:329–35.

410. Alvarez-Molina A, Cobo-Díaz JF, Alexa EA, Crispie F, Prieto M, López M, et al. Sequencing-based analysis of the microbiomes of Spanish food processing facilities reveals environment-specific variation in the dominant taxa and antibiotic resistance genes. Food Res Int. 2023;173:113442.

411. Cobo-Díaz JF, Alvarez-Molina A, Alexa EA, Walsh CJ, Mencía-Ares O, Puente-Gómez P, et al. Microbial colonization and resistome dynamics in food processing environments of a newly opened pork cutting industry during 1.5 years of activity. Microbiome. 2021;9:204.

412. Valentino V, Sequino G, Cobo-Díaz JF, Álvarez-Ordóñez A, De Filippis F, Ercolini D. Evidence of virulence and antibiotic resistance genes from the microbiome mapping in minimally processed vegetables producing facilities. Food Res Int. 2022;162:112202.

413. Haastrup MK, Johansen P, Malskær AH, Castro-Mejía JL, Kot W, Krych L, et al. Cheese brines from Danish dairies reveal a complex microbiota comprising several halotolerant bacteria and yeasts. Int J Food Microbiol. 2018;285:173–87.

414. Marino M, Innocente N, Maifreni M, Mounier J, Cobo-Díaz JF, Coton E, et al. Diversity within Italian Cheesemaking Brine-Associated Bacterial Communities Evidenced by Massive Parallel 16S rRNA Gene Tag Sequencing. Front Microbiol. 2017;0:2119.

415. Levante A, Bertani G, Marrella M, Mucchetti G, Bernini V, Lazzi C, et al. The microbiota of Mozzarella di Bufala Campana PDO cheese: a study across the manufacturing process. Front Microbiol. 2023;14:1196879.

416. Arias-Roth E, Bachmann H-P, Fröhlich-Wyder M-T, Schmidt RS, Wechsler D, Beuvier E, et al. Raw Milk Cheeses. Encycl Dairy Sci. 2022;299–308.

417. Delannoy S, Hoffer C, Tran ML, Madec JY, Brisabois A, Fach P, et al. High throughput qPCR analyses suggest that Enterobacterales of French sheep and cow cheese rarely carry genes conferring resistances to critically important antibiotics for human medicine. Int J Food Microbiol. 2023;403:110303.

418. Huang J, Zhang W, Sun B, Jiang Q, Cao Y, Shang J, et al. Genetic diversity, antibiotic resistance, and virulence characteristics of *Staphylococcus aureus* from raw milk over 10 years in Shanghai. Int J Food Microbiol. 2023;401:110273.

419. Liu X, Zuo J, Teng J, Yang L, Guo J, Liu L, et al. Antibiofilm potential of luteolin against multidrug-resistant *Staphylococcus aureus* isolated from dairy goats and farm environments. Environ Pollut. 2023;335:122274.

420. Tyrrell C, Do TT, Leigh RJ, Burgess CM, Brennan FP, Walsh F. Differential impact of swine, bovine and poultry manure on the microbiome and resistome of agricultural grassland. Sci Total Environ. 2023;886:163926.

421. Do TT, Smyth C, Crispie F, Burgess C, Brennan F, Walsh F. Comparison of soil and grass microbiomes and resistomes reveals grass as a greater antimicrobial resistance reservoir than soil. Sci Total Environ. 2023;857:159179.

422. Fortier LC, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence. 2013;4:354.

423. Teklemariam AD, Al-Hindi RR, Qadri I, Alharbi MG, Ramadan WS, Ayubu J, et al. The Battle between Bacteria and Bacteriophages: A Conundrum to Their Immune System. Antibiotics -Basel. 2023;12:381.

424. Anika TT, Al Noman Z, Islam MS, Sultana N, Ashraf MN, Pervin M, et al. Draft genome sequence of multidrug-resistant *Escherichia coli* MAHK_SCM_BAU_30A strain isolated from a subclinical mastitis cow in Bangladesh. Microbiol Resour Announc. 2023;12:e0071323.

425. Güley Z, Fallico V, Cabrera-Rubio R, Cotter PD, Beresford T. Identification of *Streptococcus infantarius* subsp. *infantarius* as the species primarily responsible for acid production in Izmir Brined Tulum Cheese from the Aegean Region of Türkiye. Food Res Int. 2022;160:111707.

426. Eliasson T, Sun L, Lundh Å, Höjer A, Saedén KH, Hetta M, et al. Epiphytic microbiota in Swedish grass-clover herbage and the effect of silage additives on fermentation profiles and bacterial community compositions of the resulting silages. J Appl Microbiol. 2023;134:lxad196.

6. UNPUBLISHED RESULTS

The accepted or published manuscripts constituting this thesis are in **Section III: appendices.** However, in this section, **manuscript VII** is included, as it has been submitted for publication but has not yet been accepted or published.

7 | Manuscript VII

Submitted to be published

Shotgun metagenomic sequencing reveals the influence of artisanal dairy environments on the microbiomes, quality and safety of a raw milk PDO cheese

Gorka Santamarina-García[1,2,3*], Min Yap[4], Fiona Crispie[4], Gustavo Amores[1,2,3], Cathy Lordan[4], Mailo Virto[1,2,3] and Paul Cotter[4,5,6]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[2] *Bioaraba Health Research Institute-Prevention, Promotion and Health Care, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[3] *Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[4] *Department of Food Biosciences, Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, P61 C996, Ireland*

[5] *APC Microbiome Ireland, University College Cork, Cork, T12 YT57, Ireland*

[6] *VistaMilk SFI Research Centre, Moorepark, Fermoy, Co., Cork, P61 C996, Ireland*

[*] *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented or accepted to be presented at:

28th International ICFMH (International Committee on Food Microbiology and Hygiene) Conference FOOD MICRO 2024, 2024. *The influence of dairy environment and practices on the microbiome of raw ewe milk-derived PDO cheeses: a shotgun sequencing approach.* Santamarina-García, G., Yap, M., Crispie, F., Amores, G., Lordan, C., Virto, M. & Cotter, P.D. (Poster presentation).

IDF Cheese Science & Technology Symposium 2024, 2024. *Resistome analysis of Idiazabal PDO cheeses: elucidating the impact of dairy environments.* Santamarina-García, G., Yap, M., Crispie, F., Amores, G., Lordan, C., Barron, L.J.R., Virto, M. & Cotter, P.D. (Poster presentation).

Under review in **Microbiome**
ISSN: 2049-2618
IF (2022): **15.5**
Category: **Microbiology (D1, 7/135)**

Shotgun metagenomic sequencing reveals the influence of artisanal dairy environments on the microbiomes, quality and safety of a raw milk PDO cheese.

Gorka Santamarina-García[1,2,3]*, Min Yap[4], Fiona Crispie[4], Gustavo Amores[1,2,3], Cathy Lordan[4], Mailo Virto [1,2,3], Paul D. Cotter[4,5,6]

[1] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain; gustavo.amores@ehu.eus; mailo.virto@ehu.eus

[2] Bioaraba Health Research Institute-Prevention, Promotion and Health Care, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

[3] Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

[4] Department of Food Biosciences, Teagasc Food Research Centre, Teagasc - The Irish Agriculture and Food Development Authority, Moorepark, Fermoy, Co., Cork, P61 C996, Ireland; min.yap@teagasc.ie; fiona.crispie@teagasc.ie; cathy.lordan@teagasc.ie; paul.cotter@teagasc.ie

[5] APC Microbiome Ireland, University College Cork, Cork, T12 YT57, Ireland

[6] VistaMilk SFI Research Centre, Moorepark, Fermoy, Co., Cork, P61 C996, Ireland.

* Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799.

Abstract:

Background: Numerous studies have highlighted the impact of bacterial communities on the quality and safety of raw ewe milk-derived cheeses. Despite reported differences in the microbiota among cheese types and even producers, to the best of our knowledge, no study has comprehensively assessed all potential microbial sources and their contributions to any raw ewe milk-derived cheese, which could suppose great potential for benefits from research in this area. Here, using the Protected Designation of Origin Idiazabal cheese as an example, the impact of the environment and practices of artisanal dairies (including herd feed, teat skin, dairy surfaces and ingredients) on the microbiomes of the associated raw milk, whey, and derived cheeses was examined through shotgun metagenomic sequencing.

Results: The results revealed diverse microbial ecosystems across sample types, comprising more than 1300 bacterial genera and 3400 species. SourceTracker analysis revealed commercial feed and teat skin as major contributors to the raw milk microbiota (45.6 % and 33.5 %, respectively), being a source of, for example, *Lactococcus* and *Pantoea*, along with rennet contributing to the composition of whey and cheese (17.4 % and 41.0 %, respectively), including taxa such as *Streptococcus, Pseudomonas_E* or *Lactobacillus_H*. Functional analysis linked microbial niches to cheese quality- and safety-related metabolic pathways, with brine and food contact surfaces being most relevant, related to genera like *Brevibacterium, Methylobacterium* or *Halomonas*. With respect to the virulome (virulence-associated gene profile), in addition to whey and cheese, commercial feed and grass were the main reservoirs (related to, e.g., *Brevibacillus_B* or *CAG-196*). Similarly, grass, teat skin or rennet were the main contributors of antimicrobial resistance genes (e.g., *Bact-11* or *Bacteriodes_B*). In terms of cheese aroma and texture, apart from the microbiome of the cheese itself, brine, grass and food contact surfaces were key reservoirs for hydrolase-encoding genes, originating from, for example, *Lactococcus, Lactobacillus, Listeria* or *Chromohalobacter*. Furthermore, over 300 metagenomic assembled genomes (MAGs) were generated, including 60 high-quality MAGs, yielding 28 novel putative species from several genera, e.g., *Citricoccus, Corynebacterium* or *Dietzia*.

Conclusion: This study emphasizes the role of the artisanal dairy environments in determining cheese microbiota and, consequently, quality and safety.

Keywords: metagenomics; shotgun sequencing; virulence factors; antimicrobial resistance genes; hydrolase-encoding genes; metagenome-assembled genomes; cheese quality; cheese safety.

1. Background

Dairy products, particularly cheese, are manufactured worldwide using a wide range of production systems and technologies [1]. This leads to a myriad of cheeses with different aromas and textures, making them a versatile and enjoyable product that enriches cultures and, consequently, gastronomic experiences [2]. In particular, raw milk cheeses are regarded as premium dairy products [3], due to their richer and more intense aromatic profiles than pasteurized milk cheeses [4,5]. Thus, a preference among consumers for raw milk cheeses has been reported [6]. Particular sensory properties of raw milk cheeses are primarily attributed to the microbiota, given their contribution to numerous biochemical reactions that determine cheese quality [7,8], including lactose metabolism, proteolysis and lipolysis [8–11]. The microbiota produces a broad range of enzymes (e.g., proteases, lipases or esterases) and consequent metabolites (e.g., free amino acids, free fatty acids or volatile compounds), which, together with their subsequent metabolism, determine the aroma and texture of cheese [8,12]. The microbiota also contributes to ensuring cheese safety, by reducing pathogens through competitive inhibition mechanisms (e.g., bacteriocins or free fatty acids) [10,13]. Nevertheless, microbial communities can also compromise cheese safety, contributing to the presence of virulence factors (VFs) [14,15] or antimicrobial resistance genes (ARGs), and thereby serve as potential reservoirs for the transfer to other bacteria, which could include pathogens, in the food chain, including the gastrointestinal tract [16]. Likewise, several undesirable compounds can also be synthetized by the microbiota of cheeses and associated environments [17], such as biogenic amines [10,18].

Next-generation sequencing (NGS) technologies, such as amplicon (commonly targeting hypervariable regions of 16S rRNA gene) and shotgun sequencing (sequencing of all genetic material), have emerged as indispensable tools for characterizing the microbiota of raw milk cheeses [19–21]. The advantages of NGS for cheese microbiota characterization are the high resolution at species level, semi-quantitative analysis, and the ability to cover non-cultivable species [21,22]. Although amplicon sequencing offers cost-effective and rapid profiling of microbial community structure and taxonomy [23], shotgun sequencing facilitates a more comprehensive assessment of the microbiome [21]. This approach not only yields more accurate taxonomic profiling, including identification of new species [20,24], but also provides an understanding of the functional potential of microbial communities (e.g., metabolic pathways or genes) [20]. However, using this technology involves increased data complexity, bioinformatics requirements, and its associated costs [21].

Overall, the microbiota of raw milk cheeses is composed of a wide range of bacteria, encompassing lactic acid bacteria (LAB), other environmental bacteria and undesirable bacteria [25]. LAB, including species of several genera, such as *Lactococcus, Lactobacillus, Streptococcus, Enterococcus, Leuconostoc* or *Carnobacterium* [26], are essential during cheese-making and ripening because of their contribution to the aforementioned biochemical processes that affect cheese flavour and texture, or pathogen inhibition [8,9,25,26]. Nevertheless, in some cases, they have also been related to the production of biogenic amines (BAs) or antimicrobial resistances (AMRs) [10,18,27]. The cheese microbiota is also composed of undesirable bacteria, such as particular species of *Staphylococcus, Clostridium* or *Listeria*, which possess pathogenic or spoilage potential, and other environmental bacteria, which are primarily derived from the cheese production environment [13,25]. Great differences in the microbial composition among types of cheeses have been reported [28–31] and even among producers of the same type of cheese [25,32]. Sources of bacteria in milk and cheese are diverse and complex [33], which may include geographical location [34], flock/herd management and feeding [35], microorganisms contaminating the teat surface [35], or practices, materials and ingredients employed during milking or in the dairy environment [26,36,37]. Although the impact of the aforementioned factors has been studied, in all cases, the impact of a single or few factors has been analysed [26,33,35–37], none comprehensively studying all or the majority of the potential factors that could have affected. Furthermore, most studies have focused only on cow milk-derived cheeses, with little information relating to cheeses made from the milk of small ruminants, such as sheep [33,35].

The present work was conducted with the Protected Designation of Origin (PDO) Idiazabal cheese, a traditional semi-hard or hard cheese manufactured from autochthonous raw ewe milk in the Basque Country (southwestern Europe). The aim of this study was to (1) investigate the microbiota inhabiting the artisanal dairy environments (e.g., herd feed, ingredients and materials), (2) identify the extent to which these factors influence the microbiota in raw ewe milk and derived cheeses, and (3) explore their functional potential , resistome, virulome and enzymatic potential, in order to unravel the impact of the microbiomes of artisanal dairies on the quality and safety of raw milk cheeses. To the best of our knowledge, no such comprehensive study has been conducted to date on any raw ewe milk-derived cheese, which would greatly contribute to this expanding field of research, providing novel knowledge directly applicable to the sector.

2. Methods

2.1 Area of study

This case study was carried out with the European PDO Idiazabal cheese, a semi-hard or hard cheese manufactured from the raw milk of the autochthonous Latxa and/or Carranzana sheep breeds. It is primarily produced by small-scale artisanal dairies that oversee the entire process, from flock management to cheese-making and sales. The entire production chain, from flock management to milking and cheese-making, occurs in Southern Basque Country (southwestern Europe), covering an area of 17,213.06 km^2 (43° 27'-41° 54' N and 1° 5'-3° 37' W). This territory corresponds to the natural habitat of the autochthonous sheep breeds [38]. Flock management involves indoor foraging in winter and extensive grazing in spring. Milking and cheese-making processes occur primarily between January and June, following the traditional seasonal approach dictated by the biological rhythms of the sheep (BOE, 1993). Milking is performed mainly by mechanical systems, and it is refrigerated for subsequent cheese-making. For cheese-making, each producer employs the milk of its own flock and follows the specifications issued by the Idiazabal Designation of Origin Regulatory Board [39]. Briefly, the raw milk collected on the same day is warmed to 25 °C and the starter culture Choozit MM 100 LYO 50 DCU (mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*) (DuPont NHIB Ibérica S.L., Barcelona, Spain) is added. Milk coagulation occurs at 28-32 °C for 20-45 min, using artisanal rennet (stomachs of Latxa or Carranzana lambs; extracted during the first month of lactation, cleaned, dried, salted and ground). The resulting curds are cut to a grain size (5-10 mm in diameter), heated to 36-38 °C, moulded and pressed for 10-12 hours. Then, the cheeses are salted in saturated brine for 24-48 hours and finally, ripened in chambers maintained at 80-95 % relative humidity and 8-14 °C temperature, at least, for 2 months.

2.2 Sample collection

For sampling, two Idiazabal PDO dairies, identified as A and B, located geographically close to each other were selected to avoid or minimize the impact of geographical conditions (temperature, precipitation, humidity, etc.) on the microbiota [34]. The sampling took place between January and April 2023, both producers managing flocks consisting of approximately 350-400 Latxa sheep and following similar herd management and cheese-making conditions, as stated before. Several sample types were collected from each producer to identify all the potential microbial reservoirs within artisanal dairies. According to herd management and feeding methods, 250 g of grass obtained from fresh pastures and 500 g of commercial feed were collected from both producers. Additionally, from Producer A, straw obtained from their own fields (150 g) and homemade feed (500 g), comprising a blend of grass, corn, and beet, were also collected. The day before milking, 30 swab samples, each from 10 to 15 sheep, of teat skin surfaces were also collected with sterile gauze swabs (7.5 cm × 7.5 cm) (Medrull, Dortmund, Deutschland) moistened in 0.9 % (w/v) NaCl sterile solution (Scharlab, Barcelona, Spain); the swabs were subsequently placed in individual sterile 100 mL bottles (Deltalab). After milking, 1 L of raw milk was collected in sterilized 1 L borosilicate glass bottles (Deltalab). From the dairies, 30 swab samples of food contact surfaces (e.g., equipment, materials, trays, shelves, etc.) and 30 swab samples of non-food contact surfaces (e.g., floors, walls, etc.) were taken with sterile gauze swabs (7.5 cm × 7.5 cm) (Medrull) moistened in 0.9 % (w/v) NaCl sterile solution (Scharlab), which were subsequently placed in individual sterile 100 mL bottles (Deltalab). Moreover, 50 g of artisanal rennet and 1 L of brine were collected in sterile 100 mL bottles (Deltalab) and sterilized 1 L borosilicate glass bottles (Deltalab), respectively. After cheese-making, 1 L of the generated whey was collected in sterilized 1 L borosilicate glass bottles (Deltalab). The cheeses were ripened for 60 days prior to collecting 2 individual cheeses (1-1.5 kg each) from each producer. All the samples were collected aseptically by producers and researchers, using appropriate personal protective equipment, disinfected gloves and sterile materials to avoid cross-contamination. Samples derived from animals were collected by producers, consequently, the approval of the Ethics Committee for Animal Experimentation was not needed. The samples were transported under refrigeration (3 °C) to the laboratory.

2.3 DNA extraction

Genomic DNA extraction was performed from fresh samples according to the method previously described [25], with some modifications based on previous studies [40–44]. To extract DNA from the cheese samples, 10 g of cheese was suspended in 90 mL of 2 % (w/v) sterile sodium citrate (pH 8.0) and homogenized 6 times, each for 20 s on and 10 s off, in a stomacher (Masticator Basic 400, IUL Instruments, Königswinter, Germany). The resulting suspension was centrifuged at 6,500 × *g* for 8 min at 4 °C. The supernatant that contained the fat layer was discarded, and the pellet was washed with 50 mL of sodium citrate and centrifuged again (6,500 × *g* for 8 min at 4 °C) to harvest the microbiota. The obtained pellet was washed with 800 µL of sodium citrate and centrifuged three times (6,500 × *g* for 8 min at 4 °C). For the milk and whey samples, 100 mL were mixed with 200 mL of 2 % (w/v) sterile sodium citrate (pH 8.0) and

processed as described for the cheese samples without the homogenization step. To extract the DNA from commercial feed samples, 200 g was suspended in 90 mL of buffered peptone water (BPW) and sonicated for 6 min. Then, the commercial feed was removed, and the resulting suspension was centrifuged at 100 × *g* for 1 min at 4 °C to sediment solid impurities that would interfere with sequencing. The resulting suspension was subsequently centrifuged at 15,000 × *g* for 8 min at 4 °C. The supernatant was discarded, and the pellet was washed with 50 mL of BPW and centrifuged again (15,000 × *g* for 8 min at 4 °C). The obtained pellet was washed with 800 µL of BPW and centrifuged thrice (15,000 × *g* for 8 min at 4 °C). The procedure for extracting the DNA from the homemade feed, grass and straw was the same but the initial starting weights were changed to 200 g, 100 g and 50 g, respectively. For the teat skin, food contact and non-food contact surface samples, pools of seven to eight gauzes from each sample type were suspended in 90 mL of BPW and vigorously shaken for 1 min thrice to dislodge microbial communities. Then, the resulting suspension was centrifuged at 100 × *g* for 1 min at 4 °C to sediment solid impurities that would interfere with sequencing, and subsequently processed similarly to the feed samples. For the rennet samples, 10 g of artisanal rennet was suspended in 90 mL of BPW and homogenized 6 times, each for 20 s on and 10 s off, in a stomacher. The resulting suspension was centrifuged at 100 × *g* for 1 min at 4 °C to sediment solid impurities and processed as for the feed samples. For the brine samples, 300 mL of brine was suspended in 600 mL of BPW and it was processed as for the rennet samples but without the homogenization step. The DNA of all the samples was extracted using QIAamp® PowerFecal® Pro DNA Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, but the elution volume was reduced to 60 µL, and a double elution step was used to increase the DNA yield. Extracted DNA was stored at −80 °C until sequencing.

The quantity and quality of the extracted DNA were verified by means of a TryCell 2.0 system (Hellma, Southend-on-Sea, United Kingdom) coupled to a Cary 50 UV–Vis spectrophotometer with Varian UV RNA–DNA estimation application software (version 3.00 (399), Agilent Technologies, Inc., Santa Clara, United States). DNA integrity and purity were checked by 1 % agarose gel electrophoresis using GelRed dye (Biotium, Inc., Fremont, CA, United States), 10X BlueJuice gel loading buffer (Invitrogen, Waltham, United States), FastGene 100 bp DNA Marker (NIPPON Genetics EUROPE GmbH, Düren, Germany), and the U:Genius 3 system (Synoptics, Cambridge, United Kingdom).

2.4 Library construction and shotgun sequencing

Prior to preparing the libraries, accurate DNA quantification was performed in a Qubit® fluorimeter, using Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) and broad-range (BR) assay kits (Bio-Sciences, Dublin, Ireland), following the manufacturer's instructions. From the extracted DNA, 150 bp paired-end sequencing libraries were prepared for shotgun metagenomic sequencing using the Illumina DNA prep kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions and indexed using unique dual indices (UDIs) (Integrated DNA Technologies, Coralville, IA, USA). Following indexing and clean up, the quantity and quality were checked using a Qubit® fluorimeter and an Agilent 2100 BioAnalyzer system with a high-sensitivity DNA kit (Agilent Technologies, Inc.), respectively. The DNA was pooled equimolarly, a further 0.8X bead:product clean up was performed with ampure beads (Beckman Coulter), and finally sequenced on a NextSeq 2000 using a P1 300 cycle cartridge, according to manufacturer's guidelines (Illumina Inc.), at the Teagasc DNA sequencing facility (Moorepark, Cork, Ireland).

2.5 Quality filtering and trimming

All bioinformatic processing was executed with the Teagasc high-performance computing cluster. First, the raw paired-end FASTQ files were trimmed using Cutadapt version 1.18 (https://github.com/marcelm/cutadapt/) to remove adapter sequences. The quality of the reads was assessed using FastQC version 0.11.8 (https://github.com/s-andrews/FastQC), and low-quality reads were removed. The reads were aligned to the ovine genome (*Ovis aries*) using Bowtie2 version 2.4.4 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), and all unmapped nonhost reads were assumed to be microbial. Unmapped reads were extracted with samtools version 1.9 (https://github.com/samtools/samtools) and split into paired-end fastq files with bamtofastq from bedtools version 2.27.1 (https://github.com/arq5x/bedtools2).

2.6 Taxonomic classification

Taxonomic classification was performed using Kraken2 (https://github.com/DerrickWood/kraken2), which classifies DNA sequences with high sensitivity and speed by assigning taxonomic labels based on a classification algorithm and exact k-mer matches with reference genomes, against the the genome taxonomy database (GTDB) release 89 (https://gtdb.ecogenomic.org/), focusing only on the bacterial reads.

2.7 Diversity analysis

Alpha diversity was calculated in RStudio version 2023.03.1 and R version 4.3.0 (R Core Team, Vienna, Austria, 2023). Data cleaning and preparation for analysis were conducted with the "tidyverse" package (https://github.com/tidyverse). The Shannon, Simpson, Inverse Simpson, Berger and Shannon evenness (Jevenness and Eevenness) diversity indices were calculated through the "BiodiversityR" package (https://github.com/cran/BiodiversityR), and the Chao1 and ACE diversity indices were calculated with the "vegan" package (https://github.com/vegandevs/vegan). Significant differences between producers for each diversity index were analysed by means of Kruskal-Wallis analysis in the IBM SPSS statistical package version 26.0 (IBM SPSS Inc., Chicago, IL, USA, 2019). Beta diversity based on Bray–Curtis dissimilarities was also calculated in R through the "vegan" package and plotted into a Principal Coordinate Analysis (PCoA) model based on Bray–Curtis dissimilarities with the "APE" package (https://github.com/cran/ape).

2.8 Source tracker analysis

The sources of the bacterial communities in the raw milk, whey and cheese samples (sinks) were analysed by means of Bayesian-based SourceTracker2 version 2.0.1 (https://github.com/caporaso-lab/sourcetracker2). The microbiota of feed, teat skin surface and food contact and non-food contact surface samples were defined as potential bacterial sources for raw milk, together with rennet for whey, and rennet and brine for cheese. Default settings were selected for the analysis, including a rarefaction depth of 1000, burn-in 100, restart 10, alpha 0.001 and beta 0.01. The percentage influence of each potential bacterial source on each sink was calculated through SourceTracker2.

2.9 Functional potential

The bacterial functional potential of the shotgun metagenomic reads was assessed by means of SUPERFOCUS version 0.34 (https://github.com/metageni/SUPER-FOCUS), using DIAMOND aligner (https://github.com/bbuchfink/diamond) against the SEED database (https://github.com/topics/seed-database), which assigns reads to homologous gene families to determine functional potential. It aggregates these gene families into higher levels of organization to serve a broader function. The highest level of organization in SUPERFOCUS is subsystem level 1, followed by levels 2 and 3, with level 3 representing the most specific functions. The relationship between microbial communities and functional potential was calculated by an orthogonal partial least squares (OPLS) approach applied to log-transformed, when necessary, and UV-scaled data in SIMCA. The microbial communities were selected as X variables, and the functional pathways were selected as Y variables. The model was validated by R2 and Q2 values, Permutation test or Inner Relation plot. The key bacterial communities were identified based on VIP values and loading weights, together with Spearman's rank correlations calculated in SPSS and interpreted in a heatmap with hierarchical clustering analysis (HCA) performed in R with the "pheatmap" package (https://github.com/raivokolde/pheatmap).

2.10 Resistome analysis

Antimicrobial resistome analysis involving the identification and quantification of antibiotic resistance genes (ARGs) was performed by aligning metagenomic reads using ShortBRED version 1.0 (https://github.com/biobakery/shortbred) against resistance gene markers from the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/). The relative abundance of ARGs was expressed as the normalized reads per kilobase per million reads (RPKM) [45]. The relationship between microbial communities and ARGs was calculated by an OPLS approach, as described before.

2.11 Virulome analysis

The identification and quantification of virulence factors (VFs) were performed using ShortBRED (https://github.com/biobakery/shortbred), where shotgun metagenomic reads were mapped against the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm). The relative abundance of VFs was expressed as the normalized reads per kilobase per million reads (RPKMs) [45]. The relationship between microbial communities and VFs was calculated by an OPLS approach, as described before.

2.12 Analysis of enzyme-encoding genes

Hydrolase-encoding gene analysis was performed through alignment of reads against ESTerases and alpha/beta-Hydrolase Enzymes and Relatives (ESTHER) database (https://bioweb.supagro.inrae.fr/ESTHER/general?what=index), using DIAMOND version 2.1.7 (https://github.com/bbuchfink/diamond). The best hits were selected for further analysis,

and an e-value threshold of 10^5 was established for the mapped contigs. The relationship between microbial communities and hydrolase-encoding genes was calculated by an OPLS approach, as described before.

2.13 Metagenome-assembled genome (MAG) analysis

The metagenome-assembled genomes (MAGs) were assembled using metaSPAdes version 3.13 (https://github.com/ablab/spades). Genome binning was performed with MetaBAT2 version 2.12.1 (https://bitbucket.org/berkeleylab/metabat), with default settings. The quality of the MAGs was checked by means of CheckM version 1.0.18 (https://github.com/Ecogenomics/CheckM). Low-quality MAGs (< 50 % completeness and/or > 5 % contamination) were removed from downstream analysis, and only medium-quality (50-90 % completeness, < 5 % contamination) and high-quality (> 90 % completeness, < 5 % contamination) MAGs were retained for further analysis. Taxonomic classification of MAGs was performed against the Genome Taxonomy Database with GTDB-tk version 2.1.1 (https://github.com/Ecogenomics/GTDBTk).

2.14 Statistical analysis

IBM SPSS statistical package version 26.0 (IBM SPSS Inc., Chicago, IL, USA, 2019) was used for data preparation and analysis. The Mann–Whitney *U* test and Kruskal-Wallis analysis of variance with Bonferroni correction were performed with the SPSS package. The objective was to study differences in the reads and relative abundances of bacterial communities or genetic potential (pathway-related genes, ARGs, VFs and enzyme-encoding genes), according to the producer and sample type factors. To determine the number of common bacterial genera between the samples, Venn diagrams were generated in R using the "ggvenn" package (https://github.com/yanlinlin82/ggvenn). Permutational Multivariate Analysis of Variance (PERMANOVA) was computed in RStudio version 1.3.959 and R version 3.6.3 by the "vegan" package, to analyse the overall effect of producer and sample type factors on the reads and relative abundance of the bacterial communities or genetic potential. The main bacterial genera and species were selected, their abundances were log-transformed when necessary, and UV-scaled, and the corresponding Principal Component Analysis (PCA) was performed and plotted by SIMCA software version 15.0.0.4783 (Umetrics AB, Umeå, Sweden). The number of principal components (PCs) was determined by the eigenvalues (greater than 1.5) and cross-validation. The aim was to study the microbial community distribution according to the producer and sample type. An Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed in SIMCA to confirm whether the microbial communities of the samples differed according to the producer and sample type.

3. Results

3.1. Characteristics of shotgun metagenomic sequencing data

Shotgun metagenomic sequencing yielded 71.3 GB of data, with 414,367,387 high-quality paired-end reads and an average of 9,417,440.61 (± 9,993,465.12) reads per sample (Figure 1A, Supplementary Table 1). Trimming of the raw reads yielded an average of 1,553.07 (± 1,723.97) reads removed, and 1,156,348.16 (± 1,987,677.59) reads were associated with the ovine (*Ovis aries*) reference genome. The percentage of microbial reads was 82.1 % (± 31.3) (Supplementary Table 1). Differences were not observed in the number of microbial reads obtained among the producers ($P > 0.05$) (Figure 1B). However, the microbial reads differed among sample types ($P \leq 0.05$), with raw milk samples showing the lowest number (45,179 ± 15,983.59) and cheese samples the greatest (26,775,204.75 ± 28,466,876.45) (Figure 1C, Supplementary Table 1). Significant differences were also detected in the host reads aligned to the *Ovis aries* genome among sample types ($P \leq 0.001$), with raw milk, rennet, whey or teat skin surface samples presenting the highest number of reads (Supplementary Table 1). PERMANOVA confirmed the differences in metagenomic reads among sample types ($P \leq 0.001$).

3.2. Diversity analyses

In terms of α-diversity (Figure 1D, Supplementary Table 2), PERMANOVA confirmed clear differences among sample types ($P \leq 0.01$), but not among producers ($P > 0.05$). Richness indices revealed that the lowest number of bacteria was detected in whey and cheese, followed by commercial feed and brine, whereas artisanal rennet and teat skin surface samples presented the highest richness. Bacterial abundance was greatest in brine, cheese and whey samples, and lowest in feed (commercial feed, homemade feed, straw and grass) and teat skin surface samples. Cheese, whey and brine samples were also the least uniform, indicating that the microbial population was dominated by a few bacteria; whereas feed (straw, homemade feed, commercial feed and grass), teat skin surface and artisanal rennet samples reported the greatest uniformity. Considering the number and abundance of bacteria, the greatest biodiversity was observed in the feed (straw, homemade feed, commercial feed and grass), teat skin swab and artisanal rennet

samples, followed by the raw milk, food-contact and non-food contact surfaces. Bacteria biodiversity was lowest in the brine, whey, and cheese samples.

In terms of β-diversity (Figure 1E), PCoA divided the samples into three clusters according to microbial composition, and aligned with the α-diversity results. Whey and cheese samples were clustered together since they presented similar bacterial compositions but differed from the rest of the samples. Samples corresponding to teat skin surface and artisanal rennet also clustered, and third cluster contained all other samples (raw milk, food contact and non-food contact swabs and feed samples [straw, homemade feed, commercial feed and grass]). It should be noted that within the last cluster greater differences among sample types were observed, indicating greater differences.

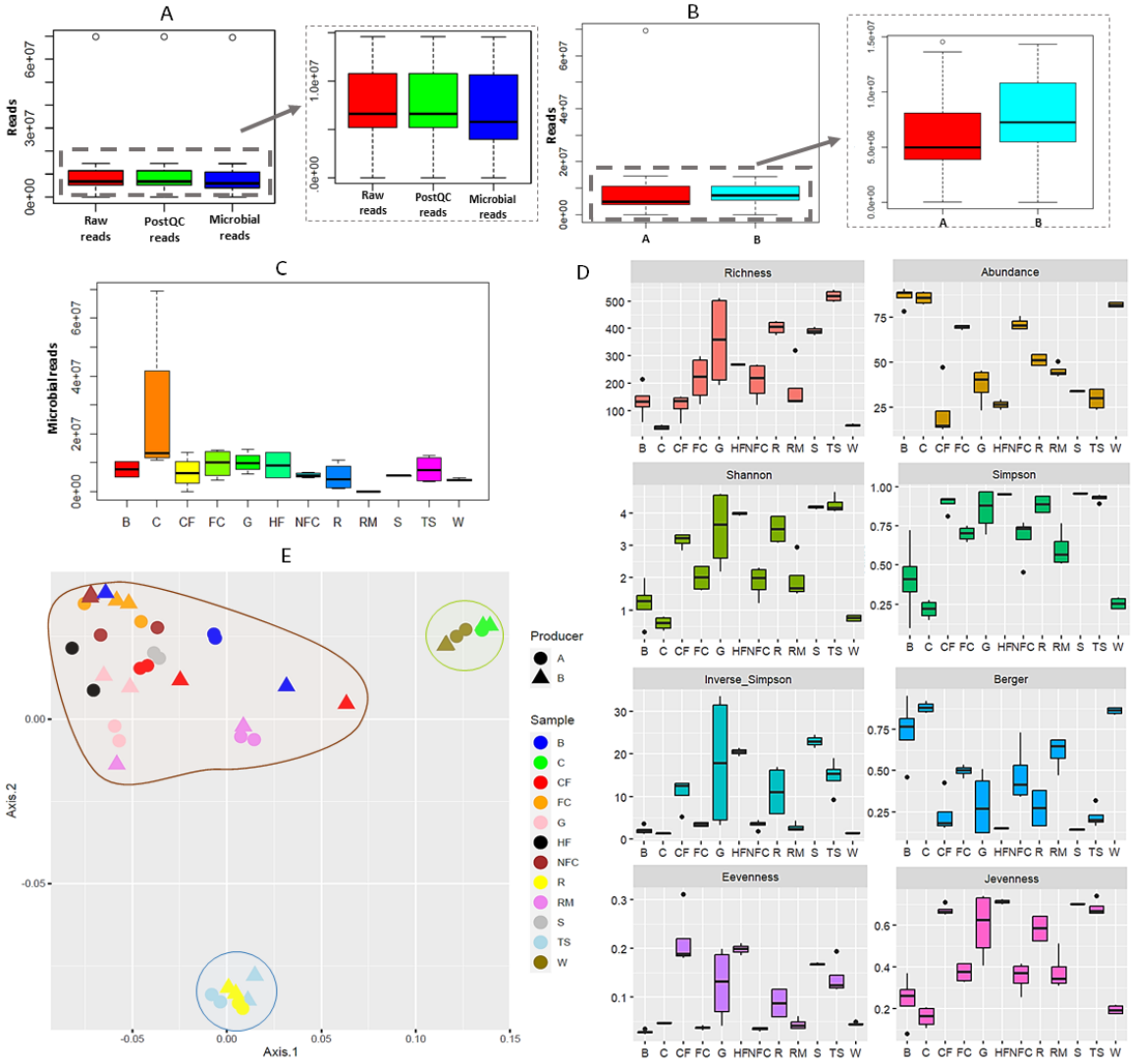


Fig. 1. Characteristics of shotgun metagenomic sequencing data, and alpha and beta diversity analyses. Boxplot representation of average raw reads, post-quality control (postQC) reads and microbial reads obtained from dairy and environmental samples (A), boxplot representation of average microbial reads obtained from dairy and environmental samples according to the producer (B), boxplot representation of microbial reads according to the sample type (dairy and environmental) (C), α-diversity indices calculated for dairy and environmental samples (D) and PCoA of β-diversity of dairy and environmental samples (E). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey.

3.3. Taxonomic analysis

A total of 56 bacterial phyla, 94 classes, 180 orders, 370 families, 1312 genera and 3467 species were detected among all the samples. An abundance greater than 1 % was observed for 8 phyla, of which *Firmicutes, Proteobacteria, Actinobacteriota, Firmicutes_A* and *Bacteroidota* were greater than 5 % (Figure 2A). Similarly, 53 genera were present at average relative abundances greater than 1 %, and 14 were present above 5 %; namely, *Lactococcus, Staphylococcus, Brevibacterium, Pseudomonas_E, Chromohalobacter, Escherichia, Lactobacillus_H, Psychrobacter, Brachybacterium,*

Pantoea, Jeotgalicoccus, Lactobacillus, Lactobacillus_G and *Streptococcus* (Figure 2B). In line with the findings for α- and β-diversity, PERMANOVA confirmed the difference in microbiota among the sample types ($P \leq 0.01$), as indicated by PCA and OPLS-DA analyses (Supplementary Figures 1 and 2). Overall, *Lactococcus* dominated in commercial feed; *Sphingomonas* and *Methylobacterium* in straw; and *Pantoea* and *Pseudomonas_E* in grass and homemade feed. Regarding the teat skin surface, *Jeotgalicoccus* and *Psychrobacter* prevailed; *Brevibacterium, Staphylococcus* or *Brachybacterium* were some of the most abundant bacteria on food contact surfaces, while *Pseudomonas_E, Staphylococcus*, *Brevibacterium* or *Psychrobacter* dominated on non-food contact surfaces. In the case of raw milk, *Escherichia* dominated, followed by *Enterococcus* and *Lactococcus*. The rennet was mainly composed of *Lactobacillus_H, Lactobacillus, Lactobacillus_G, Prevotella* and *Streptococcus*, whereas in brine samples, a clear dominance of *Chromohalobacter* was observed, followed by *Brevibacterium* and *Lactococcus*. In whey, *Lactococcus* and *Staphylococcus_A* dominated, and *Lactococcus, Streptococcus,* and *Lactobacillus_C* were the most abundant genera in the cheese (Figure 2B).

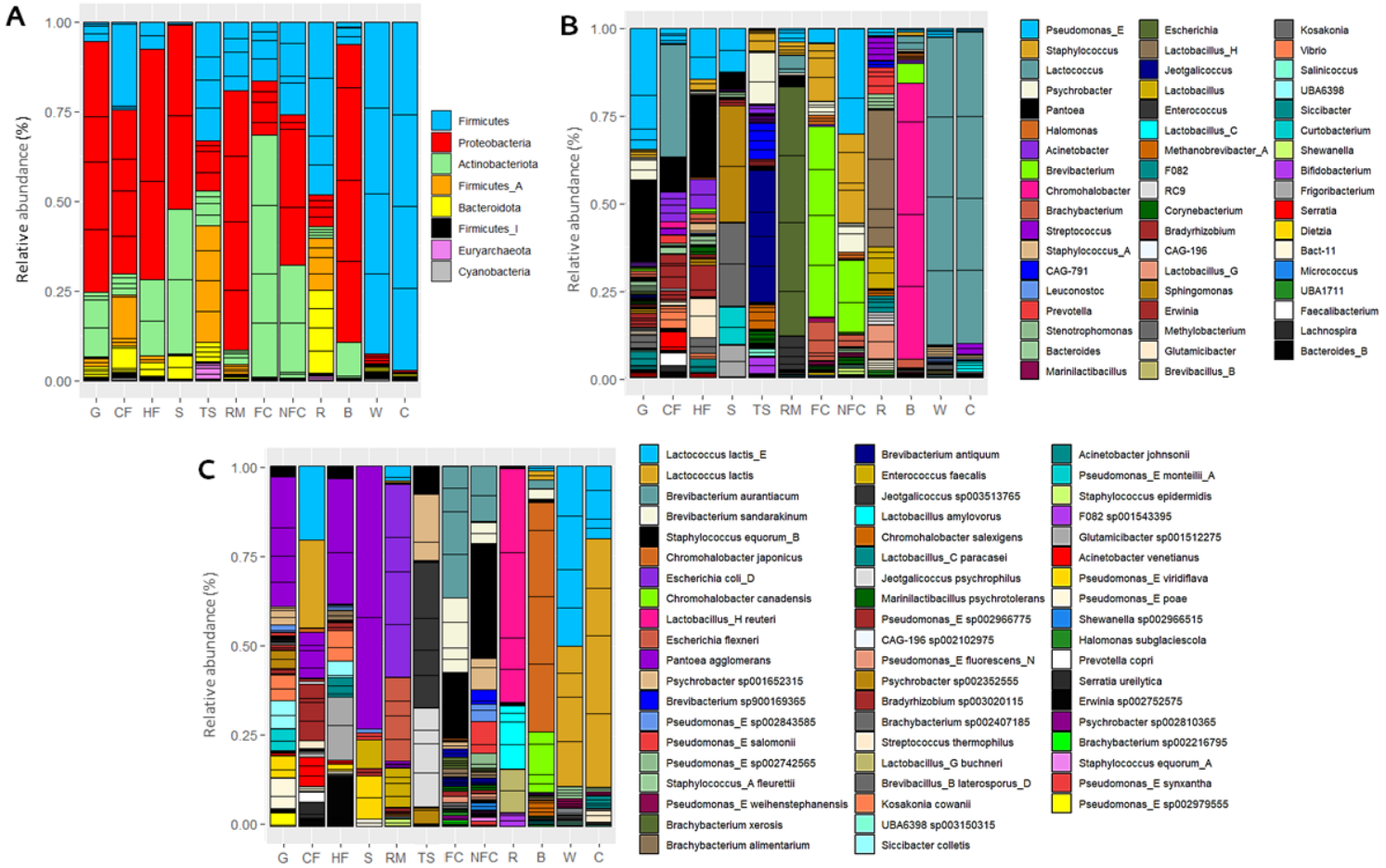


Fig. 2. Microbiota of Latxa raw ewe milk, whey, Idiazabal cheese and environmental samples.
Stacked bar chart representation of the taxonomic profiles of the microbiota in dairy and environmental samples at relative abundances greater than 1.00%, at phylum (A), genus (B) and species (C) ranks. Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey.

At the species level (Figure 2C), 58 species had an average relative abundance greater than 1 %, and 16 were present above 5 %; specifically, *Lactococcus lactis_E, Lactococcus lactis, Brevibacterium aurantiacum, Brevibacterium sandarakinum, Staphylococcus equorum_B, Chromohalobacter japonicus, Escherichia coli_D, Chromohalobacter canadensis, Lactobacillus_H reuteri, Escherichia flexneri, Pantoea agglomerans, Psychrobacter sp001652315, Brevibacterium sp900169365, Pseudomonas_E sp002843585, Pseudomonas_E salomonii* and *Pseudomonas_E sp002742565.* The abundance of the most dominant species also differed among sample types ($P \leq 0.01$). *L. lactis* dominated in commercial feed, while *P. agglomerans* dominated in grass and homemade feed. *B. aurantiacum, B. sandarakinum* and *S. equorum_B* dominated in food contact surfaces, while *S. equorum_B, B. aurantiacum, P. sp001652315, P. salomonii, B. sandarakinum* and *P. sp002843585* dominated on non-food contact surfaces. *J. sp003513765, J. psychrophilus and P. sp001652315* dominated in teat skin surface, whereas *E. coli, E. flexneri* and *E.*

faecalis did so in the raw milk. In the rennet, *L. reuteri, L. amylovorus and L. buchneri* dominated, while *C. japonicus, C. canadensis,* and *C. salexigens* did so in brine. In the whey and chesee samples, *L. lactis_E* and *L. lactis* also dominated, together with other species such as *S. fleurettii* in whey, and *S. thermophilus* and *L. paracasei* in cheese. A detailed taxonomic description of the samples can be found in the Supplementary Results.

3.4. Relationship between the environment of artisanal dairies and the microbiota of dairy products

Subsequently, an analysis was done to determine the extent at which microbial communities inhabiting the environment of dairies could contribute to the microbiota of raw milk, whey, and cheese. As shown in Figure 3A, the Venn diagrams showed that the raw milk, whey and cheese samples shared many bacterial genera with the environmental samples collected. In the case of raw milk, teat skin surface and grass samples presented the highest number of common bacterial genera (43 and 42, respectively). However, after cheese-making, the brine and teat skin surface samples had the highest number of bacteria in common with the whey (19 and 18, respectively) and cheeses (15 and 14, respectively). The food contact surfaces and straw samples exhibited the fewest number of bacteria in common with the dairy samples.

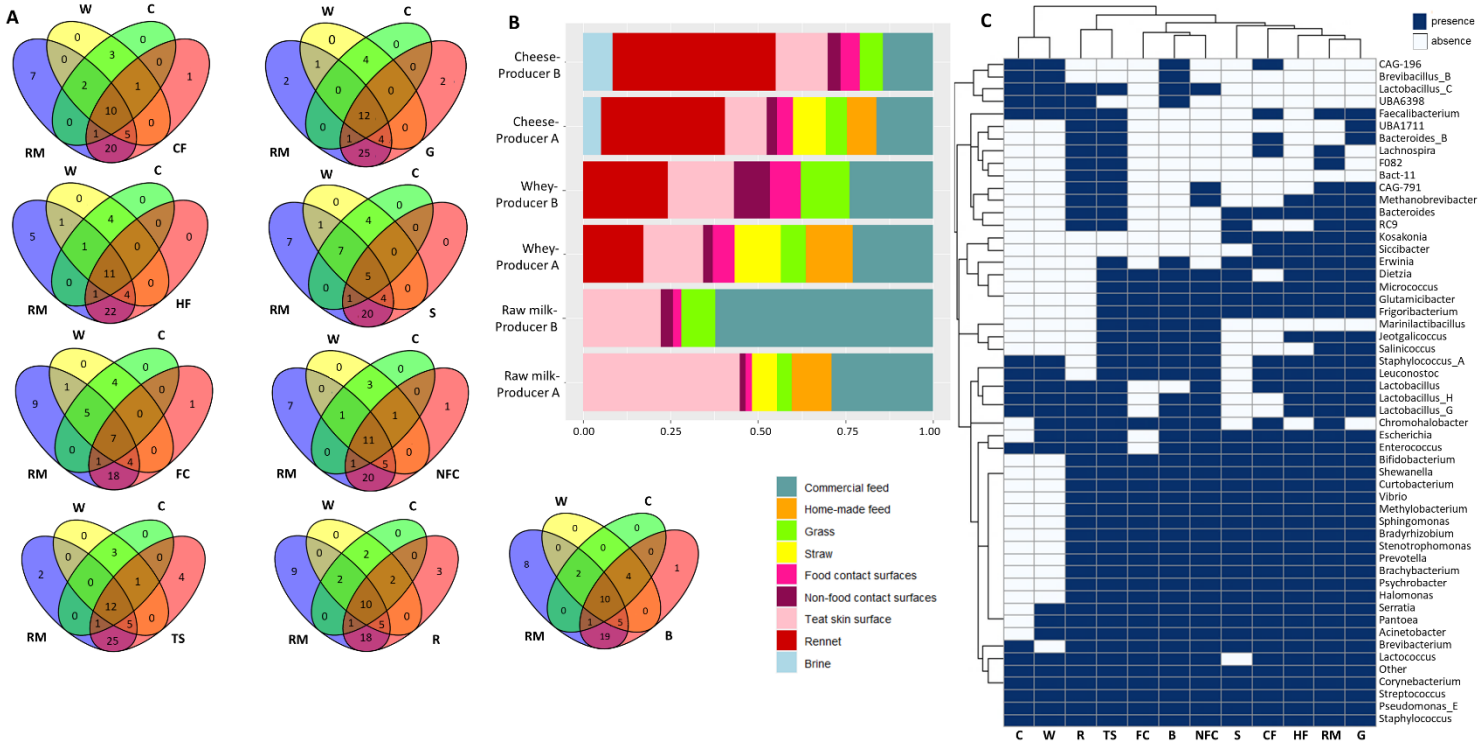


Fig. 3. Microbial sources of raw ewe milk, whey and Idiazabal cheese samples in artisanal dairy environments. Venn diagrams based on the shared bacterial communities among dairy and environmental samples (A), stacked bar chart representation of SourceTracker analysis results (unknown proportions are not shown) (B) and heatmap representation of the presence or absence of bacterial genera in each of the collected dairy and environmental samples (C). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey.

However, overlap at the genus level does not necessarily reflect the origin of the microorganism. Further investigation through SourceTracker revealed commercial feed and the teat skin surface as the main bacterial sources for raw milk (45.6 ± 21.6 % and 33.5 ± 14.2 %, respectively) (Figure 3B). More specifically, commercial feed was identified as a likely source of *Lactococcus* and *Pantoea,* together with *Bradyrhizobium* or *Acinetobacter,* among others (Figure 2B). The teat skin was identified as a source of *Staphylococcus,* as well as *Jeotgalicoccus, Psychrobacter, CAG-791, Methanobrevibacter_A* or *Bifidobacterium,* for example (Figure 2B). After cheese production, rennet was reported as the main bacterial source of the microbes found in the cheese (41.0 ± 7.58 %). Commercial feed (15.3 ± 2.47 %) and the teat skin surface (13.4 ± 2.74 %) were also identified as important sources. A similar trend was observed for the origin of whey-associated microbes, even though the contribution of the commercial feed (20.2 ± 4.18 %) was greater, followed by rennet (17.4 ± 2.59 %) and teat skin (15.4 ± 2.80 %) (Figure 3B). Rennet was identified as a source of *Streptococcus, Pseudomonas_E, Lactobacillus_H, Lactobacillus* or *Lactobacillus_G,* among others (Figure 2B). Notably, even if the remaining environmental samples (the rest of the herd feed, food contact and non-food contact surfaces and brine) were, in general, less common sources of milk and cheese microbes, they nonetheless made some notable

contributions to the raw milk and cheese microbiota (Figure 3B and 3C). For instance, food contact and non-food contact surfaces were a great source of *Brevibacterium, Staphylococcus* and *Pseudomonas_E* for dairy samples, and *Chromohalobacter* originated, primarily, from the brine (Figure 2B). The proportion of unknown bacterial sources was low in the raw milk, accounting for 1.24 %. This proportion increased considerably in the whey and cheese samples (92.9 %) due to the impact of the starter culture used (*Lactococcus lactis*) and its great abundance in whey and cheese (Figure 2C).

3.5. Functional potential analysis

Regarding the functional potential of the microbiomes associated with dairy and environmental samples, a total of 35 functional groups were detected at subsystem level 1, 194 were found at level 2, and 1280 were identified at level 3 (Figure 4A-C, Supplementary Tables 3-5). The genes involved in the metabolism of carbohydrates, amino acids and derivatives, and proteins were the most abundant at level 1, followed by DNA, cofactors, vitamins, prosthetic groups and pigments, and clustering-based subsystems metabolisms (Figure 4A, Supplementary Table 3). At level 2, genes involved in the metabolism of protein biosynthesis, central carbohydrate metabolism and di- and oligosaccharides, followed by genes involved in DNA repair, resistance to antibiotics and toxic compounds, and monosaccharides metabolism were the most abundant (Figure 4B, Supplementary Table 4). The most common functional groups at level 3 were DNA-replication, phage head and packaging, ABC transporter oligopeptide (TC 3.A.1.5.1), lactose and galactose uptake and utilization, purine conversions, maltose and maltodextrin utilization or fatty acid biosynthesis (FASII).

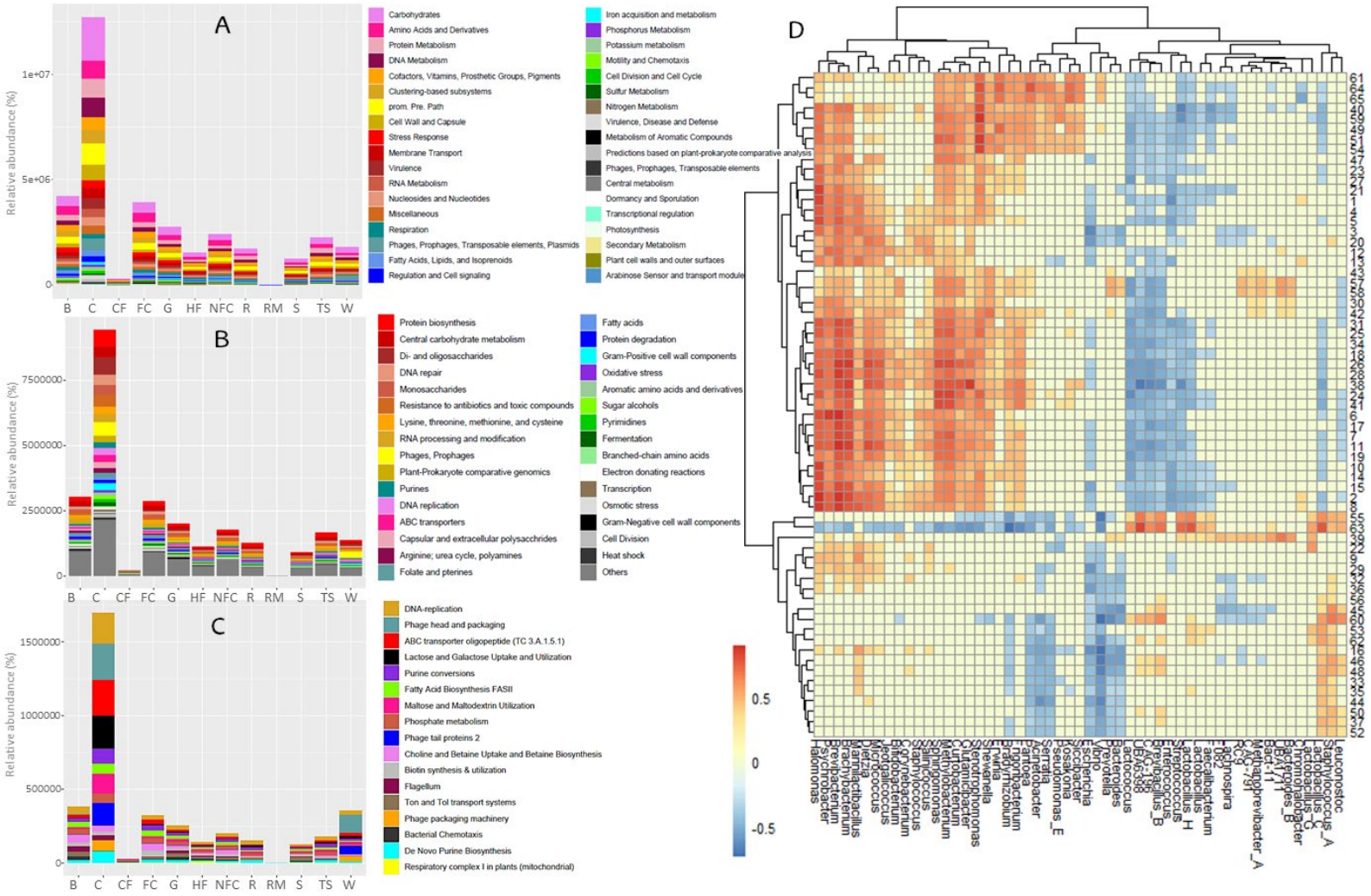


Fig. 4. Functional potential of Latxa raw ewe milk, whey, Idiazabal cheese and environmental samples. Bar chart representation of metabolic pathways at subsystem level 1 (A), and main metabolic pathways at subsystem level 2 (B) and 3 (C), and correlation heatmap between selected food quality and safety metabolic pathways and key bacterial genera resulted from OPLS model (D). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey. The numbers on the heat map correspond to abbreviations of the metabolic pathways detailed in Supplementary Table S6.

Significant differences were observed among samples for the majority of functional groups ($P \leq 0.05$) (Supplementary Tables 3 and 4), as reflected in the HCA (Supplementary Figures 3-5). Overall, cheese samples presented the greatest abundance of the most abundant pathways, followed by brine and food contact surfaces (Figure 4A-C, Supplementary Tables 3-5). Thus, *Lactobacillus_C, Brevibacillus_B, UBA6398, Staphylococcus_A, CAG-196, Lactobacillus_H, Marinilactibacillus* and *Leuconostoc* were the main genera correlated with the main metabolic pathways.

Specifically, in terms of colonization, various metabolic pathways related to biofilm formation were observed, mainly in non-food contact surfaces, grass and home-made feed, related to *Brevibacterium, Brachybacterium, Dietzia, Micrococcus* or *Psychrobacter*, among others. In terms of competitive interactions among bacteria, various metabolic pathways related to bacteriocin production were detected, mainly in cheese, brine and grass, mainly related to *Sphingomonas, Methylobacterium, Lactobacillus_H, Lactobacillus_C, Brachybacterium, Brevibacterium* or *UBA6398* (Figure 4D).

Additionally, various metabolic pathways related to cheese safety were identified. Pathways related to pathogenicity islands and the virulome of pathogens were identified, primarily in cheese, teat skin and brine (Figure 4D, Supplementary Tables 5-7), related to *Halomonas, Brevibacterium, Psychrobacter, Brachybacterium, Sphingomonas, Methylobacterium, Dietzia* or *Micrococcus*. Pathways related to AMRs were also detected, mainly in cheese, brine, grass, and homemade feed, with the strongest correlations observed for *Stenotrophomonas, Halomonas, Sphingomonas* and *Methylobacterium,* especially with multidrug, fosfomycin, erythromycin or vancomycin resistance. Pathways related to BAs degradation were identified in cheese, brine and food-contact surfaces, which were strongly related to *Halomonas, Brevibacterium, Stenotrophomonas, Sphingomonas* or *Methylobacterium* (Figure 4D).

Regarding cheese quality, several metabolic pathways related to the metabolism of flavour and texture compounds were identified (Figure 4D, Supplementary Tables 5-7). Among others, genes associated with the biosynthesis of amino acids and derivatives (e.g., isoleucine, thiamine and betaine) were observed, mainly in grass, food contact surfaces, homemade feed or brine, for which *Halomonas* and *Brevibacterium*, among others, were strongly correlated. Similarly, genes related to catabolism of amino acids (e.g., isoleucine, lysine or aromatic amino acids) were also detected, mainly in cheese, food contact surfaces and brine; these genes were also related to *Halomonas* and *Brevibacterium*, among others. Genes involved in the synthesis of fatty acids (FAs) and, especially, polyunsaturated FAs were also detected, mainly in cheese, grass, food contact and non-food contact surfaces; these genes were strongly correlated with *Brevibacterium, Sphingomonas* or *Methylobacterium*, among others. Carbohydrate metabolism-related genes, such as those involved in the degradation of L-fucose, fructose and mannose, were also identified, mainly in cheese, brine and food contact surfaces; these genes were strongly related to *Halomonas* and *Brevibacterium,* among others. The metabolism of other compounds, such as alcohols (e.g., mannitol), was mainly observed in cheese, whey and food contact surfaces, related to *Staphylococcus_A, Erwinia, Leuconostoc, Prevotella, Brevibacillus_B,* or *Lactobacillus_C*. Genes associated with the metabolism of nitrates and nitrites were identified in brine, grass and food contact surfaces related to *Halomonas, Brevibacterium* or *Stenotrophomonas*. The metabolism of vitamins (e.g., folate, thiamine or biotin) was observed in cheese, food contact surfaces and brine related to *Lactobacillus_C.* Genes associated with terpene metabolism were identified in cheese, food contact and non-food contact surfaces related to *Sphingomonas, Methylobacterium, Stenotrophomonas, Halomonas, Brachybacterium* or *Psychrobacter,* whereas genes related to the synthesis of carotenoid pigments were identified in food contact, brine and non-food contact surfaces, related to *Brevibacterium*, *Brachybacterium, Halomonas*, and *Methylobacterium,* among others.

Furthermore, genes related to the generation of volatile compounds were identified and related to cheese, food contact surfaces and brine (Figure 4D, Supplementary Tables 5-7), mainly benzoate degradation, sulfur oxidation, alkane synthesis, and to a lesser extent, alkanesulfonate assimilation, toluene degradation or menaquinone biosynthesis, detected mainly in grass, non-food contact and food contact surfaces. *Halomonas, Brevibacterium, Brachybacterium, Psychrobacter, Sphingomonas, Methylobacterium* and *Dietzia* were the main bacteria related to these genes. Within LAB, *Marinilactibacillus* was correlated with chlorobenzoate degradation, toluene degradation and butanol biosynthesis; *Leuconostoc* to formaldehyde assimilation and polyprenyl diphosphate biosynthesis; and *Lactobacillus_C* to formaldehyde assimilation and synthesis of aromatic compounds. Moreover, genes related to the synthesis of diverse enzymes, especially aminoglycoside adenylyltransferases or metalloendopeptidases, were also identified, and found to be related to *Brevibacterium, Psychrobacter* or *Halomonas*.

3.6. Resistome analysis

A total of 478 ARGs were detected among all the samples, belonging to 86 ARG families (Supplementary Table 7). ARGs were predicted to confer resistance to antimicrobial peptides and 12 antibiotic classes, i.e., aminoglycosides,

fluoroquinolones, fusidanes, glycopeptides, lincosamides, macrolides, nucleoside antibiotics, phenicols, sulfonamides, tetracyclines, β-lactams and multiple drugs. The highest number of ARG families was related to multidrug (30.2 % of ARG families) (primarily against cephamycins, cephalosporins, aminoglycosides and penicillins), tetracycline (24.4 %) and aminoglycoside (18.6 %) classes. However, in terms of abundance, lincosamide and aminoglycoside ARG families were the most abundant (total abundance of 4,197,149.04 RPKM and 1,498,605.30 RPKM, respectively) (Supplementary Table 7). Individually, *tet*(K) (accounting for 10.9 % of all ARGs), *lmrC* (7.32 %) and *lmrD* (6.69 %) were the top 3 ARG families detected, whereas in terms of abundance, *lmrC* (2,245,572.17 RPKM), *lmrD* (1,910,857.10 RPKM) and *APH*(3_)-IIa (1,125,938.83 RPKM) were the most abundant (Figure 5A, Supplementary Table 7). Additionally, ARGs belonged to 6 mechanisms of resistance, namely, efflux, drug inactivation, target protection, target alteration, target replacement and the combination of efflux and target alteration. Efflux and drug inactivation mechanisms were the most detected (40.7 % and 31.4 %, respectively) and the most abundant ones (60,489.48 ± 84,275.56 RPKM and 85,679.81 ± 131,558.84 RPKM, respectively).

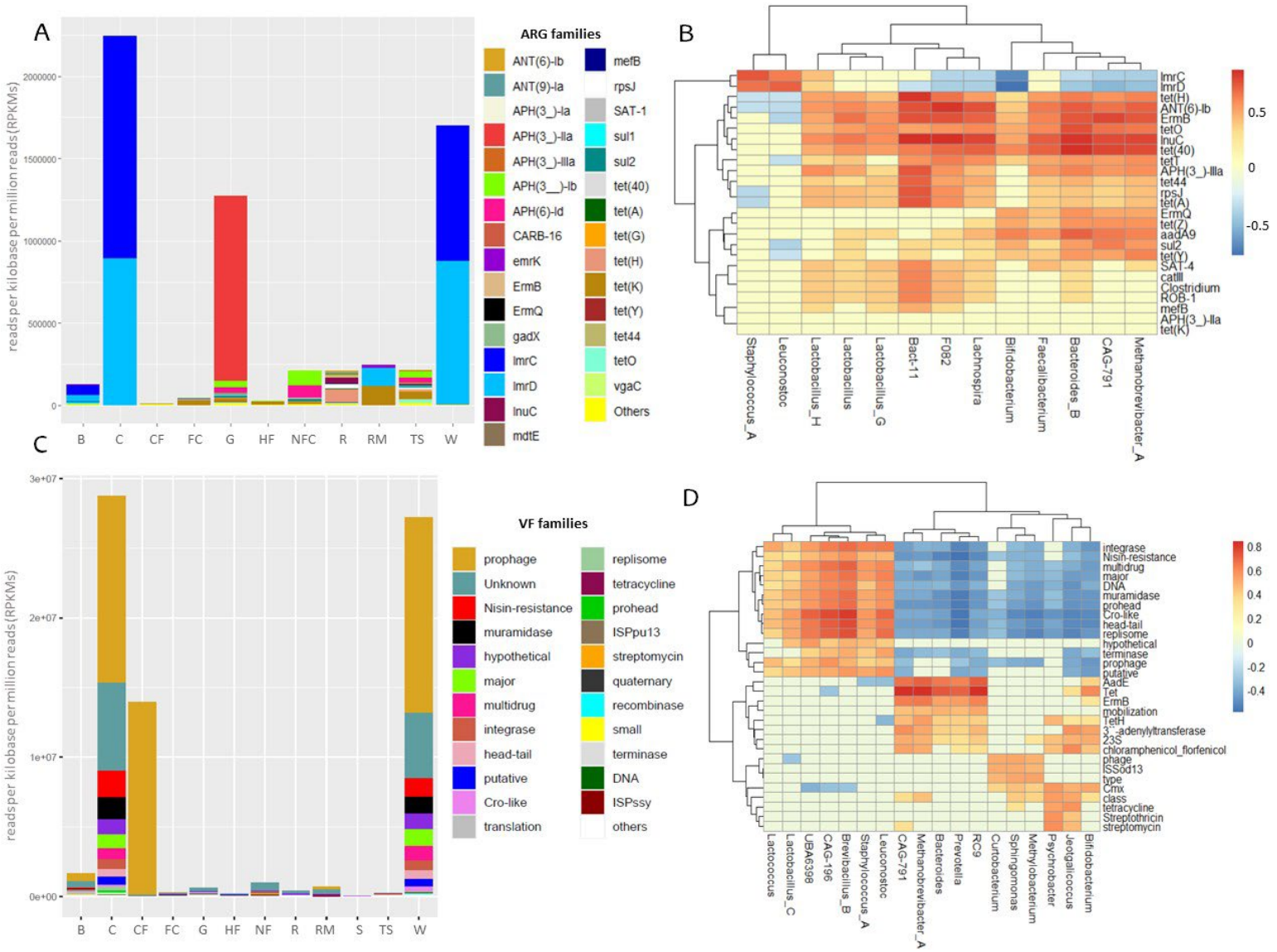


Fig. 5. Resistome and virulome of Latxa raw ewe milk, whey, Idiazabal cheese and environmental samples. Bar chart representation of main ARGs and VFs according to sample type (A and C), and correlation heatmap between ARGs and VFs, and key bacterial genera resulted from OPLS model (B and D). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey.

PERMANOVA confirmed statistically significant differences in the resistome among sample types ($P \leq 0.001$). The largest number of ARGs was detected on the teat skin surfaces (100 ARGs were detected, with *tet*(K), *sul*2 and *tet*(40) ARG families being the most frequently detected), followed by grass (95 ARGs, with *tet*(K), *tet*(Y) and *sul*2 being the most detected) and rennet (72 ARGs, with *ANT*(6)-Ib and *mef*B being the most detected). Thus, teat skin surfaces, grass

and rennet samples presented the greatest number of ARG families (21, 21 and 19, respectively) (Supplementary Table 7). However, in terms of abundance, cheese presented the greatest abundance of ARG families (2,245,798.91 RPKM), followed by whey (1,701,117.38 RPKM) that were specially related to *lmrC* and *lmrD*, and grass (1,276,130.97 RPKM) due to the abundance of *APH*(3_)-IIa (Figure 5A, Supplementary Table 7). *lmrC* and *lmrD* were detected in several samples, such as cheese, whey, commercial feed and brine, whereas *APH*(3_)-IIa was only detected in grass samples (Figure 5A, Supplementary Table 7).

A total of 13 bacterial genera were strongly related to ARGs, namely, *Staphylococcus_A, Leuconostoc, Bifidobacterium, Bacteroides_B, Lachnospira, Lactobacillus_G, Faecalibacterium, Lactobacillus_H, Lactobacillus, Bact-11, CAG-791, F082,* and *Methanobrevibacter_A* (Figure 5B). *Staphyloccus_A* and *Leuconostoc* were strongly related to *lmrC* and *lmrD*, while no relationship was observed for *tet*(K) or *APH*(3_)-IIa. For the other ARGs, *tet*(40) was related to *Bacteriodes_B, CAG-791, Methanobrevibacter_A, Faecalibacterium* and *Lachnospira*; *sul*2 to *CAG-791* and, to a lesser extent, *Methanobrevibacter_A* and *Bacteriodes_B*; *tet*(Y) to *Methanobrevibacter_A* and, to a lesser extent, *Bacteriodes_B* and *CAG-791*; and *ANT*(6)-Ib to *F082, Lachnospira, Bact-11, Bacteriodes_B* and *Methanobrevibacter_A*. Overall, *Bact-11* and *Bacteriodes_B,* followed by *CAG-791* and *Methanobrevibacter_A*, were the main bacteria related to the ARGs.

3.7. Virulome analysis

A total of 3193 VFs were detected among all the samples, belonging to 159 VF families (Supplementary Table 8), related to regulatory and genetic elements, metabolism and nucleic acids, enzymes and structural factors, bacterial motility and attachment, transport and secretion, bacteriophages and virus-related factors, antibiotic resistance, molecules and compounds, proteins, toxins and toxin-related genes, and antimicrobials and siderophores classes. VF families were primarily related to enzymes and structural factors (19.5 %), followed by antibiotic resistance (13.8 %), regulatory and genetic elements (13.2 %) and metabolism and nucleic acids (13.2 %). Prophage (756 VFs detected), tetracycline (52 VFs) and CP4-6 (36 VFs) were the most common VF families detected. However, in terms of abundance, prophage (total abundance of 42,213,014.74 RPKM), nisin resistance (3,337,734.67 RPKM) and muramidase (2,830,057.90 RPKM) dominated (Figure 5C, Supplementary Table 8).

Clear differences were observed in the detected VFs and their abundance among sample types ($P \leq 0.001$), with the highest number of VFs detected in cheese samples (605 VFs), grass (558 VFs) and whey (436 VFs), whereas raw milk, straw and commercial feed presented the lowest (15, 73 and 140 VFs, respectively). In terms of abundance, the highest abundance of VF families was observed in cheese (total abundance of 28,773,668.41 RPKM), followed by whey (27,237,094.02 RPKM) and commercial feed (13,960,998.20 RPKM), where prophage was the dominant VF family, together with nisin resistance in whey and cheese samples (Figure 5C, Supplementary Table 8).

A total of 18 bacterial genera were strongly related to VFs (Figure 5D), namely, *Psychrobacter, Lactococcus, CAG-791, Staphylococcus_A, Leuconostoc, Prevotella, Bacteroides, RC9, Methanobrevibacter_A, Lactobacillus_C, Jeotgalicoccus, CAG-196, Sphingomonas, Methylobacterium, Bifidobacterium, UBA6398, Curtobacterium,* and *Brevibacillus_B*. These bacteria were related to several VF families, especially tetracycline resistance (Tet), aminoglycoside resistance (AadE), Cro-like proteins, head-tail related virulence, replisome, multidrug resistance, integrase, erythromycin resistance (ErmB), major, prohead and muramidase (Figure 5D). Specifically, *Brevibacillus_B, CAG-196,* and, to a lesser extent, *Methanobrevibacter_A, RC9, Prevotella, Bacteroides* and *CAG-791* were among the most related bacteria. Among the dominant VFs, prophages were related mainly to *CAG-196*, and nisin resistance was related to *Brevibacillus_B* and *CAG-196.*

3.8. Enzymatic potential analysis

A total of 17,913,657 genes encoding hydrolases were identified, belonging to 58,593 gene families. The most abundant gene families encode ten types of enzymes, namely, alpha/beta hydrolase abh_upf00227, abhydrolase_5, and abhydrolase_6; bifunctional feruloyl and acetyl xylan esterase (BD-FAE), hydrolase of unknown function (duf_915), lysophospholipase/carboxylesterase, pancreatic lipase, peptidase_S9, peptidase_S15, and proline_iminopeptidase (Supplementary Table 9). Among all the samples, hydrolase-encoding genes from *Lactococcus* (i.e., *L. lactis*) and *Lactobacillus* species (i.e., *L. delbrueckii* subsp*. lactis*) were the most abundant, followed by *Listeria* (i.e., *L. monocytogenes*). The *pepX* gene family from *Lactococcus* species (e.g., *L. lactis* subsp*. lactis*), encoding an Xaa-Pro dipeptidyl aminopeptidase, was the most commonly detected (193,277), followed by cocaine esterases and alpha/beta hydrolases of the family Abhydrolase_5 encoding *YMGC* and *YBCH* gene families from *Lactococcus* (i.e., *L. lactis*) (76,240 and 73,445, respectively).

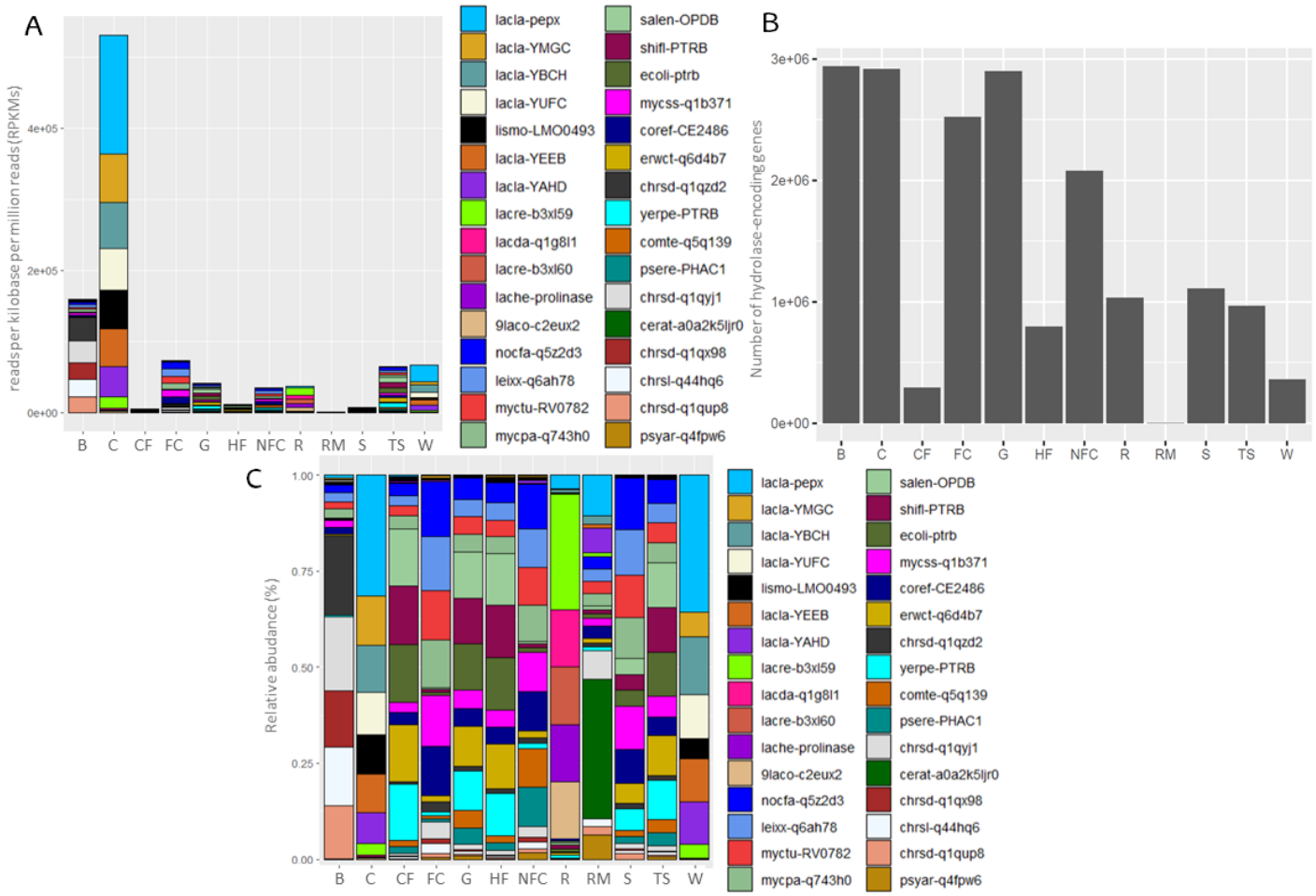


Fig. 6. Hydrolase-encoding genes of Latxa raw ewe milk, whey, Idiazabal cheese and environmental samples. Bar chart representation of main hydrolase-encoding gene families according to sample type (A), bar chart representation of the total number of hydrolase-encoding genes according to sample type (B) and stacked bar chart representation of main hydrolase-encoding gene families according to sample type (C). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey. Abbreviations of genes encoding hydrolases are detailed in Supplementary Table S9.

Clear differences were observed among sample types ($P \leq 0.001$), with brine presenting the highest number of genes (2,940,987), followed by cheese and grass samples (2,918,313 and 2,898,076, respectively) (Figure 6B). Thus, the dominant gene families also differed depending on the microbiota of each sample type (Figure 6C). Protease II-encoding gene families from *Escherichia*, *Shigella* or *Pectobacterium* and oligopeptidase B-encoding gene families from *Salmonella* or *Yersinia* dominated in the commercial feed, whereas oligopeptidase B-encoding gene families from *Salmonella* and protease II-encoding gene families from *Escherichia* and *Shigella* were notable in grass, home-made feed and teat skin surfaces. Putative protease-encoding gene families from *Nocardia,* protease II-encoding gene families from *Leifsonia* or oligopeptidase B-encoding gene families from *Mycobacterium* were found to be dominant on straw and food contact surfaces. In non-food contact surfaces, putative protease-encoding gene families from *Nocardia* or putative protease II-encoding gene families from *Corynebacterium* dominated. Gene families encoding Xaa-Pro dipeptidyl aminopeptidases from *Lactococcus* dominated in the raw milk. Gene families encoding cocaine esterase and oligopeptidase B from *Chromohalobacter* dominated in the brine, whereas Xaa-Pro dipeptidyl aminopeptidase and prolyl aminopeptidase-encoding gene families from *Lactobacillus* dominated in the rennet. Finally, gene families encoding Xaa-Pro dipeptidyl aminopeptidases from *Lactococcus* dominated in whey and cheese.

3.9. Metagenome-assembled genomes (MAGs) analysis

A total of 60 high-quality MAGs and 392 medium-quality MAGs were obtained among all the samples (Figure 7 and Supplementary Figure 6). The MAGs were classified into 63 genera, mainly *Lactococcus* (31 MAGs), *Staphylococcus* (23 MAGs), *Brevibacterium* (20 MAGs), *Brachybacterium* (18 MAGs), *Pseudomonas_E* (14 MAGs), *Psychrobacter* (14 MAGs) and *Pantoea* (11 MAGs). Similarly, MAGs were classified into 53 species, with *Staphylococcus equorum* (21 MAGs), *Lactococcus cremoris* (15 MAGs), *Lactococcus lactis* (14 MAGs), *Pantoea aglomerans* (9 MAGs), *Psychrobacter faecalis* (7 MAGs), *Brevibacterium aurantiacum* (7 MAGs) and *Chromohalobacter japonicus* (7 MAGs) dominating. A total of 123 MAGs could not be classified at the species rank, belonging to 38 genera, primarily *Brevibacterium* (13 MAGs) and *Brachybacterium* (13 MAGs). Moreover, 7 MAGs belonging to the *Carnobacteriaceae* family could not be classified at genera and species rank and 89 MAGs remained unclassified at the phylum rank.

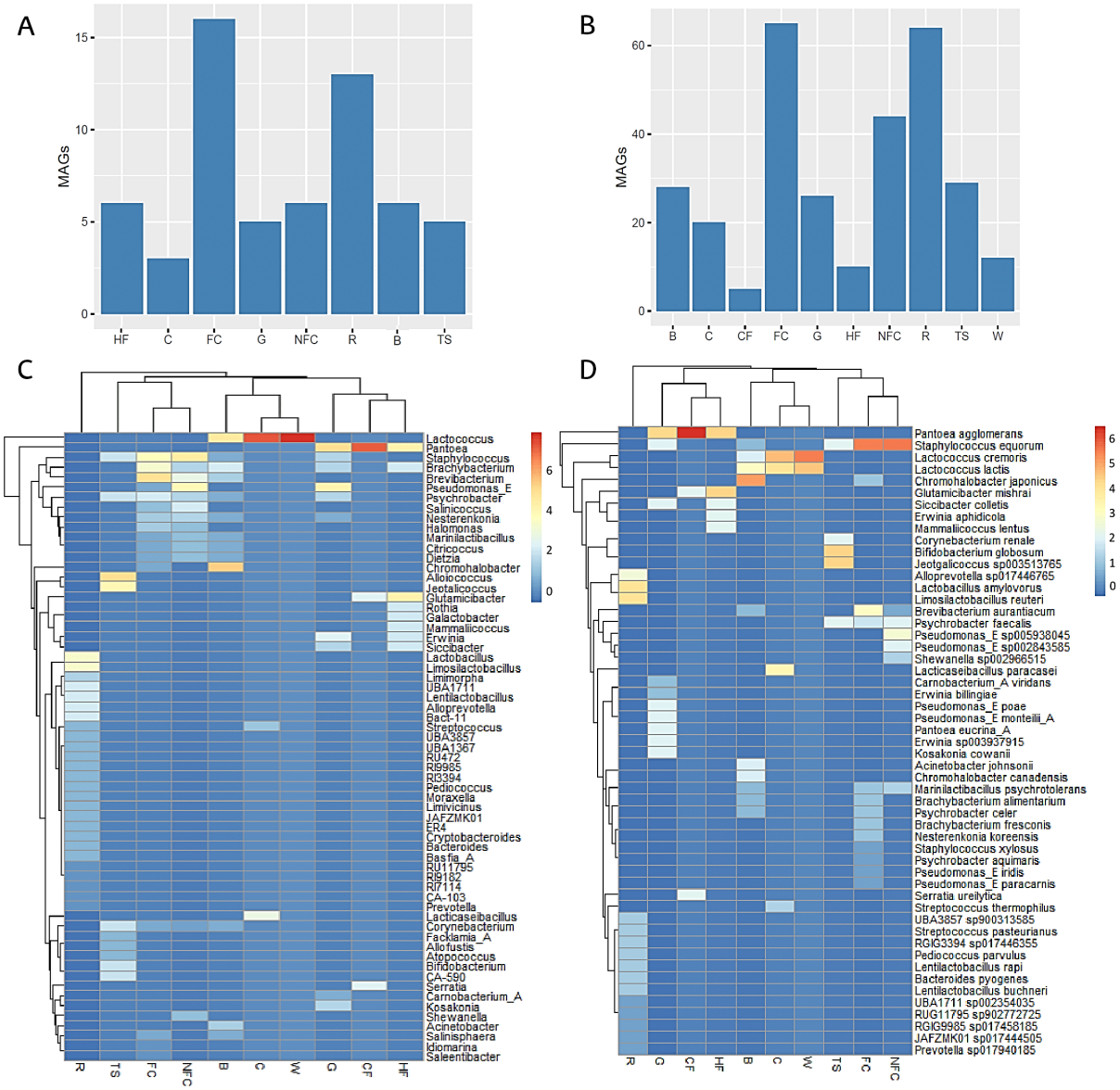


Fig. 7. Obtained MAGs from Latxa raw ewe milk, whey, Idiazabal cheese and environmental samples. Bar chart representation of the obtained high quality and medium quality MAGs according to sample type (A and B, respectively), and heatmap representation of the distribution of MAGs at genus and species taxonomic ranks according to sample type (C and D, respectively). Abbreviations: B: brine, C: cheese, CF: commercial feed, FC: food contact surfaces, G: grass, HF: home-made feed, NFC: non-food contact surfaces, R: rennet, RM: raw milk, S: straw, TS: teat skin surface, W: whey.

Clear differences were noted in the number of MAGs obtained according to sample type ($P \leq 0.001$) (Figure 7A-B, Supplementary Figure 6), with the greatest number of MAGs obtained from food contact surfaces (65 MAGs), rennet (64 MAGs) and non-food contact surfaces (44 MAGs) (Figure 7B, Supplementary Figure 6C-D). MAGs obtained from food

contact surfaces belonged, mainly, to *Staphylococcus, Brachybacterium* and *Psychrobacter* and, specifically, to *S. equorum* and *B. aurantiacum* species, whereas MAGs from rennet samples belonged to *Lactobacillus,* specifically, *L. amylovorus* and *L. reuteri*. The non-food contact surface MAGs belonged to *Staphylococcus, Pseudomonas_E* and *Brevibacterium* and, specifically, to *S. equorum* and *P. sp005938045* (Figure 7C-D and Supplementary Figure 6C-D).

Among the high-quality MAGs, 28 could not be classified at species level, which could correspond to putative new species. These MAGs were collected mainly from food contact surfaces and rennet samples (10 and 6 MAGs, respectively) and, to a lesser extent, from non-food contact surfaces (4), teat skin surfaces (4), brine (3) and homemade feed (1). These MAGs included 2 species from the genus *Basfia_A*, closely related to *Basfia_A succinogenes*; 2 of the genus *Brachybacterium*, related to *Brachybacterium endophyticum* and *Brachybacterium faecium*; 5 *Citricoccus*, related to *Citricoccus muralis* and *Citricoccus zhacaiensis*; 4 *Corynebacterium*, related to *Corynebacterium sp002363255*; 1 *Cryptobacteroides*, related to *Cryptobacteroides sp905234795*; 3 *Dietzia*, related to *Dietzia sp012841845*; 1 *Facklamia_A*, related to *Facklamia_A tabacinasalis*; 1 *Idiomarina*, related to *Idiomarina ramblicola*; 2 *Limimorpha*, related to *Limimorpha sp905234275;* 1 *Moraxella*, related to *Moraxella oblonga*; 1 *Salinicoccus*, related to *Salinicoccus sediminis;* and 2 *Salinisphaera*, related to *Salinisphaera sp002729955*. Moreover, 3 MAGs representative of the family *Carnobacteriaceae* could not be assigned at the genus or species level.

4. Discussion

In this study, shotgun metagenomic sequencing was applied to determine to what extent the environment and practices carried out in artisanal dairies could determine the microbiome of raw ewe milk and derived cheeses. The sequencing output of dairy (raw milk, whey, cheese) and environmental samples (homemade feed, commercial feed, straw, grass, teat skin surfaces, food contact surfaces, non-food contact, rennet and brine) indicated high recovery rates from all sample types, confirming the potential of this methodology to unravel the microbiota of various sample types [24]. Large differences were reported in microbial reads among the sample types, which were confirmed by diversity indices and taxonomic analyses.

Intermediate microbial richness, abundance and uniformity values were observed in the raw ewe milk, with more than 380 bacterial genera and 450 species, mainly *Escherichia, Enterococcus, Lactococcus, Pseudomonas_E, Staphylococcus* and *Pantoea*. These findings partially agree with previous analyses of Latxa raw ewe milk [25] and of raw milk collected from other breeds [31,46,47], since differences in composition and abundance have been noted. Diversity analyses reported that the whey and cheese samples contained greater bacterial abundance, but lower bacterial richness and uniformity (approximately 60 genera and 80 species), which was related to the great abundance of the starter LAB (SLAB) *L. lactis* and the reduction in the relative abundance of most other bacteria. These findings are partially consistent with previous work on raw ewe milk-derived cheeses [25,28–30,48,49]. Other than SLAB, non-starter LAB (NSLAB), such as *Streptococcus* or *Lactobacillus*, dominated in ripened cheeses, as stated in previous works, although there were differences in the identified NSLAB and their abundance [25,28,48,49]. The presence of undesirable bacteria (e.g., *Staphylococcus_A* and *Pseudomonas_E*) is also in accordance with the findings of previous studies of Idiazabal cheese [25] and other raw ewe milk cheeses, but also at different composition [28,32,49,50]. Notably, there was a greater abundance of these bacteria in the whey, suggesting greater dissemination of undesirable bacteria in the whey during cheese-making, which has not been previously reported. The presence of SLAB, NSLAB and undesirable bacteria in whey has also been reported for whey derived from the manufacturing of other raw ewe milk-derived cheeses, although information is scarce [50].

Many of the most abundant bacteria in whey or cheese were not present in the raw milk (e.g., *Lactobacillus_C, Leuconostoc* or *Staphylococcus_A*), indicating the presence of other potential microbial sources within artisanal dairies [5,47,51], as speculated before [25]. With the widely used tool Venn diagram [52] and SourceTracker analysis, which consists of a Bayesian approach model that provides an estimate of the proportion of the community originating from known or unknown source environments [53–55], it was confirmed that all the environmental samples represented notable bacterial reservoirs [5,25,47,51], albeit to different extents. Commercial feed and teat skin surfaces were identified as the main potential sources of bacteria for raw milk, together with the rennet for whey and cheese samples, without taking into account the unavoidable impact of the starter culture, as aforementioned [25,28–30,48,49]. To date, no study has comprehensively assessed all potential microbial sources and their contributions to any raw ewe milk-derived cheese. Individually, the feed used with the herd has been reported to affect the composition and quality of raw milk and derived cheeses [56,57]. However, metagenomic studies on the feed microbiota and its contribution to the microbial composition of raw milk are scarce [57]. In this study, the analysed grass, straw, commercial feed and homemade feed presented intermediate values of microbial richness, abundance and uniformity, suggesting that these are important reservoirs of bacteria present in the raw milk and cheese (e.g., *Pseudomonas_E, Pantoea* or *Lactococcus*), which was confirmed by the SourceTracker analysis. Only Tzora et al. [57] have reported that the sheep diet (meal-

based diet or flaxseed and lupin-based diet) affects the milk microbiota, including *Corynebacterium* or *Staphylococcus* species. Nonetheless, there is little information relating to the presence or absence of the feed bacterial communities in milk [58], and no work has been conducted on cheese.

In the present study, samples from the teat skin surface, together with the artisanal rennet, contained the greatest microbial richness and uniformity. The teat skin surface is considered an important source of raw milk microbes [35,53,58,59], which depends on factors such as animal feed and housing conditions [35]. In previous studies, *Corynebacterium*, *Staphylococcus*, *Moraxella*, *Mannheimia, Jeotgalicoccus* or *Methanobrevibacter* are reported to be dominant taxa in the teat skin microbiota [35,59–61]. However, those studies were carried out on cow teat skin [35,59–61], while information on sheep is scarce [62]. Bi et al. [62] have reported *Bacteroides* and *Prevotella* as dominant genera on the teat skin of ewes, which is not reflected in the present study. Moreover, no study has reported to date if those bacterial communities colonizing the skin surface of sheep are also present in derived raw milk cheeses.

Artisanal rennet, derived from the stomachs of lambs [63,64], is reported to influence the quality and aroma of cheese through the release of free fatty acids mediated by pregastric lipase [64,65]. However, the microbial communities of artisanal rennet and their potential impact on cheese have rarely been studied [66]. Hence, only Cruciata et al. [66] have reported through metagenomic techniques that artisanal rennet employed for the production of some raw ewe milk cheeses could be an important source of LAB, such as *Streptococcus* and *Lactobacillus*, even if clear differences are noted in the identified species compared to the present study. Culture-dependent studies of artisanal rennet are also scarce [67–69]. Cosentino & Palmas [67] have reported the presence of coliforms, psychrotrophs, *E. coli* or *S. aureus*, similar to Gil et al. [69] that reported aerobic mesophiles, Enterotoxigenic staphylococci and *Clostridium* sulphur–reducing. Notably, apart from LAB, the presence of other major bacterial genera (e.g., *Bacteroides, F082* or *RC9*) has not been reported to date. Likewise, no study has analysed the transfer of bacterial communities from the rennet to raw milk cheeses.

The rest of the samples, including surfaces in the dairy environment and brine, also contributed to the dairy microbiota, although to a lesser extent. The microbiota of food processing surfaces has been widely studied [70–72], as they are potential microbial niches even after disinfection due to biofilm formation [70]. The microbiota of food processing surfaces has been described as specific to each dairy facility [37]. Several genera and species, such as *Pseudomonas, Psychrobacter* and *Lactococcus* [70]; *Escherichia, Salmonella* and *Acinetobacter* [73]; or *Brevibacterium* and *Halomonas* [37], have been described. However, there is no information on dairies producing raw ewe milk-derived cheeses. Similarly, the microbiota of brines is also described as specific to processing facilities [74], with the dominance of LAB and halophilic bacteria (e.g., *Lactococcus, Chromohalobacter, Halomonas* or *Tetragenococcus*) mainly reported within the microbiota of brines used for cheese-making [74–76]. Nevertheless, there is no information on the brines used for the production of raw ewe milk-derived cheeses.

In addition to taxonomic profiling, shotgun metagenomic sequencing enables functional potential analysis of the microbiota [77]. More than 1200 metabolic pathways were detected in this study, primarily related to DNA, carbohydrate, protein or fatty acid metabolism. Information on the functional potential of the microbiota in raw ewe milk and derived cheeses is scarce [78], and no study has reported the functional contribution of the microbiota related to the dairy environment. Clear differences were observed among sample types, confirming the functional impact of the bacterial communities inhabiting artisanal dairies on cheese quality and safety, as suggested previously [25,79]. Metabolic pathways related to biofilm formation were identified and related to several bacterial communities inhabiting artisanal dairies, including surfaces (e.g., *Brevibacterium* or *Brachybacterium*), confirming that biofilm formation is one of the potential reasons for the specific communities of dairies [70]. Furthermore, pathways related to competitive inhibition mechanisms, such as bacteriocin production [13], related to bacteria such as *Sphingomonas, Methylobacterium* or *Lactobacillus_H*, could be the reason for the great abundance of these genera in ripened cheeses or environmental samples [25,80,81,82]. Moreover, several pathways related to cheese safety, such as those associated with pathogenicity or AMRs were identified and related to different dairy and environment samples bacteria (e.g., *Stenotrophomonas, Halomonas* or *Sphingomonas*), confirming their implication in the safety of raw milk cheeses [25,79,83]. Likewise, several pathways related to the metabolism of aroma compounds were identified, related to several genera, such as *Halomonas* and *Brevibacterium,* confirming the impact of environmental and non-desirable bacteria on the aroma of cheese, as suggested previously [9–11,84].

Several studies have reported the occurrence of ARGs in dairy products [85,86] or in the dairy environment (e.g., animal faeces and soil) [87]. However, information on the contribution of the dairy environment as a source of ARGs is scarce, with tetracycline, aminoglycoside, multidrug and β-lactam ARGs mainly reported from processing surfaces [70]. Among the more than 470 ARGs detected in this work, multidrug, tetracycline and aminoglycoside ARG families were the main detected, although lincosamide and aminoglycoside ARG families were the most abundant, partially

confirming the functional potential results. Nevertheless, there is no information regarding raw ewe milk and derived cheeses, and the related dairy environment. Grass samples were the main abundant source of ARG families, together with teat skin surface and rennet in terms of number of ARG families. There is no information on the resistome of any type of teat skin or rennet nor on its contribution to the resistome of milk or cheese. Aminoglycosides, β-lactams, quinolones, tetracyclines or vancomycin ARG families are reported as most abundant in grass [88,89], even if there is no information on the grass used for sheep feeding. In dairy surfaces, aminoglycoside, tetracycline, multidrug and β-lactam ARG families have been detected as dominant [70], but there is no information on the surfaces of dairies producing raw ewe milk-derived cheeses. Similarly, no study has analysed the resistome of the brine used for cheese-making, with a unique study concerning sea brine, where tetracycline and macrolide ARG families dominate [90]. Similarly, 13 bacterial genera (e.g., *Staphylococcus_A* or *Leuconostoc*) were reported to be primarily related to ARG families in this work. Even if LAB and *Enterobacteriaceae* (e.g., *Staphylococcus* or *Escherichia*) are reported as main reservoirs of ARG families [1], no study has comprehensively analysed all the ARG families present and their related hosts in dairy products and related dairy environments by metagenomic techniques. Thus, this work demonstrates the value of sequencing-based methodology for comprehensively identifying potential ARGs reservoirs [77].

Virulence determinants or factors have been widely analysed in isolates from raw milk or cheese [91,92]. Here, an exhaustive sequencing-based identification of all VFs present in the microbiome of dairy products and dairies environment is reported for the first time, revealing the potential of this methodology, in terms of food safety, to identify all the VFs present and their relative microbial hosts [77]. More than 3000 VFs were identified, primarily related to prophage or nisin resistance VF families, among others. Phages play a significant role in competitive mechanisms among bacteria and, consequently, in shaping bacterial communities, closely related to the virulence and evolution of numerous critical bacterial pathogens [93,94]. Phages also affect dairy fermentation by suppressing the growth of lactic acid bacteria through cellular lysis [95]. The presence of genes encoding proteins related to resistance to antimicrobials, primarily nisin and tetracycline, partially agreed with the results for the resistome. Within the environmental samples, grass presented the greatest number of VF families and commercial feed the greatest abundance, indicating potential reservoirs of pathogenic bacteria for raw milk. These results agree with the findings of previous studies of grass microbiota, where several pathogens to humans, for example, *Pseudomonas* species, have been detected [87,96]. However, there is no information on commercial feed. Moreover, 18 bacterial genera were primarily related to harbour VF families, e.g., *Psychrobacter, Lactococcus, CAG-791* or *Staphylococcus_A*. For many of these genera pathogenic species, including opportunistic or emerging pathogens, have been previously reported [25].

In terms of cheese quality and flavour, the most noteworthy enzymes related to the metabolism of aroma compounds are hydrolases (EC 3), such as lipases, proteases or esterases [97]. These enzymes contribute to the most important biochemical processes in terms of aroma development [9], namely, lipolysis and proteolysis [9,98,99], with the last also contributing to texture [9]. To elucidate the contribution of the microbiota onto this regard, several studies have been published reporting the correlation between microbial communities and cheese quality compounds [10,11,32,48,100]. However, the genetic potential of the microbiota in this regard has not yet been studied, this work providing the first exhaustive sequencing-based identification of hydrolase-encoding genes of the microbiomes of dairy (raw ewe milk, whey and cheese) and environmental samples. Thus, the main hydrolase-encoding genes (e.g., Xaa-Pro dipeptidyl aminopeptidase-encoding gene, i.e., the *pepX* gene) were identified, where *Lactococcus* and *Lactobacillus* were among the main related bacteria. This would confirm the implication of SLAB and NSLAB on the aroma development [8–11]. However, hydrolase-encoding genes from other environmental or undesirable bacteria (e.g., *Chromohalobacter* and *Listeria*) were also notable, confirming the potential of these bacteria on cheese aroma [10,11], and, indeed, the implying impact of the dairy environments [8–11].

Finally, over 300 medium-quality MAGs and 60 high-quality MAGs were generated, 28 of which corresponded to putative novel species, primarily collected from food contact surfaces and rennet samples. Overall, there is little information on the MAGs of raw milk cheeses and dairy environments [78,101], and information on raw ewe milk-derived cheeses is scarce [78]. The reconstruction of MAGs is evolving into an important tool in food microbiology, due to its capability to identify potential new species and infer their functional potential [102].

5. Conclusions

This study on PDO Idiazabal cheese emphasizes the substantial impact of artisanal dairy practices on the microbiomes of raw milk, whey, and derived cheeses. The shotgun sequencing results revealed diverse microbial ecosystems with more than 1300 bacterial genera and 3400 species. SourceTracker analysis identified the most significant contributions from commercial feed and teat skin to the raw milk microbiota, together with rennet to whey and cheese. Functional analysis linked microbial niches to quality- and safety-related metabolic pathways, highlighting, mainly, the apparent contribution of brine and food contact surfaces. Additionally, the study revealed key reservoirs for

731 virulence factors, i.e., commercial feed and grass, and antimicrobial resistance genes, i.e., grass, teat skin and rennet.
732 Similarly, reservoirs of hydrolase-encoding genes related to cheese aroma and texture were identified, i.e., brine, grass
733 or food contact surfaces. Furthermore, more than 300 metagenomic assembled genomes (MAGs) were generated,
734 suggesting 28 putative novel species.

735 **Declarations**

736 *Ethics approval and consent to participate*

737 Not applicable

738 *Consent for publication*

739 Not applicable

740 *Availability of data and material*

741 The datasets generated and/or analysed during the current study are available in the European Nucleotide Archive
742 (ENA) repository, https://www.ebi.ac.uk/ena/browser/view/PRJEB73723.

743 *Competing interests*

744 The authors declare that they have no competing interests

745 *Funding*

746 This work was financially supported by the grant number IT1568-22 funded by the Basque Government. GS-G
747 received a predoctoral fellowship from the University of the Basque Country (call 2019).

748 *Authors' contributions*

749 GS-G, GA, and MV conceived and designed the study and conducted the sampling. GS-G performed DNA
750 extractions. GS-G and FC conducted library preparation and shotgun sequencing. GS-G, MY, and CL performed
751 bioinformatic analyses. GS-G analysed and interpreted the data and performed the statistical analysis. GS-G wrote the
752 first draft of the manuscript. MY, FC, GA, CL, MV, and PDC reviewed the manuscript. PDC supervised the project. All the
753 authors gave their approval for the final version to be published.

754 *Acknowledgements*

755 The authors thank the collaboration of Idiazabal PDO cheesemakers and the assistance of J. Leech during sample

756 preparation.

757 **Supplementary files**

758 The following supplementary files can be downloaded from: https://upvehueus-
759 my.sharepoint.com/:f:/g/personal/gorka_santamarina_ehu_eus/Er6SsGTICzlHsr8sLN8NZsAB1q5g6uHTz1cWtit6aqBHfA?e=9lcGdj
760

6. References

1. Virto M, Santamarina-García G, Amores G, Hernández I. Antibiotics in dairy production: Where is the problem? Dairy. 2022;3:541–64.
2. Mozaffarian D. Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity – A Comprehensive Review. Circulation. 2016;133:187–225.
3. Yeluri Jonnala BR, McSweeney PLH, Sheehan JJ, Cotter PD. Sequencing of the cheese microbiome and its relevance to industry. Front. Microbiol. 2018;9:1020.
4. Barron LJR, Redondo Y, Aramburu M, Gil P, Pérez-Elortondo FJ, Albisu M, et al. Volatile composition and sensory properties of industrially produced Idiazabal cheese. Int. Dairy J. 2007;17:1401–14.
5. O'Sullivan O, Cotter PD. Microbiota of Raw Milk and Raw Milk Cheeses. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Cheese: Chemistry, Physics and Microbiology. 4th ed. San Diego: Academic Press; 2017. p. 301–16.
6. Meunier-Goddik L, Waite-Cusic J. Consumers Acceptance of Raw Milk and its Products. In: Nero LA, de Carvalho AF, editors. Raw Milk: Balance Between Hazards Benefits. San Diego: Academic Press; 2019. p. 311–50.
7. Afshari R, Pillidge CJ, Read E, Rochfort S, Dias DA, Osborn AM, et al. New insights into cheddar cheese microbiota-metabolome relationships revealed by integrative analysis of multi-omics data. Sci Rep. 2020;10:3164.
8. McSweeney PLH. Biochemistry of Cheese Ripening. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. Volume 3. 3rd ed. San Diego: Academic Press; 2022. p. 22–9.
9. Le Quéré J-L, Buchin S. Cheese Flavor. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. Volume 3. 3rd ed. San Diego: Academic Press; 2022. p. 79–90.
10. Santamarina-García G, Amores G, López de Armentia E, Hernández I, Virto M. Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese. Animals. 2022;12:3224.
11. Santamarina-García G, Amores G, Hernández I, Morán L, Barrón LJR, Virto M. Relationship between the dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese. Curr. Res. Food Sci. 2023;6:100425.
12. Zheng X, Shi X, Wang B. A Review on the General Cheese Processing Technology, Flavor Biochemical Pathways and the Influence of Yeasts in Cheese. Front. Microbiol. 2021;12:703284.
13. Mayo B, Rodríguez J, Vázquez L, Flórez AB. Microbial interactions within the cheese ecosystem and their application to improve quality and safety. Foods. 2021 ;10:602.
14. Castro RD, Pedroso SHSP, Sandes SHC, Silva GO, Luiz KCM, Dias RS, et al. Virulence factors and antimicrobial resistance of *Staphylococcus aureus* isolated from the production process of Minas artisanal cheese from the region of Campo das Vertentes, Brazil. J. Dairy Sci. 2020;103:2098–110.
15. Tirloni E, Bernardi C, Celandroni F, Mazzantini D, Massimino M, Stella S, et al. Prevalence, Virulence Potential, and Growth in Cheese of *Bacillus cereus* Strains Isolated from Fresh and Short-Ripened Cheeses Sold on the Italian Market. Microorganisms. 2023;11:521.
16. Caniça M, Manageiro V, Abriouel H, Moran-Gilad J, Franz CMAP. Antibiotic resistance in foodborne bacteria. Trends Food Sci. Technol. 2019;84:41–4.
17. Ruiz-Capillas C, Herrero AM. Impact of Biogenic Amines on Food Quality and Safety. Foods. 2019;8:62.
18. Barbieri F, Montanari C, Gardini F, Tabanelli G. Biogenic Amine Production by Lactic Acid Bacteria: A Review. Foods. 2019;8:17.
19. Papadimitriou K, Anastasiou R, Georgalaki M, Bounenni R, Paximadaki A, Charmpi C, et al. Comparison of the Microbiome of Artisanal Homemade and Industrial Feta Cheese through Amplicon Sequencing and Shotgun Metagenomics. Microorganisms. 2022;10:1073.
20. Anastasiou R, Kazou M, Georgalaki M, Aktypis A, Zoumpopoulou G, Tsakalidou E. Omics Approaches to Assess Flavor Development in Cheese. Foods. 2022;11:188.
21. Zotta T, Ricciardi A, Condelli N, Parente E. Metataxonomic and metagenomic approaches for the study of undefined strain starters for cheese manufacture. Crit. Rev. Food Sci. Nutr. 2022;62:3898–912.
22. Wensel CR, Pluznick JL, Salzberg SL, Sears CL. Next-generation sequencing: insights to advance clinical investigations of the microbiome. J. Clin. Invest. 2022;132:e154944.
23. Notario E, Visci G, Fosso B, Gissi C, Tanaskovic N, Rescigno M, et al. Amplicon-Based Microbiome Profiling: From Second- to Third-Generation Sequencing for Higher Taxonomic Resolution. Genes. 2023;14:1567.
24. Yap M, Ercolini D, Álvarez-Ordóñez A, O'Toole PW, O'Sullivan O, Cotter PD. Next-Generation Food Research: Use of Meta-Omic Approaches for Characterizing Microbial Communities Along the Food Chain. 2022;13:361–84.
25. Santamarina-García G, Hernández I, Amores G, Virto M. Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing. Biology-Basel. 2022;11:769.
26. Coelho MC, Malcata FX, Silva CCG. Lactic Acid Bacteria in Raw-Milk Cheeses: From Starter Cultures to Probiotic Functions. Foods. 2022;11:2276.

27. Yao J, Gao J, Guo J, Wang H, Zhang E, Lin Y, et al. Characterization of Bacteria and Antibiotic Resistance in Commercially Produced Cheeses Sold in China. J. Food Prot. 2022;85:484–93.
28. Dimov SG, Gyurova A, Zagorchev L, Dimitrov T, Georgieva-Miteva D, Peykov S. NGS-Based Metagenomic Study of Four Traditional Bulgarian Green Cheeses from Tcherni Vit. LWT-Food Sci. Technol. 2021;152:112278.
29. Ramezani M, Hosseini SM, Ferrocino I, Amoozegar MA, Cocolin L. Molecular investigation of bacterial communities during the manufacturing and ripening of semi-hard Iranian Liqvan cheese. Food Microbiol. 2017;66:64–71.
30. Cardinali F, Osimani A, Taccari M, Milanović V, Garofalo C, Clementi F, et al. Impact of thistle rennet from *Carlina acanthifolia* All. subsp. *acanthifolia* on bacterial diversity and dynamics of a specialty Italian raw ewes' milk cheese. Int. J. Food Microbiol. 2017;255:7–16.
31. Endres CM, Castro ÍMS, Trevisol LD, Severo JM, Mann MB, Varela APM, et al. Molecular characterization of the bacterial communities present in sheep's milk and cheese produced in South Brazilian Region via 16S rRNA gene metabarcoding sequencing. LWT-Food Sci. Technol. 2021;147:111579.
32. Cardinali F, Ferrocino I, Milanović V, Belleggia L, Corvaglia MR, Garofalo C, et al. Microbial communities and volatile profile of Queijo de Azeitão PDO cheese, a traditional Mediterranean thistle-curdled cheese from Portugal. Food Res. Int. 2021;147:110537.
33. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, et al. The complex microbiota of raw milk. Fems Microbiol. Rev. 2013;37:664–98.
34. Zheng X, Liu F, Li K, Shi X, Ni Y, Li B, et al. Evaluating the microbial ecology and metabolite profile in Kazak artisanal cheeses from Xinjiang, China. Food Res. Int. 2018;111:130–6.
35. Frétin M, Martin B, Rifa E, Isabelle VM, Pomiès D, Ferlay A, et al. Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci Rep. 2018;8:1–11.
36. Cruciata M, Sannino C, Ercolini D, Scatassa ML, De Filippis F, Mancuso I, et al. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 2014;80:2050–61.
37. Bokulich NA, Mills DA. Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. Appl. Environ. Microbiol. 2013;79:5214–23.
38. Official Journal of the European Communities. Commission Regulation (EC) No 1107/96 of 12 June 1996 on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) No 2081/92. Q. J. Electron. Commer. 1996;148:1–10
39. Boletín Oficial del Estado. Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen "Idiazabal" y su Consejo Regulador. BOE. 1993;289:34591–6.
40. Allard SM, Walsh CS, Wallis AE, Ottesen AR, Brown EW, Micallef SA. *Solanum lycopersicum* (tomato) hosts robust phyllosphere and rhizosphere bacterial communities when grown in soil amended with various organic and synthetic fertilizers. Sci. Total Environ. 2016;573:555–63.
41. Arriola KG, Kim SC, Adesogan AT. Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. J. Dairy Sci. 2011;94:1511–6.
42. Sharma R, Damgaard D, Alexander TW, Dugan MER, Aalhus JL, Stanford K, et al. Detection of transgenic and endogenous plant DNA in digesta and tissues of sheep and pigs fed roundup ready canola meal. J. Agric. Food Chem. 2006;54:1699–709.
43. Mormile MR, Hong BY, Benison KC. Molecular Analysis of the Microbial Communities of Mars Analog Lakes in Western Australia. Astrobiology. 2009;9:919–30.
44. Daniels S, Hepworth J, Moore-Colyer M. The haybiome: Characterising the viable bacterial community profile of four different hays for horses following different pre-feeding regimens. PLoS One. 2020;15:e0242373.
45. Kaminski J, Gibson MK, Franzosa EA, Segata N, Dantas G, Huttenhower C. High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. PLOS Comput. Biol. 2015;11:e1004557.
46. Biçer Y, Telli AE, Sönmez G, Telli N, Uçar G. Comparison of microbiota and volatile organic compounds in milk from different sheep breeds. J. Dairy Sci. 2021;104:12303–11.
47. Esteban-Blanco C, Gutiérrez-Gil B, Puente-Sánchez F, Marina H, Tamames J, Acedo A, et al. Microbiota characterization of sheep milk and its association with somatic cell count using 16s rRNA gene sequencing. J. Anim. Breed. Genet. 2020;137:73–83.
48. De Pasquale I, Calasso M, Mancini L, Ercolini D, La Storia A, De Angelis M, et al. Causal relationship between microbial ecology dynamics and proteolysis during manufacture and ripening of Protected Designation of Origin (PDO) cheese Canestrato Pugliese. Appl. Environ. Microbiol. 2014;80:4085–94.
49. Gaglio R, Franciosi E, Todaro A, Guarcello R, Alfeo V, Randazzo CL, et al. Addition of selected starter/non-starter lactic acid bacterial inoculums to stabilise PDO Pecorino Siciliano cheese production. Food Res. Int. 2020;136:109335.
50. Yang J, Yu P, Liu X, Zhao J, Zhang H, Chen W. Shifts in diversity and function of bacterial community during manufacture of Rushan. J. Dairy Sci. 2021;104:12375–93.
51. Zhao Z, Ning C, Chen L, Zhao Y, Yang G, Wang C, et al. Impacts of manufacture processes and geographical regions on the microbial profile of traditional Chinese cheeses. Food Res. Int. 2021;148:110600.
52. Jia A, Xu L, Wang Y. Venn diagrams in bioinformatics. Brief. Bioinform. 2021;22:bbab108.

53. Doyle CJ, Gleeson D, O'Toole PW, Cotter PD. Impacts of seasonal housing and teat preparation on raw milk microbiota: A high-throughput sequencing study. Appl. Environ. Microbiol. 2016;83:e02694-16.
54. Sun L, D'Amico DJ. Composition, Succession, and Source Tracking of Microbial Communities throughout the Traditional Production of a Farmstead Cheese. mSystems. 2021;6:e00830-21.
55. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian community-wide culture-independent microbial source tracking. Nat. Methods. 2011;8:761–3.
56. Caprioli G, Kamgang Nzekoue F, Fiorini D, Scocco P, Trabalza-Marinucci M, Acuti G, et al. The effects of feeding supplementation on the nutritional quality of milk and cheese from sheep grazing on dry pasture. Int. J. Food Sci. Nutr. 2020;71:50–62.
57. Tzora A, Nelli A, Kritikou AS, Katsarou D, Giannenas I, Lagkouvardos I, et al. The "Crosstalk" between Microbiota and Metabolomic Profile of Kefalograviera Cheese after the Innovative Feeding Strategy of Dairy Sheep by Omega-3 Fatty Acids. Foods. 2022;11:3164.
58. Verdier-Metz I, Delbès C, Bouchon M, Rifa E, Theil S, Chaucheyras-Durand F, et al. Dietary Live Yeast Supplementation Influence on Cow's Milk, Teat and Bedding Microbiota in a Grass-Diet Dairy System. Microorganisms. 2023;11:673.
59. Ruegg PL. The bovine milk microbiome – an evolving science. Domest. Anim. Endocrinol. 2022;79:106708.
60. Dahlberg J, Williams JE, McGuire MA, Peterson HK, Östensson K, Agenäs S, et al. Microbiota of bovine milk, teat skin, and teat canal: Similarity and variation due to sampling technique and milk fraction. J. Dairy Sci. 2020;103:7322–30.
61. Guo W, Bi SS, Wang WW, Zhou M, Neves ALA, Degen AA, et al. Maternal rumen and milk microbiota shape the establishment of early-life rumen microbiota in grazing yak calves. J. Dairy Sci. 2023;106:2054–70.
62. Bi Y, Cox MS, Zhang F, Suen G, Zhang N, Tu Y, et al. Feeding modes shape the acquisition and structure of the initial gut microbiota in newborn lambs. Environ Microbiol. 2019;21:2333–46.
63. Bustamante M, Chávarri F, Santisteban A, Ceballos G, Hernández I, José Miguélez M, et al. Coagulating and lipolytic activities of artisanal lamb rennet pastes. J. Dairy Res. 2000;67:393–402.
64. Amores G, Pérez-Elortondo FJ, Albisu M, Barron LJR. Short communication: To what extent do environmental or technological conditions affect the sensory differentiation of raw ewe milk cheeses produced in valley or mountain farms? J. Dairy Sci. 2021;104:301–7.
65. Virto M, Chávarri F, Bustamante MA, Barron LJR, Aramburu M, Vicente MS, et al. Lamb rennet paste in ovine cheese manufacture. Lipolysis and flavour. Int. Dairy J. 2003;13:391–9.
66. Cruciata M, Sannino C, Ercolini D, Scatassa ML, De Filippis F, Mancuso I, et al. Animal Rennets as Sources of Dairy Lactic Acid Bacteria. Appl. Environ. Microbiol. 2014;80:2050.
67. Cosentino S, Palmas F. Hygienic conditions and microbial contamination in six ewe's-milk-processing plants in Sardinia, Italy. J. Food Prot. 1997;60:283–7.
68. Etayo I, Pérez Elortondo FJ, Gil PF, Albisu M, Virto M, Conde S, et al. Hygienic quality, lipolysis and sensory properties of Spanish Protected Designation of Origin ewe's milk cheeses manufactured with lamb rennet paste. Lait. 2006;86:415–34.
69. Gil PF, Conde S, Albisu M, Pérez-Elortondo FJ, Etayo I, Virto M, et al. Hygienic quality of ewes' milk cheeses manufactured with artisan-produced lamb rennet pastes. J. Dairy Res. 2007;74:329–35.
70. Alvarez-Molina A, Cobo-Díaz JF, Alexa EA, Crispie F, Prieto M, López M, et al. Sequencing-based analysis of the microbiomes of Spanish food processing facilities reveals environment-specific variation in the dominant taxa and antibiotic resistance genes. Food Res. Int. 2023;173:113442.
71. Cobo-Díaz JF, Alvarez-Molina A, Alexa EA, Walsh CJ, Mencía-Ares O, Puente-Gómez P, et al. Microbial colonization and resistome dynamics in food processing environments of a newly opened pork cutting industry during 1.5 years of activity. Microbiome. 2021;9:1–19.
72. Valentino V, Sequino G, Cobo-Díaz JF, Álvarez-Ordóñez A, De Filippis F, Ercolini D. Evidence of virulence and antibiotic resistance genes from the microbiome mapping in minimally processed vegetables producing facilities. Food Res. Int. 2022;162:112202.
73. Alexa E, Walsh CJ, Coughlan LM, Awad A, Simon CA, Ruiz L, et al. Dairy Products and Dairy-Processing Environments as a Reservoir of Antibiotic Resistance and Quorum-Quenching Determinants as Revealed through Functional Metagenomics. mSystems. 2020;5:e00723-19.
74. Haastrup MK, Johansen P, Malskær AH, Castro-Mejía JL, Kot W, Krych L, et al. Cheese brines from Danish dairies reveal a complex microbiota comprising several halotolerant bacteria and yeasts. Int. J. Food Microbiol. 2018;285:173–87.
75. Marino M, Innocente N, Maifreni M, Mounier J, Cobo-Díaz JF, Coton E, et al. Diversity within Italian Cheesemaking Brine-Associated Bacterial Communities Evidenced by Massive Parallel 16S rRNA Gene Tag Sequencing. Front. Microbiol. 2017;8:2119.
76. Levante A, Bertani G, Marrella M, Mucchetti G, Bernini V, Lazzi C, et al. The microbiota of Mozzarella di Bufala Campana PDO cheese: a study across the manufacturing process. Front. Microbiol. 2023;14:1196879.
77. Yap M, Gleeson D, O'Toole PW, O'Sullivan O, Cotter PD. Seasonality and Geography Have a Greater Influence than the Use of Chlorine-Based Cleaning Agents on the Microbiota of Bulk Tank Raw Milk. Appl. Environ. Microbiol. 2021;87:e01081-21.
78. Fontana F, Longhi G, Alessandri G, Lugli GA, Mancabelli L, Tarracchini C, et al. Multifactorial Microvariability of the Italian Raw Milk Cheese Microbiota and Implication for Current Regulatory Scheme. mSystems. 2023;8:e01068-22.
79. Arias-Roth E, Bachmann H-P, Fröhlich-Wyder M-T, Schmidt RS, Wechsler D, Beuvier E, et al. Raw Milk Cheeses. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. Volume 3. 3rd ed. San Diego: Academic Press; 2022. p. 299–308.

80. Mayo B, Rodríguez J, Vázquez L, Flórez AB. Microbial Interactions within the Cheese Ecosystem and Their Application to Improve Quality and Safety. Foods. 2021;10:602.
81. Gontijo MTP, Silva J de S, Vidigal PMP, Martin JGP. Phylogenetic distribution of the bacteriocin repertoire of lactic acid bacteria species associated with artisanal cheese. Food Res. Int. 2020;128:108783.
82. Kaya HI, Simsek O. Characterization of pathogen-specific bacteriocins from lactic acid bacteria and their application within cocktail against pathogens in milk. LWT-Food Sci. Technol. 2019;115:108464.
83. Hayaloglu AA. Microbiology of Cheese. In: McSweeney PLH, McNamara JP, editors. Encyclopedia of Dairy Sciences. Volume 3. 3rd ed. San Diego: Academic Press; 2022. p. 225–37.
84. Meng Y, Chen X, Sun Z, Li Y, Chen D, Fang S, et al. Exploring core microbiota responsible for the production of volatile flavor compounds during the traditional fermentation of Koumiss. LWT-Food Sci. Technol. 2021;135:110049.
85. Delannoy S, Hoffer C, Tran ML, Madec JY, Brisabois A, Fach P, et al. High throughput qPCR analyses suggest that Enterobacterales of French sheep and cow cheese rarely carry genes conferring resistances to critically important antibiotics for human medicine. Int. J. Food Microbiol. 2023:403-110303;
86. Huang J, Zhang W, Sun B, Jiang Q, Cao Y, Shang J, et al. Genetic diversity, antibiotic resistance, and virulence characteristics of *Staphylococcus aureus* from raw milk over 10 years in Shanghai. Int. J. Food Microbiol. 2023;401:110273.
87. Liu X, Zuo J, Teng J, Yang L, Guo J, Liu L, et al. Antibiofilm potential of luteolin against multidrug-resistant *Staphylococcus aureus* isolated from dairy goats and farm environments. Environ. Pollut. 2023;335:122274.
88. Tyrrell C, Do TT, Leigh RJ, Burgess CM, Brennan FP, Walsh F. Differential impact of swine, bovine and poultry manure on the microbiome and resistome of agricultural grassland. Sci. Total Environ. 2023;886:163926.
89. Do TT, Smyth C, Crispie F, Burgess C, Brennan F, Walsh F. Comparison of soil and grass microbiomes and resistomes reveals grass as a greater antimicrobial resistance reservoir than soil. Sci. Total Environ. 2023;857:159179.
90. Elbehery AHA, Beason E, Siam R. Metagenomic profiling of antibiotic resistance genes in Red Sea brine pools. Arch. Microbiol. 2023;205:195.
91. Anika TT, Al Noman Z, Islam MS, Sultana N, Ashraf MN, Pervin M, et al. Draft genome sequence of multidrug-resistant *Escherichia coli* MAHK_SCM_BAU_30A strain isolated from a subclinical mastitis cow in Bangladesh. Microbiol. Resour. Ann. 2023;12:e00713-23.
92. Güley Z, Fallico V, Cabrera-Rubio R, Cotter PD, Beresford T. Identification of *Streptococcus infantarius* subsp. *infantarius* as the species primarily responsible for acid production in Izmir Brined Tulum Cheese from the Aegean Region of Türkiye. Food Res. Int. 2022;160:111707.
93. Fortier LC, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence. 2013;4:354.
94. Teklemariam AD, Al-Hindi RR, Qadri I, Alharbi MG, Ramadan WS, Ayubu J, et al. The Battle between Bacteria and Bacteriophages: A Conundrum to Their Immune System. Antibiotics-Basel. 2023;12:381.
95. Briggiler Marcó MB, Quiberoni A, Suárez V. Virulence of *Leuconostoc* phages: Influence of stress conditions associated to dairy processes on their host-phage interactions. Int. J. Food Microbiol. 2019;303:26–31.
96. Eliasson T, Sun L, Lundh Å, Höjer A, Saedén KH, Hetta M, et al. Epiphytic microbiota in Swedish grass-clover herbage and the effect of silage additives on fermentation profiles and bacterial community compositions of the resulting silages. J. Appl. Microbiol. 2023;134:lxad196.
97. McSweeney PLH, McNamara JP. Encyclopedia of Dairy Sciences. 3rd ed. San Diego: Academic Press; 2022.
98. Ardö Y, McSweeney PLH, Magboul AAA, Upadhyay VK, Fox PF. Biochemistry of Cheese Ripening: Proteolysis. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Cheese: Chemistry, Physics and Microbiology. 4th ed. San Diego: Academic Press; 2017. p. 445–82.
99. Thierry A, Collins YF, Mukdsi, M. C. A. McSweeney PLH, Wilkinson, M. G. Spinnler HE. Lipolysis and metabolism of fatty acids in cheese. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW, editors. Cheese: Chemistry, Physics and Microbiology. 4th ed. San Diego: Academic Press; 2017. p. 423– 444.
100. De Pasquale I, Di Cagno R, Buchin S, De Angelis M, Gobbetti M. Spatial distribution of the metabolically active microbiota within Italian PDO ewes' milk cheeses. PLoS One. 2016;11:e0153213.
101. Shangpliang HNJ, Tamang JP. Metagenome-assembled genomes for biomarkers of bio-functionalities in Laal dahi, an Indian ethnic fermented milk product. Int. J. Food Microbiol. 2023;402:110300.
102. Leech J, Cabrera-Rubio R, Walsh AM, Macori G, Walsh CJ, Barton W, et al. Fermented-Food Metagenomics Reveals Substrate-Associated Differences in Taxonomy and Health-Associated and Antibiotic Resistance Determinants. mSystems. 2020;5:e00522-20.

7. OTHER CONTRIBUTIONS

This section details further contributions, beyond manuscripts, related to the results derived from this research project. These contributions are divided into contributions to scientific meetings (7.1) and contributions to scientific divulgation (7.2).

7.1. Scientific meetings

The results of this doctoral thesis have given rise to several contributions in different national or international scientific **congresses, conferences, meetings and symposiums**. These contributions are listed below:

1. Title: **Estudio de la microbiota del queso Idiazabal por técnicas de metagenómica. Influencia del tiempo de curado y de los sistemas de manejo en pastoreo de ovinos**.
   Authors: **Santamarina García, G.**, Hernández, I., Amores, G., Virto. M.
   Conference: *I. Congreso Anual Internacional de Estudiantes de Doctorado (CAIED)*
   Type: Poster communication
   Place: Online
   Year: 2021

2. Title: **High-throughput sequencing for characterization of microbial shifts throughout production and ripening of Idiazabal (ewe's raw milk) cheese**.
   Authors: **Santamarina García, G.**, Hernández, I., Amores, G., Virto. M.
   Conference: *IDF International Cheese Science and Technology Symposium*
   Type: Poster communication.
   Place: Online
   Year: 2021

3. Title: **Idiazabal gaztaren ikerketa metagenomikoa: gaztagilearen eta heltze denboraren eragina mikrobiotan**.
   Authors: **Santamarina García, G.**, Hernández, I., Amores, G., Virto. M.
   Conference: *IV. IkerGazte: Nazioarteko ikerketa euskaraz Kongresua*
   Type: Oral communication
   Place: Vitoria-Gasteiz (Spain)
   Year: 2021

4. Title: **Secuenciación de alto rendimiento para la caracterización de la sucesión microbiana durante la elaboración y maduración del queso de leche cruda de oveja Idiazábal**.
   Authors: **Santamarina García, G.**, Hernández, I., Amores, G., Virto. M.
   Conference: *II. Congreso Anual Internacional de Estudiantes de Doctorado* (CAIED).

Type: Oral communication.
Place: Online
Year: 2022

5. Title: **El impacto de la microbiota en varios parámetros de calidad y seguridad del queso de leche cruda de oveja Idiazabal**.
   Authors: **Santamarina García, G**., Amores, G., López de Armentia, E., Hernández, I., Virto. M.
   Conference: *VII. Jornadas Doctorales de la Universidad de Murcia*
   Type: Oral communication.
   Place: Online
   Year: 2022

6. Title: **The impact of bacterial shifts on several quality and safety parameters of a ewe's raw milk cheese**.
   Authors: **Santamarina García, G**., Amores, G., Hernández, I., Virto. M.
   Conference: *EAAP (European Federation of Animal Science) – 73rd Annual Meeting*
   Type: Poster communication
   Place: Porto (Portugal)
   Year: 2022

7. Title: **Relationship between bacterial communities and volatile aroma compounds of a ewe's raw milk cheese**.
   Authors: **Santamarina García, G**., Amores, G., Hernández, I., Virto. M.
   Conference: *EAAP (European Federation of Animal Science) – 73rd Annual Meeting*
   Type: Poster communication
   Place: Porto (Portugal)
   Year: 2022

8. Title: **Zenbateraino eragiten diote bakterio-komunitateek esne gordineko gazten kalitateari eta kaltegabetasunari?: Idiazabal gaztaren kasua.**
   Authors: **Santamarina-García, G**., Amores, G., Virto, M.
   Type: Oral communication
   Conference: *V. IkerGazte: Nazioarteko ikerketa euskaraz Kongresua*
   Place: Donostia-San Sebastián (Spain)
   Year: 2023

9. Title: **Volatile profile differentiation among producers of a raw milk PDO cheese: a metagenomic approach**.
   Authors: **Santamarina-García, G**., Amores, G., Hernández, I., Virto, M.
   Type: Poster communication
   Conference: *3rd Food Chemistry Conference-Shaping a healthy and sustainable food chain through knowledge*.

Place: Dresden (Germany)
Year: 2023

10. **Title**: **Producers' knowledge and attitudes on antibiotics' utilisation in artisanal dairies producing a raw ewe milk-derived cheese (Idiazabal).**
Authors: **Santamarina-García, G**., Amores, G., Hernández, I., Virto, M.
Type: Oral communication
Conference: *ISAS 2023 – International Symposium on Animal Sciences*
Place: Novi Sad (Serbia)
Year: 2023

11. Title: **Elucidating the prevalence of antibiotic residues throughout a raw milk cheese production chain by LC-MS/MS and commercial testing: a farm-to-fork study.**
Authors: **Santamarina-García, G**., Amores, G., Virto, M.
Type: Poster communication
Conference: *3rd Food Chemistry Conference-Shaping a healthy and sustainable food chain through knowledge*.
Place: Dresden (Germany)
Year: 2023

12. Title: **Antimicrobial-resistant lactic acid bacteria in sheep flocks and in their raw milk used for cheese making.**
Authors: **Santamarina-García, G**., Amores G., Hernández, I., Virto M.
Type: Oral communication
Conference: 37th EFFoST *(European Federation of Food Science and Technology)* International Conference 2023 – Sustainable Food and Industry 4.0: Towards the 2030 Agenda
Place: Valencia, Spain
Year: 2023

13. Title: **Distribution of antimicrobial resistant LAB along the production of a raw ewe milk-derived cheese.**
Authors: **Santamarina-García, G**., Amores G., Azcona L., Hernández, I., Virto M.
Type: Oral communication
Conference: *ECM 2023 - 2nd International Electronic Conference on Microbiology*
Place: Online
Year: 2023

14. Title: **High Throughput Quantitative PCR to elucidate the occurrence of antimicrobial resistance genes along the production-chain of raw ewe milk-derived Idiazabal cheese.**
Authors: **Santamarina-García, G**., Amores G., Llamazares D., Hernández, I., Virto M.
Type: Poster communication
Conference: *EAR LTC-Sarea-ENLIGHT CONGRESS–Strengthening Antibiotic Resistance Networks*

Place: Bordeaux, France
Year: 2023

15. Title: **The influence of dairy environment and practices on the microbiome of raw ewe milk-derived PDO cheeses: a shotgun sequencing approach.**
Authors: **Santamarina-García, G**., Yap, M., Crispie, F., Amores G., Lordan, C., Virto M., Cotter, P.D.
Type: Poster communication
Conference: *28th International ICFMH Conference FOOD MICRO 2024*
Place: Burgos, Spain
Year: 2024

16. Title: **Resistome analysis of Idiazabal PDO cheeses: elucidating the impact of dairy environments.**
Authors: **Santamarina-García, G**., Yap, M., Crispie, F., Amores G., Lordan, C., Barron, L.J.R., Virto M., Cotter, P.D.
Type: Poster communication
Conference: *IDF Cheese Science and Technology Symposium 2024*
Place: Bergen, Norway
Year: 2024

7.2. Scientific divulgation

Additionally, the results of this doctoral thesis have also been disseminated to society and, specifically, to the production sector, through **written, radio, video and television interviews**, as well as **workshops**. These contributions are listed below:

1. Title: "**Cultivos iniciadores, ¿cómo influyen en el queso Idiazabal? Más allá de la metagenómica.**"
Authors: **Santamarina García, G**., Hernández, I.
Type: Workshop for Idiazabal PDO producers.
Year: 2020

2. Title: **Gorka Santamarina: "Ikergazte oso aukera ona iruditzen zait ikertzaile gehiago ezagutzeko eta ikertzaile-komunitate osoak Gasteizen egiten dugun lana ezagutzeko"**
Author: **Santamarina García, G**.
Media: *Udako Euskal Unibertsitatea* portal
Type: Written interview
URL:
https://www.ueu.eus/komunikazioa/albisteak/gorka-santamarina-ikergazte-oso-aukera-ona-iruditzen-zait-ikertzaile-gehiago-ezagutzeko-eta-ikertzaile-komunitate-osoak-gasteizen-egiten-dugun-lana-ezagutzeko
Year: 2021

3. Title: "Gaur egungo proiektuetan lankidetza eskatzen da eta Ikergaztek aukera hori ematen du".
   Author: **Santamarina García, G**.
   Media: *Euskal Irratia eta Telebista (EITB)* television
   Type: Telebision interview
   URL:
   https://www.eitb.eus/eu/telebista/programak/egunon-euskadi/bideoak/osoa/8116649/bideoa-ikergazte-kongresuan-hartu-du-parte-gorka-santamarinak-gazten-ikerketa-batekin/
   Year: 2021

4. Title: **"Ikertzaile gazteak ikerkuntzatik euskaraz eragiten."**
   Author: **Santamarina García, G**.
   Media: *TTAP aldizkaria* digital magazine
   Type: Video interview
   Number: 127
   Year: 2021

5. Title: **"Idiazabal gaztaren ikerketa metagenomikoa."**
   Author: **Santamarina García, G**.
   Type: Divulgative poster
   Year: 2022

6. Title: **"Idiazabal gaztaren microbiota argitzen: bere inpaktua kalitatean"**
   Author: **Santamarina García, G**.
   Media: *Udako Euskal Unibertsitatea* portal
   Type: Science divulgation competition through social media
   URL:
   https://www.ueu.eus/txiotesia/txiotesia-6/idiazabal-gaztaren-mikrobiota-argitzen-bere-inpaktua-kalitatean
   Year: 2022

7. Title: **"La microbiota del queso Idiazabal, clave para su sabor y calidad"**
   Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
   Media: *CAMPUSA*, The University of the Basque Country magazine
   Type: Written interview
   URL:
   https://www.ehu.eus/es/-/la-microbiota-del-queso-idiazabal?redirect=%2Feu%2Fcampusa%2Fikerketa%2F-%2Fasset_publisher%2FMICMqglnC3fZ%2Frss
   Year: 2024

8. Title: **"La clave del sabor del queso Idiazabal"**
   Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
   Media: *Norte Expres* digital magazine
   Type: Written interview
   URL:
   https://nortexpres.com/estudio-la-clave-del-sabor-del-queso-idiazabal/
   Year: 2024

9. Title: **"Un estudio prueba que la microbiota del queso Idiazabal determina su sabor y su calidad"**
   Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
   Media: *Diario de Álava* magazine
   Type: Written interview
   URL:
   https://www.noticiasdealava.eus/alava/2024/01/22/estudio-prueba-microbiota-queso-idiazabal-7771135.html
   Year: 2024

10. Title: **" Idiazabal gaztaren mikrobiota, funtsezkoa haren zaporean eta kalitatean"**
    Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
    Media: *Naiz* magazine
    Type: Written interview
    URL:
    https://www.naiz.eus/eu/gaiak/noticia/20240122/idiazabal-gaztaren-mikrobiota-funtsezkoa-haren-zaporean-eta-kalitatean
    Year: 2024

11. Title: **"La microbiota del queso Idiazabal determina sus características de sabor y calidad"**
    Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
    Media: *Oviespaña* digital journal
    Type: Written interview
    URL:
    https://www.oviespana.com/articulos/538633-La-microbiota-del-queso-Idiazabal-determina-sus-caracteristicas-de-sabor-y-calidad.html
    Year: 2024

12. Title: **" La microbiota del queso Idiazabal, clave para su sabor y calidad"**
    Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
    Media: *Real Federación Española De Asociaciones De Ganado Selecto* portal
    Type: Written interview
    URL:
    https://rfeagas.es/microbiota-queso-idiazabal-sabor-calidad/

Year: 2024

13. Title: **"Idiazabal gaztaren mikrobiota, funtsezkoa haren zaporean eta kalitatean"**
    Authors: **Santamarina García, G.**, Amores, G., Hernández, I., Virto, M.
    Media: *Noticias de Álava* magazine
    Type: Written interview
    URL:
    https://www.noticiasdealava.eus/mundo/2024/01/25/idiazabal-gaztaren-mikrobiota-funtsezkoa-haren-7784155.html
    Year: 2024

14. Title: **"La fórmula del queso Idiazabal: ¿Qué determina su sabor y calidad?"**
    Author: **Santamarina García, G.**
    Media: *Radio Popular de Bilbao* radio
    Type: Radio interview
    URL:
    https://radiopopular.com/podcast/las-claves-del-queso-idiazabal-que-determina-su-sabor-y-calidad
    Year: 2024

15. Title: **" Bakterioak, begi hutsez ikusten ez diren gaztagile txikiak"**
    Author: **Santamarina García, G.**
    Media: *Zientzia Kaiera*, UPV/EHU Chair of Scientific Culture blog
    Type: Written interview
    URL:
    https://zientziakaiera.eus/2024/02/27/bakterioak-begi-hutsez-ikusten-ez-diren-gaztagile-txikiak/
    Year: 2024

16. Title: **"Asteon zientzia begi-bistan #475"**
    Author: **Santamarina García, G.**
    Media: *Zientzia Kaiera*, UPV/EHU Chair of Scientific Culture blog
    Type: Written interview
    URL:
    https://zientziakaiera.eus/2024/03/03/asteon-zientzia-begi-bistan-475/
    Year: 2024

17. Title: **"Gazta mikroskopio azpian"**
    Author: **Santamarina García, G.**
    Media: *Berria* magazine
    Type: Written interview
    URL:
    https://www.berria.eus/bizigiro/gazta-mikroskopio-azpian_2122611_102.html

Year: 2024

18. Title: **"La microbiota define el queso de Idiazabal."**
    Author: **Santamarina García, G.**
    Media: *Radio Euskadi* radio
    Type: Radio interview
    URL:
    https://www.eitb.eus/es/radio/radio-euskadi/programas/la-mecanica-del-caracol/detalle/9461720/la-microbiota-define-queso-de-idiazabal-la-narrativa-de-seresmitologicos/
    Year: 2024

19. Title: **"El sabor del queso Idiazabal, cosa de bacterias."**
    Author: **Santamarina García, G.**
    Media: *Noticias de Gipuzkoa* magazine
    Type: Written interview
    URL:
    https://www.noticiasdegipuzkoa.eus/sociedad/2024/04/02/sabor-queso-idiazabal-cosa-bacterias-8063745.html
    Year: 2024

20. Title: **"Idiazabal gaztaren mikrobiota."**
    Author: **Santamarina García, G.**
    Media: *Euskadi Irratia* radio
    Type: Radio interview
    URL:
    https://www.eitb.eus/eu/irratia/euskadi-irratia/programak/ekosfera/audioak/osoa/9484823/ekosfera-20240501/
    Year: 2024

Section II
Conclusions

8. CONCLUSIONS

Based on the research carried out (**manuscripts I-VII**), whose aimed to elucidate the microbiota of raw ewe milk-derived Idiazabal cheese, its implication on several quality and safety aspects, and the main factors affecting it, the following **conclusions** were drawn:

1. Metataxonomic, diversity and statistical analyses confirmed the **impact of the cheese making and ripening processes on the microbiota** of Idiazabal cheese. Some environmental or undesirable genera (such as *Hafnia, Brevibacterium* or *Psychrobacter*) presented notable abundances at the beginning of the ripening (7, 14, 30 days). However, in general, their abundance was reduced along cheese making and ripening processes, accounting for low abundance in the ripened cheese (120 days). According to LAB, a clear dynamic was observed. After the cheese making and until 30 or 60 days of ripening, the SLAB *Lactococcus* presented the greatest abundance. However, thereafter, the abundance of the NSLAB *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Enterococcus* was notable.

2. Although a general dynamic of the microbiota was described throughout the cheese making and ripening of Idiazabal cheese, **clear differences in the microbial composition** of Latxa raw ewe milk and Idiazabal cheese samples were observed **among producers**, related to LAB, environmental and undesirable bacteria, and their abundances. Therefore, the practices adopted by producers, which differed among dairies, were suggested as having a great impact on the microbiota of cheese.

3. Correlation analyses reported that **gross composition evolution during ripening**, including NaCl concentrations or pH, influenced the microbial composition of cheese, promoting NSLAB proliferation, especially *Lactobacillus, Streptococcus* and *Enterococcus*, while inhibiting the growth of environmental or undesirable bacteria, such as *Psychrobacter, Erwinia, Pseudomonas* and *Pantoea.* Consequently, physico-chemical changes during ripening were confirmed as **determining microbial dynamics**.

4. Correlation analyses confirmed **the impact of the microbiota on quality parameters of Idiazabal cheese, especially FFAs and volatile compounds** related to **cheese aroma.** The SLAB *Lactococcus* was related to particular ketones production, while NSLAB (*Enterococcus, Streptococcus* and *Leuconostoc*) were related to FFAs release and the production of acids, esters or alcohols. Undesirable bacteria (e.g., *Psychrobacter, Brevibacterium* and *Chromohalobacter*), instead, were related to ketones, sulphur compounds or hydrocarbons synthesis. Likewise, several negative correlations were observed, related to **compounds degradation abilities**, such as volatile compounds, or indicating **competitive inhibition mechanisms** among bacteria, such as FFAs that have antimicrobial potential.

5. The **influence of the microbiota on the cheese safety** was also corroborated, due to BAs formation abilities. *Lactobacillus* NSLAB was described as the main genera related to **BAs production**, both those with toxigenic potential, e.g. TYR or CAD, and GABA related to several health benefits. Additionally, *Lactobacillus,* together with other environmental and undesirable bacteria, such as *Erwinia, Chromohalobacter* or *Pantoea*, were related to **BAs degradation abilities**, which should be further explored.

6. **Antibiotics residues** were detected throughout the production of Idiazabal cheese, which was related to the observed **lack of producers' knowledge on antibiotic use**. However, a discrepancy was noted among commercial and analytical approaches, since screening tests reported only positive samples for fresh and ripened cheese, whereas chromatographically residues were only identified in raw milk and whey, as well as faeces, as published previously. Thus, the need for improving these techniques was suggested, in order to avoid consequent impacts, such as on human or environmental health.

7. The **influence of the microbiota on the prevalence of AMRs** was also confirmed, with, in general, LAB showing high resistance rates to those antibiotics commonly used in dairies. However, for the first time, it was described that **LAB dynamics during cheese making and ripening favoured a reduction in AMRs,** both phenotypically (resistance rates of isolates) and genotypically (ARGs and MGEs abundance). Thus, *Lactococcus, Enterococcus* and *Bacillus* species that dominated faeces, raw milk, whey or fresh cheeses showed the greatest resistance rates, whereas *Lacticaseibacillus* and *Lactobacillus* species that dominate in ripened

cheeses were the most susceptible. This is of special interest since these are the species consumed and could come into contact with the intestinal microbiota.

8. **Dairy environments and practices** clearly impacted the microbiota of Latxa raw ewe milk and derived Idiazabal cheese, with commercial feed and teat skin as the main contributors for raw milk microbiota, together with the rennet for the cheese. Nevertheless, all the collected samples around dairy environments, such as dairy surfaces or brine, were microbial sources influencing the microbiota, although to a different extent. Consequently, the **dairy environment and practices** were confirmed as **the main reason for the distinction** in the microbial communities **among producers**.

9. Shotgun sequencing allowed the evaluation of the **functional potential of the microbial communities inhabiting artisanal dairies**. Functional analysis associated microbial niches with metabolic pathways relevant to cheese quality and safety, particularly brine and food contact surfaces, related to genera such as *Brevibacterium, Methylobacterium,* and *Halomona*s. In terms of **cheese safety**, in addition to whey and cheese, commercial feed and grass were the main reservoirs of **VFs**, related to, e.g. *Brevibacillus_B* or *CAG-196*. Similarly, grass, teat skin or rennet were the main contributors of **ARGs**, related to, e.g. *Bact-11* or *Bacteriodes_B*. In terms of **cheese quality**, such as aroma and texture, apart from the microbiome of the cheese itself, brine, grass and food contact surfaces were key reservoirs for hydrolase-encoding genes, originating from, for example, *Lactococcus, Lactobacillus, Listeria* or *Chromohalobacter*. Thus**, the functional impact of dairy environments and practices on Idiazabal cheese quality and safety was confirmed**.

Considering all the foregoing points**,** it can be concluded that, in general, **the stablished hypothesis was confirmed**, namely, **each Idiazabal cheese has its own microbiota**, which has an **essential and unavoidable effect on the composition and characteristics** of the final product and which **depends on several factors**, such as the flock management, the ingredients used during cheese making and the dairy environment.

Section III
Appendices

1 Appendix I
Manuscript I

Biology (2022), 11(5), 769

Characterization of microbial shifts during the production and ripening of raw ewe milk-derived Idiazabal cheese by high-throughput sequencing

Gorka Santamarina-García[1,*], Igor Hernández[1], Gustavo Amores[1] and Mailo Virto[1]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[*] *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented at:

IDF International Cheese Science and Technology Symposium, 2021. *High-throughput sequencing for characterization of microbial shifts throughout production and ripening of Idiazabal (ewe's raw milk) cheese.* Santamarina-García, G., Hernández, I., Amores, G. and Virto, M. (Poster presentation).

IV. Ikergazte. Nazioarteko Ikerketa Euskaraz, 2021. *Idiazabal gaztaren ikerketa metagenomikoa: gaztagilearen eta heltze denboraren eragina mikrobiotan.* Santamarina-García, G., Hernández, I., Amores, G. and Virto, M. (Oral presentation).

II. Congreso Anual Internacional de Estudiantes de Doctorado de la Universidad Miguel Hernández de Elche, 2022. *Secuenciación de alto rendimiento para la caracterización de la sucesión microbiana durante la elaboración y maduración del queso de leche cruda de oveja Idiazábal.* Santamarina-García, G., Hernández, I., Amores, G. and Virto, M. (Oral presentation).

Published in **Biology**
ISSN: 2079-7737
IF (2022): **4.2**
Category: **Biology (Q2, 25/92)**
Citations: 5 (updated: 04/2024)
doi.org/10.3390/biology11050769

biology

MDPI

Article

Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing

Gorka Santamarina-García *, Igor Hernández, Gustavo Amores and Mailo Virto *

Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain; igor.hernandezo@ehu.eus (I.H.); gustavo.amores@ehu.eus (G.A.)

* Correspondence: gorka.santamarina@ehu.eus (G.S.-G.); mailo.virto@ehu.eus (M.V.); Tel.: +34-945013799 (G.S.-G.); +34-945013099 (M.V.)

Simple Summary: Idiazabal is a traditional cheese produced from raw ewe milk in the Basque Country (Southwestern Europe). The sensory properties of raw milk cheeses have been attributed, among other factors, to microbial shifts that occur during the production and ripening processes. In this study, we used high-throughput sequencing technologies to investigate the microbiota of Latxa ewe raw milk and the dynamics during cheese production and ripening processes. The microbiota of raw milk was composed of lactic acid bacteria (LAB), environmental bacteria and non-desirable bacteria. Throughout the cheese making and ripening processes, the growth of LAB was promoted, whereas that of non-desirable and environmental bacteria was inhibited. Moreover, some genera not reported previously in raw ewe milk were detected and clear differences were observed in the bacterial composition of raw milk and cheese among producers, in relation to LAB and environmental or non-desirable bacteria, some of which could be attributed to the production of flavour related compounds.

Abstract: In this study, we used high-throughput sequencing technologies (sequencing of V3–V4 hypervariable regions of 16S rRNA gene) to investigate for the first time the microbiota of Latxa ewe raw milk and the bacterial shifts that occur during the production and ripening of Idiazabal cheese. Results revealed several bacterial genera not reported previously in raw ewe milk and cheese, such as *Buttiauxella* and *Obesumbacterium*. Both the cheese making and ripening processes had a significant impact on bacterial communities. Overall, the growth of lactic acid bacteria (LAB) (*Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus* and *Carnobacterium*) was promoted, whereas that of non-desirable and environmental bacteria was inhibited (such as *Pseudomonas* and *Clostridium*). However, considerable differences were observed among producers. It is noteworthy that the starter LAB (*Lactococcus*) predominated up to 30 or 60 days of ripening and then, the growth of non-starter LAB (*Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Streptococcus*) was promoted. Moreover, in some cases, bacteria related to the production of volatile compounds (such as *Hafnia*, *Brevibacterium* and *Psychrobacter*) also showed notable abundance during the first few weeks of ripening. Overall, the results of this study enhance our understanding of microbial shifts that occur during the production and ripening of a raw ewe milk-derived cheese (Idiazabal), and could indicate that the practices adopted by producers have a great impact on the microbiota and final quality of this cheese.

Keywords: cheese quality; ripening; microbiota; bacterial diversity; 16S rRNA sequencing; PCoA

Citation: Santamarina-García, G.; Hernández, I.; Amores, G.; Virto, M. Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing. *Biology* **2022**, *11*, 769. https://doi.org/10.3390/biology11050769

Academic Editor: Huizhong Chen

Received: 13 April 2022
Accepted: 11 May 2022
Published: 18 May 2022

1. Introduction

Idiazabal cheese is a semi-hard or hard cheese made exclusively from the raw milk of Latxa and/or Carranzana sheep, with a minimum ripening time of 60 days. Its production is located in the Basque Country (Southwestern Europe) and has a Protected Designation

of Origin (PDO) [1]. Most of the producers attached to the Idiazabal PDO are small family dairies that lead the whole process, from livestock management to cheese making and final sales. Although Idiazabal cheese production is a strictly regulated process, producers may use different practices that may affect the characteristics of the final product. The most considerable differences in production practices are noticed in the management and feeding of the herd, leading to differences in milk quality [2]; in the use of artisanal or commercial rennet, or in the parameters selected during cheese making and ripening, since the Idiazabal PDO specifications establish ranges [3].

Idiazabal cheese, as other cheeses prepared from raw milk, has a richer and more intense aromatic profile compared with those produced from pasteurized milk [4,5]. Such interesting sensory properties of raw milk cheeses have previously been attributed, among other factors, to the complex dynamics of microbial composition during cheese making and ripening [5,6]. The quality of raw milk, use of starters and their intrinsic characteristics, type of rennet used and ripening time are some factors that determine the cheese microbiota [5,7–9]. The microbiota of milk has a diverse and complex composition, but it is mainly composed of lactic acid bacteria (LAB) [5,10]. In Idiazabal cheese, the most common LAB are *Lactococcus*, *Lactobacillus* and *Leuconostoc* [11,12]. These bacteria metabolize the lactose present in milk, generating lactic acid and other compounds, such as acetic acid, ethanol and diacetyl. These compounds, along with others produced during ripening, determine the sensory properties of cheese [13]. Although LAB are predominant, other low-abundance microorganisms are also part of the microbial ecosystem of cheese [10,14,15], and consequently contribute to the quality of the final product [16,17].

The characteristics of LAB and other microorganisms present in Idiazabal cheese have been described and related to its sensory properties in several studies [11,12,18,19]. However, these studies were performed 20 years ago using culture-dependent methods. Nowadays, high-throughput sequencing (HTS) technologies are used to monitor microbial communities in different fermented products [20–22], including cheese [23–25]. The HTS techniques allow the detection of a large number of bacteria, including those present in relatively small numbers [26–29], those present in a viable but non-cultivable state (VBNC) [30] and those not detected by other culture-dependent or independent methods [30–34].

The vast majority of studies on cheese focus on cheese produced from cow milk [24,35,36], and only a few studies have been carried out on cheese produced from the raw milk of ewe [37–39]. Moreover, little is known about the bacterial composition of raw ewe milk [40–42] and how it changes during cheese making and ripening processes [26,43,44].

Therefore, this study aimed to (1) characterize the bacterial communities of the raw milk of Latxa ewe; (2) analyse the effect of cheese making and ripening processes on bacterial populations; and (3) study the potential differences among producers producing the same type of cheese. To the best of our knowledge, no comprehensive metagenomic study has been conducted to date on raw ewe milk-derived cheeses. Moreover, although Idiazabal cheese has an internationally recognized PDO [1], no HTS studies have been performed to characterize its bacterial populations.

2. Materials and Methods

2.1. Milk and Cheese Sampling

To analyse the microbiota of Latxa ewe raw milk and Idiazabal cheeses, samples were collected from four artisanal Idiazabal PDO cheese producers (identified as A, B, C and D), whose dairies were situated in different geographic locations throughout the Basque Country. Milk was kept in refrigeration tanks before cheese making. Cheeses were produced from the collected milk samples, according to specifications issued by the Idiazabal Designation of Origin Regulatory Board [3], using Choozit MM 100 LYO 50 DCU (mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*) (DuPont NHIB Ibérica S.L., Barcelona, Spain) as the starter. Milk was coagulated using artisanal rennet prepared from the stomachs of Latxa lambs (extracted during the first month of lactation, cleaned, dried, salted and ground, as described

previously [45]) or commercial rennet NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). Cheese ripening was carried out in chambers maintained at 8–14 °C temperature and 80–95% relative humidity. Cheeses were collected in duplicate at six time points during ripening (1, 7, 14, 30, 60 and 120 days). Therefore, a total of 4 raw milk samples and 48 cheese samples were analysed. Samples were collected and transported to the laboratory under refrigerated conditions (3 °C) for analysis.

2.2. DNA Extraction

DNA extraction was performed immediately after sample arrival, following the method described by Erkus et al. [46], with some modifications. To extract DNA from cheese samples, 10 g of each sample was suspended in 90 mL of 2% (*w*/*v*) sterile sodium citrate (pH 8.0), and homogenized in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany) six times, each for 20 s ON and 10 s OFF. Then, 1.5 mL of the resulting suspension was centrifuged at 8000× *g* for 10 min at 4 °C, and the fat-containing supernatant was discarded. The obtained pellet was resuspended in 600 µL of sodium citrate, and centrifuged three times at 8000× *g* for 10 min at 4 °C. DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. To extract DNA from milk samples, 10 mL of raw milk from each sample was processed as described above, however, without the need for homogenisation in the stomacher.

2.3. Library Preparation and Sequencing

HTS analysis was performed in the Sequencing and Genotyping Unit of the Genomic Facility/SGIker (supported by UPV/EHU, MICINN, GV/EJ, FSE) of the University of the Basque Country. The 16S rRNA gene library was prepared using Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA), according to the 16S rRNA gene metagenomics workflow of Illumina. The V3–V4 regions of the 16S rRNA gene were amplified by PCR (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC-3′) as described by Klindworth et al. [47]. Then, 16S rRNA gene sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 (2 × 300 bp) (Illumina Inc.).

2.4. Bioinformatic Analysis

Quality filtering and trimming of raw reads were performed using the MiSeq Reporter software (Illumina), and taxonomic classification was performed using the MG-RAST web data analysis tool [48], based on the Silva SSU database [49]. Since the sequencing of most variable regions of the 16S rRNA gene is effective up to the genus level, and seldom discriminates among species adequately [50], the taxonomic classification was performed up to the genus rank. Rarefaction curves were also generated using MG-RAST.

2.5. Statistical Analysis

Relative bacterial abundance (%) was calculated based on the identified sequences, and three significant figures were used to express the results. The IBM SPSS statistical package version 26.0 (IBM SPSS Inc., Chicago, IL, USA, 2019) was used for data preparation and analysis. Mann–Whitney *U* test and Kruskal-Wallis analysis of variance with Bonferroni correction were performed using the SPSS package. The objective was to estimate differences in reads and operational taxonomic units (OTUs) between milk and cheese samples, and to analyse the influence of producer, cheese making and ripening time factors on bacterial phyla and genera abundance. To determine the direction and strength of correlations among the main bacterial genera, Spearman's rank correlation coefficients were calculated using SPSS, and displayed as a heat map in RStudio version 1.3.959 and R version 3.6.3 [51] using the "gplots" package [52]. To analyse the effect of producer and ripening time factors on the abundance of the main bacterial genera, Permutational Multivariate

Analysis of Variance (PERMANOVA) was computed in R using the "vegan" package [53]. Principal Component Analysis (PCA) of the main bacterial genera was performed using their log-transformed, when necessary, and Unit Variance scaled abundance data, and plotted using the SIMCA software (version 15.0.0.4783; Umetrics AB, Umeå, Sweden). The number of principal components (PCs) was determined by eigenvalues (greater than 1.5) and cross validation. The aim was to study microbial dynamics in cheeses according to producer and ripening time factors. An Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed in SIMCA to confirm whether microbial communities of samples differed according to the producer.

Alpha and beta diversity indices were calculated by taking into account the sequence abundance of all bacterial genera present in milk and cheese samples. Alpha diversity was assessed in R using different packages, depending on the objective: "tidyverse" package for data cleaning and preparation for analysis [54]; "BiodiversityR" package for calculating Shannon, Simpson, Inverse Simpson, Berger and Shannon evenness (Jevenness and Eevenness) diversity indices [55]; and "vegan" package for calculating Chao1 and ACE diversity indices. Significant differences among producers for each diversity index were analysed in SPSS using Kruskal-Wallis test. Beta diversity indices (Bray–Curtis and Jaccard dissimilarities) were calculated using the "vegan" package of R, and plotted into a Principal Coordinate Analysis (PCoA) model using the "APE" package of R [56].

3. Results and Discussion

3.1. Characteristics of 16S rRNA Gene Sequencing Data

A total of 10,798,992 16S rRNA gene sequences were obtained from Latxa ewe raw milk and Idiazabal cheese samples (n = 52), with an average sequence length of 348 ± 101 bp, mean GC content of 53 ± 5% and 10,388 OTUs. Altogether, 24 bacterial phyla, 209 families and 645 genera were identified. Further details of the reads, OTUs and number of identified phyla, families and/or genera are summarised in Table 1. The number of sequences obtained from cheese samples was significantly greater than those obtained from milk samples ($p \leq 0.001$), although no significant differences were observed in the number of identified OTUs between the two sample types ($p > 0.05$). Moreover, both milk and cheese samples obtained from different producers showed significant dissimilarities in the number of reads ($p \leq 0.01$) and identified OTUs ($p \leq 0.001$), with producer A being clearly distinct from the other three producers. In general, the rarefaction curves showed a clear and strong stabilizing tendency (Figure S1), indicating sufficient sampling of microbial communities. Overall, this study reports a greater number of sequence reads, OTUs and taxonomic identifications in raw ewe milk and cheese than previous studies [14,38,39,44].

3.2. In-Depth Analysis of Microbial Shifts

3.2.1. Bacterial Composition of the Raw Milk of Ewe

Milk is an important source of microorganisms in cheese [5,57]. A total of 21 bacterial phyla, 165 families and 455 genera were identified in raw milk samples. At the phylum level (Figure 1A, Table S1), Firmicutes (10.5–54.1%) and Proteobacteria (16.9–40.7%) were the most dominant, followed by Bacteroidetes (5.44–19.6%). Other phyla, with abundances higher than 1%, were detected only in milk samples obtained from some producers: Actinobacteria and Verrucomicrobia in samples obtained from producer C (3.75% and 1.33%, respectively) and D (2.97% and 2.75%, respectively), and Planctomycetes in samples from producer D (1.28%). In general, the predominance of Firmicutes and Proteobacteria in raw ewe milk is consistent with previous studies [26,40,42], although differences in the abundance of each phylum have been reported among milk samples collected from different breeds [26,40–42]. However, raw milk samples of Latxa ewe were characterized by the high-level abundance of Bacteroidetes and a notable presence of Verrucomicrobia and Planctomycetes in comparison with milk collected from other breeds [26,40,42]. This indicates a differential characteristic of Latxa ewe raw milk used for Idiazabal cheese production.

Table 1. Metataxonomic data of Latxa ewe raw milk and Idiazabal cheese samples at 6 ripening times (1, 7, 14, 30, 60 and 120 days) from 4 producers (A, B, C and D) (n = 52).

Producer	Milk/Cheese Ripening Time (Day)	Sample ID	Bacterial Diversity					
			Sequences	OTUs	Mbp Count	Phyla	Families	Genera
A	Milk	MA	66,989	8450	2.70	8	103	221
	1	1	321,148	21,102	6.84	10	102	244
		2	331,816	17,948	5.96	10	107	242
	7	9	238,804	12,412	4.21	10	82	182
		10	251,225	13,472	4.55	10	92	203
	14	17	263,231	13,305	4.51	10	81	176
		18	334,305	16,504	5.95	11	90	209
	30	25	269,207	14,266	5.18	8	89	195
		26	280,285	13,701	4.65	8	84	191
	60	33	330,729	15,810	5.50	10	79	179
		34	341,419	16,324	5.92	10	79	183
	120	41	370,181	22,557	7.61	14	83	187
		42	332,471	16,882	6.15	12	78	170
B	Milk	MB	6092	2504	0.769	10	66	136
	1	3	257,407	12,967	4.39	9	90	178
		4	184,670	10,745	3.66	7	80	158
	7	11	160,436	9174	3.15	7	70	124
		12	148,700	8429	3.09	9	67	117
	14	19	193,164	8672	2.94	9	57	107
		20	134,695	7270	2.48	7	55	107
	30	27	140,484	2919	1.31	7	40	60
		28	129,491	6422	2.33	6	43	88
	60	35	295,377	13,046	4.38	10	64	125
		36	157,505	7103	2.46	6	47	91
	120	43	294,909	12,781	4.61	10	54	107
		44	175,689	8629	3.13	10	53	95
C	Milk	MC	10,632	4889	1.41	10	66	135
	1	5	237,563	11,167	3.76	10	83	165
		6	200,211	9565	3.24	10	73	156
	7	13	172,573	9232	3.16	9	72	136
		14	105,377	2947	1.32	5	39	65
	14	21	281,503	11,879	4.03	10	76	152
		22	162,633	7438	2.54	8	57	112
	30	29	374,652	14,534	5.20	8	75	164
		30	178,935	7641	2.65	9	66	125
	60	37	390,610	14,658	5.06	12	83	170
		38	218,602	8265	2.84	8	57	105
	120	45	116,160	5909	2.05	5	47	76
		46	188,779	8633	2.99	7	51	83
D	Milk	MD	52,040	11,547	3.80	21	151	378
	1	7	210,147	11,128	3.71	10	97	217
		8	175,444	8825	3.03	11	91	197
	7	15	136,796	6512	2.38	7	65	120
		16	126,129	6062	2.11	8	56	113
	14	23	194,767	8284	2.99	11	66	131
		24	201,259	9138	3.09	14	73	139
	30	31	161,519	7050	2.39	13	68	119
		32	187,889	8164	2.78	10	66	136
	60	39	86,466	4525	1.68	8	45	84
		40	202,656	8933	3.28	7	65	127
	120	47	205,505	9667	3.36	11	67	119
		48	209,716	10,185	3.51	15	63	123

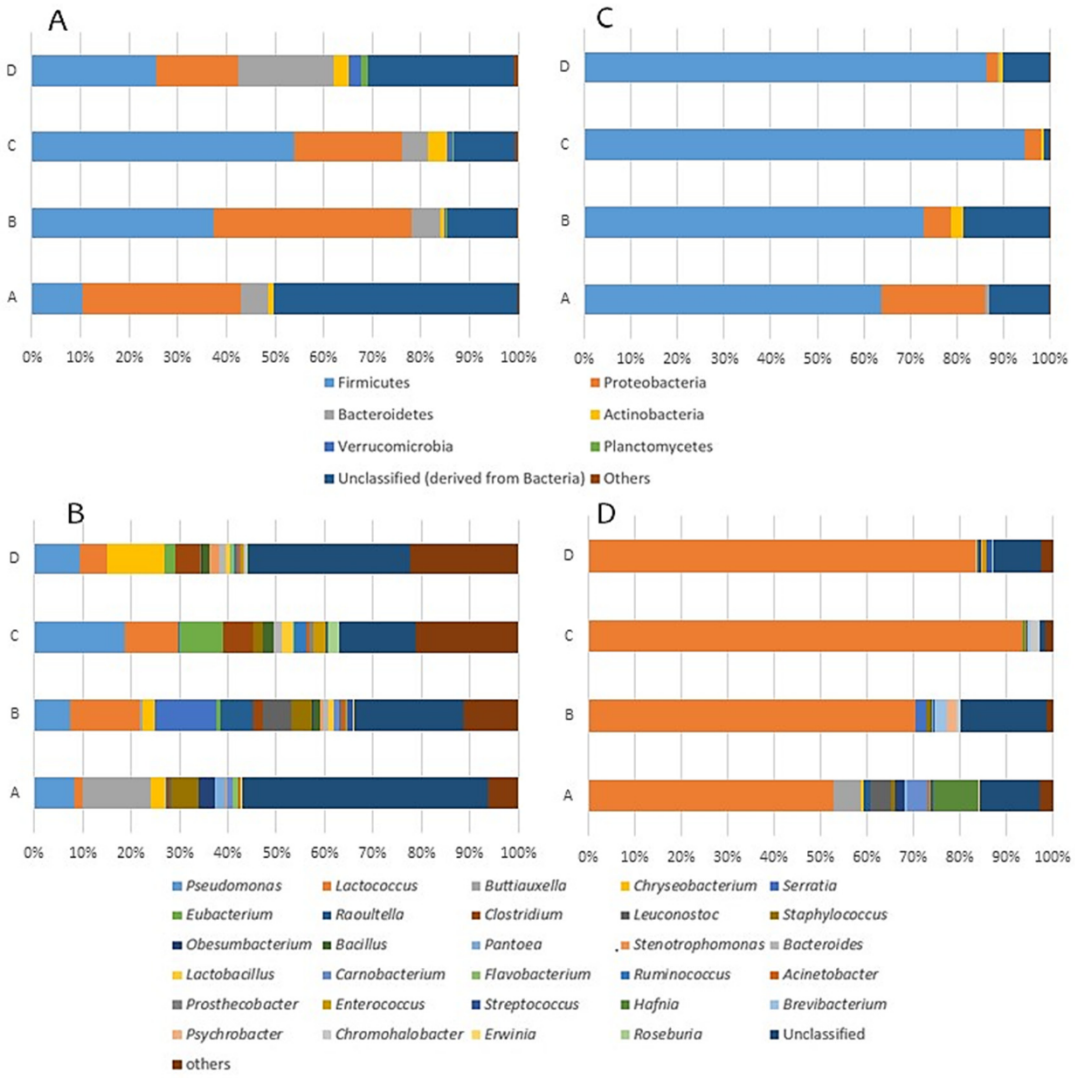


Figure 1. Relative abundance (%) of bacterial phyla and genera of Latxa ewe raw milk ((**A**,**B**), respectively) and 1-day-old ripened Idiazabal cheese samples ((**C**,**D**), respectively) produced by four producers (A, B, C, D).

A total of 24 genera with abundance greater than 1% were identified, and 10 of these genera showed abundance higher than 5% (Figure 1B, Table S2). *Lactococcus* (1.64–14.5%), *Eubacterium* (0.0766–9.18%), *Clostridium* (0.183–6.09%), *Leuconostoc* (0–6.10%) and *Staphylococcus* (0.291–5.48%) were the most abundant genera within Firmicutes. Similarly, *Pseudomonas* (7.36–18.5%), *Buttiauxella* (0–14.1%), *Serratia* (0.0245–12.6%) and *Raoultella* (0–6.86%) showed the highest abundance within Proteobacteria, and *Chryseobacterium* (0–11.7%) within Bacteroidetes. Differences were observed among milk samples obtained from different producers (Table S2). While *Pseudomonas* and *Lactococcus* were identified as the main genera common to all analysed raw milk samples, the remaining genera were characteristic of each producer. The abundance of the rest of genera classified as "others" and unclassified sequences was remarkable (6.25–22.4% and 15.6–50.8%, respectively).

Differences observed in the microbial composition of milk samples at the phylum and genus levels among producers (Tables S1 and S2) could be caused by various factors such as differences in lactation stage, flock management and feeding, or sources of microorganisms, for instance, mammary gland diseases or microorganisms contaminating the teat surface, practices and materials employed during milking or dairy environment [5,10,15,41]. Moreover, these factors could explain the differences observed in bacterial communities between the raw milk of Latxa ewe and that of other ewe breeds [40–42].

The identified bacterial genera were divided into three groups: LAB, comprising genera previously classified as LAB [57]; environmental bacteria, including bacteria derived from the natural environment [58]; and non-desirable bacteria, containing genera exhibiting a pathogenic potential [59] or related to spoilage [60]. The LAB identified in this study included the genera *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Lactobacillus*, *Carnobacterium* and *Streptococcus*. These gram-positive bacteria have frequently been identified in dairy products [57,61], and their presence in the raw milk of ewe breeds, other than Latxa, has been confirmed by HTS, albeit at different abundances [42–44].

The environmental bacterial genera identified in this study included *Obesumbacterium*, *Roseburia* and *Prosthecobacter*. These genera have been isolated from different natural sources, such as soil, fresh and salt water as well as animal and human gut [62–64]; however, to the best of our knowledge, no study has reported their presence in raw ewe milk.

The non-desirable bacterial genera identified in this study included *Pseudomonas*, *Clostridium*, *Staphylococcus* and *Bacillus*, which are widely known pathogens [59,65]. For instance, *Pseudomonas* is the most important psychrotrophic bacteria in raw milk, which may even predominate in refrigerated milk [10]. It comes from natural environment [66] and has been related to hygiene conditions [67,68]. Some species belonging to the genera *Buttiauxella*, *Serratia*, *Chryseobacterium*, *Eubacterium*, *Raoultella*, *Ruminococcus*, *Pantoea*, *Stenotrophomonas*, *Bacteroides*, *Flavobacterium* and *Acinetobacter* have also been described as opportunistic or as emerging pathogens [69–79]. Moreover, some of these genera, such as *Serratia* and *Clostridium*, are also related to milk spoilage, resulting in off-flavours [80] and to the cheese blowing defect because of CO_2 production [81]. The presence of these bacteria in raw ewe milk has been reported only in a few studies [42,59,82,83]. To the best of our knowledge, the genera *Buttiauxella*, *Serratia*, *Eubacterium*, *Raoultella*, *Ruminococcus* and *Bacteroides* have not been identified in raw ewe milk so far.

3.2.2. Bacterial Shifts during the Cheese Making Process

Next, the effect of the cheese making process, which encompasses all the production stages from milk to 1-day-old ripened cheeses, on microbiota was analysed. In this way, the bacterial composition of Latxa ewe raw milk and 1-day-old ripened Idiazabal cheese was compared. In 1-day-old ripened cheese samples, bacteria belonging to 19 phyla, 160 families and 450 genera were detected; thus, the number of identified bacterial families and genera were similar between 1-day-old ripened cheese and raw milk samples, but the number of bacterial phyla identified in cheese was less than that identified in raw milk. However, the cheese making process had a great impact on the abundance of bacterial communities (Figure 1C,D, Tables S1 and S2). At the phylum rank (Figure 1C, Table S1), the relative abundance of Firmicutes increased remarkably in 1-day-old ripened Idiazabal cheese samples (63.7–94.7%), while that of Proteobacteria decreased (2.61–22.4%), although remaining as the second most important phyla. In general, the abundances of the rest of phyla decreased, although the effect of the cheese making process was not statistically significant in all cases. The abundance of sequences classified as "others" was considerably reduced (<0.01%), and unidentified sequences accounted for lower, yet remarkable, abundance (1.21–18.6%). To date, very few HTS studies have analysed the effect of the cheese making process on bacterial communities in raw ewe milk cheeses [43,44], and even fewer at the phylum rank [26]. In comparison to raw ewe milk-derived cheeses, more HTS studies have been conducted on cow milk-derived cheeses [29,84]. De Pasquale et al. [26] have reported an increase in Firmicutes abundance and a decrease in Proteobacteria abundance in Canestrato Pugliese raw ewe milk-derived cheese, but the changes were more drastic than those observed in this study. No information could be found in the literature concerning the effect of the manufacturing process on the remaining phyla.

The effect of the cheese making process on the main bacterial genera is shown in Figure 1D and Table S2. Within LAB, *Lactococcus* was the most abundant genus in 1-day-old ripened cheese samples collected from all producers (52.5–93.2%), although a notably lower abundance was observed for producer A. The effect of the cheese making process

on the remaining genera in the LAB group varied with the producer. The abundance of *Lactobacillus* decreased in cheese samples collected from all producers, except producer A (<0.200% in all producers); abundances of *Leuconostoc* and *Carnobacterium* were higher in producer A samples (4.48% and 4.40%, respectively); abundances of *Streptococcus* and *Enterococcus* were slightly higher in the cheese samples of producer A (0.507% and 0.458%, respectively) and producer D (0.993% and 0.892%, respectively). The genus *Lactococcus* was predominant during the cheese making process because of its presence in the starter culture, confirming that bacteria comprising the starter culture grow and predominate, as has been previously observed for Pecorino Siciliano cheese [39]. The proliferation of non-starter LAB (NSLAB) has been reported previously, although there are clear differences according to the type of cheese. For instance, *Lactococcus* and *Lactobacillus* have been reported as predominant in Caciofiore della Sibilla cheese [43], whereas *Lactococcus*, *Carnobacterium* and *Enterococcus* predominate in Canestrato Pugliese cheese [26].

In general, the abundance of non-desirable bacteria was less than 1% after cheese making, although the abundance of some bacterial genera, namely *Buttiauxella* (0–5.79%), *Serratia* (0.00179–2.16%) and *Raoultella* (0.0151–1.43%), was maintained at a remarkable level or even increased in cheese obtained from some producers (Figure 1D, Table S2). The opportunistic bacteria *Hafnia*, *Brevibacterium* and *Psychrobacter* [85–87], which had low abundance in milk (<1%), also increased their abundances in the cheese of some producers (0.00282–9.62%, 0.0210–2.43% and 0.00168–2.28%, respectively). Notably, these bacterial genera exhibit lipase and/or protease activities [88–90], and produce interesting volatile compounds (such as 1-hexanol, 1-propanol, propyl butanoate or butyl butanoate), affecting cheese quality [91–93]. Overall, the abundance of environmental bacteria decreased during cheese making, although *Obesumbacterium* maintained a remarkable abundance in samples from producer A (1.89%). Moreover, the environmental genus *Chromohalobacter* [94], which showed low abundance in milk, exhibited higher abundance in cheese, especially that obtained from producer C (1.78%). The abundance of bacteria classified as "others" and of unidentified bacteria in cheese (1.31–2.74% and 1.26–18.6%, respectively) was lower than that in milk (Figure 1D, Table S2). Suppression of the growth of environmental and non-desirable bacteria, such as *Pseudomonas* or *Staphylococcus*, during cheese making has been reported previously [26,43]. Nonetheless, little has been reported about the prevalence of opportunistic or emerging pathogens and environmental bacteria after the cheese making process using raw ewe milk. De Pasquale et al. [26] have detected *Raoultella* in Canestrato Pugliese cheese but not in raw milk and Alegría et al. [32] have reported a prevalence of *Chromohalobacter* in fresh Oscypek cheese. *Hafnia* and *Psychrobacter* have been identified in other cheeses prepared from raw ewe milk [38,95], although the effect of the cheese making process on the abundance of these bacteria is unknown.

The cheese making process adds other factors that can influence the bacterial communities [9], in addition to factors that determine the milk microbiota (Section 3.2.1). Briefly, the conversion of milk to cheese decreases the pH to 4.5–5.3, which interferes with the growth of most bacteria, except LAB [9,57]. The NaCl concentration of the brine and low salt tolerance of most bacteria only facilitate the growth of LAB [57] and halophiles, such as *Psychrobacter* [96] and *Chromohalobacter* [94]. The decrease in moisture content and water activity (a_w) also suppresses the proliferation of most bacteria, except LAB, because of their resistance to reduced a_w values [9,57]. Moreover, variation that occurs in the redox potential during the conversion of milk to cheese only allows the growth of facultative or obligate anaerobic bacteria [57]. It is worth mentioning that artisanal rennet employed for the production of some raw ewe milk cheeses could be an important source of microorganisms, for example LAB [8]. The use of lamb rennet paste containing pregastric lipase results in higher lipolysis and the development of the characteristic flavour of Idiazabal cheese [97]. Although artisanal rennet contains high levels of a wide range of microorganisms, including aerobic mesophilic bacteria [98], no significant differences have been detected in microbial counts in Idiazabal cheeses prepared using artisanal or commercial rennet [99]; however, it would be interesting to elucidate this aspect using

culture-independent methods, such as HTS. Finally, it has been observed that small differences in the environment of dairy facilities producing artisanal cheeses can lead to the development of site-specific "household" microbiota [100]. Therefore, these factors could explain the differences in bacterial composition observed among different raw ewe milk cheeses and among producers producing the same type of cheese.

3.2.3. Bacterial Shifts during the Cheese Ripening Process

Finally, the effect of the ripening process on the bacterial composition of cheese was studied. A total of 23 phyla, 197 families and 583 genera were identified throughout the cheese ripening process; thus, the number of bacterial families and genera was higher during cheese ripening than in raw milk and in cheese after the cheese making process. At the phylum level (Table S1), the abundance of Firmicutes increased (from a mean of 79.4% at 1 day of ripening to 97.7% at 120 days of ripening), while that of Proteobacteria decreased sharply (from 8.56% to 0.116%). In general, abundance of the remaining phyla not predominant during the cheese making process and that of "others" and unidentified bacteria were reduced, except Actinobacteria in the cheese of some producers; nonetheless, the change in abundance levels was not significant for all phyla. Overall, the predominance of Firmicutes and reduction in the abundance of Proteobacteria and remaining phyla have previously been reported in other raw ewe milk cheeses such as Liqvan cheese [38,44].

The cheese ripening time had a considerable impact on bacterial abundance at the genus level, resulting in large differences among producers (Figure 2, Table S2). Within LAB, *Lactococcus* remained the most dominant genus in Idiazabal cheese during ripening at all times and for all producers (mean abundance: 74.9% at 1 day of ripening, and 74.5% at 120 days of ripening), except producer A, which showed notably lower proportions of *Lactococcus*. The effect of ripening time on bacterial abundance was significant only for *Lactobacillus*, with an increase in its abundance for all producers (from 0.0949% to 8.96%), while the evolution of the abundance of the remaining genera varied with the producer. In cheeses from producer A, the abundance of *Leuconostoc*, which increased after cheese making, was unquestionably promoted by ripening time (from 4.48% to 31.0%), whereas that of *Carnobacterium* decreased (from 4.40% to 0.330%). In cheeses obtained from producers A and D, the abundances of *Streptococcus* and *Enterococcus*, which increased during the cheese making process, also increased during ripening (from 0.750% to 4.52% and from 0.675% to 2.12%, respectively). Overall, taking into account LAB dynamics (Table S2) and their correlations during ripening time (Figure 3), a clear pattern was observed. The abundance of *Lactococcus* decreased over 30 or 60 days of ripening, depending on the producer, when NSLAB (*Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Enterococcus*) began to proliferate. In other words, from the first ripening month on these NSLAB begin to proliferate and become an important part of the final microbiota of the cheese.

The predominance of bacteria added as part of the starter culture has also been previously reported during the ripening of other raw ewe milk cheeses, such as Pecorino Siciliano cheese [38,39]. However, lactose depletion, salt concentration, and low pH and temperature decrease the viability of starter LAB, and depending on lysis rates, the NSLAB gain importance [101]. The proliferation of *Lactobacillus*, *Leuconostoc*, *Streptococcus* or *Enterococcus* has also been observed in other raw ewe milk cheeses [26,38,39,95]; however, the NSLAB composition of other types of raw ewe milk cheeses is different from that of Idiazabal cheese [38,39,43,44]. These differences are important, since NSLAB affect, among others, the proteolysis and lipolysis of cheese, and consequently, its final properties, including flavour and texture [102–104].

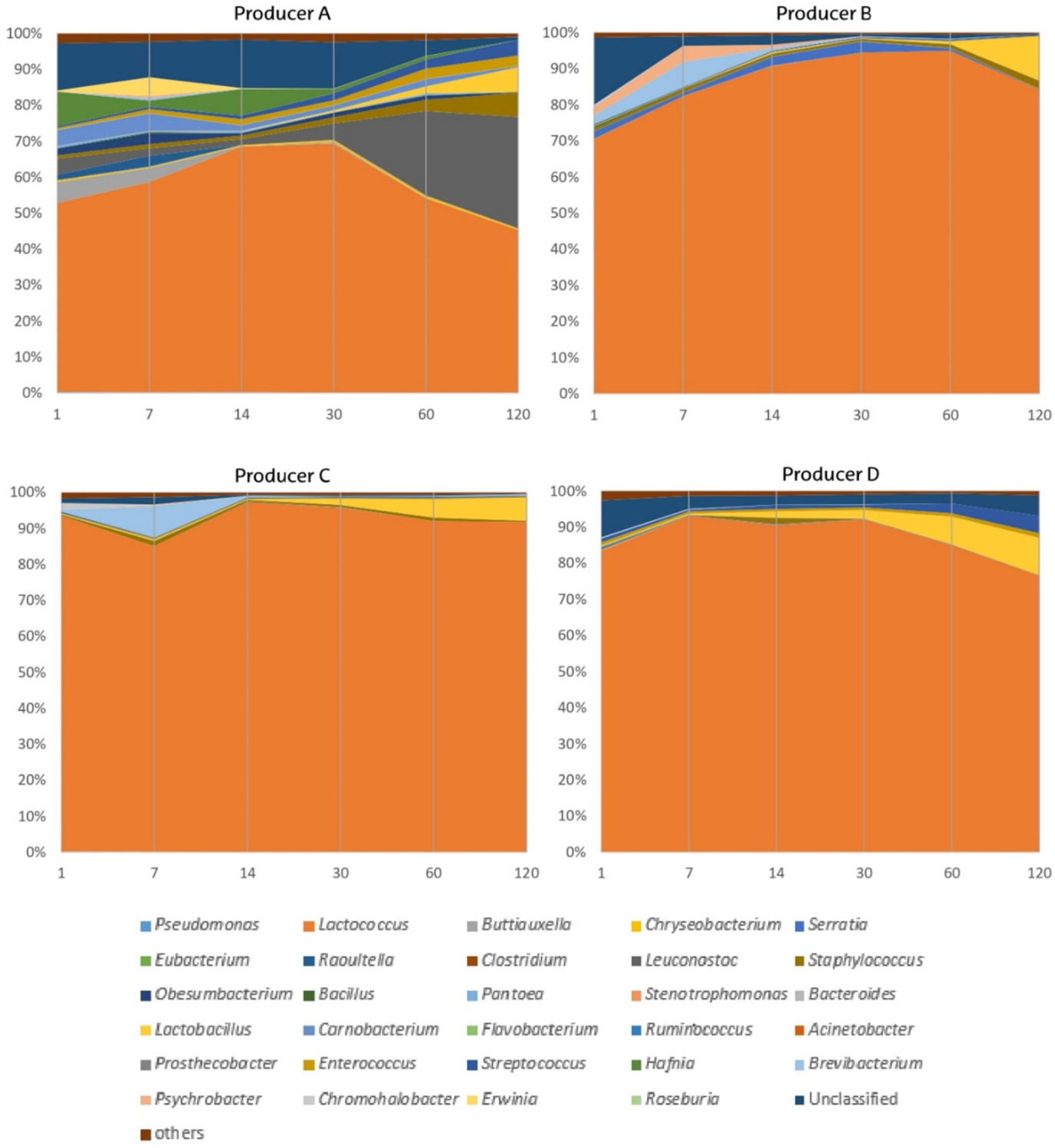


Figure 2. Microbial shifts at genus rank during ripening time (from 1 to 120 days) of Idiazabal cheese samples from different producers (**A**–**D**).

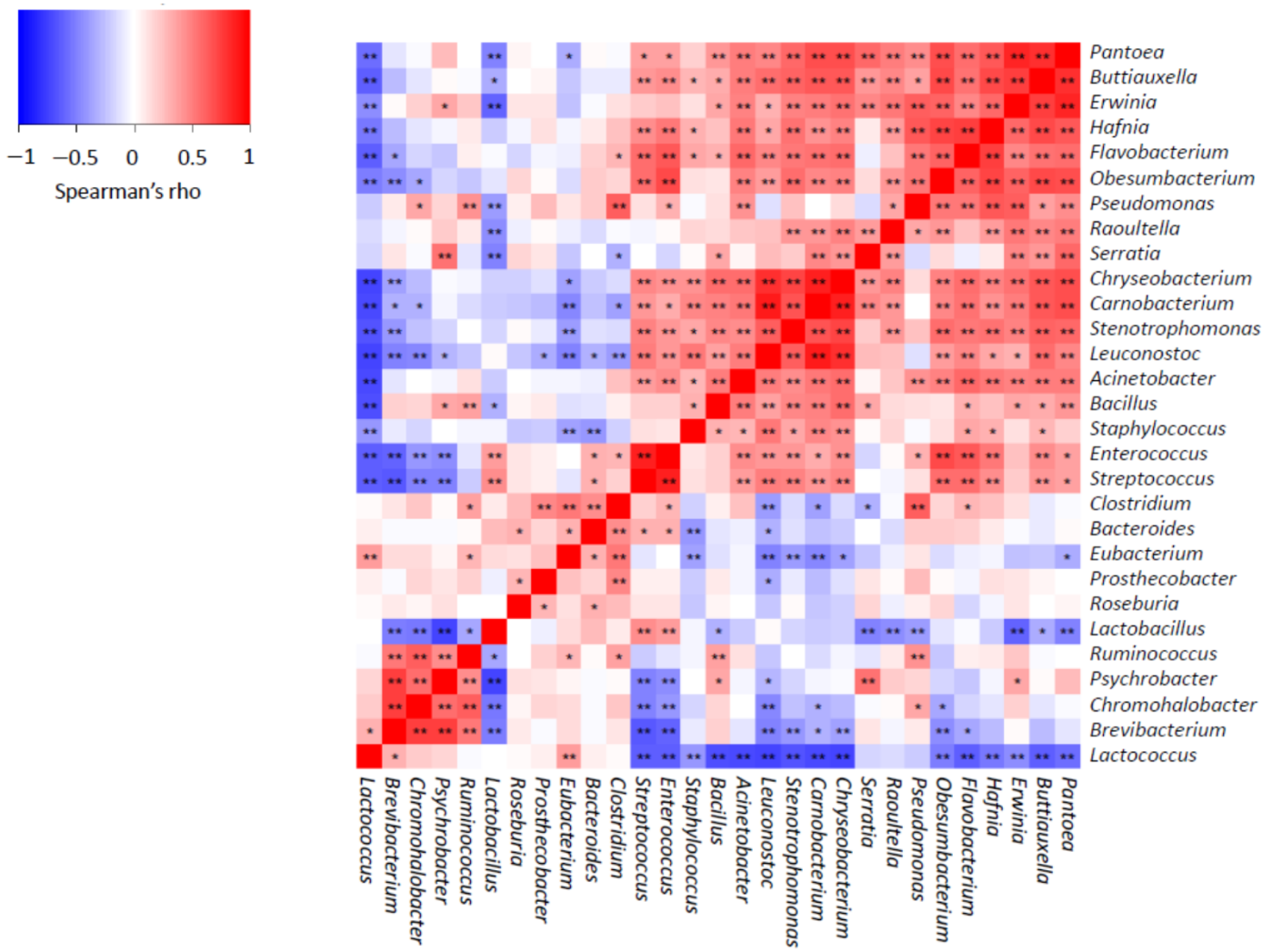


Figure 3. Spearman's rank correlations between main bacterial genera found in Idiazabal cheese samples. Significant correlations are represented by ** $p \leq 0.01$ and * $p \leq 0.05$.

Overall, the abundance of environmental bacteria, except *Obesumbacterium*, decreased throughout the ripening period; *Obesumbacterium* showed an increase in abundance at 7 days of ripening (3.03%) in samples from producer A (Figure 2, Table S2). Among the non-desirable bacteria, *Hafnia*, *Staphylococcus*, *Buttiauxella*, *Psychrobacter*, *Raoultella*, *Serratia* and *Brevibacterium* remained abundant during cheese ripening (>1%), depending on the producer. Nonetheless, their dynamics differed during ripening. The abundance of *Buttiauxella* decreased throughout the ripening phase, while that of *Staphylococcus* increased until 120 days of ripening. The remaining genera showed an increase in abundance at intermediate time points (at 7, 14 or 30 days of ripening). Moreover, the emerging pathogen *Erwinia* [105], whose abundance was minor in milk (<1%), also showed an increase in abundance in samples from producer A (5.15% at 7 days) (Table S2). Considering that some of these genera, including *Hafnia*, *Brevibacterium* and *Psychrobacter*, are related to the production of volatile compounds [91–93], the results of this study suggest that their contribution to the sensory properties of cheese would occur at beginning of the ripening process. The abundance of bacteria classified as "others" and that of unidentified bacteria decreased (from 2.08% to 0.665% and from 10.8% to 1.69%, respectively). Most of these environmental and non-desirable genera have previously been reported in raw ewe milk cheeses [26,37–39,43,106,107], including the notable presence of *Staphylococcus* and the increase in its abundances during ripening [26,38,39]. Nonetheless, *Obesumbacterium* and *Hafnia* have only been found in Alberquilla cheese prepared from a mixture of ewe and

goat milk [31]. To the best of our knowledge, information on the evolution of most of these bacteria during the ripening period is scarce.

Furthermore, we examined the correlation among the main bacterial genera during ripening (Figure 3). Spearman's rank correlations showed some positive relationships between LAB and non-desirable or environmental genera, for example, *Streptococcus–Stenotrophomonas*, *Enterococcus–Pseudomonas* and *Leuconostoc–Bacillus*. However, a remarkable number of negative correlations were detected, confirming that LAB tend to predominate and limit the proliferation of non-desirable or environmental bacteria, as observed previously [44]. Moreover, this supports the idea that the growth of aroma-related bacteria (such as *Hafnia*, *Brevibacterium* and *Psychrobacter*) is inhibited during the first few weeks of ripening [57]. Changes in the physicochemical properties of cheese throughout the ripening process could explain LAB predominance, similar to the cheese making process. Overall, reduced a_w, high NaCl concentration, refrigeration temperatures during ripening, evolution of oxidation-reduction potential to a more reduced state and the decline in pH may affect the proliferation of most bacteria, and LAB are almost the unique that could proliferate [57]. Moreover, it is well known that competitive interaction mechanisms exist between bacteria [44,108]; for example, LAB produce organic acids or bacteriocins [57,109,110]. However, different parameters, such as temperature and relative humidity, could also affect bacterial proliferation during cheese ripening [57,111] and explain the differentiation observed among the cheeses from different producers.

3.3. Overall Effect of Producer and Ripening Time Factors

To examine the effect of producer and ripening time on the main bacterial genera of Idiazabal cheese, a multivariate analysis was performed. PERMANOVA showed that producer and ripening time factors had a statistically significant effect on modulating the microbial composition of Idiazabal cheeses ($p \leq 0.001$ and $p \leq 0.01$, respectively) (data not shown), thus confirming the results of univariate analysis (Kruskal-Wallis test). Moreover, the F statistic indicated a higher influence of the producer than that of the ripening time (17.3 and 7.17, respectively) on cheese microbiota.

PCA of the main bacterial genera identified in cheese samples revealed five PCs (PC1–5), which accounted for 77.0% of the total variance in cheese microbiota due to the producer and ripening time. According to the scores plot (Figure 4A), PC1 (accounting for 35.5% of the explained variance) was related to the producer factor, thus leading to a clear differentiation between the samples of producer A and those of the other producers. According to the loadings plot (Figure 4B), *Lactococcus*, *Carnobacterium*, *Leuconostoc*, *Chryseobacterium*, *Hafnia*, *Buttiauxella*, *Obesumbacterium*, *Pantoea*, *Erwinia*, *Enterococcus*, *Raoultella*, *Streptococcus*, *Pseudomonas* and *Staphylococcus* were highly correlated to PC1 (Figure 4B), indicating that these genera were the most responsible for the differentiation of cheese microbiota among producers. On the other hand, PC2 (accounting for 16.9% of the explained variance) was correlated with the ripening time factor. Therefore, samples were distributed from positive (for less ripened cheeses) to negative (for more ripened cheeses) values (Figure 4A). *Psychrobacter*, *Brevibacterium*, *Chromohalobacter*, *Bacillus* and *Serratia* showed positive loadings in PC2, indicating their disappearance along ripening. Instead, *Lactobacillus* showed negative loadings, indicating that its abundance increased during the ripening phase (Figure 4B). This would confirm the results of the PERMANOVA and indicate that producer factor has a greater impact on cheese microbiota than ripening time.

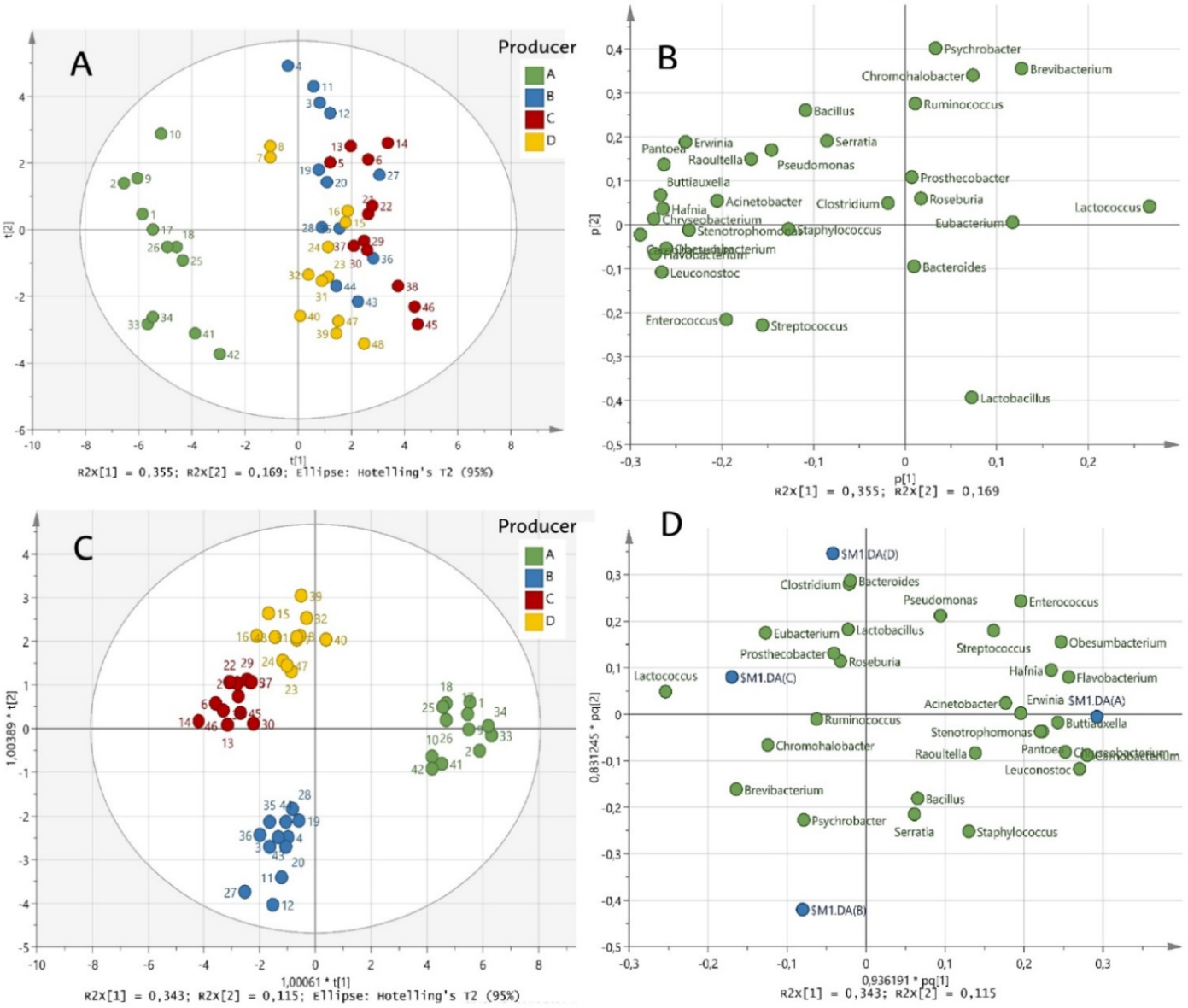


Figure 4. Scores and loadings plots of PCA ((**A**,**B**), respectively) and OPLS-DA ((**C**,**D**), respectively) based on main bacterial genera of Idiazabal cheese samples from four producers (A, B, C and D). Samples are coloured according to the producer and labels indicate samples ID.

Compared with PC1 and PC2, the other three PCs, PC3, PC4 and PC5, explained lesser variance in cheese microbiota (11.9%, 6.84% and 5.89% respectively) (Figure S2). Nonetheless, taking together the five PCs provided by the PCA, an idea of the cheeses' microbiota evolution during the ripening time was obtained for each producer. For producer A, the microbial composition of less ripened cheeses was characterized by *Hafnia*, *Buttiauxella*, *Carnobacterium*, *Obesumbacterium*, *Raoultella*, *Pantoea*, *Chryseobacterium* and *Erwinia* genera. As the ripening progressed, *Lactococcus* proliferated, and the cheese microbiota was finally characterized by high abundance of *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Enterococcus*. For producer B, less ripened cheeses were characterized by *Serratia*, *Psychrobacter*, *Brevibacterium*, *Chromohalobacter* and *Bacillus*, but as ripening progressed, the microbiota was simplified, with *Lactococcus*, *Lactobacillus* and *Staphylococcus* as the predominating genera. For producer C, less ripened cheeses were characterized by *Chromohalobacter*, *Brevibacterium* and *Pseudomonas*, and throughout ripening, the microbiota was simplified by the predominating genera *Lactococcus* and *Lactobacillus*. For producer D, *Pseudomonas*, *Serratia*, *Bacillus* and *Raoultella* characterized the less ripened cheeses, but the microbiota

was predominated by *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus* as the ripening progressed. In general, the microbial dynamics described in Section 3.2 were confirmed by multivariate analysis.

Finally, OPLS-DA, which yielded 3 + 2 + 0 components and with the parameters $R2X = 0.740$ and $R2Y = 0.898$, confirmed the differentiation among producers (Figure 4C). Producer A was clearly distinguished from the other producers, as observed before. However, producer B also showed a clear differentiation from producers C and D. The loadings plot (Figure 4D) revealed the characteristic genera in the cheeses of each producer, corroborating the results of the in-depth analysis of microbial shifts and the PCA.

3.4. Alpha and Beta Diversity Analyses

To analyse alpha diversity, different indices were employed, and the evolution of bacterial richness, evenness and biodiversity was examined (Table S3). Chao1 and ACE richness estimators showed a negative trend during the transition from milk to 120-day-old ripened cheese, which was either more pronounced or less pronounced depending on the producer. This implies that a non-negligible number of bacterial genera originally present in raw ewe milk disappeared during cheese making and ripening. Overall, these results are consistent with what has been previously observed for other raw ewe milk cheeses, such as Liqvan cheese [43,44]. According to the uniformity, Shannon evenness and Berger indices showed a decreasing trend throughout the cheese making and ripening processes, indicating that the microbial population of cheese was dominated by a few genera. Nevertheless, uniformity increased after 30 or 60 days, depending on the producer, since other genera gained importance. To the best of our knowledge, there has been no report to date on the shifts in bacterial uniformity during production and ripening of raw ewe milk-derived cheeses. Finally, combining the measurement of the number of genera and their abundance, the Shannon, Simpson and Inverse Simpson biodiversity indices confirmed a downward trend from milk to 30- or 60-day-old ripened cheeses, depending on the producer. However, subsequently, biodiversity increased until 120 days of ripening. In other words, it was confirmed that up to the first or second month of ripening, a few bacterial genera predominated; however, subsequently, other bacteria proliferated and acquired importance. Ramezani et al. [44] have also reported a greater complexity of biodiversity in raw milk than in curd or cheese, and an increase in biodiversity during the conversion of curd into Liqvan cheese. However, De Pasquale et al. [26] have reported a higher biodiversity in curd after moulding than in milk or final cheese. In general, statistically significant differences were observed in alpha diversity between producer A and the others (Figure 5). Differences in alpha diversity among producers of other raw ewe milk and cheeses have rarely been studied [37].

Subsequently, to clarify differences in the microbial composition of cheeses among producers, beta diversity was calculated. At genus level, cheese samples were distributed into three clusters corresponding to cheeses from producer A, producer B and producers C and D, which were very similar (Figure 6). Samples from producer A were tightly clustered, indicating less microbial changes during the ripening time compared with cheeses from other producers. Samples collected from producers A and B at 120 days of ripening grouped close to those collected from producers C and D at the same time point, indicating similar bacterial composition among cheese samples of different producers at the end of ripening. In addition, milk samples were far from the general dispersion of cheese samples, indicating clear differences in bacterial composition between the two sample types. To the best of our knowledge, very few studies have been published comparing beta diversity of the same raw ewe milk and cheese among different producers [37,39,40,44]. Endres et al. [40] have reported differences in raw ewe milk samples among different dairies, and Cardinali et al. [37] have reported differences among the producers of Queijo de Azeitão cheese. Beta diversity has also been used to differentiate among the different types of ewe cheeses [95] and to analyse the effect of specific starters on cheese microbiota [39].

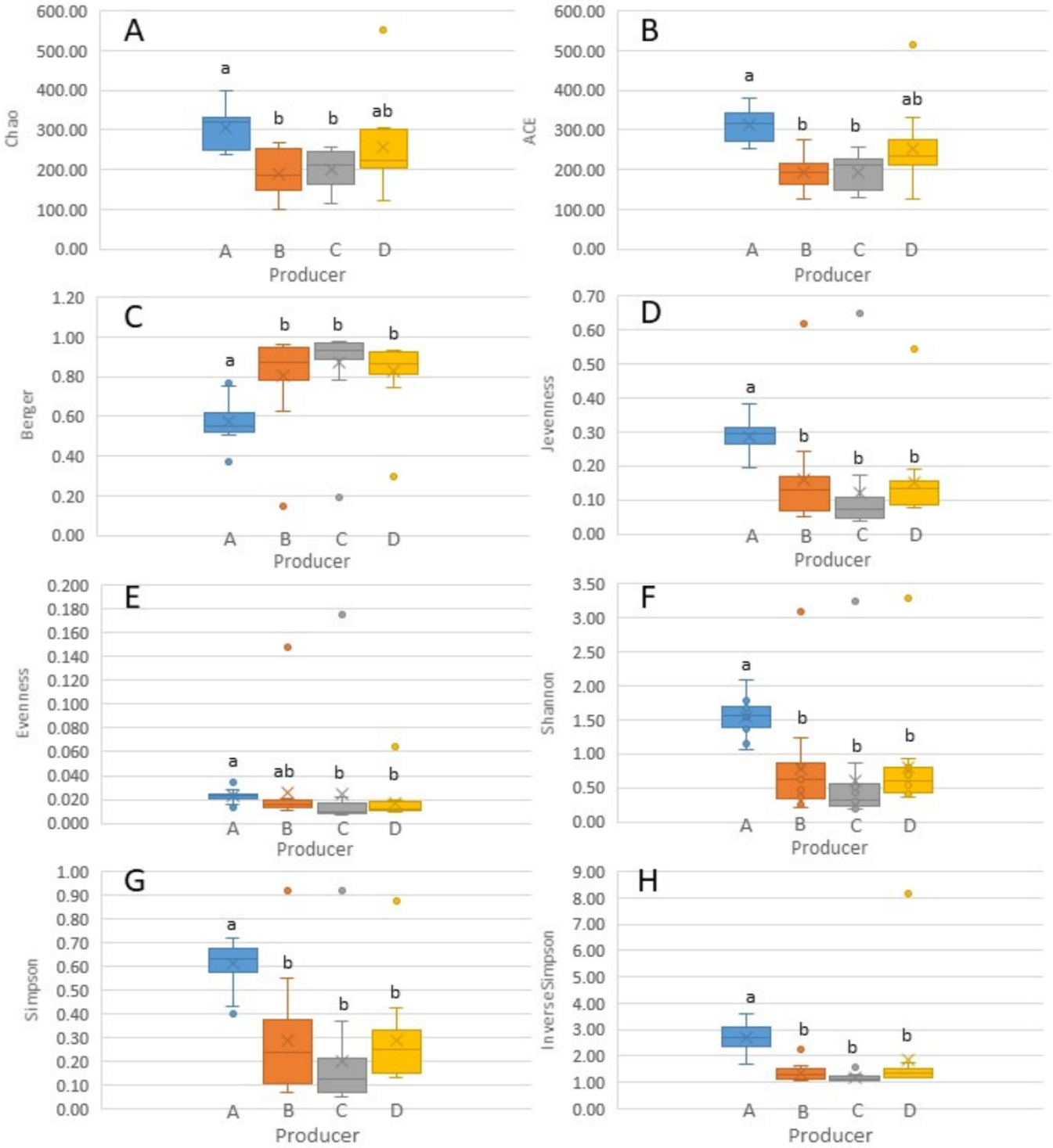


Figure 5. Box plot representation of bacterial alpha diversity indices ((**A**) Chao1; (**B**) ACE; (**C**) Berger; (**D**) Jevenness; (**E**) Eevenness; (**F**) Shannon; (**G**) Simpson; (**H**) Inverse Simpson) of Latxa ewe raw milk and Idiazabal cheese samples obtained from four producers (A, B, C and D). For each diversity index, different letters indicate significant differences between producers at $p \leq 0.05$.

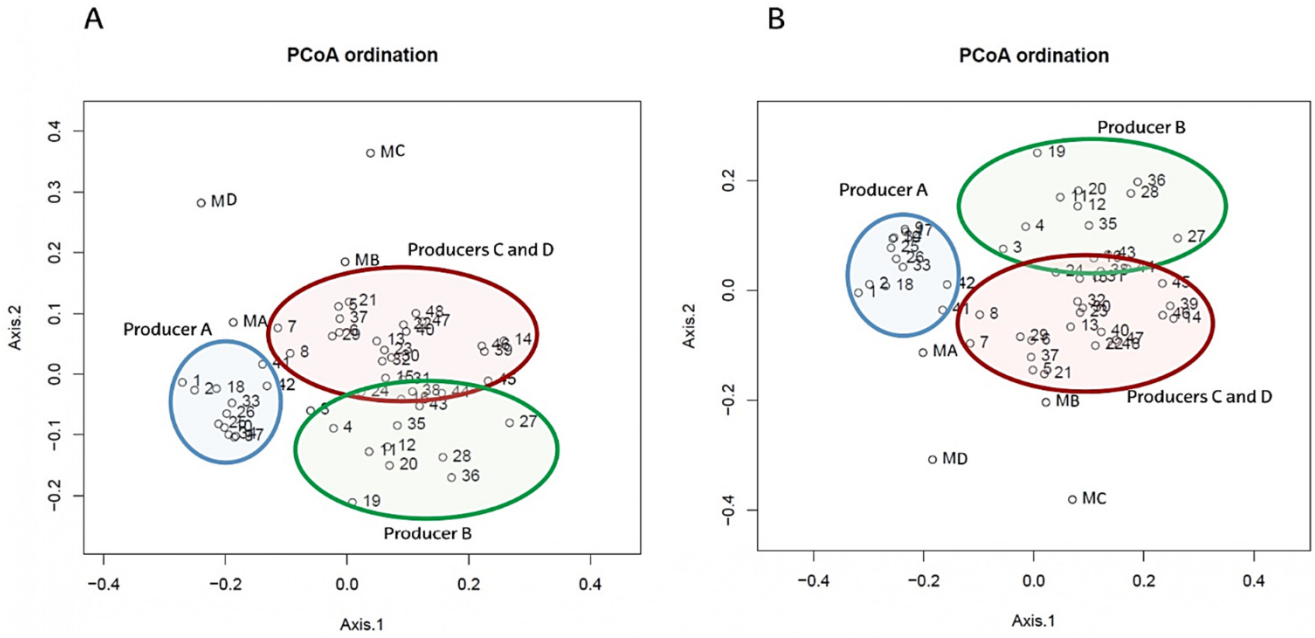


Figure 6. PCoA of bacterial beta diversity at genus rank based on Bray-Curtis (**A**) and Jaccard (**B**) dissimilarities.

Taking together, alpha and beta diversity indices confirmed the results of the in-depth analysis of microbial shifts, univariate analysis (Kruskal-Wallis test) and multivariate analyses (PERMANOVA, PCA and OPLS-DA). The cheese making and ripening processes had an undoubted impact on the bacterial communities. Overall, bacteria from the starter culture predominated at the beginning of ripening, but after 30 or 60 days of ripening, the bacteria from raw milk, especially NSLAB, began to proliferate and become noticeable. Nonetheless, clear differences in the microbial composition of raw ewe milk and cheese samples were observed among producers, which could indicate that differences in practices, such as flock management and milking, as well as parameters selected during cheese making and ripening processes would determine the final microbiota.

4. Conclusions

This is the first HTS study carried out with the objective of characterizing the microbiota of Latxa ewe raw milk and examining the bacterial shifts that occur during the production and ripening of Idiazabal cheese. This research confirms that HTS techniques allow a better understanding of the microbial communities, which could not be achieved previously using culture-dependent techniques. Several bacterial genera were detected for the first time in raw ewe milk and cheese. Both the cheese making process and ripening time had a remarkable impact on bacterial communities, although considerable differences were observed among producers. Thus, the use of raw milk and the practices and conditions employed by each producer for flock management, milking and cheese making and ripening could determine the microbiota. The growth of LAB was promoted throughout the cheese making and ripening processes, whereas that of non-desirable and environmental bacteria was inhibited. However, LAB composition differed among producers, and the growth of NSLAB was promoted after 30 or 60 days of ripening. In addition, in some cases, bacteria related to the production of volatile compounds (such as *Hafnia*, *Brevibacterium* and *Psychrobacter*) showed notable abundance during the first few weeks of ripening.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology11050769/s1, Figure S1: Rarefaction curves of microbial populations of the studied samples from each producer. Each graph represents a producer (A, B, C and D) and each line is coloured according to the Sample ID; Figure S2: Scores and loadings plots of

PCA based on main bacterial genera of Idiazabal cheeses from 4 producers (A, B, C and D). Samples are coloured according to the producer and labels indicate samples identification; Table S1: Mean and standard deviation of bacterial phyla of Latxa ewe raw milk and Idiazabal cheese samples at 6 ripening times (1, 7, 14, 30, 60 and 120 days) from 4 producers (A, B, C and D) (n = 52).; Table S2: Mean and standard deviation of bacterial genera of Latxa ewe raw milk and Idiazabal cheese samples at 6 ripening times (1, 7, 14, 30, 60 and 120 days) from 4 producers (A, B, C and D) (n = 52): Table S3: α-diversity indices of Latxa ewe raw milk and Idiazabal cheese samples at 6 ripening times (1, 7, 14, 30, 60 and 120 days) from 4 producers (A, B, C and D) (n = 52).

Author Contributions: Conceptualization, methodology, validation, investigation and resources, G.S.-G., I.H., G.A. and M.V; formal analysis and data curation, G.S.-G., I.H. and G.A. writing—original draft preparation, G.S.-G.; writing—review and editing, G.S.-G., I.H., G.A. and M.V.; visualization, G.S.-G.; supervision, project administration and funding acquisition, M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Basque Government, grant to Research Groups number IT944-16. G. Santamarina-García received a predoctoral grant from the University of the Basque Country (UPV/EHU).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are not publicly available yet as some data sets are being used for additional publications.

Acknowledgments: The authors thank the Idiazabal PDO cheesemakers for collaborating with this study and the technical support provided by SGIker (UPV/EHU, ERDF, EU).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Diario Oficial de las Comunidades Europeas. Reglamento (CE) N° 1107/96 de La Comisión de 12 de Junio de 1996 Relativo Al Registro de Las Indicaciones Geográficas y de Las Denominaciones de Origen Con Arreglo Al Procedimiento Establecido En El Artículo 17 Del Reglamento (CEE) No 2081/92 Del Consejo. *DOCE* **1996**, *148*, 1–10.
2. Valdivielso, I.; Bustamante, M.A.; Aldezabal, A.; Amores, G.; Virto, M.; Ruiz De Gordoa, J.C.; De Renobales, M.; Barron, L.J.R. Case Study of a Commercial Sheep Flock under Extensive Mountain Grazing: Pasture Derived Lipid Compounds in Milk and Cheese. *Food Chem.* **2016**, *197*, 622–633. [CrossRef] [PubMed]
3. Boletín Oficial del Estado. Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen «Idiazabal» y su Consejo Regulador. *BOE* **1993**, *289*, 34591–34596.
4. Barron, L.J.R.; Redondo, Y.; Aramburu, M.; Gil, P.; Pérez-Elortondo, F.J.; Albisu, M.; Nájera, A.I.; de Renobales, M.; Fernández-García, E. Volatile Composition and Sensory Properties of Industrially Produced Idiazabal Cheese. *Int. Dairy J.* **2007**, *17*, 1401–1414. [CrossRef]
5. O'Sullivan, O.; Cotter, P.D. Microbiota of Raw Milk and Raw Milk Cheeses. In *Cheese: Chemistry, Physics and Microbiology*, 4th ed.; McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds.; Academic Press: San Diego, CA, USA, 2017; Volume 1, pp. 301–316. [CrossRef]
6. Peláez, C.; Requena, T. Exploiting the Potential of Bacteria in the Cheese Ecosystem. *Int. Dairy J.* **2005**, *15*, 831–844. [CrossRef]
7. Cosentino, S.; Palmas, F. Hygienic Conditions and Microbial Contamination in Six Ewe's-Milk-Processing Plants in Sardinia, Italy. *J. Food Prot.* **1997**, *60*, 283–287. [CrossRef]
8. Cruciata, M.; Sannino, C.; Ercolini, D.; Scatassa, M.L.; De Filippis, F.; Mancuso, I.; La Storia, A.; Moschetti, G.; Settanni, L. Animal Rennets as Sources of Dairy Lactic Acid Bacteria. *Appl. Environ. Microbiol.* **2014**, *80*, 2050–2061. [CrossRef]
9. Possas, A.; Bonilla-Luque, O.M.; Valero, A. From Cheese-Making to Consumption: Exploring the Microbial Safety of Cheeses through Predictive Microbiology Models. *Foods* **2021**, *10*, 355. [CrossRef]
10. Quigley, L.; O'Sullivan, O.; Stanton, C.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. The Complex Microbiota of Raw Milk. *FEMS Microbiol. Rev.* **2013**, *37*, 664–698. [CrossRef]
11. Pérez-Elortondo, F.J.; Albisu, M.; Barcina, Y. Changes in the Microflora of Idiazabal Cheese with the Addition of Commercial Lactic Starters. *Aust. J. Dairy Technol.* **1993**, *48*, 10–14.
12. Pérez-Elortondo, F.J.; Aldámiz Echobarria, P.; Albisu, M.; Barcina, Y. Indigenous Lactic Acid Bacteria in Idiazabal Ewes' Milk Cheese. *Int. Dairy J.* **1998**, *8*, 725–732. [CrossRef]
13. Thierry, A.; Pogacic, T.; Weber, M.; Lortal, S. Production of Flavor Compounds by Lactic Acid Bacteria in Fermented Foods. In *Biotechnology of Lactic Acid Bacteria: Novel Applications*, 2nd ed.; Mozzi, F., Raya, R.R., Vignolo, G.M., Eds.; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2015; pp. 314–340. ISBN 9781118868386.

14. Kamilari, E.; Anagnostopoulos, D.A.; Papademas, P.; Kamilaris, A.; Tsaltas, D. Characterizing Halloumi Cheese's Bacterial Communities through Metagenomic Analysis. *LWT* **2020**, *126*, 109298. [CrossRef]
15. Zhao, Z.; Ning, C.; Chen, L.; Zhao, Y.; Yang, G.; Wang, C.; Chen, N.; Zhang, Z.; Li, S. Impacts of Manufacture Processes and Geographical Regions on the Microbial Profile of Traditional Chinese Cheeses. *Food Res. Int.* **2021**, *148*, 110600. [CrossRef] [PubMed]
16. Niro, S.; Fratianni, A.; Tremonte, P.; Sorrentino, E.; Tipaldi, L.; Panfili, G.; Coppola, R. Innovative Caciocavallo Cheeses Made from a Mixture of Cow Milk with Ewe or Goat Milk. *J. Dairy Sci.* **2014**, *97*, 1296–1304. [CrossRef] [PubMed]
17. Zheng, X.; Liu, F.; Shi, X.; Wang, B.; Li, K.; Li, B.; Zhuge, B. Dynamic Correlations between Microbiota Succession and Flavor Development Involved in the Ripening of Kazak Artisanal Cheese. *Food Res. Int.* **2018**, *105*, 733–742. [CrossRef]
18. Mendia, C.; Ibañez, F.C.; Torre, P.; Barcina, Y. Influence of the Season on Proteolysis and Sensory Characteristics of Idiazabal Cheese. *J. Dairy Sci.* **2000**, *83*, 1899–1904. [CrossRef]
19. Pérez-Elortondo, F.J.; Albisu, M.; Barcina, Y. Physicochemical Properties and Secondary Microflora Variability in the Manufacture and Ripening of Idiazabal Cheese. *Lait* **1999**, *79*, 281–290. [CrossRef]
20. Hu, Y.; Huang, X.; Yang, B.; Zhang, X.; Han, Y.; Chen, X.-X.; Han, B.-Z. Contrasting the Microbial Community and Metabolic Profile of Three Types of Light-Flavor Daqu. *Food Biosci.* **2021**, *144*, 101395. [CrossRef]
21. Liang, T.; Xie, X.; Zhang, J.; Ding, Y.; Wu, Q. Bacterial Community and Composition of Different Traditional Fermented Dairy Products in China, South Africa, and Sri Lanka by High-Throughput Sequencing of 16S RRNA Genes. *LWT* **2021**, *144*, 111209. [CrossRef]
22. Zhang, Q.Q.; Li, D.; Zhang, W.; Jiang, M.; Chen, X.H.; Dong, M.S. Comparative Analysis of the Bacterial Diversity of Chinese Fermented Sausages Using High-Throughput Sequencing. *LWT* **2021**, *150*, 111975. [CrossRef]
23. Biolcati, F.; Ferrocino, I.; Bottero, M.T.; Dalmasso, A. Short Communication: High-Throughput Sequencing Approach to Investigate Italian Artisanal Cheese Production. *J. Dairy Sci.* **2020**, *103*, 10015–10021. [CrossRef] [PubMed]
24. Nero, L.A.; Andretta, M.; Almeida, T.T.; Ferreira, L.R.; Camargo, A.C.; Yamatogi, R.S.; Carvalho, A.F.; Call, D.R. Lactic Microbiota of the Minas Artisanal Cheese Produced in the Serro Region, Minas Gerais, Brazil. *LWT* **2021**, *148*, 111698. [CrossRef]
25. Salazar, J.K.; Carstens, C.K.; Ramachandran, P.; Shazer, A.G.; Narula, S.S.; Reed, E.; Ottesen, A.; Schill, K.M. Metagenomics of Pasteurized and Unpasteurized Gouda Cheese Using Targeted 16S RDNA Sequencing. *BMC Microbiol.* **2018**, *18*, 189. [CrossRef] [PubMed]
26. De Pasquale, I.; Calasso, M.; Mancini, L.; Ercolini, D.; La Storia, A.; De Angelis, M.; Di Cagno, R.; Gobbetti, M. Causal Relationship between Microbial Ecology Dynamics and Proteolysis during Manufacture and Ripening of Protected Designation of Origin (PDO) Cheese Canestrato Pugliese. *Appl. Environ. Microbiol.* **2014**, *80*, 4085–4094. [CrossRef]
27. Ercolini, D.; De Filippis, F.; La Storia, A.; Iacono, M. "Remake" by High-Throughput Sequencing of the Microbiota Involved in the Production of Water Buffalo Mozzarella Cheese. *Appl. Environ. Microbiol.* **2012**, *78*, 8142–8145. [CrossRef]
28. Michailidou, S.; Pavlou, E.; Pasentsis, K.; Rhoades, J.; Likotrafiti, E.; Argiriou, A. Microbial Profiles of Greek PDO Cheeses Assessed with Amplicon Metabarcoding. *Food Microbiol.* **2021**, *99*, 103836. [CrossRef]
29. Jiang, Y.; Yu, P.; Liu, X.; Zhao, J.; Zhang, H.; Chen, W. Shifts in Diversity and Function of Bacterial Community during Manufacture of Rushan. *J. Dairy Sci.* **2021**, *104*, 12375–12393. [CrossRef]
30. Zhang, F.; Wang, Z.; Lei, F.; Wang, B.; Jiang, S.; Peng, Q.; Zhang, J.; Shao, Y. Bacterial Diversity in Goat Milk from the Guanzhong Area of China. *J. Dairy Sci.* **2017**, *100*, 7812–7824. [CrossRef]
31. Abriouel, H.; Martín-Platero, A.; Maqueda, M.; Valdivia, E.; Martínez-Bueno, M. Biodiversity of the Microbial Community in a Spanish Farmhouse Cheese as Revealed by Culture-Dependent and Culture-Independent Methods. *Int. J. Food Microbiol.* **2008**, *127*, 200–208. [CrossRef]
32. Alegría, Á.; Szczesny, P.; Mayo, B.; Bardowski, J.; Kowalczyk, M. Biodiversity in Oscypek, a Traditional Polish Cheese, Determined by Culture-Dependent and -Independent Approaches. *Appl. Environ. Microbiol.* **2012**, *78*, 1890–1898. [CrossRef]
33. Masoud, W.; Vogensen, F.K.; Lillevang, S.; Abu Al-Soud, W.; Sørensen, S.J.; Jakobsen, M. The Fate of Indigenous Microbiota, Starter Cultures, *Escherichia Coli*, *Listeria Innocua* and *Staphylococcus Aureus* in Danish Raw Milk and Cheeses Determined by Pyrosequencing and Quantitative Real Time (QRT)-PCR. *Int. J. Food Microbiol.* **2012**, *153*, 192–202. [CrossRef] [PubMed]
34. Yarza, P.; Yilmaz, P.; Pruesse, E.; Glöckner, F.O.; Ludwig, W.; Schleifer, K.H.; Whitman, W.B.; Euzéby, J.; Amann, R.; Rosselló-Móra, R. Uniting the Classification of Cultured and Uncultured Bacteria and Archaea Using 16S RRNA Gene Sequences. *Nat. Rev. Microbiol.* **2014**, *12*, 635–645. [CrossRef] [PubMed]
35. Bertani, G.; Levante, A.; Lazzi, C.; Bottari, B.; Gatti, M.; Neviani, E. Dynamics of a Natural Bacterial Community under Technological and Environmental Pressures: The Case of Natural Whey Starter for Parmigiano Reggiano Cheese. *Food Res. Int.* **2020**, *129*, 108860. [CrossRef] [PubMed]
36. De Paula, A.C.L.; Medeiros, J.D.; Fernandes, G.R.; da Silva, V.L.; Diniz, C.G. Microbiome of Industrialized Minas Frescal Cheese Reveals High Prevalence of Putative Bacteria: A Concern in the One Health Context. *LWT* **2021**, *139*, 110791. [CrossRef]
37. Cardinali, F.; Ferrocino, I.; Milanović, V.; Belleggia, L.; Corvaglia, M.R.; Garofalo, C.; Foligni, R.; Mannozzi, C.; Mozzon, M.; Cocolin, L.; et al. Microbial Communities and Volatile Profile of Queijo de Azeitão PDO Cheese, a Traditional Mediterranean Thistle-Curdled Cheese from Portugal. *Food Res. Int.* **2021**, *147*, 110537. [CrossRef]
38. Dimov, S.G.; Gyurova, A.; Zagorchev, L.; Dimitrov, T.; Georgieva-Miteva, D.; Peykov, S. NGS-Based Metagenomic Study of Four Traditional Bulgarian Green Cheeses from Tcherni Vit. *LWT* **2021**, *152*, 112278. [CrossRef]

39. Gaglio, R.; Franciosi, E.; Todaro, A.; Guarcello, R.; Alfeo, V.; Randazzo, C.L.; Settanni, L.; Todaro, M. Addition of Selected Starter/Non-Starter Lactic Acid Bacterial Inoculums to Stabilise PDO Pecorino Siciliano Cheese Production. *Food Res. Int.* **2020**, *136*, 109335. [CrossRef]
40. Endres, C.M.; Castro, Í.M.S.; Trevisol, L.D.; Severo, J.M.; Mann, M.B.; Varela, A.P.M.; Frazzon, A.P.G.; Mayer, F.Q.; Frazzon, J. Molecular Characterization of the Bacterial Communities Present in Sheep's Milk and Cheese Produced in South Brazilian Region via 16S RRNA Gene Metabarcoding Sequencing. *LWT* **2021**, *147*, 111579. [CrossRef]
41. Esteban-Blanco, C.; Gutiérrez-Gil, B.; Puente-Sánchez, F.; Marina, H.; Tamames, J.; Acedo, A.; Arranz, J.J. Microbiota Characterization of Sheep Milk and Its Association with Somatic Cell Count Using 16s RRNA Gene Sequencing. *J. Anim. Breed. Genet.* **2020**, *137*, 73–83. [CrossRef]
42. Biçer, Y.; Telli, A.E.; Sönmez, G.; Telli, N.; Uçar, G. Comparison of Microbiota and Volatile Organic Compounds in Milk from Different Sheep Breeds. *J. Dairy Sci.* **2021**, *104*, 12303–12311. [CrossRef]
43. Cardinali, F.; Osimani, A.; Taccari, M.; Milanović, V.; Garofalo, C.; Clementi, F.; Polverigiani, S.; Zitti, S.; Raffaelli, N.; Mozzon, M.; et al. Impact of Thistle Rennet from *Carlina Acanthifolia* All. Subsp. *Acanthifolia* on Bacterial Diversity and Dynamics of a Specialty Italian Raw Ewes' Milk Cheese. *Int. J. Food Microbiol.* **2017**, *255*, 7–16. [CrossRef] [PubMed]
44. Ramezani, M.; Hosseini, S.M.; Ferrocino, I.; Amoozegar, M.A.; Cocolin, L. Molecular Investigation of Bacterial Communities during the Manufacturing and Ripening of Semi-Hard Iranian Liqvan Cheese. *Food Microbiol.* **2017**, *66*, 64–71. [CrossRef] [PubMed]
45. Bustamante, M.; Chávarri, F.; Santisteban, A.; Ceballos, G.; Hernández, I.; José Miguélez, M.; Aranburu, I.; Barrón, L.J.R.; Virto, M.; De Renobales, M. Coagulating and Lipolytic Activities of Artisanal Lamb Rennet Pastes. *J. Dairy Res.* **2000**, *67*, 393–402. [CrossRef] [PubMed]
46. Erkus, O.; de Jager, V.C.L.; Geene, R.T.C.M.; van Alen-Boerrigter, I.; Hazelwood, L.; van Hijum, S.A.F.T.; Kleerebezem, M.; Smid, E.J. Use of Propidium Monoazide for Selective Profiling of Viable Microbial Cells during Gouda Cheese Ripening. *Int. J. Food Microbiol.* **2016**, *228*, 1–9. [CrossRef] [PubMed]
47. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef]
48. Meyer, F.; Paarmann, D.; D'Souza, M.; Olson, R.; Glass, E.M.; Kubal, M.; Paczian, T.; Rodriguez, A.; Stevens, R.; Wilke, A.; et al. The Metagenomics RAST Server—A Public Resource for the Automatic Phylogenetic and Functional Analysis of Metagenomes. *BMC Bioinform.* **2008**, *9*, 386. [CrossRef]
49. Pruesse, E.; Quast, C.; Knittel, K.; Fuchs, B.M.; Ludwig, W.; Peplies, J.; Glöckner, F.O. SILVA: A Comprehensive Online Resource for Quality Checked and Aligned Ribosomal RNA Sequence Data Compatible with ARB. *Nucleic Acids Res.* **2007**, *35*, 7188–7196. [CrossRef]
50. Johnson, J.S.; Spakowicz, D.J.; Hong, B.Y.; Petersen, L.M.; Demkowicz, P.; Chen, L.; Leopold, S.R.; Hanson, B.M.; Agresta, H.O.; Gerstein, M.; et al. Evaluation of 16S RRNA Gene Sequencing for Species and Strain-Level Microbiome Analysis. *Nat. Commun.* **2019**, *10*, 5029. [CrossRef]
51. R Core Team. The R Project for Statistical Computing. Available online: https://www.r-project.org/ (accessed on 15 March 2020).
52. Warnes, G.R.; Bolker, B.; Bonebakker, L.; Gentleman, R.; Huber, W.; Liaw, A.; Lumley, T.; Maechler, M.; Magnusson, A.; Moeller, S.; et al. Package "Gplots" Title Various R Programming Tools for Plotting Data [R Package Gplots Version 3.1.1]. Available online: https://cran.r-project.org/package=gplots (accessed on 15 January 2021).
53. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; Mcglinn, D.; Minchin, P.R.; O'hara, R.B.; Simpson, G.L.; Solymos, P.; et al. Package "Vegan". Available online: https://cran.r-project.org/web/packages/vegan/vegan.pdf (accessed on 15 January 2021).
54. Wickham, H.; Averick, M.; Bryan, J.; Chang, W.; McGowan, L.; François, R.; Grolemund, G.; Hayes, A.; Henry, L.; Hester, J.; et al. Welcome to the Tidyverse. *J. Open Source Softw.* **2019**, *4*, 1686. [CrossRef]
55. Kindt, R.; Coe, R. *Tree Diversity Analysis: A Manual and Software for Common Statistical Methods for Ecological and Biodiversity Studies*; World Agroforestry Centre (ICRAF): Nairobi, Kenya, 2005; ISBN 92 9059 179 X.
56. Paradis, E.; Claude, J.; Strimmer, K. APE: Analyses of Phylogenetics and Evolution in R Language. *Bioinformatics* **2004**, *20*, 289–290. [CrossRef]
57. Fox, P.F.; Guinee, T.P.; Cogan, T.M.; McSweeney, P.L.H. *Fundamentals of Cheese Science*, 2nd ed.; Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., Eds.; Springer: New York, NY, USA, 2017; ISBN 9781489976819.
58. Quintana, Á.R.; Seseña, S.; Garzón, A.; Arias, R. Factors Affecting Levels of Airborne Bacteria in Dairy Farms: A Review. *Animals* **2020**, *10*, 526. [CrossRef] [PubMed]
59. Van den Brom, R.; de Jong, A.; van Engelen, E.; Heuvelink, A.; Vellema, P. Zoonotic Risks of Pathogens from Sheep and Their Milk Borne Transmission. *Small Rumin. Res.* **2020**, *189*, 106123. [CrossRef] [PubMed]
60. Lu, M.; Wang, N.S. Spoilage of Milk and Dairy Products. In *The Microbiological Quality of Food: Foodborne Spoilers*; Bevilacqua, A., Corbo, M.R., Sinigaglia, M., Eds.; Woodhead Publishing: Duxford, UK, 2017; pp. 151–178. ISBN 9780081005033.
61. Wang, Y.; Wu, J.; Lv, M.; Shao, Z.; Hungwe, M.; Wang, J.; Bai, X.; Xie, J.; Wang, Y.; Geng, W. Metabolism Characteristics of Lactic Acid Bacteria and the Expanding Applications in Food Industry. *Front. Bioeng. Biotechnol.* **2021**, *9*, 612285. [CrossRef] [PubMed]
62. Paradh, A.D. Gram-Negative Spoilage Bacteria in Brewing. In *Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste*; Hill, A.E., Ed.; Elsevier Ltd.: Amsterdam, The Netherlands, 2015; pp. 175–194. ISBN 9781782423492.

63. Staley, J.T. The Genus *Prosthecobacter*. In *The Prokaryotes*; Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G., Eds.; Springer: New York, NY, USA, 1981; pp. 477–479. ISBN 9783662131879.
64. Suchodolski, J.S. Gastrointestinal Microbiota. In *Canine and Feline Gastroenterology*; Washabau, R.J., Day, M.J., Eds.; W.B. Saunders: St. Louis, MO, USA, 2012; pp. 32–41. ISBN 9781416036616.
65. Vataščinová, T.; Pipová, M.; Fraqueza, M.J.R.; Mal'a, P.; Dudriková, E.; Drážovská, M.; Lauková, A. Short Communication: Antimicrobial Potential of *Lactobacillus plantarum* Strains Isolated from Slovak Raw Sheep Milk Cheeses. *J. Dairy Sci.* **2020**, *103*, 6900–6903. [CrossRef]
66. Falardeau, J.; Keeney, K.; Trmčić, A.; Kitts, D.; Wang, S. Farm-to-Fork Profiling of Bacterial Communities Associated with an Artisan Cheese Production Facility. *Food Microbiol.* **2019**, *83*, 48–58. [CrossRef]
67. Bellassi, P.; Rocchetti, G.; Nocetti, M.; Lucini, L.; Masoero, F.; Morelli, L. A Combined Metabolomic and Metagenomic Approach to Discriminate Raw Milk for the Production of Hard Cheese. *Foods* **2021**, *10*, 109. [CrossRef]
68. Yeluri Jonnala, B.R.; McSweeney, P.L.H.; Sheehan, J.J.; Cotter, P.D. Sequencing of the Cheese Microbiome and Its Relevance to Industry. *Front. Microbiol.* **2018**, *9*, 1020. [CrossRef]
69. Actor, J.K. Clinical Bacteriology. In *Elsevier's Integrated Review Immunology and Microbiology*; Actor, J.K., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2012; pp. 105–120. ISBN 978-0-323-07447-6.
70. Antonello, V.S.; Dallé, J.; Domingues, G.C.; Ferreira, J.A.S.; Fontoura, M.d.C.Q.; Knapp, F.B. Post-Cesarean Surgical Site Infection Due to *Buttiauxella agrestis*. *Int. J. Infect. Dis.* **2014**, *22*, 65–66. [CrossRef]
71. Bergogne-Bérézin, E. Bacteria: *Acinetobacter*. In *Encyclopedia of Food Safety*; Motarjemi, Y., Ed.; Academic Press: San Diego, CA, USA, 2014; Volume 1, pp. 337–341. ISBN 9780123786128.
72. Fusco, V.; Abriouel, H.; Benomar, N.; Kabisch, J.; Chieffi, D.; Cho, G.-S.; Franz, C.M.A.P. Opportunistic Food-Borne Pathogens. In *Food Safety and Preservation*; Grumezescu, A.M., Holban, A.M., Eds.; Academic Press: San Diego, CA, USA, 2018; pp. 269–306. ISBN 9780128149560.
73. Guiu, A.; Buendía, B.; Llorca, L.; Gómez Punter, R.M.; Girón, R. *Chryseobacterium* spp., a New Opportunistic Pathogen Associated with Cystic Fibrosis? *Enferm. Infecc. Microbiol. Clin.* **2014**, *32*, 497–501. [CrossRef]
74. Li, N.; Wang, Y.; You, C.; Ren, J.; Chen, W.; Zheng, H.; Liu, Z. Variation in Raw Milk Microbiota throughout 12 Months and the Impact of Weather Conditions. *Sci. Rep.* **2018**, *8*, 2371. [CrossRef]
75. Morin, A. Pantoea. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Academic Press: San Diego, CA, USA, 2014; pp. 1028–1032. ISBN 9780123847331.
76. Murphy, E.C.; Mörgelin, M.; Cooney, J.C.; Frick, I.M. Interaction of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* with the Kallikrein-Kinin System. *Microbiology* **2011**, *157*, 2094–2105. [CrossRef] [PubMed]
77. Titécat, M.; Wallet, F.; Vieillard, M.H.; Courcol, R.J.; Loïez, C. *Ruminococcus gnavus*: An Unusual Pathogen in Septic Arthritis. *Anaerobe* **2014**, *30*, 159–160. [CrossRef] [PubMed]
78. Waśkiewicz, A.; Irzykowska, L. Flavobacterium spp.—Characteristics, Occurrence, and Toxicity. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Academic Press: San Diego, CA, USA, 2014; pp. 938–942. ISBN 9780123847331.
79. Appel, T.M.; Quijano-Martínez, N.; De La Cadena, E.; Mojica, M.F.; Villegas, M.V. Microbiological and Clinical Aspects of *Raoultella* spp. *Front. Public Health* **2021**, *9*, 686789. [CrossRef] [PubMed]
80. Lo, R.; Xue, T.; Weeks, M.; Turner, M.S.; Bansal, N. Inhibition of Bacterial Growth in Sweet Cheese Whey by Carbon Dioxide as Determined by Culture-Independent Community Profiling. *Int. J. Food Microbiol.* **2016**, *217*, 20–28. [CrossRef]
81. Morandi, S.; Battelli, G.; Silvetti, T.; Tringali, S.; Nunziata, L.; Villa, A.; Acquistapace, A.; Brasca, M. Impact of Salting and Ripening Temperatures on Late Blowing Defect in Valtellina Casera PDO Cheese. *Food Control* **2021**, *120*, 107508. [CrossRef]
82. Fotou, K.; Tzora, A.; Voidarou, C.; Alexopoulos, A.; Plessas, S.; Avgeris, I.; Bezirtzoglou, E.; Akrida-Demertzi, K.; Demertzis, P.G. Isolation of Microbial Pathogens of Subclinical Mastitis from Raw Sheep's Milk of Epirus (Greece) and Their Role in Its Hygiene. *Anaerobe* **2011**, *17*, 315–319. [CrossRef]
83. Fuka, M.M.; Engel, M.; Skelin, A.; Redžepović, S.; Schloter, M. Bacterial Communities Associated with the Production of Artisanal Istrian Cheese. *Int. J. Food Microbiol.* **2010**, *142*, 19–24. [CrossRef]
84. Ruvalcaba-Gómez, J.M.; Delgado-Macuil, R.J.; Zelaya-Molina, L.X.; Maya-Lucas, O.; Ruesga-Gutiérrez, E.; Anaya-Esparza, L.M.; de la Mora, Z.V.; López-De la Mora, D.A.; Arteaga-Garibay, R.I. Bacterial Succession through the Artisanal Process and Seasonal Effects Defining Bacterial Communities of Raw-Milk Adobera Cheese Revealed by High Throughput Dna Sequencing. *Microorganisms* **2021**, *9*, 24. [CrossRef]
85. Bowman, J.P. The Genus *Psychrobacter*. In *The Prokaryotes*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrandt, E., Eds.; Springer: New York, NY, USA, 2006; pp. 920–930. ISBN 9780387307466.
86. Shweta, F.N.U.; Gurram, P.R.; O'Horo, J.C.; Khalil, S. *Brevibacterium* Species: An Emerging Opportunistic Cause of Bloodstream Infections. *Mayo Clin. Proc.* **2021**, *96*, 1093–1094. [CrossRef]
87. Yin, Z.; Yuan, C.; Du, Y.; Yang, P.; Qian, C.; Wei, Y.; Zhang, S.; Huang, D.; Liu, B. Comparative Genomic Analysis of the *Hafnia* Genus Reveals an Explicit Evolutionary Relationship between the Species *Alvei* and *Paralvei* and Provides Insights into Pathogenicity. *BMC Genom.* **2019**, *20*, 768. [CrossRef]
88. Anast, J.M.; Dzieciol, M.; Schultz, D.L.; Wagner, M.; Mann, E.; Schmitz-Esser, S. *Brevibacterium* from Austrian Hard Cheese Harbor a Putative Histamine Catabolism Pathway and a Plasmid for Adaptation to the Cheese Environment. *Sci. Rep.* **2019**, *9*, 6164. [CrossRef] [PubMed]

89. Ozturkoglu-Budak, S.; Wiebenga, A.; Bron, P.A.; de Vries, R.P. Protease and Lipase Activities of Fungal and Bacterial Strains Derived from an Artisanal Raw Ewe's Milk Cheese. *Int. J. Food Microbiol.* **2016**, *237*, 17–27. [CrossRef] [PubMed]
90. Zhang, D.; Palmer, J.; Teh, K.H.; Flint, S. Identification and Selection of Heat-Stable Protease and Lipase-Producing Psychrotrophic Bacteria from Fresh and Chilled Raw Milk during up to Five Days Storage. *LWT* **2020**, *134*, 110165. [CrossRef]
91. De Pasquale, I.; Di Cagno, R.; Buchin, S.; De Angelis, M.; Gobbetti, M. Spatial Distribution of the Metabolically Active Microbiota within Italian PDO Ewes' Milk Cheeses. *PLoS ONE* **2016**, *11*, e0153213. [CrossRef]
92. Deetae, P.; Bonnarme, P.; Spinnler, H.E.; Helinck, S. Production of Volatile Aroma Compounds by Bacterial Strains Isolated from Different Surface-Ripened French Cheeses. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 1161–1171. [CrossRef]
93. Irlinger, F.; In Yung, S.A.Y.; Sarthou, A.S.; Delbès-Paus, C.; Montel, M.C.; Coton, E.; Coton, M.; Helinck, S. Ecological and Aromatic Impact of Two Gram-Negative Bacteria (*Psychrobacter celer* and *Hafnia alvei*) Inoculated as Part of the Whole Microbial Community of an Experimental Smear Soft Cheese. *Int. J. Food Microbiol.* **2012**, *153*, 332–338. [CrossRef]
94. Quillaguamán, J.; Delgado, O.; Mattiasson, B.; Hatti-Kaul, R. *Chromohalobacter sarecensis* sp. Nov., a Psychrotolerant Moderate Halophile Isolated from the Saline Andean Region of Bolivia. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 1921–1926. [CrossRef]
95. Fuka, M.M.; Wallisch, S.; Engel, M.; Welzl, G.; Havranek, J.; Schloter, M. Dynamics of Bacterial Communities during the Ripening Process of Different Croatian Cheese Types Derived from Raw Ewe's Milk Cheeses. *PLoS ONE.* **2013**, *8*, e80734. [CrossRef]
96. García-López, M.L.; Santos, J.A.; Otero, A.; Rodríguez-Calleja, J.M. Psychrobacter. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Elsevier: Amsterdam, The Netherlands, 2014; pp. 261–268. ISBN 9780123847331.
97. Hernández, I.; Barrón, L.J.R.; Virto, M.; Pérez-Elortondo, F.J.; Flanagan, C.; Rozas, U.; Nájera, A.I.; Albisu, M.; Vicente, M.S.; de Renobales, M. Lipolysis, Proteolysis and Sensory Properties of Ewe's Raw Milk Cheese (Idiazabal) Made with Lipase Addition. *Food Chem.* **2009**, *116*, 158–166. [CrossRef]
98. Gil, P.F.; Conde, S.; Albisu, M.; Pérez-Elortondo, F.J.; Etayo, I.; Virto, M.; De Renobales, M. Hygienic Quality of Ewes' Milk Cheeses Manufactured with Artisan-Produced Lamb Rennet Pastes. *J. Dairy Res.* **2007**, *74*, 329–335. [CrossRef]
99. Etayo, I.; Pérez Elortondo, F.J.; Gil, P.F.; Albisu, M.; Virto, M.; Conde, S.; Rodriguez Barron, L.J.; Nájera, A.I.; Gómez-Hidalgo, M.E.; Delgado, C.; et al. Hygienic Quality, Lipolysis and Sensory Properties of Spanish Protected Designation of Origin Ewe's Milk Cheeses Manufactured with Lamb Rennet Paste. *Lait* **2006**, *86*, 415–434. [CrossRef]
100. Bokulich, N.A.; Mills, D.A. Facility-Specific "House" Microbiome Drives Microbial Landscapes of Artisan Cheesemaking Plants. *Appl. Environ. Microbiol.* **2013**, *79*, 5214–5223. [CrossRef] [PubMed]
101. Blaya, J.; Barzideh, Z.; LaPointe, G. Symposium Review: Interaction of Starter Cultures and Nonstarter Lactic Acid Bacteria in the Cheese Environment. *J. Dairy Sci.* **2018**, *101*, 3611–3629. [CrossRef] [PubMed]
102. Frantzen, C.A.; Kot, W.; Pedersen, T.B.; Ardö, Y.M.; Broadbent, J.R.; Neve, H.; Hansen, L.H.; Dal Bello, F.; Østlie, H.M.; Kleppen, H.P.; et al. Genomic Characterization of Dairy Associated *Leuconostoc* Species and Diversity of Leuconostocs in Undefined Mixed Mesophilic Starter Cultures. *Front. Microbiol.* **2017**, *8*, 132. [CrossRef]
103. Gobbetti, M.; De Angelis, M.; Di Cagno, R.; Mancini, L.; Fox, P.F. Pros and Cons for Using Non-Starter Lactic Acid Bacteria (NSLAB) as Secondary/Adjunct Starters for Cheese Ripening. *Trends Food Sci. Technol.* **2015**, *45*, 167–178. [CrossRef]
104. Li, S.; Li, Y.; Du, Z.; Li, B.; Liu, Y.; Gao, Y.; Zhang, Y.; Zhang, K.; Wang, Q.; Lu, S.; et al. Impact of NSLAB on Kazakh Cheese Flavor. *Food Res. Int.* **2021**, *144*, 110315. [CrossRef]
105. Mohamaden, W.I.; Zhen-fen, Z.; Hegab, I.M.; Shang-li, S. Experimental Infection in Mice with *Erwinia persicina*. *Microb. Pathog.* **2019**, *130*, 38–43. [CrossRef]
106. Quigley, L.; O'Sullivan, O.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. High-Throughput Sequencing for Detection of Subpopulations of Bacteria Not Previously Associated with Artisanal Cheeses. *Appl. Environ. Microbiol.* **2012**, *78*, 5717–5723. [CrossRef]
107. Sádecká, J.; Šaková, N.; Pangallo, D.; Koreňová, J.; Kolek, E.; Puškárová, A.; Bučková, M.; Valík, L.; Kuchta, T. Microbial Diversity and Volatile Odour-Active Compounds of Barrelled Ewes' Cheese as an Intermediate Product That Determines the Quality of Winter Bryndza Cheese. *LWT* **2016**, *70*, 237–244. [CrossRef]
108. D'Amico, D.J.; Donnelly, C.W. Growth and Survival of Microbial Pathogens in Cheese. In *Cheese: Chemistry, Physics and Microbiology*, 4th ed.; McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds.; Academic Press: San Diego, CA, USA, 2017; Volume 1, pp. 573–594. ISBN 9780122636530.
109. Gontijo, M.T.P.; Silva, J.d.S.; Vidigal, P.M.P.; Martin, J.G.P. Phylogenetic Distribution of the Bacteriocin Repertoire of Lactic Acid Bacteria Species Associated with Artisanal Cheese. *Food Res. Int.* **2020**, *128*, 108783. [CrossRef]
110. Kaya, H.I.; Simsek, O. Characterization of Pathogen-Specific Bacteriocins from Lactic Acid Bacteria and Their Application within Cocktail against Pathogens in Milk. *LWT* **2019**, *115*, 108464. [CrossRef]
111. Macedo, A.C.; Malcata, F.X.; Oliveira, J.C. Effect of Production Factors and Ripening Conditions on the Characteristics of Serra Cheese. *Int. J. Food Sci. Technol.* **1997**, *32*, 501–511. [CrossRef]

This article was published in Biology, Vol 11, Gorka Santamarina-García, Igor Hernández, Gustavo Amores and Mailo Virto, Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing, 769,

2 | Appendix II
Manuscript II

Animals (2022), 12(22), 3224

Relationship between dynamics of gross composition, free fatty acids and biogenic amines, and microbial shifts during the ripening of raw ewe milk-derived Idiazabal cheese

Gorka Santamarina-García[1,*], Gustavo Amores[1], Emma López de Armentia[1], Igor Hernández[1] and Mailo Virto[1]

[1]*Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[*] *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented at:

VII Jornadas Doctorales de la Universidad de Murcia-Universidad Politécnica de Cartagena-Campus Mare Nostrum, 2022. *El impacto de la microbiota en varios parámetros de calidad y seguridad del queso de leche cruda de oveja Idiazabal.* Santamarina-García, G., Hernández, I., Amores, G. and Virto, M. (Oral presentation).

73rd European Federation of Animal Science Annual Meeting, 2022. *The impact of bacterial shifts on several quality and safety parameters of a ewe's raw milk cheese.* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Poster presentation).

Ikergazte, 2023. *Zenbateraino eragiten diote bakterio-komunitateek esne gordineko gazten kalitateari eta kaltegabetasunari?: Idiazabal gaztaren kasua.* Santamarina-García, G., Amores, G., and Virto, M. (Oral presentation).

Published in **Animals** (Animal Products section)
ISSN: 2076-2615
IF (2022): **3.0**
Category: **Agriculture, Dairy & Animal Science (Q1, 12/62), Veterinary Sciences (D1, 13/144)**
Citations: 6 (updated: 04/2024)
doi.org/10.3390/ani12223224

animals

MDPI

Article

Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese

Gorka Santamarina-García *, Gustavo Amores *, Emma López de Armentia, Igor Hernández and Mailo Virto

Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Unibertsitate Ibilbidea 7, 01006 Vitoria-Gasteiz, Basque Country, Spain

* Correspondence: gorka.santamarina@ehu.eus (G.S.-G.); gustavo.amores@ehu.eus (G.A.); Tel.: +34-9-4501-3799 (G.S.-G.); +34-9-4501-3101 (G.A.)

Simple Summary: The microbiota present in cheese is of special interest as it contributes to the synthesis of different compounds related to cheese quality and safety. However, to date, no studies have been carried out in cheese to elucidate the relationship between bacterial communities, characterized by high-throughput sequencing (HTS), and the dynamics of gross composition, free fatty acids (FFAs) and biogenic amines (BAs) during ripening. In this sense, this work focused on Idiazabal PDO cheese, a semi-hard or hard cheese produced from raw ewe milk. Results revealed that the non-starter lactic acid bacteria *Lactobacillus*, *Enterococcus* and *Streptococcus* were positively associated with the changes in gross composition and the release of FFAs, while only *Lactobacillus* was positively associated with the production of BAs. Several genera of environmental or undesirable bacteria presented negative correlations, which could indicate a negative impact of gross composition on their growth, the antimicrobial effect of FFAs and/or the importance of such FFAs as metabolic substrates for these bacteria, and their capability to degrade BAs.

Abstract: This study reports for the first time the relationship between bacterial succession, characterized by high-throughput sequencing (sequencing of V3–V4 16S rRNA regions), and the evolution of gross composition, free fatty acids (FFAs) and biogenic amines (BAs) during cheese ripening. Specifically, Idiazabal PDO cheese, a raw ewe milk-derived semi-hard o hard cheese, was analysed. Altogether, 8 gross parameters were monitored (pH, dry matter, protein, fat, Ca, Mg, P and NaCl) and 21 FFAs and 8 BAs were detected. The ripening time influenced the concentration of most physico-chemical parameters, whereas the producer mainly affected the gross composition and FFAs. Through an O2PLS approach, the non-starter lactic acid bacteria *Lactobacillus*, *Enterococcus* and *Streptococcus* were reported as positively related to the evolution of gross composition and FFAs release, while only *Lactobacillus* was positively related to BAs production. Several environmental or non-desirable bacteria showed negative correlations, which could indicate the negative impact of gross composition on their growth, the antimicrobial effect of FFAs and/or the metabolic use of FFAs by these genera, and their ability to degrade BAs. Nonetheless, *Obesumbacterium* and *Chromohalobacter* were positively associated with the synthesis of FFAs and BAs, respectively. This research work provides novel information that may contribute to the understanding of possible functional relationships between bacterial communities and the evolution of several cheese quality and safety parameters.

Keywords: metagenomics; 16S rRNA gene sequencing; sheep cheese; cheese ripening; cheese quality; cheese safety; O2PLS; CCorA

check for updates

Citation: Santamarina-García, G.; Amores, G.; López de Armentia, E.; Hernández, I.; Virto, M. Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese. *Animals* **2022**, *12*, 3224. https://doi.org/10.3390/ani12223224

Academic Editor: Claudio Cipolat-Gotet

Received: 13 October 2022
Accepted: 14 November 2022
Published: 21 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Idiazabal cheese is a semi-hard or hard cheese from the Basque Country (Southwestern Europe), which is produced from the raw milk of Latxa and/or Carranzana sheep and

with at least 60 days of ripening. Its production is regulated by its Protected Designation of Origin (PDO) [1]. Like other raw milk cheeses, the aromatic profile of this cheese is richer compared to those produced from pasteurized milk [2–5], which has been mainly attributed to the indigenous milk microbiota [6–9]. Nowadays, high-throughput sequencing (HTS) is a powerful technology to characterize the bacterial communities of fermented foods, avoiding the limitations of culture-based methods [10]. However, these technologies also have their limitations, such as the accuracy in taxonomic identification at the species level or the high associated cost due to equipment, reagents or trained personnel [11]. In a previous study, the microbiota of Latxa ewe raw milk and the bacterial shifts that occur during the production and ripening of Idiazabal cheese have been successfully described by means of this technology. In fact, some bacterial genera not reported so far in raw ewe milk and cheese have been identified. It should be noted that notable differences were observed among producers, which was attributed to the different practices carried out among producers in relation to herd management, such as feeding; or cheese making, for example, the type of rennet used or the technological settings [12].

The microbiota of cheese is of great importance since it contributes to numerous biochemical reactions involved in the formation of compounds related to cheese quality and safety [13–15]. Lipolysis is one of the most important processes for flavour development during cheese ripening. It is defined as the enzymatic hydrolysis of milk triglycerides (TG) with the subsequent release and accumulation of free fatty acids (FFAs). FFAs contribute directly to cheese flavour, but also act as precursor molecules for subsequent reactions that lead to the production of volatile compounds [16,17]. The most important lipolytic agents in cheese are lipoprotein lipase (LPL) from raw milk, rennets containing pregastric lipase and microbial lipases and esterases [17–19].

Proteolysis is another important biochemical event that takes place during ripening, whereby caseins are broken down into peptides and free amino acids (FAAs). Therefore, and together with the metabolism of the resulting peptides and FAAs, it contributes to cheese texture and flavour [18,20]. However, microbial decarboxylation of FAAs could lead to the production of biogenic amines (BAs), which are non-volatile low molecular weight nitrogenous organic bases with biological activity [21–23]. BAs are not harmful at low levels, but some can have toxicological effects after ingestion in high concentrations [24,25]. In relation to dairy products and specifically cheese, histamine and tyramine are the main ones responsible for intoxication. Histamine causes the so-called "histamine poisoning", characterized by low blood pressure, skin irritation or rashes typical of allergic reactions; while tyramine causes the so-called "cheese reaction", with symptoms like migraines, headaches or blood pressure increase. Putrescine and cadaverine are also important BAs, since they can potentiate the toxic effect of other BAs due to the inhibition of detoxifying amine oxidases and are related to the production of carcinogenic nitrosamines [24,26,27]. Within fermented foods, cheese is classified as a potential source of BAs due to the high microbial activity associated [23,28]. Nevertheless, so far, no legal limits have been established [29,30].

To elucidate the relationship between bacterial communities and the chemical composition of fermented foods, complex chemometric approaches are needed [31–33]. The multivariate bidirectional orthogonal partial least squares (O2PLS) [34] is one of the most useful approaches [35–37]. It has been successfully applied to analyse the correlation between bacterial and fungal metabolisms and different metabolites, such as FAAs, of fermented foods [38–41]; including cheese [31,32]. However, its combination with other parameters, such as correlation coefficients, is considered the most appropriate approach to studying such relationships [42,43]. Recently published studies analyse the relationship between bacterial communities and the chemical composition of different types of cheeses [14,31,44,45]. However, information on raw ewe milk-derived cheeses is scarce [46–48]. Moreover, to the best of our knowledge, no study has analysed the relation between bacterial succession characterized by HTS and the evolution of gross composition, FFAs and BAs of any type of cheese. To date, no multivariate approach and only some correlations have been reported

between specific bacteria, such as *Lactobacillus*, and certain physico-chemical parameters, such as pH, NaCl, short-chain FFAs, such as C4; or histamine [49–51].

Therefore, the objective of the present study was to analyse how the gross composition, FFAs and BAs content of Idiazabal cheese evolve during the ripening time and to explore how they relate with the bacterial communities present. Moreover, the potential differences among producers producing the same type of cheese were also analysed.

2. Materials and Methods

2.1. Cheese Sampling

The Idiazabal PDO cheeses analysed in the present study were produced by four artisanal producers (identified as A, B, C and D), whose dairies were situated in different geographic locations throughout the Basque Country. Idiazabal cheeses were produced from the raw milk of Latxa sheep, each producer employing the milk of its own flock and following the specifications issued by the Idiazabal Designation of Origin Regulatory Board [52]. The producers employed the mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*) (DuPont NHIB Ibérica S.L., Barcelona, Spain). The milk was coagulated either with artisanal lamb rennet (produced as described previously [53]) or with commercial NATUREN® 195 Premium rennet (Chr. Hansen Holding A/S, Hørsholm, Denmark). Cheese ripening took place in chambers with 80–95% relative humidity and 8–14 °C temperature. The cheeses were collected in duplicate at six ripening times (1, 7, 14, 30, 60 and 120 days). Therefore, a total of 48 cheese samples were analysed. The samples were collected and transported in refrigeration (3 °C) to the laboratory. Each cheese was divided in eighths and the analyses of gross composition, except NaCl, were performed on fresh samples, while for the subsequent determination of NaCl and the analysis of FFAs and BAs the samples were stored in a freezer (−80 °C). The samples were allowed to thaw at 5 °C for 24 h and then, kept at room temperature for 1 h prior to analysis.

2.2. Analysis of Gross Composition

The pH was measured in a pH-meter micropH 2000 (Crison Instruments S. A., Barcelona, Spain). Dry matter, fat, protein, calcium, magnesium and phosphorous content was measured using the SpectraAlyzer 2.0 FOOD Near Infrared Spectrometer (NIR) (ZEUTEC GmbH, Rendsburg, Germany), as described by Aldalur et al. [54]. NaCl content was determined on the basis of the standard method ISO 5943 IDF 88 [55], but replacing the potentiometric determination of the endpoint with its colorimetric determination (Mohr titration method). These analyses were conducted in duplicate.

2.3. Analysis of Free Fatty Acids

FFAs were extracted, isolated, identified and quantified without derivatization by gas–liquid chromatography (GLC), basically as described by Chavarri et al. [56]. In short, 0.5 g of cheese sample was ground with 3.0 g of anhydrous Na_2SO_4 (reagent grade, Scharlab, Barcelona, Spain), and 0.3 mL of 2.5 M H_2SO_4 (reagent grade, Scharlab, Barcelona, Spain) and 100 µL of internal standard (IS) solution (n-pentanoic (5:0), n-nonanoic (9:0) and n-heptadecanoic (17:0) acids (GC grade, Sigma-Aldrich, Madrid, Spain), 1 mg/mL solution of each FFA in diethyl ether-heptane 1:1, *v*/*v* (GC grade, ROMIL Ltd., Cambridge, UK)) were added. The lipids were extracted three times with 3.0 mL of diethyl ether-heptane 1:1, *v*/*v* each time. After each extraction, the solution was cleared by centrifugation. The three extracts were pooled and applied to an aminopropyl-bonded phase column (Sep-Pak®, 3 cm^3, 500 mg, Waters, Barcelona, Spain), previously equilibrated with 10.0 mL of heptane. The TG were eluted with 10.0 mL of chloroform-isopropanol 2:1, *v*/*v* (GC grade, ROMIL Ltd., Cambridge, UK) and the FFAs were isolated by elution of 5.0 mL of diethyl ether-formic acid [reagent grade, Panreac Química, Barcelona, Spain (98:2, *v*/*v*)].

FFAs were separated on an FFAP capillary column (25 m length, 0.32 mm inner diameter, 0.50 μm film thickness) (Agilent Technologies, Madrid, Spain) installed on a 7890 Series II gas chromatograph (Agilent Technologies, Madrid, Spain) equipped with a flame ionization detector. Helium (99.999% purity, Air liquid, Madrid, Spain) was used as carrier gas at a flow rate of 2 mL/min. The temperature was raised from 65 °C to 240 °C at 10 °C/min, and this final temperature was maintained for 30 min. The split/splitless ratio was set at 1:5. The injector and detector temperatures were maintained at 325 °C and 275 °C, respectively. FFAs were identified by comparison of their retention times with those of high purity standards (≥90%; Sigma-Aldrich, Madrid, Spain). Quantification was performed by the internal calibration method for each of the FFAs found in the samples. Relative IS response factors were calculated for the standard solutions of FFAs. The concentrations of FFAs in the cheese samples were expressed as μmol/g of cheese.

2.4. Analysis of Biogenic Amines

BAs were extracted, separated, identified and quantified by reversed phase-high-performance liquid chromatography (RP-HPLC), based on the method described by Busto et al. [57]. In brief, 1.0 g of cheese was grounded together with 9.0 mL of trichloroacetic acid (TCA) 5%, *w*/*v* (reagent grade, Scharlab, Barcelona, Spain), after which, 1.0 mL of TCA 5% was added, which contained 1,7-diaminoheptane 1.5 mM (≥98%, Sigma-Aldrich, Madrid, Spain) employed as IS. The acid extract was sonicated for 25 min and centrifuged at 5000 rpm for 30 min at room temperature. The extract was filtered through Durapore filters (0.45 mm pore size; Millipore, Madrid, Spain). The derivate reagent was formed by reconstituting 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (reagent grade, Waters, Barcelona, Spain) with 1.0 mL of acetonitrile (reagent grade, Waters, Barcelona, Spain) according to the specifications of the AccQ-Fluor Reagent Kit (Waters, Barcelona, Spain). Then, 2.5 μL of the amine extract was mixed with 75 μL of borate buffer (pH 8.8, reagent grade, Waters, Barcelona, Spain) and 25 μL of the derivative reagent AQC (reagent grade, Waters, Barcelona, Spain). The mixture was shacked and kept at room temperature for 1 min and finally, maintained at 55 °C for 10 min.

The derivatized BAs were separated in an XTerraTM MS C18 column (XTerra MS C18 Column, 125 Å pore size, 5 μm particle size, 4.6 mm i.d. × 250 mm length) installed in an Alliance 2690 separation module, equipped with a quaternary pump system, automatic injector, vacuum degasification, thermostated oven and with a Waters 474 Scanning Fluorescence Detector (Waters, Barcelona, Spain). For separating BAs, both the column and the pre-column (XTerra MS C18 Sentry Guard Cartridge, 125 Å, 5 μm, 3.9 mm × 20 mm) were maintained at 50 °C and two solvents were used: (A) 0.05 M sodium acetate solution in 1% tetrahydrofuran pH 7.0 (>99.9%, ROMIL Ltd., Cambridge, UK) and (B) methanol (>99.9%, ROMIL Ltd., Cambridge, UK). The programme is shown in Supplementary Table S1. Subsequently, the identification was made by fluorimetry, at excitation and emission wavelength of 250 nm and 395 nm, respectively. The quantification was performed using 1,7-diaminoheptane (≥98%, Sigma-Aldrich, Madrid, Spain) as IS and analysing standard solutions with known concentrations of methylamine (MMA), α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), cadaverine (CAD), ethanolamine (MEA), ethylamine (ETY), histamine (HIS), methylbutylamine + spermidine (MBA + SPD) (those were analysed together because it was not possible to separate peaks), n-butylamine (NBA), phenylethylamine (PHE), putrescine (PUT), spermine (SPM) and tyramine (TYR) (reagent grade, Sigma-Aldrich, Madrid, Spain). The concentrations of BAs in cheese samples were expressed as μmol/g of cheese.

2.5. Statistical Analysis

Different packages and software were employed for physico-chemical parameters (gross composition, FFAs and BAs) analysis and three significant figures were used to express the results. The IBM SPSS statistical package version 26.0 (IBM SPSS Inc., Chicago, IL, USA) was used for data preparation and analysis. Kruskal–Wallis analysis of variance

with Bonferroni correction was performed using the IBM SPSS package to determine the effect of ripening time and producer on each physico-chemical parameter. Permutational multivariate analysis of variance (PERMANOVA) was performed using RStudio version 1.3.959 and R version 3.6.3 [58] through the "vegan" package [59]. The objective was to measure the overall effect of the aforementioned factors on the physico-chemical parameters. The F statistic was employed to measure the influence of each factor. Physico-chemical parameters were selected, log-transformed, when necessary, and unit variance (UV) scaled, and a heat-map with hierarchical clustering analysis (HCA) was performed using the "gplots" package of R [60]. The aim was to analyse the clustering of physico-chemical parameters during ripening time. Trends along ripening were then explored in depth by means of a principal component analysis (PCA) performed using the SIMCA software version 15.0.0.4783 (Umetrics AB, Umeå, Sweden). The number of principal components (PCs) to be taken into account was decided on the basis of eigenvalues (greater than 1.0) and cross-validation. An orthogonal partial least squares discriminant analysis (OPLS-DA) was run in SIMCA software in order to analyse whether samples differ among producers. Variable influence on projection (VIP) and loadings weights were employed to estimate the importance of each physico-chemical parameter in the model.

2.6. Correlation with Bacterial Communities

To characterize the bacterial communities and the shifts that occur during the ripening of the collected Idiazabal cheese samples, an HTS analysis was performed, as previously described [12]. Briefly, 10 g of cheese was added to 90 mL of 2% (*w*/*v*) sterile sodium citrate (pH 8.0), and homogenized in a sample homogenizer (Masticator Basic 400; IUL Instruments, Königswinter, Germany) six times (cycles of 20 s ON and 10 s OFF each). Of the resulting dispersion, 1.5 mL was centrifuged (8000 *g* for 10 min at 4 °C) and the supernatant containing the fat was discarded. The pellet obtained was resuspended in 600 µL of sodium citrate, and centrifuged thrice (8000 *g* for 10 min at 4 °C). From the resulting product, DNA was extracted by means of the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and the 16S rRNA gene library was prepared with the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). The hypervariable V3–V4 regions of the 16S rRNA gene were amplified by means of PCR (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′), as described by Klindworth et al. [61]. Next, the gene encoding 16S rRNA was sequenced with the MiSeq Reagent Kit v3 (2 × 300 bp) (Illumina Inc.) on the Illumina MiSeq platform. Quality filtering and trimming of raw reads was performed with MiSeq Reporter software, and the MG-RAST web data analysis tool [62] was used for taxonomic classification, based on the Silva SSU database [63]. Bacterial abundance was given as relative abundance (%) based on the identified sequences.

To study the relationship between the identified bacterial genera and the physico-chemical parameters, an O2PLS approach was applied to log-transformed, when necessary, and UV scaled data in SIMCA, selecting the main bacterial genera as X-variables and physico-chemical parameters as Y-variables. The model was validated, among others, by R2 and Q2 values, permutation test or inner relation plot. Correlations were also analysed by Spearman's rank correlation coefficients calculated using SPSS and displayed as a heat map with an HCA in R, as previously mentioned. The canonical correlation analysis (CCorA) multivariate statistical approach was performed using the "vegan" package of R to verify the previously obtained correlations.

3. Results

3.1. Changes in Gross Composition

Firstly, the evolution of gross composition during the ripening of Idiazabal cheeses was analysed. As shown in Figure 1A and Supplementary Table S2, the pH showed a different evolution from the rest of the parameters, that is why it formed a single cluster

(A1). The pH was 5.08 ± 0.08 on the first day of ripening, which decreased and reached the lowest values at 30 days (4.91 ± 0.14). Then, up to 120 days, the pH increased close to the initial values (5.11 ± 0.14) ($p \leq 0.05$). The rest of the parameters presented an increasing trend throughout the ripening time (cluster A2), namely dry matter, protein, fat, NaCl, Ca, Mg and P content ($p \leq 0.01$). It should be noted that changes differed largely according to the producer ($p \leq 0.05$), except for the NaCl content ($p > 0.05$).

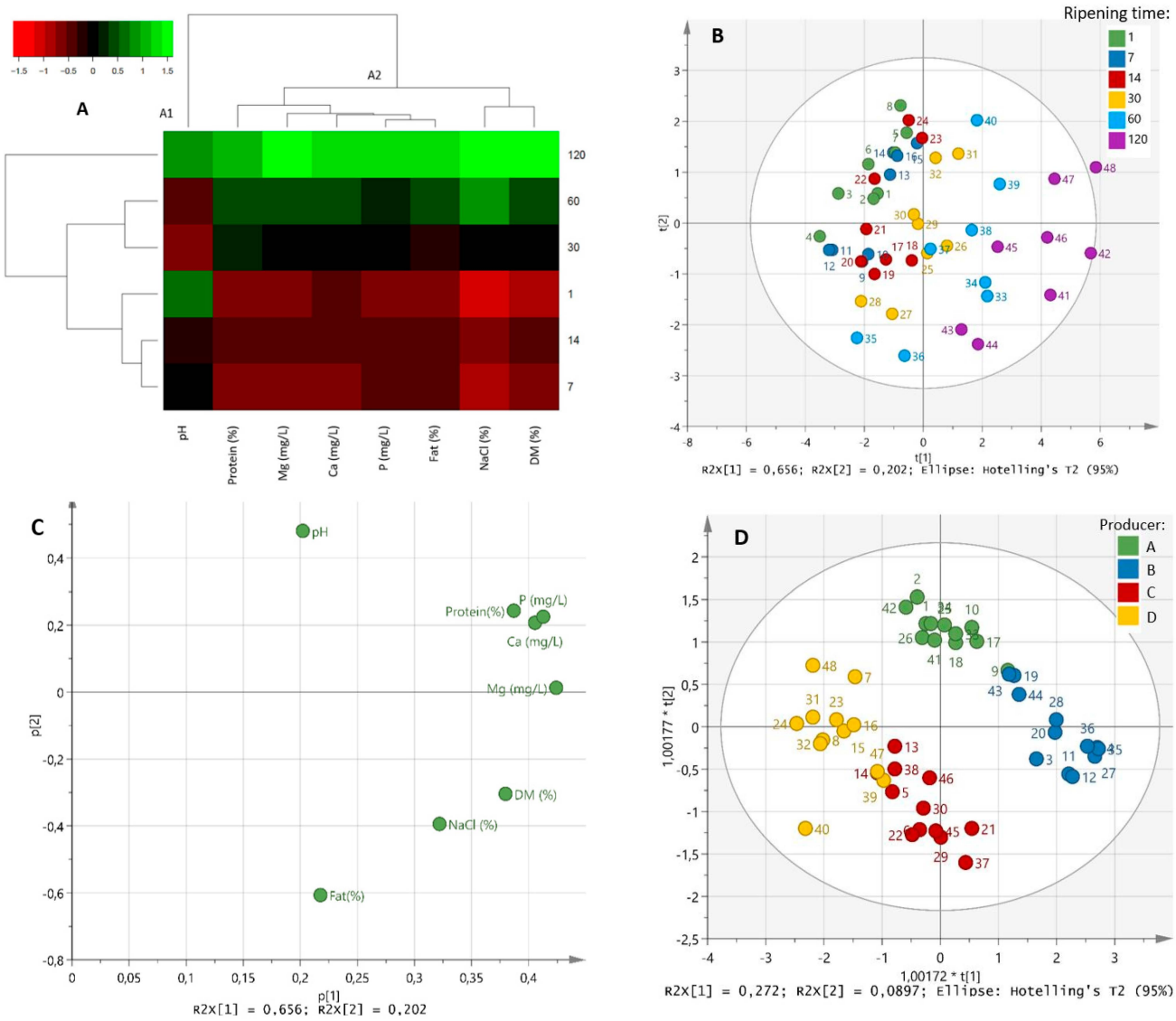


Figure 1. HCA heat map (**A**), scores and loadings plots of PCA (**B** and **C**, respectively) and scores plot of the OPLS-DA based on the producer of the analysed gross composition parameters during ripening (1, 7, 14, 30, 60 and 120 days) (**D**). The scale values of the HCA correspond to log-transformed and UV scaled data.

By means of a multivariate approach, PERMANOVA indicated that both the ripening time and the producer factors influenced the gross composition of Idiazabal cheeses ($p \leq 0.001$) (data not shown). However, the F statistic indicated that the ripening time had a greater impact than that of the producer (38.1 and 7.89, respectively). Through a PCA, which revealed three PCs and accounted for 94.8% of the variance, these results were confirmed. According to the scores plot (Figure 1B), PC1 values ranged from negative (for less ripened cheeses) to positive (for more ripened cheeses). Consequently, PC1 (accounting for 65.6% of the explained variance) was clearly correlated with the ripening

time. Gross composition parameters showed positive loadings in PC1, which confirmed that their evolution was clearly affected by the ripening time (Figure 1C). On the other hand, PC2 (accounting for 20.2% of the explained variance) was related to the producer factor, indicating that the gross composition also differed according to the dairy, although to a lesser extent (Figure 1B). Differences among producers were confirmed through an OPLS-DA, which reported a clear distinction among all producers (Figure 1D). The pH and protein content were reported as important parameters for such differentiation.

3.2. Changes in Free Fatty Acids

As shown in Table 1, the concentrations of total, unsaturated, saturated, short-, medium- and long-chain FFAs of Idiazabal cheeses increased throughout the ripening time ($p \leq 0.01$). Total FFAs varied from 12.0 ± 5.95 µmol/g in 1-day-old ripened cheeses to 49.9 ± 24.5 µmol/g in 120-day-old ripened cheeses (Table 1, Supplementary Figure S1). Along the whole process, saturated and short-chain FFAs predominated. Individually, a total of 21 FFAs were identified and C2, C4, C6 and C10 were the most abundant (Supplementary Table S3). A clear increase in the concentrations of all individual FFAs was observed during ripening time ($p \leq 0.05$), except for iC4, iC6 and 4-methyl-C8 ($p > 0.05$). That is why the HCA analysis reported two different clusters (Figure 2A). Clear differences were observed among producers, which, in general, increased as ripening progressed (Table 1, Supplementary Table S3).

Table 1. Mean concentration ± standard deviation (µmol/g) of total, unsaturated, saturated, short-, medium- and long-chain FFAs of Idiazabal cheese analysed from four producers (A, B, C and D) at six time points during ripening (1, 7, 14, 30, 60 and 120 days) (n = 48).

Producer	Ripening Time (Days)	Total FFAs	Unsaturated FFAs	Saturated FFAs	Short-Chain FFAs	Medium-Chain FFAs	Long-Chain FFAs
A	1	16.7 ± 4.36	0.331 ± 0.166	16.4 ± 4.19	15.4 ± 3.88	0.413 ± 0.0723	0.851 ± 0.412
	7	32.6 ± 2.26	0.481 ± 0.214	32.1 ± 2.05	30.6 ± 1.91	0.814 ± 0.00560	1.15 ± 0.342
	14	37.2 ± 4.92	0.486 ± 0.0122	36.7 ± 4.93	35.0 ± 4.64	1.04 ± 0.190	1.19 ± 0.0939
	30	41.2 ± 7.01	0.712 ± 0.0524	40.5 ± 6.96	38.1 ± 6.36	1.42 ± 0.429	1.66 ± 0.219
	60	56.4 ± 1.00	1.31 ± 0.497	55.1 ± 0.507	51.4 ± 0.463	2.10 ± 0.0919	2.81 ± 0.634
	120	77.8 ± 8.39	2.92 ± 0.434	74.9 ± 7.96	68.3 ± 6.96	3.50 ± 0.248	5.94 ± 1.18
B	1	6.04 ± 0.764	0.533 ± 0.0261	5.51 ± 0.738	4.47 ± 0.610	0.312 ± 0.0302	1.25 ± 0.124
	7	10.5 ± 2.51	0.584 ± 0.0288	9.93 ± 2.54	8.55 ± 2.01	0.465 ± 0.231	1.50 ± 0.267
	14	9.47 ± 0.508	0.474 ± 0.0623	8.99 ± 0.446	7.96 ± 0.563	0.321 ± 0.0689	1.19 ± 0.0141
	30	10.0 ± 0.431	0.655 ± 0.082	9.34 ± 0.513	8.21 ± 0.720	0.372 ± 0.0805	1.41 ± 0.209
	60	20.3 ± 10.6	0.831 ± 0.312	19.5 ± 10.3	17.9 ± 9.64	0.519 ± 0.207	1.90 ± 0.770
	120	18.1 ± 1.65	1.05 ± 0.0411	17.0 ± 1.61	15.3 ± 1.59	0.569 ± 0.0238	2.22 ± 0.0816
C	1	7.75 ± 1.30	0.227 ± 0.0409	7.52 ± 1.34	6.34 ± 1.65	0.476 ± 0.121	0.932 ± 0.23
	7	7.63 ± 1.96	0.273 ± 0.00353	7.36 ± 1.96	6.00 ± 1.70	0.568 ± 0.118	1.06 ± 0.145
	14	7.43 ± 1.61	0.240 ± 0.067	7.19 ± 1.55	5.92 ± 1.22	0.598 ± 0.148	0.913 ± 0.244
	30	16.3 ± 2.77	0.447 ± 0.0478	15.8 ± 2.72	14.0 ± 2.63	0.877 ± 0.00720	1.41 ± 0.142
	60	30.8 ± 4.16	0.512 ± 0.112	30.2 ± 4.05	27.8 ± 3.37	1.21 ± 0.366	1.70 ± 0.427
	120	52.1 ± 6.96	1.02 ± 0.0542	51.1 ± 7.01	47.2 ± 6.76	2.12 ± 0.123	2.83 ± 0.0777
D	1	17.5 ± 1.68	0.523 ± 0.132	17.0 ± 1.82	15.3 ± 1.72	0.719 ± 0.0749	1.51 ± 0.106
	7	21.2 ± 0.441	0.700 ± 0.107	20.5 ± 0.334	18.6 ± 0.173	0.816 ± 0.0865	1.72 ± 0.182
	14	22.6 ± 2.46	0.817 ± 0.188	21.8 ± 2.27	19.5 ± 2.23	1.09 ± 0.00966	2.03 ± 0.241
	30	36.1 ± 1.77	1.15 ± 0.427	35.0 ± 1.34	31.8 ± 1.26	1.60 ± 0.0392	2.74 ± 0.547
	60	38.4 ± 1.90	1.42 ± 0.179	37.0 ± 1.72	33.7 ± 1.53	1.73 ± 0.0656	3.02 ± 0.307
	120	51.6 ± 1.92	2.45 ± 0.0444	49.2 ± 1.87	43.4 ± 1.63	3.12 ± 0.145	5.04 ± 0.142
p-value [1]	RT	***	***	***	**	**	***
	P	***	**	***	***	***	*

[1] RT: ripening time factor effect; P: producer factor effect; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

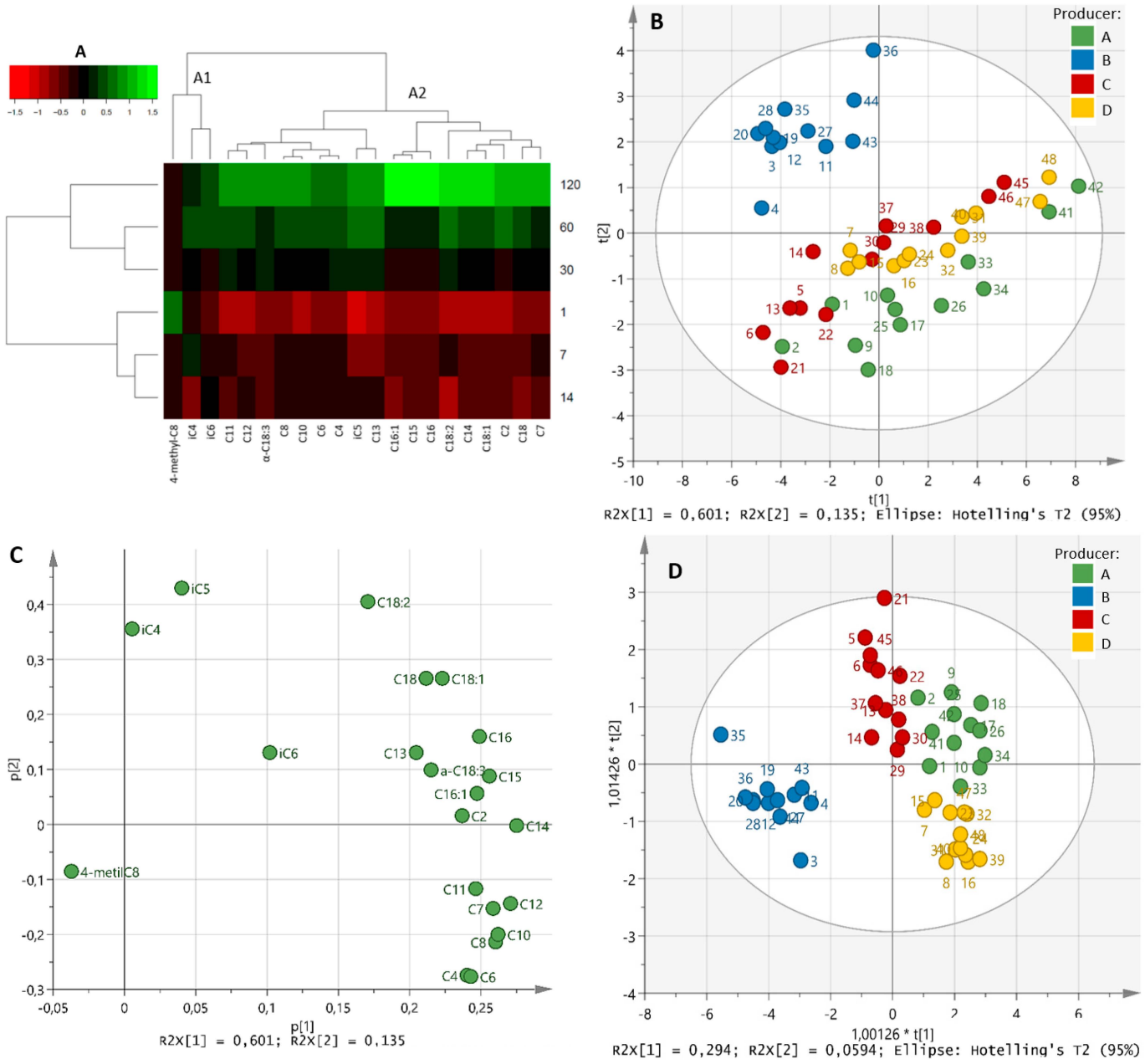


Figure 2. HCA heat map (**A**), scores and loadings plots of PCA (**B** and **C**, respectively) and scores plot of the OPLS-DA based on the producer of the identified FFAs during ripening (1, 7, 14, 30, 60 and 120 days) (**D**). The scale values of the HCA correspond to log-transformed and UV scaled data.

PERMANOVA confirmed that ripening time and producer factors influenced the lipolysis of Idiazabal cheeses ($p \leq 0.001$) (data not shown), although the ripening effect was greater (14.3 and 11.8, respectively). PCA revealed two PCs, accounting for 73.7% of the variance. PC1 values ranged from negative (for less ripened cheeses) to positive (for more ripened cheeses) (Figure 2B). Therefore, PC1 (accounting for 60.1% of the explained variance) was correlated to the ripening time. All FFAs, except 4-methyl-C8, iC4, iC5 and iC6, showed positive loadings in PC1, confirming an increase in their concentrations during the ripening time (Figure 2C). PC2 (accounting for 13.5% of the explained variance) was related to the producer, indicating a notable but lower impact than that of the ripening time. Differences among producers were confirmed by an OPLS-DA, which reported a clear differentiation between producer B, who used commercial rennet, and the rest that employed artisanal rennet (Figure 2D). C4, C6, C8, C10 and C12 were reported as important

FFAs for such differentiation. It is noteworthy that, although to a lesser extent, a distinction among the producers who used artisanal rennet (A, C and D) was also observed (Figure 2D).

3.3. Changes in Biogenic Amines

Of the 14 BAs analysed, only AABA, GABA, CAD, HIS, MEA, MMA, PUT and TYR were detected during the ripening of Idiazabal cheese (Figure 3 and Supplementary Table S4). Total BAs concentration varied from a mean of 4.29 ± 0.375 µmol/g at 1 day of ripening, to 14.9 ± 3.73 µmol/g at 120 days. As shown in Figure 3A, the concentration of most individual BAs increased during ripening (cluster A1). However, MEA presented a different trend, remaining constant at 0.836 ± 0.0573 µmol/g until 30 days of ripening and, in general, decreasing from that moment on (cluster A2). Individually, MMA was the most abundant (3.16 ± 0.323 µmol/g at 120 days of ripening), followed by PUT (2.72 ± 1.39 µmol/g), CAD (2.58 ± 0.380 µmol/g) and GABA (2.35 ± 1.12 µmol/g). The ripening time effect was significant for all BAs ($p \leq 0.01$), except for HIS ($p > 0.05$). Differences among producers were only observed for HIS ($p \leq 0.001$).

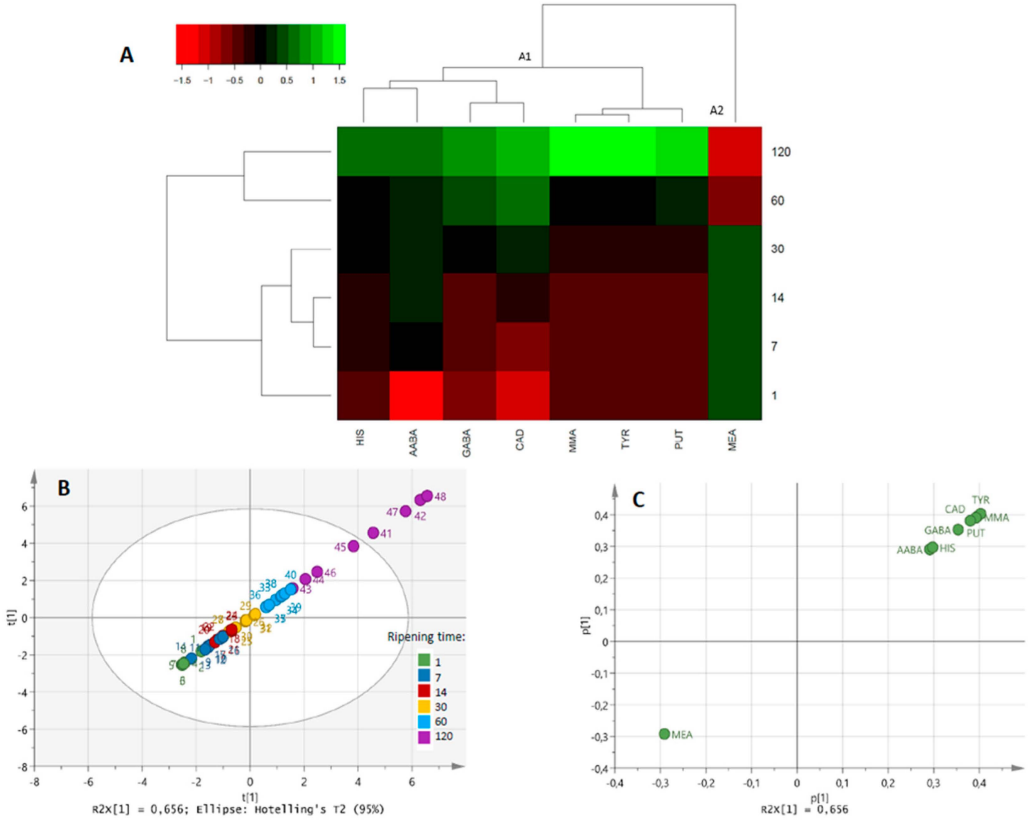


Figure 3. HCA (**A**) and scores and loadings plots of PCA (**B** and **C**, respectively) of the identified BAs during ripening (1, 7, 14, 30, 60 and 120 days) of Idiazabal cheese. The scale values of the HCA correspond to log-transformed and UV scaled data.

Overall, PERMANOVA confirmed that the ripening time had a significant effect on the BAs concentrations of the analysed Idiazabal cheeses ($p \leq 0.001$). The effect of the producer was not significant ($p > 0.05$) (data not shown). PCA revealed a unique PC accounting for 65.6% of the variance, which was highly correlated with the ripening time (Figure 3B). Consequently, samples were distributed from the third quadrant to the first one as the ripening progressed. All BAs, except MEA, were distributed together with the more ripened cheeses (Figure 3C), confirming the aforementioned dynamics. Nonetheless, a differentiation between the samples could be observed at 120 days of ripening (Figure 3B).

3.4. Correlation between Bacterial Dynamics and Physico-Chemical Parameters Evolution

From our previous publication, in general, it was observed that the starter LAB (SLAB) *Lactococcus* was predominant during ripening. However, after 30 or 60 days of ripening, its abundance decreased and the non-starter LAB (NSLAB) began to proliferate. Specifically, the proliferation of *Lactobacillus* was promoted in all producers (from a mean of 0.0949% at 1 day of ripening to 8.96% at 120 days), while for the rest it depended on the producer. That is, *Leuconostoc* proliferation was promoted in producer A (from 4.48% to 31.0%), while in producers A and D, *Streptococcus* and *Enterococcus* growth was promoted (from 0.750% to 4.52% and from 0.675% to 2.12%, respectively). On the other hand, the abundance of those bacteria classified as environmental or non-desirable decreased during ripening, except for some genera that remained abundant (>1%), namely *Obesumbacterium*, *Hafnia*, *Staphylococcus*, *Buttiauxella*, *Psychrobacter*, *Raoultella*, *Serratia*, *Brevibacterium* and *Erwinia*. Among these genera, the abundance of *Buttiauxella* decreased during ripening, while that of *Staphylococcus* increased and the rest showed an increase at intermediate points (at 7, 14 or 30 days of ripening). More details can be found in Santamarina-García et al. [12].

An O2PLS approach with Spearman's rank correlation coefficients was applied to analyse the relationship between the identified main bacterial genera and the evolution of gross composition, FFAs and BAs during ripening. The key functional microbiota was identified based on: (i) the VIP value of main bacterial genera higher than 1; (ii) the loading weights of main bacterial genera and (iii) the significant ($p \leq 0.05$) Spearman's rank correlation coefficients, which were interpreted as follows: $|\rho| < 0.500$, low correlation; $0.500 \leq |\rho| < 0.600$, moderate correlation; $0.600 \leq |\rho| < 0.700$, high correlation; and $|\rho| \geq 0.700$, strong correlation. It is noteworthy that many factors influence the physico-chemical parameters of Idiazabal cheese, such as the raw milk or the rennet used. Hence, the coefficients obtained are not as high as those reported for other fermented foods where microbial metabolisms are solely responsible [31,64].

3.4.1. Correlation between Main Bacterial Genera and Gross Composition

The key bacterial genera related to gross composition evolution were *Lactobacillus*, *Psychrobacter*, *Erwinia*, *Enterococcus*, *Pseudomonas*, *Pantoea* and *Streptococcus* (Figure 4A). The HCA analysis divided these bacteria into two clusters: the A1, which includes lactic acid bacteria (LAB) that were positively correlated to gross composition parameters; and A2, which includes the environmental or non-desirable genera that were negatively correlated. Within LAB, *Lactobacillus* presented the greatest positive correlations, especially with protein ($\rho = 0.842$), Ca ($\rho = 0.809$), P ($\rho = 0.805$) and Mg content ($\rho = 0.781$) ($p \leq 0.01$). Moreover, *Lactobacillus* was the unique LAB that presented a strong positive correlation with NaCl ($\rho = 0.709$, $p \leq 0.01$), whereas *Streptococcus* and *Enterococcus* were correlated to the pH ($\rho \geq 0.528$, $p \leq 0.01$). On the other hand, environmental and non-desirable bacteria showed diverse negative correlations, but all were negatively correlated with NaCl ($\rho \leq -0.531$, $p \leq 0.01$). In general, *Psychrobacter* was the most negatively correlated bacteria, with strong negative correlations with protein ($\rho = -0.762$), Ca ($\rho = -0.750$), Mg ($\rho = -0.747$), P ($\rho = -0.718$) and dry matter content ($\rho = -0.705$) ($p \leq 0.01$). Overall, pH and fat content were the parameters least related to bacterial genera. Through a CCorA analysis, the aforementioned correlations were confirmed (Supplementary Figure S2).

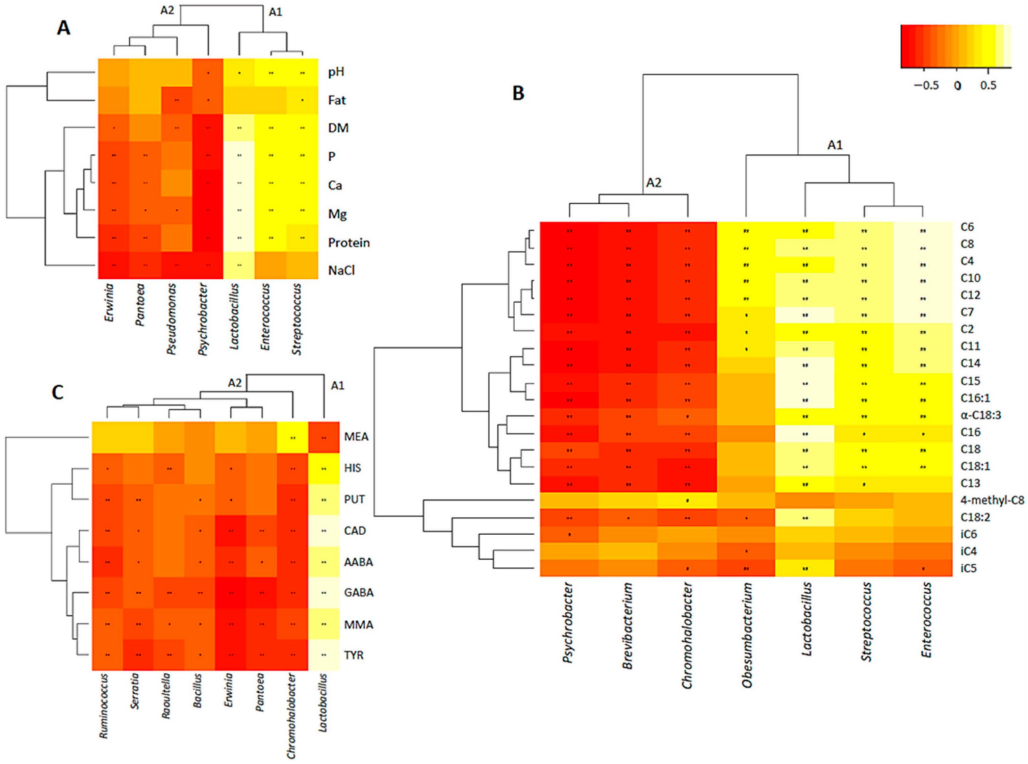


Figure 4. Spearman's rank correlations with HCA analysis between key bacterial genera and gross composition parameters (**A**), FFAs (**B**) and BAs (**C**). Significant correlations are represented by ** $p \leq 0.01$ and * $p \leq 0.05$.

3.4.2. Correlation between Main Bacterial Genera and FFAs

Seven bacterial genera were reported as the key microbiota related to FFAs evolution, namely, *Psychrobacter*, *Brevibacterium*, *Lactobacillus*, *Enterococcus*, *Chromohalobacter*, *Streptococcus* and *Obesumbacterium* (Figure 4B). As for gross composition, the HCA analysis reported two clusters: A1 grouped those LAB and the environmental *Obesumbacterium* that were positively correlated to most FFAs concentrations and A2 those non-desirable and environmental bacteria that were negatively correlated. Within LAB, *Lactobacillus* and *Enterococcus* showed the highest correlations with FFAs concentrations (Figure 4B). *Enterococcus* showed strong correlations with short-chain FFAs, such as C4 (ρ = 0.819), C6 (ρ = 0.800), C8 (ρ = 0.780) and C10 (ρ = 0.762) ($p \leq 0.01$). *Lactobacillus* presented the strongest correlations with C7 (ρ = 0.718) and medium- and long-chain FFAs, such as C14 (ρ = 0.745), C15 (ρ = 0.729) and C16 (ρ = 0.762) ($p \leq 0.01$). Within environmental and non-desirable bacteria, *Obesumbacterium* showed positive correlations with short-chain FFAs, such as C4 (ρ = 0.534) and C6 (ρ = 0.502) ($p \leq 0.01$), while the rest presented several negative correlations ($p \leq 0.01$). As for gross composition, *Psychrobacter* was reported as the most negatively related. It showed strong negative correlations with C4 (ρ = –0.759), C6 (ρ = –0.765), C8 (ρ = –0.757) and C11 (ρ = –0.762) ($p \leq 0.01$), for example. In addition, iC4, iC5, iC6 and 4-methyl-C8 were the FFAs least related to bacterial genera. These correlations were confirmed by means of a CCorA model (Supplementary Figure S3).

3.4.3. Correlation between Main Bacterial Genera and BAs

Eight bacterial genera were reported as key bacteria related to BAs evolution, namely, *Lactobacillus*, *Erwinia*, *Chromohalobacter*, *Pantoea*, *Bacillus*, *Ruminococcus*, *Serratia* and *Raoultella* (Figure 4C). *Lactobacillus* was the only genus that presented several positive correlations, so it formed a single cluster (A1) differentiated from the rest that were mainly negatively related (A2). Individually, *Lactobacillus* was strongly correlated with the concentrations of TYR (ρ = 0.865), GABA (ρ = 0.840) and CAD (ρ = 0.752), and to a lesser extent, AABA (ρ = 0.729)

and MMA (ρ = 0.704) ($p \leq 0.01$) (Figure 4C). Within environmental and non-desirable bacteria, *Chromohalobacter* presented a high positive correlation with MEA (ρ = 0.625, $p \leq 0.01$) and the rest showed only several negative correlations. *Erwinia* was reported as the most negatively related, mainly with GABA (ρ = –0.768), MMA (ρ = –0.651), CAD (ρ = –0.643) and TYR (ρ = –0.635) ($p \leq 0.01$). In general, MEA was the BA correlated with fewer bacterial genera. The CCorA model confirmed the observed correlations (Supplementary Figure S4).

4. Discussion

It is well known that the microbiota inhabiting cheese affects the quality and safety of the final product [65]. In recent years, several studies have been published focused on elucidating the relationship between the microbiota and the production of metabolites, such as volatile compounds or FAAs, in fermented foods [38,39,66], including cheese [32,67]. However, only a few studies have analysed raw ewe milk-derived cheeses [46–48]. The present study aimed to elucidate the relationship between bacterial communities characterized by HTS and the evolution of gross composition, FFAs and BAs during the ripening of raw ewe milk-derived Idiazabal cheese. To the best of our knowledge, this approach has not been applied so far for any type of cheese.

Within the gross composition, the decrease in the pH until 30 days of ripening and the subsequent increase, together with the concentration of the rest of the parameters during ripening, is in agreement with what has been previously reported for Idiazabal cheese [19,50,68,69]. However, it is noteworthy that the evolution of Ca, Mg and P content during ripening has not been studied until now, only Aldalur et al. [70] have reported an increase after pressing up to two months of ripening. Through HTS analysis, it has been observed that the starter *Lactococcus* remains dominant until the first month of ripening, but from that moment on other bacteria proliferate, mainly NSLAB [12]. Therefore, the lactic acid production by *Lactococcus* could lead to a decrease in the pH until 30 days of ripening, but the subsequent catabolism of lactate and amino acids by other microorganisms, such as NSLAB, would favour the aforementioned increase [18].

Compared to other raw ewe milk-derived cheeses, clear differences can be observed in gross composition [71,72]. Anyway, notable differences were observed even among the Idiazabal cheese samples elaborated by different producers, with pH and protein as the most differentiating parameters. This differentiation could be due to several reasons, such as differences in milk composition [73,74] or different cheese-making and ripening conditions [54,70]. For instance, Aldalur et al. [75] have reported differences in cooking pH and whey-draining pH between Idiazabal cheese producers. The type of rennet used or the concentration of coagulant enzyme employed have also been described as affecting the gross composition of cheese, including pH or protein content [76–78]. This could also explain the differences observed among producers, although it should be further studied in Idiazabal cheese.

Changes in the gross composition of cheese during ripening have been described as influencing the bacterial succession that takes place [18,79]. Based on the results obtained, gross composition evolution promoted LAB proliferation in Idiazabal cheese, especially that of *Lactobacillus* and to a lesser extent, that of *Streptococcus* and *Enterococcus*. However, the growth of environmental or non-desirable bacteria was negatively affected, mainly, that of *Psychrobacter* and to a lesser extent, that of *Erwinia*, *Pseudomonas* and *Pantoea*. In general, this would confirm that the evolution of the gross composition determines the bacterial dynamics during ripening, as previously proposed [12]. Specifically, the NaCl concentrations used are considered an important factor that controls the microbiological quality of cheese [18] and the results obtained could indicate that it is the most limiting factor for the proliferation of environmental and non-desirable bacteria in Idiazabal cheese. Within LAB, NaCl tolerance varies among genera and species. *Streptococcus* are able to grow until 2.5% of NaCl, whereas *Enterococcus* and some *Lactobacillus* species can grow up to 6.5% [18,80]. Consequently, the results obtained could indicate a great abundance of halotolerant *Lactobacillus* species and could explain why in a previous study for these

same cheese samples the ripening time effect was only significant for this LAB [12]. On the other hand, *Streptococcus* and *Enterococcus* showed the highest positive correlations with the pH, confirming the previously observed succession between SLAB and NSLAB [12]. These correlations have also been described in other fermented products, although clear differences exist [81–83]. For the Suan zuo rou fermented meat, Wang et al. [82] have reported a positive correlation between *Lactobacillus* and pH and salt content, and a negative relation between pH and *Psychrobacter*, in line with the results of this study.

Regarding lipolysis, the concentration of most FFAs increased during ripening and saturated and short-chain FFAs predominated, which agrees with previous studies in Idiazabal cheese [19,69,84–86]. Individually, the predominance of C2, C4, C6 or C10 is also in accordance with what has been reported before [19,56,85,87]. Nevertheless, 4-methyl-C8, iC4 and iC6 were identified for the first time in Idiazabal cheese, as Amores et al. [88] have reported for iC5, C7, C11, C13 and C15. There are differences in the lipolysis process during ripening or in the predominant FFAs compared to other raw ewe milk-derived cheeses [89–91]. For instance, Esmaeilzadeh et al. [89] have reported a higher abundance of long-chain FFAs than short- or medium-chain FFAs during ripening of Kope cheese.

Clear differences were also observed in the lipolysis of Idiazabal cheese between producers, which could be due to several reasons. The use of artisanal rennet resulted in, mainly, higher concentrations of short- and medium-chain FFAs, with C4, C6, C8, C10 and C12 as characteristic. These observations agree with previous studies [19,69,85,86,92]. The lamb rennet employed for Idiazabal cheese production contains higher rennet pregastric lipase activity than commercial rennet [19]. This lipase has *sn*-3 stereospecificity and therefore, releases preferentially short-chain fatty acids since they are esterified on the *sn*-3 position of the TG of milk fat [17,88]. The lipase activity in artisanal rennets is variable among Idiazabal cheese producers [88], which could lead to differences in the concentrations of most individual FFAs in cheeses from different dairies. On the other hand, the LPL of raw milk is also an important lipolytic agent in raw milk cheeses, which releases preferentially short- and medium-chain fatty acids due to *sn*-1 and *sn*-3 stereospecificity [17]. However, LPL activity is very low in Idiazabal cheese [93]. For Friesian cross-bred sheep, it has been observed that an undernourishment or overfeeding of the herd affects the relative transcription accumulation of the genes involved in LPL biosynthesis [94].

Microbial lipases and esterases are considered important lipolytic agents in cheese [17,18]. The results obtained in the present study indicate that *Lactobacillus*, *Enterococcus* and *Streptococcus* are important lipolytic LAB in Idiazabal cheese. *Lactobacillus* was related to the release of medium- and long-chain FFAs, that is to say, it could present high lipase activity; whereas *Enterococcus* could present high esterase activity, as it was related to the release of short-chain FFAs [17,95]. Several lipases and esterases have been characterized from *Lactobacillus*, *Streptococcus* and *Enterococcus* species [96–100], which would support the results obtained. Nonetheless, no information has been found on the correlation between bacterial communities and the FFAs profile of fermented products, which has been more analysed with volatile or organic acids [40,41,46,101]. Furthermore, considering the importance that FFAs have in the sensory properties of Idiazabal cheese [19,85,86] and the differences observed in LAB composition among producers [12], these results could indicate an important role of LAB in sensory differentiation between producers that should be worth studying in depth.

Within environmental and non-desirable bacteria, the results obtained indicate an interesting lipolytic activity of *Obesumbacterium* that has not been described so far. This would make sense, since lipolytic activity has been previously described for other environmental or non-desirable bacteria, such as *Flavobacterium* or *Pseudomonas* [16,102–104]. Several negative correlations were observed between FFAs and environmental or non-desirable bacteria. FFAs can act as high-spectrum antimicrobial agents, comparable even with antimicrobial peptides [105–107]. Therefore, the results obtained could indicate that the release of FFAs in Idiazabal cheese could be part of the competitive inhibition mechanisms among bacteria. LAB could be the main bacterial genera responsible for the FFAs release and could have an

inhibitory effect against environmental and non-desirable bacteria, mainly *Psychrobacter*, *Brevibacterium* and *Chromohalobacter*. This competitive inhibition mechanism could be one of the reasons why most environmental and non-desirable bacteria are inhibited during the first weeks of ripening [12]. Nonetheless, these correlations could also indicate the metabolic use of FFAs by *Psychrobacter*, *Brevibacterium* and *Chromohalobacter*, which has been little studied so far [108].

The increase in the total concentrations of BAs and the predominance of MMA, PUT, CAD and GABA during ripening partially agree with what has been reported for Idiazabal cheese [109] and other raw ewe milk-derived cheeses [110–112], since there are differences in the identified BAs and/or in their evolution during ripening. For instance, Ordóñez et al. [109] have identified isopenty1amine, spermidine, phenylethylamine and tryptamine in Idiazabal cheese and Tofalo et al. [110] have not identified HIS in Pecorino di Farindola cheese. The differences observed compared to previous studies and other raw ewe milk-derived cheeses, could be attributed, apart from the different BAs analysed, to the microbiota or to the different cheese-making or ripening parameters used by each producer [113,114]. Within the predominant BAs detected, information about MMA is scarce, but it has been described as harmful to livestock in high concentrations [115]. PUT and CAD, which are widely known toxic BAs, are in general below the limits considered toxic in cheese [26]. Finally, GABA predominance is of special interest due to its several beneficial effects, such as modulating sleep disorders, temporal and spatial memory [116], epilepsy [117], diabetes [118], depression [119] or cancer [120]. It is noteworthy that spermidine and spermine were not detected, which are endogenous BAs formed inherently by animals, plants or microorganisms and that are important for several physiological functions, such as neurotransmitter, vasoactive or regulating gene expression [24]. This could indicate that the identified BAs in Idiazabal cheese were mainly produced by bacterial decarboxylation [24]. It is well known that during cheese ripening there is an accumulation of FAAs as a result of secondary proteolysis [18], which has been observed for Idiazabal cheese [85,121] and other ewe milk-derived cheeses [110,122]. These FAAs serve as substrates for bacterial and/or endogenous decarboxylases, leading to an accumulation of BAs [123–125].

In terms of the functional relationship between bacterial communities and BAs production, *Lactobacillus* was strongly positively correlated with most of the identified BAs, while *Chromohalobacter* was positively correlated with MEA. SLAB, NSLAB and other microorganisms have been reported as decarboxylase-producing bacteria [28,126], which has been proven for several *Lactobacillus* strains, for example, *L. acidophilus* PNW3 [127–130], including halotolerant *Lactobacillus* species [131,132]. Body et al. [133] have reported the higher the salt concentration (up to 3%) the higher BAs production by *L. reuteri* strains, which is consistent with the results obtained in the present study. For other fermented foods, positive correlations between *Lactobacillus* and different BAs have also been reported, such as TYR, PUT or HIS [134,135], but results differ according to the product [136]. For *Chromohalobacter*, Jung et al. [137] have reported a positive correlation with PUT that would support the decarboxylase activity of this genus, but no information related to MEA has been found.

On the other hand, *Erwinia*, *Pantoea*, *Serratia*, *Ruminococcus*, *Bacillus*, *Raoultella* and also, *Chromohalobacter* and to a lesser extent *Lactobacillus*, showed some negative correlations with BAs, which could be related to BAs degradation abilities [138]. Although not for cheese, negative correlations have also been reported for other fermented products, for example, between *Erwinia* and HIS or *Pantoea* and TYR during spontaneous fermentation of pickled mustard tubers [134,135]. Nonetheless, BAs degradation ability has only been demonstrated for *Lactobacillus* and *Bacillus* species [139–142].

It is worth noting that the SLAB *Lactococcus*, which is the predominant genera during ripening [12], was not reported as key bacteria for any of the physico-chemical parameters studied. In other words, the evolution of the cheese quality and safety parameters studied

would be related to the autochthonous microbiota of raw milk and not to the starter bacteria. Overall, these results could suppose interesting novel insights into dairy microbiology.

5. Conclusions

The present study investigated the dynamics of gross composition, FFAs and BAs and their relationship with bacterial communities during the ripening of raw ewe milk-derived Idiazabal cheese. All gross parameters (DM, protein, fat, Ca, Mg, P, and NaCl) except pH, FFAs and BAs showed an increasing trend during ripening. However, the producer also affected the gross composition and FFAs. In terms of functional relationships, *Lactobacillus*, *Streptococcus* and *Enterococcus* were positively correlated to gross composition and FFAs. This could indicate that their proliferation was favoured during ripening and that they were the main bacterial genera responsible for FFAs release, which could be important due to their aromatic impact. *Lactobacillus* was the unique LAB positively related to BAs production. Within environmental and non-desirable bacteria, 11 genera were reported as negatively correlated to gross composition, FFAs and BAs. This could be related, respectively, to the negative effect of gross composition evolution during ripening on their proliferation, to the antimicrobial effect of FFAs and/or the metabolic use of FFAs by these genera, and to BAs degradation capacities. Nonetheless, *Obesumbacterium* and *Chromohalobacter* were positively related to FFAs and BAs formation, respectively. Overall, this study presents novel knowledge to help understand the possible functional relationship between cheese microbiota and several physico-chemical parameters related to cheese quality and safety.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12223224/s1, Figure S1: Total concentrations of FFAs (μmol/g of cheese) accumulated during ripening time of Idiazabal cheese from four producers (A, B, C and D). Error bars represent the standard deviation of the measurements; Figure S2: CCorA analysis between key bacterial genera and gross composition parameters; Figure S3: CCorA analysis between key bacterial genera and FFAs; Figure S4: CCorA analysis between key bacterial genera and BAs; Table S1: Followed program for the separation of biogenic amines (BAs) from raw ewe milk-derived Idiazabal cheese samples (n = 48); Table S2: Mean concentration ± standard deviation of physico-chemical properties of the analysed Idiazabal cheeses from four producers (A, B, C and D) at six time points during ripening (1, 7, 14, 30, 60 and 120 days) (n = 48); Table S3: Mean concentration ± standard deviation (μmol/g) of individual FFAs identified in Idiazabal cheeses from four producers (A, B, C and D) at six time points during ripening (1, 7, 14, 30, 60 and 120 days) (n = 48); Table S4: Mean concentration ± standard deviation (μmol/g) of total and individual BAs identified in Idiazabal cheeses from four producers (A, B, C and D) at six time points during ripening (1, 7, 14, 30, 60 and 120 days) (n = 48).

Author Contributions: Conceptualization, G.S.-G., G.A., I.H. and M.V.; methodology, G.S.-G., G.A., E.L.d.A. and M.V.; software, G.S.-G.; validation, G.S.-G., G.A., E.L.d.A. and M.V.; formal analysis, G.S.-G. and G.A.; investigation, G.S.-G., G.A., E.L.d.A. and M.V.; resources, G.S.-G., G.A., I.H. and M.V.; data curation, G.S.-G., G.A. and E.L.d.A.; writing—original draft preparation, G.S.-G.; writing—review and editing, G.A., I.H. and M.V.; visualization, G.S.-G.; supervision, G.A. and M.V.; project administration, M.V.; funding acquisition, M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Basque Government, grant number IT944-16. G. Santamarina-García received a predoctoral grant from the University of the Basque Country (UPV/EHU).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank the collaboration of Idiazabal PDO cheesemakers and the technical and human support provided by SGIker (UPV/EHU/ ERDF, EU).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Official Journal of the European Communities. Reglamento (CE) N° 1107/96 de La Comisión de 12 de Junio de 1996 Relativo Al Registro de Las Indicaciones Geográficas y de Las Denominaciones de Origen Con Arreglo Al Procedimiento Establecido En El Artículo 17 Del Reglamento (CEE) No 2081/92 Del Consejo. *Off. J. Eur. Union* **1996**, *148*, 1–10.
2. Ordoñez, A.I.; Ibañez, F.C.; Torre, P.; Barcina, Y.; Pérez-Elortondo, F.J. Application of multivariate analysis to sensory characterization of ewes' milk cheese. *J. Sens. Stud.* **1998**, *13*, 45–55. [CrossRef]
3. Grappin, R.; Beuvier, E. Possible Implications of Milk Pasteurization on the Manufacture and Sensory Quality of Ripened Cheese. *Int. Dairy J.* **1997**, *7*, 751–761. [CrossRef]
4. Leroy, F.; De Vuyst, L. Lactic Acid Bacteria as Functional Starter Cultures for the Food Fermentation Industry. *Trends Food Sci. Technol.* **2004**, *15*, 67–78. [CrossRef]
5. Barron, L.J.R.; Redondo, Y.; Aramburu, M.; Gil, P.; Pérez-Elortondo, F.J.; Albisu, M.; Nájera, A.I.; de Renobales, M.; Fernández-García, E. Volatile Composition and Sensory Properties of Industrially Produced Idiazabal Cheese. *Int. Dairy J.* **2007**, *17*, 1401–1414. [CrossRef]
6. Madrau, M.A.; Mangia, N.P.; Murgia, M.A.; Sanna, M.G.; Garau, G.; Leccis, L.; Caredda, M.; Deiana, P. Employment of Autochthonous Microflora in Pecorino Sardo Cheese Manufacturing and Evolution of Physicochemical Parameters during Ripening. *Int. Dairy J.* **2006**, *16*, 876–885. [CrossRef]
7. Ballesteros, C.; Poveda, J.M.; González-Viñas, M.A.; Cabezas, L. Microbiological, Biochemical and Sensory Characteristics of Artisanal and Industrial Manchego Cheeses. *Food Control* **2006**, *17*, 249–255. [CrossRef]
8. Ortigosa, M.; Arizcun, C.; Oneca, M.; Irigoyen, A.; Torre, P. Effects of Pasteurisation on Microorganisms and Sensory Attributes in Roncal Type Cheese from Ewe's Milk. *Milchwissenschaft* **2004**, *59*, 392–396.
9. Rehman, S.U.; Banks, J.M.; Brechany, E.Y.; Muir, D.D.; McSweeney, P.L.H.; Fox, P.F. Influence of Ripening Temperature on the Volatiles Profile and Flavour of Cheddar Cheese Made from Raw or Pasteurised Milk. *Int. Dairy J.* **2000**, *10*, 55–65. [CrossRef]
10. Balkir, P.; Kemahlioglu, K.; Yucel, U. Foodomics: A New Approach in Food Quality and Safety. *Trends Food Sci. Technol.* **2021**, *108*, 49–57. [CrossRef]
11. Rizal, N.S.M.; Neoh, H.M.; Ramli, R.; Periyasamy, P.R.A.L.K.; Hanafiah, A.; Samat, M.N.A.; Tan, T.L.; Wong, K.K.; Nathan, S.; Chieng, S.; et al. Advantages and Limitations of 16S RRNA Next-Generation Sequencing for Pathogen Identification in the Diagnostic Microbiology Laboratory: Perspectives from a Middle-Income Country. *Diagnostics* **2020**, *10*, 816. [CrossRef] [PubMed]
12. Santamarina-García, G.; Hernández, I.; Amores, G.; Virto, M. Characterization of Microbial Shifts during the Production and Ripening of Raw Ewe Milk-Derived Idiazabal Cheese by High-Throughput Sequencing. *Biology* **2022**, *11*, 769. [CrossRef]
13. Bertuzzi, A.S.; McSweeney, P.L.H.; Rea, M.C.; Kilcawley, K.N. Detection of Volatile Compounds of Cheese and Their Contribution to the Flavor Profile of Surface-Ripened Cheese. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 371–390. [CrossRef] [PubMed]
14. Afshari, R.; Pillidge, C.J.; Read, E.; Rochfort, S.; Dias, D.A.; Osborn, A.M.; Gill, H. New Insights into Cheddar Cheese Microbiota-Metabolome Relationships Revealed by Integrative Analysis of Multi-Omics Data. *Sci. Rep.* **2020**, *10*, 1–13. [CrossRef]
15. McSweeney, P.L.H.; Sousa, M.J. Biochemical Pathways for the Production of Flavour Compounds in Cheeses during Ripening: A Review. *Lait* **2000**, *80*, 293–324. [CrossRef]
16. Collins, Y.F.; McSweeney, P.L.H.; Wilkinson, M.G. Lipolysis and Free Fatty Acid Catabolism in Cheese: A Review of Current Knowledge. *Int. Dairy J.* **2003**, *13*, 841–866. [CrossRef]
17. Thierry, A.; Collins, Y.F.; Mukdsi, M.C.A.; McSweeney, P.L.H.; Wilkinson, M.G.; Spinnler, H.E. Lipolysis and Metabolism of Fatty Acids in Cheese. In *Cheese: Chemistry, Physics, and Microbiology*; McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds.; Academic Press: San Diego, CA, USA, 2017; pp. 423–444.
18. Fox, P.F.; Guinee, T.P.; Cogan, T.M.; McSweeney, P.L.H. *Fundamentals of Cheese Science*, 2nd ed.; Springer: New York, NT, USA, 2017; pp. 1–803.
19. Virto, M.; Chávarri, F.; Bustamante, M.A.; Barron, L.J.R.; Aramburu, M.; Vicente, M.S.; Pérez-Elortondo, F.J.; Albisu, M.; de Renobales, M. Lamb Rennet Paste in Ovine Cheese Manufacture. Lipolysis and Flavour. *Int. Dairy J.* **2003**, *13*, 391–399. [CrossRef]
20. Ardö, Y.; McSweeney, P.L.H.; Magboul, A.A.A.; Upadhyay, V.K.; Fox, P.F. Biochemistry of Cheese Ripening: Proteolysis. In *Cheese: Chemistry, Physics and Microbiology*, 4th ed.; McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds.; Elsevier Inc.: London, UK, 2017; Volume 1, pp. 445–482.
21. Fernández, M.; Hudson, J.A.; Korpela, R.; De Los Reyes-Gavilán, C.G. Impact on Human Health of Microorganisms Present in Fermented Dairy Products: An Overview. *Biomed. Res. Int.* **2015**, *2015*, 1–13. [CrossRef] [PubMed]
22. Şanli, T.; Şenel, E. Chapter 27: Formation of Biogenic Amines in Cheese. In *Processing and Impact on Active Components in Food*; Preedy, V., Ed.; Elsevier Inc.: London, UK, 2015; pp. 223–230.
23. Spano, G.; Russo, P.; Lonvaud-Funel, A.; Lucas, P.; Alexandre, H.; Grandvalet, C.; Coton, E.; Coton, M.; Barnavon, L.; Bach, B.; et al. Biogenic Amines in Fermented Foods. *Eur. J. Clin. Nutr.* **2010**, *64*, S95–S100. [CrossRef] [PubMed]
24. Ruiz-Capillas, C.; Herrero, A.M. Impact of Biogenic Amines on Food Quality and Safety. *Foods* **2019**, *8*, 62. [CrossRef] [PubMed]
25. de Almeida Møller, C.O.; Ücok, E.F.; Rattray, F.P. Histamine Forming Behaviour of Bacterial Isolates from Aged Cheese. *Food Res. Int.* **2020**, *128*, 108719. [CrossRef] [PubMed]

26. Özogul, Y.; Özogul, F. Chapter 1: Biogenic Amines Formation, Toxicity, Regulations in Food. In *Food Chemistry, Function and Analysis*; Saad, B., Tofalo, R., Eds.; Royal Society of Chemistry: London, UK, 2020; pp. 1–17.
27. Linares, D.M.; MartÍn, M.C.; Ladero, V.; Alvarez, M.A.; Fernández, M. Biogenic Amines in Dairy Products. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 691–703. [CrossRef] [PubMed]
28. Barone, C.; Barbera, M.; Barone, M.; Parisi, S.; Zaccheo, A. Biogenic Amines in Cheeses: Types and Typical Amounts. In *Chemical Evolution of Nitrogen-Based Compounds in Mozzarella Cheeses*; Barone, C., Barbera, M., Barone, M., Parisi, S., Zaccheo, A., Eds.; Springer: Cham, Switzerland, 2018; pp. 1–18.
29. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on Risk Based Control of Biogenic Amine Formation in Fermented Foods. *EFSA J.* **2011**, *9*, 1–93.
30. Mah, J.H.; Park, Y.K.; Jin, Y.H.; Lee, J.H.; Hwang, H.J. Bacterial Production and Control of Biogenic Amines in Asian Fermented Soybean. *Foods* **2019**, *8*, 85. [CrossRef] [PubMed]
31. Zheng, X.; Liu, F.; Shi, X.; Wang, B.; Li, K.; Li, B.; Zhuge, B. Dynamic Correlations between Microbiota Succession and Flavor Development Involved in the Ripening of Kazak Artisanal Cheese. *Food Res. Int.* **2018**, *105*, 733–742. [CrossRef]
32. Zheng, X.; Ge, Z.; Lin, K.; Zhang, D.; Chen, Y.; Xiao, J.; Wang, B.; Shi, X. Dynamic Changes in Bacterial Microbiota Succession and Flavour Development during Milk Fermentation of Kazak Artisanal Cheese. *Int. Dairy J.* **2021**, *113*, 104878. [CrossRef]
33. Wang, L.; Zhong, K.; Luo, A.; Chen, J.; Shen, Y.; Wang, X.; He, Q.; Gao, H. Dynamic Changes of Volatile Compounds and Bacterial Diversity during Fourth to Seventh Rounds of Chinese Soy Sauce Aroma Liquor. *Food Sci. Nutr.* **2021**, *9*, 3500–3511. [CrossRef] [PubMed]
34. Trygg, J.; Wold, S. O2-PLS, a two-block (X-Y) latent variable regression (LVR) method with an integral OSC filter. *J. Chemometr.* **2003**, *17*, 53–64. [CrossRef]
35. El Bouhaddani, S.; Houwing-Duistermaat, J.; Salo, P.; Perola, M.; Jongbloed, G.; Uh, H.-W. Evaluation of O2PLS in Omics Data Integration. *BMC Bioinforma.* **2016**, *17*, 117–132. [CrossRef]
36. Eriksson, L.; Toft, M.; Johansson, E.; Wold, S.; Trygg, J. Separating Y-Predictive and Y-Orthogonal Variation in Multi-Block Spectral Data. *J. Chemom.* **2006**, *20*, 352–361. [CrossRef]
37. Trygg, J. O2-PLS for Qualitative and Quantitative Analysis in Multivariate Calibration. *J. Chemom.* **2002**, *16*, 283–293. [CrossRef]
38. Zhao, D.; Hu, J.; Chen, W. Analysis of the Relationship between Microorganisms and Flavour Development in Dry-Cured Grass Carp by High-Throughput Sequencing, Volatile Flavour Analysis and Metabolomics. *Food Chem.* **2022**, *368*, 130889. [CrossRef] [PubMed]
39. Zang, J.; Xu, Y.; Xia, W.; Regenstein, J.M.; Yu, D.; Yang, F.; Jiang, Q. Correlations between Microbiota Succession and Flavor Formation during Fermentation of Chinese Low-Salt Fermented Common Carp (*Cyprinus Carpio* L.) Inoculated with Mixed Starter Cultures. *Food Microbiol.* **2020**, *90*, 103487. [CrossRef] [PubMed]
40. Xu, X.; Wu, B.; Zhao, W.; Pang, X.; Lao, F.; Liao, X.; Wu, J. Correlation between Autochthonous Microbial Communities and Key Odorants during the Fermentation of Red Pepper (*Capsicum Annuum* L.). *Food Microbiol.* **2020**, *91*, 103510. [CrossRef] [PubMed]
41. Che, H.; Yu, J.; Sun, J.; Lu, K.; Xie, W. Bacterial Composition Changes and Volatile Compounds during the Fermentation of Shrimp Paste: Dynamic Changes of Microbial Communities and Flavor Composition. *Food Biosci.* **2021**, *43*, 101169. [CrossRef]
42. Kubinyi, H. *3D QSAR in Drug Design: Volume 1: Theory Methods and Applications*; Springer: Berlin, Germany, 2000; pp. 1–759.
43. Galindo-Prieto, B.; Eriksson, L.; Trygg, J. Variable Influence on Projection (VIP) for Orthogonal Projections to Latent Structures (OPLS). *J. Chemom.* **2014**, *28*, 623–632. [CrossRef]
44. Unno, R.; Suzuki, T.; Matsutani, M.; Ishikawa, M. Evaluation of the Relationships Between Microbiota and Metabolites in Soft-Type Ripened Cheese Using an Integrated Omics Approach. *Front. Microbiol.* **2021**, *12*, 681185. [CrossRef]
45. Bertuzzi, A.S.; Walsh, A.M.; Sheehan, J.J.; Cotter, P.D.; Crispie, F.; McSweeney, P.L.H.; Kilcawley, K.N.; Rea, M.C. Omics-Based Insights into Flavor Development and Microbial Succession within Surface-Ripened Cheese. *mSystems* **2018**, *3*, e00211–e00217. [CrossRef]
46. Cardinali, F.; Ferrocino, I.; Milanović, V.; Belleggia, L.; Corvaglia, M.R.; Garofalo, C.; Foligni, R.; Mannozzi, C.; Mozzon, M.; Cocolin, L.; et al. Microbial Communities and Volatile Profile of Queijo de Azeitão PDO Cheese, a Traditional Mediterranean Thistle-Curdled Cheese from Portugal. *Food Res. Int.* **2021**, *147*, 110537. [CrossRef]
47. De Pasquale, I.; Calasso, M.; Mancini, L.; Ercolini, D.; La Storia, A.; De Angelis, M.; Di Cagno, R.; Gobbetti, M. Causal Relationship between Microbial Ecology Dynamics and Proteolysis during Manufacture and Ripening of Protected Designation of Origin (PDO) Cheese Canestrato Pugliese. *Appl. Environ. Microbiol.* **2014**, *80*, 4085–4094. [CrossRef]
48. De Pasquale, I.; Di Cagno, R.; Buchin, S.; De Angelis, M.; Gobbetti, M. Spatial Distribution of the Metabolically Active Microbiota within Italian PDO Ewes' Milk Cheeses. *PLoS ONE* **2016**, *11*, e0153213. [CrossRef] [PubMed]
49. Tungjaroenchai, W.; White, C.H.; Holmes, W.E.; Drake, M.A. Influence of Adjunct Cultures on Volatile Free Fatty Acids in Reduced-Fat Edam Cheeses. *J. Dairy Sci.* **2004**, *87*, 3224–3234. [CrossRef]
50. Pérez Elortondo, F.J.; Albisu, M.; Barcina, Y. Brining Time Effect on Physicochemical and Microbiological Parameters in Idiazabal Cheese. *Int. J. Food Microbiol.* **1999**, *49*, 139–149. [CrossRef]
51. Cleide, C.O.; Castro-Mejía, J.L.; Krych, L.; Rattray, F.P. Histamine-Forming Ability of *Lentilactobacillus parabuchneri* in Reduced Salt Cheddar Cheese. *Food Microbiol.* **2021**, *98*, 103789.
52. Boletín Oficial del Estado. Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen «Idiazabal» y su Consejo Regulador. *BOE* **1993**, *289*, 34591–34596.

53. Bustamante, M.A.; Chávarri, F.; Santisteban, A.; Ceballos, G.; Hernández, I.; José Miguélez, M.; Aranburu, I.; Barrón, L.J.R.; Virto, M.; de Renobales, M. Coagulating and Lipolytic Activities of Artisanal Lamb Rennet Pastes. *J. Dairy Res.* **2000**, *67*, 393–402. [CrossRef]
54. Aldalur, A. Technical Factors Affecting Cheese Properties, Yield and Whey Generated from Raw Sheep Milk during Cheesemaking in Small Rural Dairies. Ph.D. Thesis, University of the Basque Country, Vitoria-Gasteiz, Spain, 2019.
55. *ISO 5943/IDF 88: 2006*; Cheese and Processed Cheese Products—Determination of Chloride Content—Potentiometric Titration Method. International Standardisation Organisation: Geneva, Switzerland, 2006.
56. Chavarri, F.; Virto, M.; Martin, C.; Nájera, A.I.; Santisteban, A.; Barrón, L.J.R.; de Renobales, M. Determination of Free Fatty Acids in Cheese: Comparison of Two Analytical Methods. *J. Dairy Res.* **1997**, *64*, 445–452. [CrossRef]
57. Busto, O.; Miracle, M.; Guasch, J.; Borrull, F. Determination of Biogenic Amines in Wines by High-Performance Liquid Chromatography with on-Column Fluorescence Derivatization. *J. Chromatogr. A* **1997**, *757*, 311–318. [CrossRef]
58. R Core Team. The R Project for Statistical Computing. Available online: https://www.r-project.org/ (accessed on 17 May 2021).
59. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; Mcglinn, D.; Minchin, P.R.; O'hara, R.B.; Simpson, G.L.; Solymos, P.; et al. Package "Vegan". Available online: https://cran.r-project.org/web/packages/vegan/vegan.pdf (accessed on 15 January 2021).
60. Warnes, G.R.; Bolker, B.; Bonebakker, L.; Gentleman, R.; Huber, W.; Liaw, A.; Lumley, T.; Maechler, M.; Magnusson, A.; Moeller, S.; et al. Package "Gplots" Title Various R Programming Tools for Plotting Data [R Package Gplots Version 3.1.1]. Available online: https://cran.r-project.org/package=gplots (accessed on 15 January 2021).
61. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Res.* **2013**, *41*, 1–11. [CrossRef]
62. Meyer, F.; Paarmann, D.; D'Souza, M.; Olson, R.; Glass, E.M.; Kubal, M.; Paczian, T.; Rodriguez, A.; Stevens, R.; Wilke, A.; et al. The Metagenomics RAST Server-A Public Resource for the Automatic Phylogenetic and Functional Analysis of Metagenomes. *BMC Bioinform.* **2008**, *9*, 386. [CrossRef]
63. Pruesse, E.; Quast, C.; Knittel, K.; Fuchs, B.M.; Ludwig, W.; Peplies, J.; Glöckner, F.O. SILVA: A Comprehensive Online Resource for Quality Checked and Aligned Ribosomal RNA Sequence Data Compatible with ARB. *Nucleic Acids Res.* **2007**, *35*, 7188–7196. [CrossRef] [PubMed]
64. Liu, Z.; Wang, Z.; Sun, J.; Ni, L. The Dynamics of Volatile Compounds and Their Correlation with the Microbial Succession during the Traditional Solid-State Fermentation of Gutian Hong Qu Glutinous Rice Wine. *Food Microbiol.* **2020**, *86*, 103347. [CrossRef] [PubMed]
65. Mayo, B.; Rodríguez, J.; Vázquez, L.; Flórez, A.B. Microbial Interactions within the Cheese Ecosystem and Their Application to Improve Quality and Safety. *Foods* **2021**, *10*, 602. [CrossRef]
66. Lv, J.; Yang, Z.; Xu, W.; Li, S.; Liang, H.; Ji, C.; Yu, C.; Zhu, B.; Lin, X. Relationships between Bacterial Community and Metabolites of Sour Meat at Different Temperature during the Fermentation. *Int. J. Food Microbiol.* **2019**, *307*, 108286. [CrossRef]
67. Zheng, X.; Xu, X.; Ma, Y.; Zhu, L.; Xiao, J.; Deng, L.; Shi, X.; Wang, B. Diversity and Potential Function of Bacterial Communities during Milk Fermentation of Kazak Artisanal Cheese. *Process Biochem.* **2021**, *111*, 191–200. [CrossRef]
68. Nájera, A.I.; Barcina, Y.; de Renobales, M.; Barron, L.J.R. Influence of Salt Content on the Triglyceride Composition of Idiazabal Cheese during Ripening. *Lait* **1999**, *79*, 527–534. [CrossRef]
69. Etayo, I.; Pérez Elortondo, F.J.; Gil, P.F.; Albisu, M.; Virto, M.; Conde, S.; Barron, L.J.R.; Nájera, A.I.; Gómez-Hidalgo, M.E.; Delgado, C.; et al. Hygienic Quality, Lipolysis and Sensory Properties of Spanish Protected Designation of Origin Ewe's Milk Cheeses Manufactured with Lamb Rennet Paste. *Lait* **2006**, *86*, 415–434. [CrossRef]
70. Aldalur, A.; Bustamante, M.A.; Barron, L.J.R. Effects of Technological Settings on Yield, Curd, Whey, and Cheese Composition during the Cheese-Making Process from Raw Sheep Milk in Small Rural Dairies: Emphasis on Cutting and Cooking Conditions. *J. Dairy Sci.* **2019**, *102*, 7813–7825. [CrossRef]
71. Pappa, E.C.; Kondyli, E.; Samelis, J. Microbiological and Biochemical Characteristics of Kashkaval Cheese Produced Using Pasteurised or Raw Milk. *Int. Dairy J.* **2019**, *89*, 60–67. [CrossRef]
72. De Pasquale, I.; Di Cagno, R.; Buchin, S.; De Angelis, M.; Gobbetti, M. Use of Autochthonous Mesophilic Lactic Acid Bacteria as Starter Cultures for Making Pecorino Crotonese Cheese: Effect on Compositional, Microbiological and Biochemical Attributes. *Food Res. Int.* **2019**, *116*, 1344–1356. [CrossRef]
73. Valdivielso, I.; Bustamante, M.A.; Buccioni, A.; Franci, O.; Ruiz de Gordoa, J.C.; de Renobales, M.; Barron, L.J.R. Commercial Sheep Flocks–Fatty Acid and Fat-Soluble Antioxidant Composition of Milk and Cheese Related to Changes in Feeding Management throughout Lactation. *J. Dairy Res.* **2015**, *82*, 334–343. [CrossRef]
74. Valdivielso, I.; Bustamante, M.A.; Aldezabal, A.; Amores, G.; Virto, M.; Ruiz De Gordoa, J.C.; de Renobales, M.; Barron, L.J.R. Case Study of a Commercial Sheep Flock under Extensive Mountain Grazing: Pasture Derived Lipid Compounds in Milk and Cheese. *Food Chem.* **2016**, *197*, 622–633. [CrossRef] [PubMed]
75. Aldalur, A.; Bustamante, M.A.; Salmerón, J.; Barron, L.J.R. Relationships between cheese-processing conditions and curd and cheese properties to improve the yield of Idiazabal cheese made in small artisan dairies: A multivariate approach. *J. Dairy Sci.* **2021**, *104*, 253–269. [CrossRef] [PubMed]

76. Hayaloglu, A.A.; Karatekin, B.; Gurkan, H. Thermal Stability of Chymosin or Microbial Coagulant in the Manufacture of Malatya, a Halloumi Type Cheese: Proteolysis, Microstructure and Functional Properties. *Int. Dairy J.* **2014**, *38*, 136–144. [CrossRef]
77. Soltani, M.; Sahingil, D.; Gokce, Y.; Hayaloglu, A.A. Effect of Blends of Camel Chymosin and Microbial Rennet (*Rhizomucor Miehei*) on Chemical Composition, Proteolysis and Residual Coagulant Activity in Iranian Ultrafiltered White Cheese. *J. Food Sci. Technol.* **2019**, *56*, 589–598. [CrossRef] [PubMed]
78. Sanjuán, E.; Saavedra, P.; Millán, R.; Castelo, M.; Fernández-Salguero, J. Effect of Ripening and Type of Rennet on the Mineral Content of Los Pedroches Cheese. *J. Food Qual.* **1998**, *21*, 187–200. [CrossRef]
79. Possas, A.; Bonilla-Luque, O.M.; Valero, A. From Cheese-Making to Consumption: Exploring the Microbial Safety of Cheeses through Predictive Microbiology Models. *Foods* **2021**, *10*, 355. [CrossRef]
80. Narvhus, J.A.; Axelsson, L. Lactic acid bacteria. In *Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Elsevier Inc.: London, UK, 2003; pp. 3465–3472.
81. An, F.; Li, M.; Zhao, Y.; Zhang, Y.; Mu, D.; Hu, X.; You, S.; Wu, J.; Wu, R. Metatranscriptome-Based Investigation of Flavor-Producing Core Microbiota in Different Fermentation Stages of Dajiang, a Traditional Fermented Soybean Paste of Northeast China. *Food Chem.* **2021**, *343*, 128509. [CrossRef]
82. Wang, H.; Su, W.; Mu, Y.; Zhao, C. Correlation Between Microbial Diversity and Volatile Flavor Compounds of Suan Zuo Rou, a Fermented Meat Product from Guizhou, China. *Front. Microbiol.* **2021**, *12*, 3216. [CrossRef]
83. Li, C.; Zhao, Y.; Wang, Y.; Li, L.; Yang, X.; Chen, S.; Zhao, Y.; Zhou, W. Microbial Community Changes Induced by *Pediococcus pentosaceus* Improve the Physicochemical Properties and Safety in Fermented Tilapia Sausage. *Food Res. Int.* **2021**, *147*, 110476. [CrossRef] [PubMed]
84. Chávarri, F.; Bustamante, M.A.; Santisteban, A.; Virto, M.; Barrón, L.J.R.; de Renobales, M. Changes in Free Fatty Acids During Ripening of Idiazabal Cheese Manufactured at Different Times of the Year. *J. Dairy Sci.* **1999**, *82*, 885–890. [CrossRef]
85. Hernández, I.; Barrón, L.J.R.; Virto, M.; Pérez-Elortondo, F.J.; Flanagan, C.; Rozas, U.; Nájera, A.I.; Albisu, M.; Vicente, M.S.; de Renobales, M. Lipolysis, Proteolysis and Sensory Properties of Ewe's Raw Milk Cheese (Idiazabal) Made with Lipase Addition. *Food Chem.* **2009**, *116*, 158–166. [CrossRef]
86. Barron, L.J.R.; Hernández, I.; Bilbao, A.; Flanagan, C.E.; Nájera, A.I.; Virto, M.; Pérez-Elortondo, F.J.; Albisu, M.; de Renobales, M. Changes in Lipid Fractions and Sensory Properties of Idiazabal Cheese Induced by Lipase Addition. *J. Dairy Res.* **2004**, *71*, 372–379. [CrossRef]
87. Hernández, I.; de Renobales, M.; Virto, M.; Pérez-Elortondo, F.J.; Barron, L.J.R.; Flanagan, C.; Albisu, M. Assessment of Industrial Lipases for Flavour Development in Commercial Idiazabal (Ewe's Raw Milk) Cheese. *Enzyme Microb. Technol.* **2005**, *36*, 870–879. [CrossRef]
88. Amores, G.; Pérez-Elortondo, F.J.; Albisu, M.; Barron, L.J.R. Short Communication: To What Extent Do Environmental or Technological Conditions Affect the Sensory Differentiation of Raw Ewe Milk Cheeses Produced in Valley or Mountain Farms? *J. Dairy Sci.* **2021**, *104*, 301–307. [CrossRef] [PubMed]
89. Esmaeilzadeh, P.; Ehsani, M.R.; Mizani, M.; Givianrad, M.H. Characterization of a Traditional Ripened Cheese, Kurdish Kope: Lipolysis, Lactate Metabolism, the Release Profile of Volatile Compounds, and Correlations with Sensory Characteristics. *J. Food Sci.* **2021**, *86*, 3303–3321. [CrossRef] [PubMed]
90. Caboni, P.; Maxia, D.; Scano, P.; Addis, M.; Dedola, A.; Pes, M.; Murgia, A.; Casula, M.; Profumo, A.; Pirisi, A. A Gas Chromatography-Mass Spectrometry Untargeted Metabolomics Approach to Discriminate Fiore Sardo Cheese Produced from Raw or Thermized Ovine Milk. *J. Dairy Sci.* **2019**, *102*, 5005–5018. [CrossRef] [PubMed]
91. Fernández, D.; Combarros-Fuertes, P.; Renes, E.; Abarquero, D.; Fresno, J.M.; Tornadijo, M.E. Influence of the Breed of Sheep on the Characteristics of Zamorano Cheese. *Dairy* **2021**, *2*, 242–255. [CrossRef]
92. Larráyoz, P.; Martínez, M.T.; Barrón, L.J.R.; Torre, P.; Barcina, Y. The Evolution of Free Fatty Acids during the Ripening of Idiazábal Cheese: Influence of Rennet Type. *Eur. Food Res. Technol.* **1999**, *210*, 9–12. [CrossRef]
93. Chávarri, F.; Santisteban, A.; Virto, M.; de Renobales, M. Alkaline Phosphatase, Acid Phosphatase, Lactoperoxidase, and Lipoprotein Lipase Activities in Industrial Ewe's Milk and Cheese. *J. Agric. Food Chem.* **1998**, *46*, 2926–2932. [CrossRef]
94. Tsiplakou, E.; Flemetakis, E.; Kouri, E.; Sotirakoglou, K.; Zervas, G. The Effect of Long Term Under- and over-Feeding on the Expression of Genes Related to Lipid Metabolism in Mammary Tissue of Sheep. *J. Dairy Res.* **2015**, *82*, 107–112. [CrossRef] [PubMed]
95. Holland, R.; Liu, S.Q.; Crow, V.L.; Delabre, M.L.; Lubbers, M.; Bennett, M.; Norris, G. Esterases of Lactic Acid Bacteria and Cheese Flavour: Milk Fat Hydrolysis, Alcoholysis and Esterification. *Int. Dairy J.* **2005**, *15*, 711–718. [CrossRef]
96. Dellali, A.; Karam, H.Z.; Karam, N.-E. Lipase and Esterase Activities of Lactic Acid Bacteria Isolated from Different Biotopes. *African J. Biotechnol.* **2020**, *19*, 156–164.
97. Katz, M.; Medina, R.; Gonzalez, S.; Oliver, G. Esterolytic and Lipolytic Activities of Lactic Acid Bacteria Isolated from Ewe's Milk and Cheese. *J. Food Prot.* **2002**, *65*, 1997–2001. [CrossRef] [PubMed]
98. Esteban-Torres, M.; Mancheño, J.M.; de las Rivas, B.; Muñoz, R. Characterization of a Halotolerant Lipase from the Lactic Acid Bacteria *Lactobacillus plantarum* Useful in Food Fermentations. *LWT-Food Sci. Technol.* **2015**, *60*, 246–252. [CrossRef]
99. Esteban-Torres, M.; Mancheño, J.M.; de las Rivas, B.; Muñoz, R. Production and Characterization of a Tributyrin Esterase from *Lactobacillus plantarum* Suitable for Cheese Lipolysis. *J. Dairy Sci.* **2014**, *97*, 6737–6744. [CrossRef] [PubMed]

100. Ramakrishnan, V.; Goveas, L.C.; Suralikerimath, N.; Jampani, C.; Halami, P.M.; Narayan, B. Extraction and Purification of Lipase from *Enterococcus faecium* MTCC5695 by PEG/Phosphate Aqueous-Two Phase System (ATPS) and Its Biochemical Characterization. *Biocatal. Agric. Biotechnol.* **2016**, *6*, 19–27. [CrossRef]
101. Han, D.M.; Chun, B.H.; Kim, H.M.; Jeon, C.O. Characterization and Correlation of Microbial Communities and Metabolite and Volatile Compounds in Doenjang Fermentation. *Food Res. Int.* **2021**, *148*, 110645. [CrossRef] [PubMed]
102. Özer, B.; Yaman, H. Milk and Milk Products: Microbiology of Liquid Milk. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Elsevier Inc.: London, UK, 2014; pp. 721–727.
103. Ozturkoglu-Budak, S.; Wiebenga, A.; Bron, P.A.; de Vries, R.P. Protease and Lipase Activities of Fungal and Bacterial Strains Derived from an Artisanal Raw Ewe's Milk Cheese. *Int. J. Food Microbiol.* **2016**, *237*, 17–27. [CrossRef]
104. Schmitz-Esser, S.; Dzieciol, M.; Nischler, E.; Schornsteiner, E.; Bereuter, O.; Mann, E.; Wagner, M. Abundance and Potential Contribution of Gram-Negative Cheese Rind Bacteria from Austrian Artisanal Hard Cheeses. *Int. J. Food Microbiol.* **2018**, *266*, 95–103. [CrossRef] [PubMed]
105. Desbois, A.P.; Smith, V.J. Antibacterial Free Fatty Acids: Activities, Mechanisms of Action and Biotechnological Potential. *Appl. Microbiol. Biotechnol.* **2009**, *85*, 1629–1642. [CrossRef] [PubMed]
106. Nieman, C. Influence of Trace Amounts of Fatty Acids on the Growth of Microorganisms. *Bacteriol. Rev.* **1954**, *18*, 147. [CrossRef] [PubMed]
107. Yoon, B.K.; Jackman, J.A.; Valle-González, E.R.; Cho, N.-J. Antibacterial Free Fatty Acids and Monoglycerides: Biological Activities, Experimental Testing, and Therapeutic Applications. *Int. J. Mol. Sci.* **2018**, *19*, 1114. [CrossRef] [PubMed]
108. Forquin, M.P.; Weimer, B.C. Brevibacterium. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Tortorello, M.L., Eds.; Elsevier Inc.: London, UK, 2014; pp. 324–330.
109. Ordóñez, A.I.; Ibáñez, F.C.; Torre, P.; Barcina, Y. Formation of Biogenic Amines in Idiazabal Ewe's-Milk Cheese: Effect of Ripening, Pasteurization, and Starter. *J. Food Prot.* **1997**, *60*, 1371–1375. [CrossRef] [PubMed]
110. Tofalo, R.; Perpetuini, G.; Battistelli, N.; Pepe, A.; Ianni, A.; Martino, G.; Suzzi, G. Accumulation γ-Aminobutyric Acid and Biogenic Amines in a Traditional Raw Milk Ewe's Cheese. *Foods* **2019**, *8*, 401. [CrossRef] [PubMed]
111. Torracca, B.; Pedonese, F.; López, M.B.; Turchi, B.; Fratini, F.; Nuvoloni, R. Effect of Milk Pasteurisation and of Ripening in a Cave on Biogenic Amine Content and Sensory Properties of a Pecorino Cheese. *Int. Dairy J.* **2016**, *61*, 189–195. [CrossRef]
112. Schirone, M.; Tofalo, R.; Fasoli, G.; Perpetuini, G.; Corsetti, A.; Manetta, A.C.; Ciarrocchi, A.; Suzzi, G. High Content of Biogenic Amines in Pecorino Cheeses. *Food Microbiol.* **2013**, *34*, 137–144. [CrossRef]
113. Linares, D.M.; del Río, B.; Ladero, V.; Martínez, N.; Fernández, M.; Martín, M.C.; Alvarez, M.A. Factors Influencing Biogenic Amines Accumulation in Dairy Products. *Front. Microbiol.* **2012**, *3*, 180. [CrossRef] [PubMed]
114. Nájera, A.I.; Nieto, S.; Barron, L.J.R.; Albisu, M. A Review of the Preservation of Hard and Semi-Hard Cheeses: Quality and Safety. *Int. J. Environ. Res. Public Health* **2021**, *18*, 9789. [CrossRef] [PubMed]
115. Mao, S.; Huo, W.; Liu, J.; Zhang, R.; Zhu, W. In Vitro Effects of Sodium Bicarbonate Buffer on Rumen Fermentation, Levels of Lipopolysaccharide and Biogenic Amine, and Composition of Rumen Microbiota. *J. Sci. Food Agric.* **2017**, *97*, 1276–1285. [CrossRef] [PubMed]
116. Sigel, E.; Steinmann, M.E. Structure, Function, and Modulation of GABAA Receptors. *J. Biol. Chem.* **2012**, *287*, 40224–40231. [CrossRef] [PubMed]
117. Bagheri, S.; Heydari, A.; Alinaghipour, A.; Salami, M. Effect of Probiotic Supplementation on Seizure Activity and Cognitive Performance in PTZ-Induced Chemical Kindling. *Epilepsy Behav.* **2019**, *95*, 43–50. [CrossRef]
118. Abdelazez, A.; Abdelmotaal, H.; Evivie, S.E.; Melak, S.; Jia, F.F.; Khoso, M.H.; Zhu, Z.T.; Zhang, L.J.; Sami, R.; Meng, X.C. Screening Potential Probiotic Characteristics of *Lactobacillus brevis* Strains in Vitro and Intervention Effect on Type i Diabetes in Vivo. *Biomed Res. Int.* **2018**, *2018*, 7356173. [CrossRef]
119. Boonstra, E.; de Kleijn, R.; Colzato, L.S.; Alkemade, A.; Forstmann, B.U.; Nieuwenhuis, S. Neurotransmitters as Food Supplements: The Effects of GABA on Brain and Behavior. *Front. Psychol.* **2015**, *6*, 1520. [CrossRef]
120. Wang, C.; Zhu, C.L.; Huang, Z.J.; Wang, G.; Huang, Q.; Liu, C.H.; Xie, F.; Wang, W. γ-Aminobutyric Acid Inhibits the Growth of Cholangiocarcinoma via CAMP/PKA Signal Pathway. *Int. J. Clin. Exp. Med.* **2016**, *9*, 9992–9998.
121. Bustamante, M.A.; Virto, M.; Aramburu, M.; Barron, L.J.R.; Pérez-Elortondo, F.J.; Albisu, M.; de Renobales, M. Lamb Rennet Paste in Ovine Cheese (Idiazabal) Manufacture. Proteolysis and Relationship between Analytical and Sensory Parameters. *Int. Dairy J.* **2003**, *13*, 547–557. [CrossRef]
122. Renes, E.; Fernández, D.; Abarquero, D.; Ladero, V.; Álvarez, M.A.; Tornadijo, M.E.; Fresno, J.M. Effect of Forage Type, Season, and Ripening Time on Selected Quality Properties of Sheep Milk Cheese. *J. Dairy Sci.* **2021**, *104*, 2539–2552. [CrossRef] [PubMed]
123. Silla Santos, M.H. Biogenic Amines: Their Importance in Foods. *Int. J. Food Microbiol.* **1996**, *29*, 213–231. [CrossRef]
124. Vasconcelos, H.; de Almeida, J.M.M.M.; Matias, A.; Saraiva, C.; Jorge, P.A.S.; Coelho, L.C.C. Detection of Biogenic Amines in Several Foods with Different Sample Treatments: An Overview. *Trends Food Sci. Technol.* **2021**, *113*, 86–96. [CrossRef]
125. Halász, A.; Baráth, Á.; Simon-Sarkadi, L.; Holzapfel, W. Biogenic Amines and Their Production by Microorganisms in Food. *Trends Food Sci. Technol.* **1994**, *5*, 42–49. [CrossRef]
126. Tittarelli, F.; Perpetuini, G.; Di Gianvito, P.; Tofalo, R. Biogenic Amines Producing and Degrading Bacteria: A Snapshot from Raw Ewes' Cheese. *LWT-Food Sci. Technol.* **2019**, *101*, 1–9. [CrossRef]

127. Barbieri, F.; Montanari, C.; Gardini, F.; Tabanelli, G. Biogenic Amine Production by Lactic Acid Bacteria: A Review. *Foods* **2019**, *8*, 17. [CrossRef] [PubMed]
128. Espinosa-Pesqueira, D.; Roig-Sagués, A.X.; Manuela Hernández-Herrero, M. Screening Method to Evaluate Amino Acid-Decarboxylase Activity of Bacteria Present in Spanish Artisanal Ripened Cheeses. *Foods* **2018**, *7*, 182. [CrossRef]
129. Diaz, M.; del Rio, B.; Ladero, V.; Redruello, B.; Fernández, M.; Martin, M.C.; Alvarez, M.A. Isolation and Typification of Histamine-Producing *Lactobacillus vaginalis* Strains from Cheese. *Int. J. Food Microbiol.* **2015**, *215*, 117–123. [CrossRef] [PubMed]
130. Rodríguez-Sánchez, S.; Ramos, I.M.; Seseña, S.; Poveda, J.M.; Palop, M.L. Potential of *Lactobacillus* Strains for Health-Promotion and Flavouring of Fermented Dairy Foods. *LWT-Food Sci. Technol.* **2021**, *143*, 111102. [CrossRef]
131. Joosten, H.M.L.J.; Northolt, M.D. Conditions Allowing the Formation of Biogenic Amines in Cheese, 2: Decarboxylative Properties of Some Non-Starter Bacteria. *Netherlands Milk Dairy J.* **1987**, *41*, 259–280.
132. Aliakbarlu, J.; Alizadeh, M.; Razavi-Rohani, S.M.; Agh, N. Biogenic Amines in Iranian White Brine Cheese: Modelling and Optimisation of Processing Factors. *Int. J. Dairy Technol.* **2011**, *64*, 417–424. [CrossRef]
133. Body, P.; Greif, G.; Greifová, G.; Sliacká, M.; Greifová, M. Effects of Cultivation Media and NaCl Concentration on the Growth Kinetics and Biogenic Amine Production of *Lactobacillus reuteri*. *Czech J. Food Sci.* **2021**, *39*, 9–16. [CrossRef]
134. Zhang, C.; Zhang, J.; Liu, D. Biochemical Changes and Microbial Community Dynamics during Spontaneous Fermentation of Zhacai, a Traditional Pickled Mustard Tuber from China. *Int. J. Food Microbiol.* **2021**, *347*, 109199. [CrossRef] [PubMed]
135. Zhao, Y.; Wang, Y.; Li, C.; Li, L.; Yang, X.; Wu, Y.; Chen, S.; Zhao, Y. Novel Insight into Physicochemical and Flavor Formation in Naturally Fermented Tilapia Sausage Based on Microbial Metabolic Network. *Food Res. Int.* **2021**, *141*, 110122. [CrossRef]
136. Santiyanont, P.; Chantarasakha, K.; Tepkasikul, P.; Srimarut, Y.; Mhuantong, W.; Tangphatsornruang, S.; Zo, Y.G.; Chokesajjawatee, N. Dynamics of Biogenic Amines and Bacterial Communities in a Thai Fermented Pork Product Nham. *Food Res. Int.* **2019**, *119*, 110–118. [CrossRef] [PubMed]
137. Jung, J.Y.; Chun, B.H.; Jeon, C.O. *Chromohalobacter* Is a Causing Agent for the Production of Organic Acids and Putrescine during Fermentation of Ganjang, a Korean Traditional Soy Sauce. *J. Food Sci.* **2015**, *80*, M2853–M2859. [CrossRef] [PubMed]
138. Li, B.; Lu, S. The Importance of Amine-Degrading Enzymes on the Biogenic Amine Degradation in Fermented Foods: A Review. *Process Biochem.* **2020**, *99*, 331–339. [CrossRef]
139. Lee, Y.C.; Kung, H.F.; Huang, C.Y.; Huang, T.C.; Tsai, Y.H. Reduction of Histamine and Biogenic Amines during Salted Fish Fermentation by *Bacillus Polymyxa* as a Starter Culture. *J. Food Drug Anal.* **2016**, *24*, 157–163. [CrossRef] [PubMed]
140. Chong, C.Y.; Bakar, F.A.; Russly, A.R.; Jamilah, B.; Mahyudin, N.A. The Effects of Food Processing on Biogenic Amines Formation. *Int. Food Res. J.* **2011**, *18*, 867–876.
141. Butor, I.; Pištěková, H.; Purevdorj, K.; Jančová, P.; Buňka, F.; Buňková, L. Biogenic Amines Degradation by Microorganisms Isolated from Cheese. *Potravin. Slovak J. Food Sci.* **2017**, *11*, 302–308. [CrossRef]
142. Herrero-Fresno, A.; Martínez, N.; Sánchez-Llana, E.; Díaz, M.; Fernández, M.; Martin, M.C.; Ladero, V.; Alvarez, M.A. *Lactobacillus casei* Strains Isolated from Cheese Reduce Biogenic Amine Accumulation in an Experimental Model. *Int. J. Food Microbiol.* **2012**, *157*, 297–304. [CrossRef]

3 Appendix III
Manuscript III

Current Research in Food Science (2023), 6, 100425

Relationship between dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese

Gorka Santamarina-García[1,*], Gustavo Amores[1], Igor Hernández[1], Lara Morán[2], Luis Javier R. Barrón[2] and Mailo Virto[1]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[2] *Lactiker Research Group, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[*] *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented at:

VII Jornadas Doctorales de la Universidad de Murcia-Universidad Politécnica de Cartagena-Campus Mare Nostrum, 2022. *El impacto de la microbiota en varios parámetros de calidad y seguridad del queso de leche cruda de oveja Idiazabal.* Santamarina-García, G., Hernández, I., Amores, G. and Virto, M. (Oral presentation).

73rd European Federation of Animal Science Annual Meeting, 2022. *Relationship between bacterial communities and volatile aroma compounds of a ewe's raw milk cheese.* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Poster presentation).

3rd Food Chemistry Conference: Shaping a Healthy and Sustainable Food Chain through Knowledge, 2023. *Volatile profile differentiation among producers of a raw milk PDO cheese: a metagenomic approach.* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Poster presentation).

Published in **Current Research in Food Science**
ISSN: 2665-9271
IF (2022): **6.3**
Category: **Food Science & Technology (Q1, 21/142)**
Citations: 8 (updated: 04/2024)
doi.org/10.1016/j.crfs.2022.100425

Current Research in Food Science 6 (2023) 100425

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Relationship between the dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese

Gorka Santamarina-García [a,*], Gustavo Amores [a], Igor Hernández [a], Lara Morán [b], Luis Javier R. Barrón [b], Mailo Virto [a,**]

[a] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006, Vitoria-Gasteiz, Spain
[b] Lactiker Research Group, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006, Vitoria-Gasteiz, Spain

ARTICLE INFO

Keywords:
Volatile composition
Odour-active compounds
Microbiota
Correlation analysis
O2PLS
CCorA

ABSTRACT

Cheese microbiota contributes to various biochemical processes that lead to the formation of volatile compounds and the development of flavour during ripening. Nonetheless, the role of these microorganisms in volatile aroma compounds production is little understood. This work reports for the first time the dynamics and odour impact of volatile compounds, and their relationship to microbial shifts during the ripening of a raw ewe milk-derived cheese (Idiazabal). By means of SPME-GC-MS, 81 volatile compounds were identified, among which acids predominated, followed by esters, ketones and alcohols. The ripening time influenced the abundance of most volatile compounds, thus the moments of greatest abundance were determined (such as 30–60 days for acids). Through Odour Impact Ratio (OIR) values, esters and acids were reported as the predominant odour-active chemical families, while individually, ethyl hexanoate, ethyl 3-methyl butanoate, ethyl butanoate, butanoic acid or 3-methyl butanal were notable odorants, which would provide fruity, rancid, cheesy or malt odour notes. Using a bidirectional orthogonal partial least squares (O2PLS) approach with Spearman's correlations, 12 bacterial genera were reported as key bacteria for the volatile and aromatic composition of Idiazabal cheese, namely *Psychrobacter, Enterococcus, Brevibacterium, Streptococcus, Leuconostoc, Chromohalobacter, Chryseobacterium, Carnobacterium, Lactococcus, Obesumbacterium, Stenotrophomonas* and *Flavobacterium*. Non-starter lactic acid bacteria (NSLAB) were highly related to the formation of certain acids, esters and alcohols, such as 3-hexenoic acid, ethyl butanoate or 1-butanol. On the other hand, the starter LAB (SLAB) was related to particular ketones production, specifically 3-hydroxy-2-butanone; and environmental and/or non-desirable bacteria to certain ketones, hydrocarbons and sulphur compounds formation, such as 2-propanone, *t*-3-octene and dimethyl sulphone. Additionally, the SLAB *Lactococcus* and *Psychrobacter, Brevibacterium* and *Chromohalobacter* were described as having a negative effect on aroma development caused by NSLAB and *vice versa*. These results provide novel knowledge to help understand the aroma formation in a raw ewe milk-derived cheese.

1. Introduction

Idiazabal cheese is a traditional semi-hard or hard cheese from the Basque Country (southwestern Europe), which is manufactured from raw milk of Latxa and/or Carranzana autochthonous breed sheep. Its production is regulated by its Protected Designation of Origin (PDO) since 1996 (Official Journal of the European Communities, 1996). The Idiazabal cheese making process is strictly regulated and establishes a minimum ripening time of 60 days (Boletín Oficial del Estado, 1993). Nonetheless, producers may employ different flock management and cheese making practices that, in turn, affect the quality of the milk and final cheese. The most notable differences are related to the flock

* Corresponding author.
** Corresponding author.
E-mail addresses: gorka.santamarina@ehu.eus (G. Santamarina-García), gustavo.amores@ehu.eus (G. Amores), igor.hernandezo@ehu.eus (I. Hernández), laramoran84@hotmail.com (L. Morán), luisjavier.rbarron@ehu.eus (L.J.R. Barrón), mailo.virto@ehu.eus (M. Virto).

https://doi.org/10.1016/j.crfs.2022.100425
Received 12 September 2022; Received in revised form 10 December 2022; Accepted 21 December 2022
Available online 22 December 2022

management and grazing practices, such as valley or mountain grazing (Abilleira et al., 2010a; Valdivielso et al., 2016); the use of artisanal or commercial rennet (Virto et al., 2003) or the technological conditions used for cheese making and ripening (Aldalur et al., 2021).

During cheese ripening, various biochemical processes take place that are responsible for the synthesis of volatile compounds and, consequently, flavour development (Fox et al., 2017; Thierry et al., 2017). Volatile compounds originate primarily from three groups of metabolic pathways: the metabolism of residual lactose, lactate and citrate; the lipolysis and subsequent metabolism of the released free fatty acids (FFAs); and the proteolysis and metabolism of the resulting peptides and free amino acids (FAAs) (Le Quéré and Buchin, 2022). It has been reported that ripening time affects the volatile composition of Idiazabal cheese (Barron et al., 2005a, 2007). However, these studies only focused on certain time points of ripening and did not comprehensively characterize how volatile chemical families and individual compounds evolve throughout this process. This aspect is of special interest since the presence or absence, abundance and proportions of each compound characterize the type of cheese and its aroma (Le Quéré and Buchin, 2022). In fact, the imbalance or excessive concentrations of several compounds have been related to off-flavours (Zabaleta et al., 2016). Moreover, it is generally recognized that not all volatile compounds contribute to cheese aroma (Starowicz, 2021), that is to say, not all are odour-active compounds (Fox et al., 2017; Natrella et al., 2020). Despite the large work done to elucidate the flavour and key aromatic compounds of Idiazabal cheese (Barron et al., 2005a, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016), it is unknown how the odour-impact of volatile compounds evolve during ripening.

Cheese microbiota contributes to numerous biochemical reactions involved in the formation of flavour compounds (Bertuzzi et al., 2018; Le Quéré and Buchin, 2022) and, indeed, has been described as responsible for the particular sensory properties of raw milk cheeses, such as Manchego or Erronkari/Roncal (Ballesteros et al., 2006). The microbiota of Idiazabal cheese has recently been characterized by means of high-throughput sequencing (HTS) (Santamarina-García et al., 2022a), allowing a better understanding of microbial communities than culture-based methods (Yeluri Jonnala et al., 2018). Overall, it has been observed that the ripening time modulates the bacterial composition. Specifically, the starter LAB (SLAB) (*Lactococcus*) predominates up to 30 or 60 days and then, non-starter LAB (NSLAB) (*Lactobacillus, Leuconostoc, Enterococcus, Streptococcus* and *Carnobacterium*) proliferate, while the relative abundance of non-desirable and/or environmental bacteria is reduced (such as *Pseudomonas, Staphylococcus* or *Chromohalobacter*). Moreover, bacterial composition differs largely among producers and several bacterial genera not reported previously in any raw ewe milk and derived cheeses have been identified (such as *Buttiauxella* or *Obesumbacterium*) (Santamarina-García et al., 2022a).

In recent times, several studies that attempt to elucidate the relationship between microbial communities and volatile compounds formation in fermented products have been published (Zhong et al., 2021; Xia et al., 2022). However, few studies have focused on cheese (Zheng et al., 2018; Chen et al., 2021a) and information on raw ewe milk-derived cheeses is scarce (Cardinali et al., 2021). In addition, although the aim of these studies was to understand the association between microbial communities and the aroma formation in fermented products, only one work has analysed the correlation to odour-active volatile compounds in fermented milk (Xia et al., 2021). In order to elucidate such correlations, complex chemometric approaches are needed and the multivariate bidirectional orthogonal partial least squares (O2PLS) (Trygg and Wold, 2003) is one of the most useful approaches (Bouhaddani et al., 2016). Nonetheless, its combination with other parameters, such as correlation coefficients, is the most appropriate approach (Galindo-Prieto et al., 2014).

Therefore, this study aimed to (1) characterize how volatile compounds and their odour impact evolve during the ripening of raw ewe milk-derived Idiazabal cheese, in order to (2) investigate how they relate to shifts during ripening of the unique microbiota found in this PDO cheese and (3) highlight the relevance of appropriate advanced statistical approaches to obtain insights and improve cheese quality. Moreover, the potential differences among producers producing the same type of cheese were also analysed. To the best of our knowledge, no such a comprehensive study has been conducted to date on any type of cheese.

2. Materials and methods

2.1. Cheese sampling

Four artisanal Idiazabal PDO cheese producers (identified as A, B, C and D) were selected for the sampling. Idiazabal cheeses were produced from raw milk of Latxa sheep, each producer employing the milk of its own flock and following the specifications issued by Idiazabal Designation of Origin Regulatory Board (Boletín Oficial del Estado, 1993). Briefly, the milk was tempered to 25 °C and the mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*) (DuPont NHIB Ibérica S.L., Barcelona, Spain) was added. Milk was coagulated using artisanal rennet or commercial rennet NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). Cheese ripening was carried out in chambers maintained at 80–95% relative humidity and 8–14 °C temperature. Cheese samples for analysis were collected in duplicate at six time points during ripening (1, 7, 14, 30, 60 and 120 days) ($n = 48$). Samples were collected and transported to the laboratory under refrigerated conditions (3 °C) and then stored in a freezer (−80 °C). Before analysis, cheese samples were defrosted for 24 h at 5 °C and kept at room temperature for 1 h.

2.2. Solid-phase microextraction (SPME) methodology

The preparation of Idiazabal cheese samples and the SPME procedure was carried out as previously described (Valdivielso et al., 2016). Briefly, removing the rind, 15 g of cheese were ground together with 20 g of anhydrous Na_2SO_4 (reagent grade, Scharlab, Barcelona, Spain). Afterwards, 5 μL of cyclohexanone (0.5 μg/L) (≥99.5%, Sigma-Aldrich, Madrid, Spain) were added as internal standard (IS) solution and it was re-homogenized. The extraction of the volatile compounds was carried out by adding 2.5 g of the aforementioned mixture to a 10 mL amber vial (Agilent Technologies). The vials were sealed with a PTFE septa and a steel magnetic cap (18 mm PTFE/SIL, Agilent Technologies) prior to placing them in the sample tray at room temperature for analysis. SPME procedure was carried out employing a PAL RSI 85 autosampler (CTC CombiPAL, Zwingen, Switzerland) equipped with a temperature-controlled air incubator. After 15 min of pre-equilibration time at 60 °C, volatile compounds were trapped onto a 50/30 μm DVB/Carboxen/PDMS fibre (57298-U, Supelco, Madrid, Spain) at 60 °C for 30 min.

2.3. Gas chromatography–mass spectrometry (GC-MS) analysis

Volatile compounds were analysed in a 7820A Gas Chromatograph system, equipped with a split/splitless injector and coupled to a 5975 series MS detector (Agilent Technologies), essentially as previously described (Barron et al., 2005b). Volatile compounds were desorbed from the fibre in the front injection port for 10 min at 240 °C in splitless mode (split valve was opened at 200 mL/min after 10 min of the injection). Then, volatile compounds were separated in a Supelcowax-10 fused silica capillary column (59.5 m length, 0.25 mm i.d.; 0.25 μm film thickness) (Supelco, Madrid, Spain) and helium (99.999% purity, Air liquid, Madrid, Spain) was employed as the carrier gas at a constant pressure of 16 psi. Oven was held at 40 °C for 10 min, then raised at a rate of 5 °C/min until 110 °C, increased again at 10 °C/min until 240 °C,

and finally held at 240 °C for 15 min. Volatile compounds were then transferred to the MS detector through a transfer line at 280 °C and MS detector operated at 150 °C in full scan mode with 70 eV as total ion current.

The chromatographic data obtained were analysed with MSD ChemStation Data Analysis version 5.52 (Agilent Technologies). Volatile compounds were tentatively identified by comparing their mass spectra (match factor >800) with those of the National Institute of Standards and Technology spectra library (NIST version 2.0, Gaithersburg, USA). Mean linear retention index (LRI) value of each chromatographic peak was calculated from the analysis of each Idiazabal cheese sample (4 replicates) and the saturated alkanes standard mixture (3 replicates × 2 times through the experiment) (certified reference material 49452-u, C7-C40, Sigma-Aldrich). Then, positive identification of volatile compounds was carried out by comparing the LRI and mass spectra with those of commercially available high purity standards (≥90%, supplied by Sigma-Aldrich and Honeywell Fluka, Madrid, Spain). The limit of detection (LOD) was established at twice the noise (arbitrary units) of the chromatogram. Peak area quantification was carried out by the total ion current (TIC) and the content of volatile compounds was expressed as relative abundance (peak area in arbitrary units relative to the internal standard (IS)), as expressed by the following equation:

$$\text{Volatile relative abundace} = \frac{\text{peak area}}{\text{IS area}} \times \frac{2,5\ \text{g}}{\text{sample weight (g)}} \times 100$$

The mean abundance of the volatile compounds of each cheese sample was obtained from the peak areas (>LOD) of each compound, as long as they were detected in at least two of the four sample replicates. The relative abundance of volatile compounds in cheese samples was expressed as mean ± standard deviation.

2.4. Statistical analysis of volatile composition

Different packages and software were employed for data analysis. The IBM SPSS statistical package version 26.0 (IBM SPSS Inc., Chicago, 2019) was used for data preparation and analysis. Kruskal-Wallis analysis of variance with Bonferroni correction was performed in IBM SPSS package to determine the effect of ripening time and producer factors on volatile chemical families and individual compounds. Hierarchical Clustering Analysis (HCA) of volatile compounds was performed using log transformed, when necessary, and Unit Variance (UV) scaled data and plotted into a heat-map with RStudio version 1.3.959 and R version 3.6.3 (R Core Team, 2020) with "gplots" package (Warnes et al., 2020). The aim was to analyse volatile compounds groupings during ripening time. Then, trends along ripening were analysed through a Principal Component Analysis (PCA) using the SIMCA software version 15.0.0.4783 (Umetrics AB, Umeå, Sweden). The number of principal components (PC) was determined by eigenvalues (greater than 2.5) and cross validation. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was also performed in SIMCA, in order to analyse whether samples differed according to the producer. Variable Influence on Projection (VIP) values and loadings weights were used to analyse the importance of each volatile compound in the model.

2.5. Calculation of Odour Impact Ratio

To obtain a measure of the odour impact of each volatile compound detected in Idiazabal cheese samples, Odour Impact Ratio (OIR) was calculated as Abilleira et al. (2010b) described, with minor modifications, and as the following equation expresses:

$$\text{OIR} = \frac{\text{Volatile relative abundance}}{\text{odour threshold}(\mu\text{g/L or } \mu\text{g/kg})}$$

Compounds with OIR values greater than 1 indicate that the abundance of the volatile compound was higher than the odour threshold (OT) and thus, were odour-active compounds. OTs are usually calculated in water, however, values could change depending on the matrix (van Gemert, 2011). Therefore, available OT values measured in cheese were taken to avoid the matrix effect (listed in Table 2).

2.6. Correlation between bacterial communities and volatile compounds

An HTS analysis was performed to characterize the bacterial communities and the shifts that occur during ripening of the collected Idiazabal cheese samples, as described by Santamarina-García et al. (2022a). Briefly, 10 g of cheese were suspended in 90 mL of 2% (w/v) sterile sodium citrate (pH 8.0), and homogenized six times (each for 20 s ON and 10 s OFF) in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany). Then, 1.5 mL of the resulting suspension was centrifuged (8000×*g* for 10 min at 4 °C) and the fat-containing supernatant was discarded. The obtained pellet was resuspended in 600 μL of sodium citrate, and centrifuged three times (8000×*g* for 10 min at 4 °C). DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) and the 16S rRNA gene library was prepared using Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). The V3–V4 regions of the 16S rRNA gene were amplified by PCR (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′), as described by Klindworth et al. (2013). Then, 16S rRNA gene sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 (2 × 300 bp) (Illumina Inc.). MiSeq Reporter software was used for quality filtering and trimming of raw reads and taxonomic classification was performed using the MG-RAST web data analysis tool (Meyer et al., 2008), based on the Silva SSU database (Pruesse et al., 2007). Bacterial abundance was presented as relative abundance (%) based on the identified sequences.

To study the relationship between the identified bacterial genera and the dynamics of volatile aroma compounds, an O2PLS approach was applied to log transformed, when necessary, and UV scaled data in SIMCA. Main bacterial genera were selected as X-variables and volatile compounds as Y-variables. The model was validated, among others, by R2 and Q2 values, Permutation test or Inner Relation plot. The key bacterial genera for the volatile composition of Idiazabal cheese were identified based on VIP values and loading weights, together with Spearman's Rank Correlations calculated in SPSS and interpreted in a heat map with an HCA analysis performed in R with "pheatmap" package (Kolde, 2019). The resulted correlations were verified by a Canonical Correlation Analysis (CCorA) multivariate statistical approach performed in R with "vegan" package (Oksanen et al., 2020).

3. Results and discussion

3.1. Changes in volatile composition

Table 1 summarizes the average relative abundance of the individual volatile compounds identified during the ripening time of the collected Idiazabal cheese samples from the four producers (A, B, C and D). The average relative abundance percentage of the identified volatile chemical families is reported in Supplementary Table 1. A total of 81 volatile compounds were identified, which could be grouped into seven families according to their chemical structure, namely acids (9 individual acids), alcohols (21), aldehydes (7), ketones (12), esters (24), hydrocarbons (5), sulphur compounds (2) and terpenes (1). Esters and alcohols constituted the highest number of individual compounds, as previously reported for Idiazabal cheese (Barron et al., 2005a, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016), although a higher number of alcohols were identified in the present study. Overall, 18 individual compounds not reported previously in Idiazabal cheese were detected. Compared to the latest works on raw ewe milk-derived cheeses, a greater number of individual compounds have been identified (Gaglio et al., 2019a; Cardinali et al., 2021).

Acids were the predominant chemical family (79.5–88.9% of the

Table 1
Average relative abundance ± standard deviation of individual volatile compounds identified throughout ripening (1, 7, 14, 30, 60 and 120 days) of Idiazabal cheese samples from 4 producers (n = 48).

ID	LRI[a]	Volatile compounds	Ripening time (days)[b]						P-value[c]	
			1	7	14	30	60	120	RT	P
Acids										
C1	1647	Acetic acid[d]	8.06 ± 22.8	353 ± 542	ND	24.3 ± 36.4	61.6 ± 101	120 ± 243	NS	NS
C2	1698	*n*- Butanoic acid[d]	1681 ± 1594	6884 ± 5928	9289 ± 7950	10674 ± 6878	12053 ± 7633	6624 ± 8670	*	**
C3	1880	*n*- Pentanoic acid[d]	10.5 ± 14.4	55.1 ± 50.4	1135 ± 3050	4166 ± 11566	48.1 ± 57.5	428 ± 942	NS	*
C4	1921	*n*- Hexanoic acid[d]	3168 ± 2911	12617 ± 9968	16782 ± 13507	24546 ± 15744	25632 ± 19485	15567 ± 22362	NS	***
C5	2067	(E)-3-Hexenoic acid[d,f]	ND	ND	ND	682 ± 1692	196 ± 365	2682 ± 5030	NS	***
C6	2105	*n*- Heptanoic acid[d]	ND	102 ± 113	435 ± 817	183 ± 199	192 ± 210	151 ± 237	NS	**
C7	2192	*n*- Octanoic acid[d]	1174 ± 1231	3727 ± 2899	5186 ± 4099	7982 ± 5158	9623 ± 8291	6362 ± 7524	*	***
C8	2395	*n*- Nonanoic acid[d]	ND	ND	ND	ND	ND	307 ± 617		
C9	2396	*n*- Decanoic acid[d]	490 ± 502	1439 ± 1119	2185 ± 1695	4073 ± 2525	4441 ± 3872	2959 ± 3862	*	**
		Total straight-chain acids	6532 ± 6275	25177 ± 20619	35012 ± 31119	52331 ± 43801	52247 ± 40014	35200 ± 49486	*	***
Alcohols										
C10	936	Ethanol[d]	171 ± 58.5	696 ± 931	743 ± 1065	1288 ± 2102	443 ± 505	145 ± 154	NS	NS
C11	1040	1-Propanol[d]	ND	ND	ND	ND	ND	287 ± 723		
C12	1145	1-Butanol[e]	ND	4.76 ± 8.93	10.6 ± 19.8	10.6 ± 19.6	20.3 ± 31.4	22.4 ± 24.9	*	***
C13	1205	3-Methyl-1-butanol[e]	7.46 ± 15.1	34.1 ± 29.8	58.2 ± 87.8	52.3 ± 55.9	29.5 ± 16.7	6.51 ± 7.76	*	***
C14	1249	1-Pentanol[e]	7.76 ± 6.20	4.60 ± 8.55	ND	19.4 ± 52.6	ND	ND	**	NS
C15	1351	1-Hexanol[e]	ND	ND	8.12 ± 15.5	8.69 ± 16.1	17.4 ± 20.8	26.0 ± 20.6	***	NS
C16	1482	2-Ethyl-1-hexanol[d]	9.66 ± 7.22	18.2 ± 22.2	5.41 ± 5.99	62.9 ± 147	15.8 ± 8.09	6.71 ± 9.78	NS	*
C17	1552	1-Octanol[e]	ND	ND	ND	ND	ND	3.15 ± 4.24		
		Total primary alcohols	196 ± 87.1	757 ± 1001	825 ± 1194	1442 ± 2393	526 ± 582	497 ± 945	NS	NS
C18	1025	2-Butanol[e]	ND	ND	ND	88.5 ± 124	812 ± 1102	424 ± 334	***	NS
C19	1120	2-Pentanol[e]	ND	ND	ND	1.60 ± 2.97	3.41 ± 8.25	27.0 ± 18.2	***	NS
C20	1122	3-Methyl-2-butanol[d,f]	ND	ND	ND	ND	4.41 ± 8.43	7.14 ± 13.3	NS	NS
C21	1218	2-Hexanol[e]	ND	ND	ND	ND	ND	1.44 ± 2.75		
C22	1316	2-Heptanol[d]	ND	ND	ND	30.7 ± 82.2	3.73 ± 6.99	12.2 ± 19.4	*	NS
C23	1509	2-Nonanol[e]	ND	ND	ND	ND	ND	7.29 ± 11.4		
C24	1640	2,3-Butanediol[d]	ND	27.1 ± 76.7	ND	32.1 ± 59.6	ND	ND	NS	*
C25	1646	Menthol[d,f]	6.65 ± 6.63	13.0 ± 10.4	27.5 ± 41.2	11.2 ± 12.6	ND	ND	**	**
		Total secondary alcohols	6.65 ± 6.63	40.1 ± 87.1	27.5 ± 41.2	164 ± 281	823 ± 1126	479 ± 399	***	NS
C26	1352	2-Methyl-3-pentanol[d]	9.41 ± 17.4	ND	ND	ND	ND	ND		
		Total tertiary alcohols	9.41 ± 17.4	ND	ND	ND	ND	ND		
C27	1115	2-Propen-1-ol[d,f]	ND	ND	10.7 ± 20.9	26.8 ± 49.7	31.8 ± 81.4	1.79 ± 5.06	NS	***
C28	1396	2-Nonen-1-ol[d,f]	0.497 ± 0.921	ND	ND	ND	ND	ND		
C29	1407	(E)-4-Hexen-1-ol[d,f]	ND	ND	ND	ND	ND	2.09 ± 3.94		
C30	1320	6-Heptene-2,4-diol[d,f]	8.00 ± 10.0	8.23 ± 18.7	ND	ND	ND	ND	**	*
		Total allyl alcohols	8.50 ± 11.0	805 ± 18.7	10.7 ± 20.9	26.8 ± 49.7	31.8 ± 81.4	3.88 ± 9.01	NS	***
		Total alcohols	221 ± 122	1602 ± 1107	863 ± 1256	1633 ± 2724	1381 ± 1789	980 ± 1353	*	NS
Aldehydes										
C31	1083	Hexanal[e]	ND	ND	1.55 ± 2.88	ND	ND	ND		
C32	1187	Heptanal[d]	3.62 ± 6.94	ND	ND	ND	ND	ND		
C33	1287	Octanal[e]	0.0911 ± 0.258	ND	ND	ND	ND	ND		
C34	1399	Nonanal[e]	7.93 ± 5.03	14.2 ± 4.22	15.2 ± 4.82	24.2 ± 35.2	14.4 ± 8.11	9.73 ± 12.1	NS	NS
		Total straight-chain aldehydes	11.6 ± 12.2	14.2 ± 4.22	16.7 ± 7.70	24.2 ± 35.2	14.4 ± 8.11	9.73 ± 12.1	NS	NS
C35	919	3-Methyl-butanal[d]	2.99 ± 5.54	3.96 ± 4.83	1.29 ± 2.44	2.44 ± 4.63	8.40 ± 10.8	43.5 ± 31.5	***	NS
		Total branched-chain aldehydes	2.99 ± 5.54	3.96 ± 4.83	1.29 ± 2.44	2.44 ± 4.63	8.40 ± 10.8	43.5 ± 31.5	***	NS
C36	1541	Benzaldehyde[e]	ND	ND	ND	3.90 ± 11.0	12.4 ± 23.0	1.80 ± 2.24	*	**
C37	1667	Benzeneacetaldehyde[d,f]	ND	ND	7.98 ± 15.6	ND	ND	ND		
		Total aromatic aldehydes	ND	ND	7.98 ± 15.6	3.90 ± 11.0	12.4 ± 23.0	1.80 ± 2.24	NS	NS
		Total aldehydes	14.6 ± 17.7	18.2 ± 9.05	26.0 ± 25.7	30.5 ± 50.8	35.2 ± 41.9	55.0 ± 45.8	*	NS
Ketones										
C38	817	2-Propanone[d]	13.8 ± 16.0	13.1 ± 16.9	10.6 ± 12.6	56.9 ± 142	ND	ND	*	***
C39	904	2-Butanone[e]	2.84 ± 5.25	ND	25.2 ± 21.3	1962 ± 2305	1465 ± 1572	243 ± 335	***	NS
C40	978	2-Pentanone[e]	ND	ND	ND	107 ± 129	57.2 ± 77.8	98.9 ± 73.9	***	NS

(*continued on next page*)

Table 1 (*continued*)

ID	LRI[a]	Volatile compounds	Ripening time (days)[b]						P-value[c]	
			1	7	14	30	60	120	RT	P
C41	1184	2-Heptanone[e]	17.5 ± 9.45	34.6 ± 12.7	39.7 ± 17.2	163 ± 226	82.1 ± 60.3	170 ± 211	**	*
C42	1288	2-Octanone[e]	1.82 ± 2.83	28.4 ± 74.6	1.57 ± 2.90	22.5 ± 57.0	1.92 ± 3.82	3.28 ± 6.14	NS	***
C43	1392	2-Nonanone[e]	12.4 ± 8.40	28.7 ± 13.1	34.5 ± 20.8	91.0 ± 69.7	64.7 ± 56.1	99.2 ± 111	*	*
C44	1447	8-Nonen-2-one[d]	ND	ND	ND	ND	ND	8.91 ± 11.2		
C45	1604	2-Undecanone[e]	ND	ND	2.72 ± 5.07	7.09 ± 10.8	6.59 ± 7.77	0.716 ± 1.35	*	NS
		Total methyl ketones	48.4 ± 42.0	105 ± 117	114 ± 79.8	2409 ± 2940	1678 ± 1778	624 ± 749	***	NS
C46	918	(E,E)-6,10-Dimethyl-5,9- dodecadien-2-one[d,f]	ND	ND	ND	ND	29.2 ± 64.9	ND		
C47	981	2,3-Butanedione[e]	60.3 ± 72.1	76.9 ± 79.1	86.2 ± 76.7	24.9 ± 46.4	22.7 ± 46.6	ND	**	**
C48	1292	3-Hydroxy-2-butanone[d]	349 ± 564	477 ± 401	533 ± 451	380 ± 377	179 ± 245	66.8 ± 110	*	***
C49	1367	2-Hydroxy-3-pentanone[d,f]	ND	ND	4.69 ± 8.77	1.45 ± 2.85	ND	ND	NS	**
		Total diketones and other ketones	409 ± 636	554 ± 480	624 ± 536	406 ± 427	230 ± 357	66.8 ± 110	*	***
		Total ketones	457 ± 678	659 ± 597	738 ± 616	2815 ± 3367	1908 ± 2135	691 ± 859	**	*
Esters										
C50	1186	Methyl hexanoate[e]	ND	3.81 ± 7.83	3.33 ± 6.17	ND	ND	ND	NS	**
C51	1187	Methyl 4-methyl pentanoate[d,f]	2.87 ± 5.34	7.40 ± 8.64	3.33 ± 6.17	ND	3.84 ± 7.20	4.53 ± 8.66	NS	**
		Total methyl esters	2.87 ± 5.34	11.2 ± 16.5	6.66 ± 12.3	ND	3.84 ± 7.20	4.53 ± 8.66	NS	**
C52	1038	Ethyl butanoate[d]	4.32 ± 3.12	27.0 ± 45.3	53.8 ± 97.8	78.3 ± 116	62.8 ± 81.3	288 ± 362	**	**
C53	1055	Ethyl 2-methyl butanoate[d]	ND	ND	ND	ND	1.77 ± 3.28	3.11 ± 5.75	NS	**
C54	1069	Ethyl 3-methyl butanoate[d]	ND	ND	ND	ND	44.2 ± 112	33.9 ± 65.4	NS	**
C55	1134	Ethyl pentanoate[e]	ND	ND	ND	ND	2.12 ± 4.92	2.42 ± 4.54	NS	**
C56	1234	Ethyl hexanoate[e]	18.7 ± 12.0	183 ± 267	343 ± 554	453 ± 584	480 ± 647	216 ± 270	*	*
C57	1292	Ethyl hex-4-enoate[d,f]	ND	ND	ND	7.24 ± 14.1	7.70 ± 15.1	0.292 ± 0.559	NS	***
C58	1335	Ethyl heptanoate[e]	ND	ND	4.78 ± 8.89	5.77 ± 10.7	3.86 ± 9.01	33.8 ± 53.2	NS	***
C59	1434	Ethyl octanoate[e]	5.27 ± 3.72	31.7 ± 45.9	71.5 ± 117	120 ± 139	101 ± 171	58.3 ± 95.9	*	**
C60	1537	Ethyl nonanoate[d,f]	ND	ND	ND	ND	ND	1.18 ± 2.20		
C61	1641	Ethyl decanoate[d]	4.47 ± 3.82	20.3 ± 24.1	458 ± 1275	1002 ± 2560	87.9 ± 140	320 ± 559	**	NS
C62	1855	Ethyl dodecanoate[d]	ND	ND	ND	ND	0.640 ± 1.80	1291 ± 2894	NS	*
		Total ethyl esters	32.7 ± 22.7	262 ± 383	930 ± 2051	1667 ± 3424	791 ± 1185	2248 ± 4312	***	**
C63	1122	Propyl butanoate[e]	ND	ND	ND	ND	3.35 ± 8.57	6.74 ± 7.88	**	***
C64	1320	Propyl hexanoate[e]	ND	ND	ND	ND	14.6 ± 19.6	27.1 ± 28.0	***	NS
C65	1521	Propyl octanoate[e]	ND	ND	ND	ND	ND	5.68 ± 8.53		
		Total propyl esters	ND	ND	ND	ND	18.0 ± 28.2	39.5 ± 44.4	***	NS
C66	1218	Butyl butanoate[d]	ND	ND	ND	ND	2.20 ± 5.05	2.98 ± 3.31	**	*
C67	1412	Butyl hexanoate[d]	ND	ND	ND	ND	3.34 ± 9.45	7.81 ± 7.31	***	NS
		Total butyl esters	ND	ND	ND	ND	5.54 ± 14.5	10.8 ± 10.6	***	NS
C68	1267	Pentyl butanoate[d,f]	ND	ND	2.11 ± 3.90	3.62 ± 6.81	2.45 ± 5.45	127 ± 237	NS	***
		Total pentyl esters	ND	ND	2.11 ± 3.90	3.62 ± 6.81	2.45 ± 5.45	127 ± 237	NS	***
C69	1414	Hexyl hexanoate[d]	ND	ND	ND	ND	ND	3.08 ± 6.05		
		Total hexyl esters	ND	ND	ND	ND	ND	3.08 ± 6.05		
C70	1131	1-Methylpropyl butanoate[d,f]	ND	ND	ND	ND	ND	5.56 ± 7.07		
C71	1323	2-Methylpropyl hexanoate[d]	ND	ND	ND	ND	11.4 ± 22.1	35.5 ± 32.4	***	NS
C72	1372	2-Propenyl hexanoate[d]	ND	ND	ND	ND	2.54 ± 5.47	0.485 ± 0.900	NS	**
C73	1461	3-Methylbutyl hexanoate[d,f]	ND	ND	ND	ND	1.55 ± 3.49	40.2 ± 74.8	NS	**
		Total branched-alkyl esters	ND	ND	ND	ND	15.5 ± 31.0	81.7 ± 115	***	NS
		Total esters	35.6 ± 28.0	273 ± 400	939 ± 2067	1671 ± 3431	836 ± 1271	2515 ± 4734	***	**
Hydrocarbons										
C74	700	Heptane[e]	7.46 ± 17.2	13.0 ± 17.6	7.10 ± 17.2	11.9 ± 14.2	5.45 ± 15.4	ND	NS	NS
		Total saturated hydrocarbons	7.46 ± 17.2	13.0 ± 17.6	7.10 ± 17.2	11.9 ± 14.2	5.45 ± 15.4	ND	NS	NS
C75	688	1,3-Pentadiene[d]	ND	ND	ND	ND	ND	2.42 ± 4.55		
C76	837	*t*-3-Octene[d]	35.6 ± 54.4	35.7 ± 44.8	36.9 ± 47.0	239 ± 621	16.3 ± 33.0	17.5 ± 33.5	NS	***
C77	956	1,3-Octadiene[d,f]	5.93 ± 11.3	7.08 ± 13.9	7.50 ± 14.7	98.5 ± 268	6.71 ± 15.3	6.22 ± 11.7	NS	***
C78	1041	Toluene[e]	20.2 ± 12.2	42.9 ± 28.4	24.9 ± 19.4	112 ± 261	19.9 ± 23.5	68.0 ± 112	NS	NS
		Total unsaturated hydrocarbons	61.7 ± 77.9	85.7 ± 87.1	69.3 ± 81.2	449 ± 1150	42.9 ± 71.8	94.1 ± 162	NS	***
		Total hydrocarbons	69.2 ± 95.1	98.7 ± 105	76.4 ± 98.4	461 ± 1164	48.4 ± 87.2	94.1 ± 162	NS	***
Sulphur compounds										

(*continued on next page*)

Table 1 (*continued*)

ID	LRI[a]	Volatile compounds	Ripening time (days)[b]						P-value[c]	
			1	7	14	30	60	120	RT	P
C79	746	Dimethyl sulphide[e]	1.55 ± 2.86	ND	ND	17.2 ± 46.3	ND	ND	NS	**
C80	1934	Dimethyl sulphone[d]	25.0 ± 34.0	8.05 ± 15.7	7.41 ± 14.0	61.4 ± 163	7.76 ± 14.5	6.77 ± 12.5	NS	***
		Total sulphur compounds	26.6 ± 36.8	8.05 ± 15.7	7.41 ± 14.0	78.6 ± 209	7.76 ± 14.5	6.77 ± 12.5	NS	***
Terpenes										
C81	1195	D-Limonene[e]	0.151 ± 0.430	1.35 ± 3.83	ND	1.02 ± 2.89	ND	ND	NS	NS
		Total terpenes	0.151 ± 0.430	1.35 ± 3.83	ND	1.02 ± 2.89	ND	ND	NS	NS

[a] LRI: linear retention index.
[b] ND: not detected.
[c] RT: ripening time factor effect; P: producer factor effect; NS: $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.
[d] Tentatively identified volatile compounds.
[e] Positively identified volatile compounds.
[f] Volatile compounds not previously described in Idiazabal cheese.

total abundance) (Supplementary Table 1), as observed previously (Barron et al., 2005a, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016), and the highest abundances were found between 30 and 60 days of ripening ($P \leq 0.05$) (Table 1). All the identified acids were straight-chain fatty acids, predominating hexanoic (44.2–51.1% of total acids), butanoic (18.8–27.3%) and octanoic acids (14.8–18.4%). These results agree with previous studies (Barron et al., 2005b, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016). However, 3-hexenoic acid was identified for the first time and no branched-chain acids were detected in this study. Volatile acids also predominate in other raw ewe milk-derived cheeses, such as Vastedda della valle del Belìce cheese, and similar changes during ripening have been reported (Delgado-Martínez et al., 2019; Gaglio et al., 2019a). Straight-chain fatty acids are also abundant in other raw ewe milk-derived cheeses (Gaglio et al., 2019a; Cardinali et al., 2021), although, in some cases, such as in Torta del Casar, acetic acid, branched-chain or long-chain FFAs predominate (Delgado-Martínez et al., 2019). Moreover, clear differences were observed among Idiazabal cheese producers ($P \leq 0.001$). Samples from producer B, who used commercial rennet, showed lower abundance of acids compared to producers A, C and D that used artisanal rennet. This was expected due to the higher pregastric lipase activity of artisanal rennet compared to commercial rennet (Virto et al., 2003) and its *sn*-3 stereospecificity (Thierry et al., 2017; Amores et al., 2021). Lipoprotein lipase (LPL) from raw milk and microbial lipases and esterases are also important lipolytic agents in raw milk cheeses (Thierry et al., 2017; Le Quéré and Buchin, 2022). However, LPL activity is very low in Idiazabal cheese (Chávarri et al., 1998).

Esters were the second family in order of importance throughout ripening (0.859–11.3%) (Supplementary Table 1), whose abundance increased until 120 days ($P \leq 0.001$) (Table 1). Ethyl esters predominated (89.4–99.8% of total esters throughout ripening), specifically, decanoic, hexanoic, dodecanoic and butanoic ethyl esters. Moreover, several esters were identified for the first time in Idiazabal cheese, such as methyl 4-methyl pentanoate or pentyl butanoate. Among minor esters, a significant increase was also observed for propyl, butyl and branched-alkyl esters during ripening ($P \leq 0.01$). These results partially agree with previous works in Idiazabal cheese, as far as identified compounds and their abundance are concerned (Barron et al., 2007; Abilleira et al., 2010b; Valdivielso et al., 2016). Compared to other raw ewe milk-derived cheeses, there are notable differences, although ethyl esters have also been described as predominant (Delgado-Martínez et al., 2019; Gaglio et al., 2019a; Cardinali et al., 2021). Significant differences among producers were only observed for methyl esters ($P \leq 0.01$), with producers B and C showing the highest abundance; and for ethyl and pentyl esters ($P \leq 0.01$ and $P \leq 0.001$, respectively), for which producer A presented the highest abundance. In general, the presence of esters has been associated to LAB esterase activity (Liu et al., 2004; Bertuzzi et al., 2018) and consequently, the observed differentiation among producers could be related to the different LAB composition.

Ketones abundance was remarkable (3.68–7.16%) (Supplementary Table 1), which also changed during ripening time ($P \leq 0.01$) (Table 1). Methyl ketones abundance increased until 30 days and decreased afterwards but remaining predominant, while diketones and other ketones showed the greatest abundance at the beginning of the ripening (Table 1). These results would explain why methyl ketones have been described as predominant in Idiazabal cheese before (Barron et al., 2005a, 2007; Valdivielso et al., 2016). Individually 2-butanone predominated, as observed in previous studies (Barron et al., 2007; Abilleira et al., 2010b; Valdivielso et al., 2016), together with 3-hydroxy-2-butanone that has previously been related to mountain grazing (Valdivielso et al., 2016). Some ketones, such as 2-hydroxy-3--pentanone or 6,10-dimethyl-5,9-dodecadien-2-one, have not been identified in Idiazabal cheese to date (Table 1). Differences among producers were only observed for diketones and other ketones ($P \leq 0.001$), with producers C and D showing the highest abundance. Compared to other raw ewe milk-derived cheeses, there are great

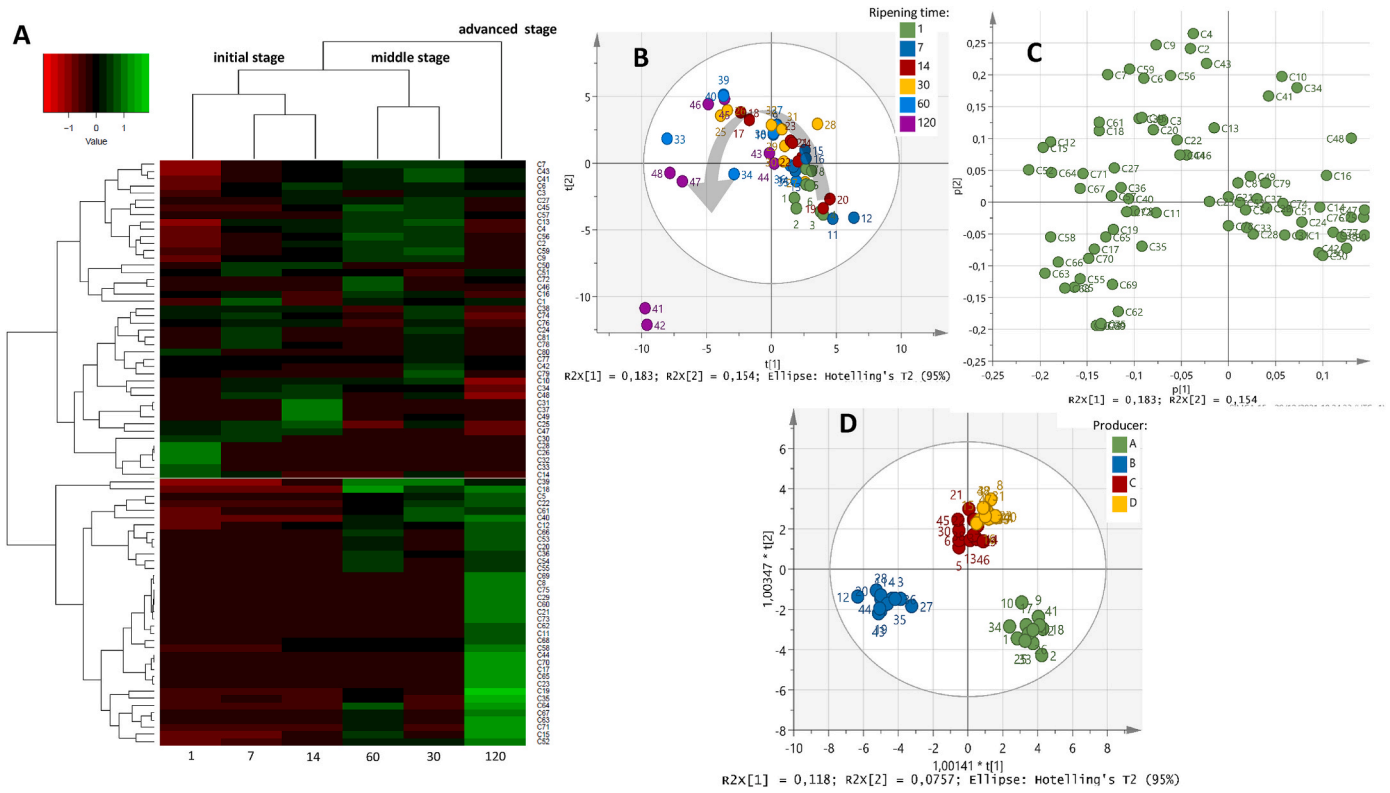


Fig. 1. "Analysis of volatile composition evolution during ripening (1, 7, 14, 30, 60 and 120 days) of Idiazabal cheese by means of HCA (A) and PCA (scores and loadings plots, B and C respectively) and scores plot of the OPLS-DA model based on the producer (D). Volatile compounds are labeled according to the ID (Table 1). The scale values of the HCA correspond to log transformed and UV scaled data."

differences in the identified ketones and their abundance during ripening (Delgado-Martínez et al., 2019; Gaglio et al., 2019a; Cardinali et al., 2021). Gezginc et al. (2021) have also reported 2-butanone as the most common ketone in Tulum cheese, whereas 2-heptanone, 2-nonanone and hydroxyacetone predominate in Gran Ovino cheese (Gaglio et al., 2019b). Ketones are formed by catabolism of FFAs, involving the oxidation to β-ketoacids and decarboxylation (Fox et al., 2017; Bertuzzi et al., 2018), mainly by mould and fungi (Fox et al., 2017; Le Quéré and Buchin, 2022).

Alcohols were another abundant chemical family (3.40–5.46%) (Supplementary Table 1), which showed the highest abundance at 30 days of ripening ($P \leq 0.05$) (Table 1). Primary alcohols were remarkable during ripening, although a decrease was observed from 30 to 120 days, when the abundance of secondary alcohols notably increased (Table 1). However, the ripening time effect was only significant for secondary alcohols ($P \leq 0.001$). These results would explain the great abundance of secondary alcohols reported before (Barron et al., 2005a, 2007; Valdivielso et al., 2016). Ethanol and 2-butanol predominated, which partially agrees with previous studies (Barron et al., 2007; Abilleira et al., 2010b) and several alcohols were identified for the first time in Idiazabal cheese, such as 3-methyl-2-butanol or 2-propen-1-ol. Differences among producers were only observed for allyl alcohols ($P \leq 0.001$), with producer A presenting the highest abundance. Compared to other raw ewe milk-derived cheeses, the evolution of alcohols and the predominant compounds differ (Fernández-García et al., 2004; Delgado-Martínez et al., 2019; Gaglio et al., 2019a; Cardinali et al., 2021). Gezginc et al. (2021) have also observed ethanol and 2-butanol as predominant in Tulum cheese, while Gaglio et al. (2019b) have reported 1-hexanol in Gran Ovino cheese. Alcohols biosynthesis has been related to lactose metabolism, reduction of carbonyl compounds (Le Quéré and Buchin, 2022) or several enzymatic and non-enzymatic reactions from amino acids or methyl ketones (Yvon and Rijnen, 2001; Bertuzzi et al., 2018; Le Quéré and Buchin, 2022).

Hydrocarbons, sulphur compounds, aldehydes and terpenes were minor compounds (<4%) (Supplementary Table 1), as observed before in Idiazabal cheese (Barron et al., 2005a, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016). However, there are differences compared to other raw ewe milk-derived cheeses (Delgado-Martínez et al., 2019; Gaglio et al., 2019a; Cardinali et al., 2021), such as the high abundance of aromatic hydrocarbons reported for Vastedda della valle del Belìce cheese (Gaglio et al., 2019a). The ripening time only had a significant effect on aldehydes ($P \leq 0.05$) (Table 1), with a predominance of straight-chain aldehydes at 30 days of ripening, aromatic aldehydes at 60 days and branched-chain aldehydes at 120 days. Nevertheless, the ripening time effect was only significant for the relative abundance of branched-chain aldehydes ($P \leq 0.001$) (Table 1). Individually, nonanal and 3-methyl-butanal were abundant, unlike in previous studies in Idiazabal cheese (Barron et al., 2007; Abilleira et al., 2010b; Valdivielso et al., 2016), and benzeneacetaldehyde was identified for the first time. The evolution of aldehydes and the predominant compounds differ for other raw ewe milk-derived cheeses (Fernández-García et al., 2004; Delgado-Martínez et al., 2019; Gaglio et al., 2019a; Cardinali et al., 2021). The presence of straight-chain aldehydes in cheese is related to the autoxidation of unsaturated fatty acids, both free and esterified (Bertuzzi et al., 2018). The presence of branched-chain and aromatic aldehydes, on the other hand, is related to the degradation of amino acids by non-enzymatic or enzymatic reactions by yeast or LAB (Yvon and Rijnen, 2001; Bertuzzi et al., 2018; Le Quéré and Buchin, 2022). Anyway, aldehydes are transitory compounds because of their rapid reduction to alcohols or oxidation to acids (Le Quéré and Buchin, 2022), which explains the low abundance observed. In addition, it should be noted that among minor compounds, significant differences among producers were observed only for unsaturated hydrocarbons and sulphur compounds ($P \leq 0.001$), with producer B showing the highest abundance in both cases. Overall, the formation of sulphur compounds is related to enzymatic reactions of sulphur-containing amino acids,

Table 2
Estimated OIR values throughout ripening time (1, 7, 14, 30, 60 and 120 days) and sensory description of each volatile compound detected in Idiazabal cheese samples ($n = 48$).

Volatile compounds	OT[a]	OIR[b] values during ripening (days)						Described odour notes	References[c]
		1	7	14	30	60	120		
Acids									
Acetic acid	22000				<1			Sour, vinegar, pungent, acid	1, 2, 3
n- Butanoic acid	50	**33.6**	**138**	**186**	**213**	**241**	**132**	Rancid, cheesy, putrid, sharp, sour, sweat	1, 2, 3
n- Pentanoic acid	137	<1	<1	**8.28**	**30.4**	<1	**3.13**	Sweat, putrid, sharp, sour, cheesy, burned	1, 3
n- Hexanoic acid	290	**10.9**	**43.5**	**57.9**	**84.6**	**88.4**	**53.7**	Sweat, sour, pungent, goat, rancid, cheesy, foot, faecal	1, 2, 3
(E)-3-Hexenoic acid	–				–			Pungent, sweat, vinegar, cheesy, green	4, 5
n- Heptanoic acid	3000				<1			Sweat, rancid, faecal	2, 3
n- Octanoic acid	450	**2.61**	**8.28**	**11.5**	**17.7**	**21.4**	**14.1**	Sweat, goat, soapy, waxy, musty, faecal, dust, cleaner	1, 2, 3
n- Nonanoic acid	4000				<1			Faecal, burned, fruity	3
n- Decanoic acid	10000				<1			Fatty, soapy, dust, waxy, burned	2, 3
Alcohols									
Ethanol	8				<1			Alcohol, winey, sweet, ethereal	1
1-Propanol	5700				<1			Alcohol, winey, sweet	1
1-Butanol	800				<1			Winey, sweet, fruity, fusel oil	1
3-Methyl-1-butanol	250				<1			Alcohol, winey, fruity, burned, herbal	1, 3
1-Pentanol	4000				<1			Alcohol, sharp, harsh	1
1-Hexanol	50				<1			Winey, oily, flower, fruity,	1, 2
2-Ethyl-1-hexanol	830				<1			Green, citrus, floral, oily, sweet	5, 6
1-Octanol	42				<1			Fatty, waxy, citrus, oily, walnut, moss, chemical, metal, burned	7, 8
2-Butanol	59	<1	<1	<1	**1.50**	**13.8**	**7.19**	Winey, alcohol, sweet, fruity, fusel oil	1, 2
2-Pentanol	41				<1			Alcohol, slightly green, winey, fruity	1, 2
3-Methyl-2-butanol	420				<1			Fruity	5
2-Hexanol	82				<1			Herbal, green, chemical, winey, fruity, fatty, terpenic, cauliflower	5, 9
2-Heptanol	70				<1			Earthy, sweet, fruity, oily, green, herbal	1, 2, 3
2-Nonanol	75				<1			Fatty, mild, green, melon, coconut	7, 9, 10
2,3-Butanediol	11000				<1			Fruity	3
Menthol	920				<1			Minty, cooling	11
2-Methyl-3-pentanol	420				<1			Grilled, bread	3
2-Propen-1-ol	5000				<1			Pungent, mustard	5
2-Nonen-1-ol	130				<1			Green, fatty, melon	5
(E)-4-Hexen-1-ol	100				<1			Green, herbal, musty, tomato	5
6-Heptene-2,4-diol	–				–			–	
Aldehydes									
Hexanal	5				<1			Green, herbal, sharp	1, 3
Heptanal	3	**1.21**	<1	<1	<1	<1	<1	Fatty, fruity, soapy, green, waxy, herbal	10, 12, 13, 14, 15
Octanal	1.5				<1			Fruity, green, citrus, fatty, fatty-fruity, lemon	10, 12, 13, 14, 15, 16
Nonanal	10	<1	**1.42**	**1.52**	**2.42**	**1.44**	<1	Sweet, fatty-floral, floral-waxy, rosy, citrus, peas, plastic	1, 2, 3
3-Methyl-butanal	0.200	**15.0**	**19.8**	**6.46**	**12.2**	**42.0**	**217**	Malt, chocolate, toffee, green	1
Benzaldehyde	325				<1			Almond, cherry stone, burned sugar	7, 14, 17
Benzeneacetaldehyde	5.50	<1	<1	**1.45**	<1	<1	<1	Floral, honey, daisy, green, violet-like, hyacinth, styrene, rosy, dry fruit, sweet	6, 10, 14, 16, 18, 19
Ketones									
2-Propanone	840				<1			Sweet, fruity, ethereal, nauseating	1
2-Butanone	30	<1	<1	<1	**65.4**	**48.8**	**8.09**	Sweet, ethereal, slightly nauseating	1
2-Pentanone	70000				<1			Sweet, fruity, ethereal	1, 2
2-Heptanone	5	**3.51**	**6.92**	**7.93**	**32.5**	**16.4**	**34.0**	Musty, blue cheese, pungent, soapy, flower	1, 2, 3
2-Octanone	41				<1			Fruity, musty, floral, green, herbal, mouldy, humidity, soapy, musty, blue-cheese	6, 8, 9, 10, 18
2-Nonanone	5	**2.48**	**5.75**	**6.91**	**18.2**	**12.9**	**19.8**	Musty, floral, fruity, soapy	1, 2
8-Nonen-2-one	–				–			Blue cheese, fruity baked	6, 8, 10
2-Undecanone	6.2	<1	<1	<1	**1.14**	**1.06**	<1	Fruity, herbal	2
(E,E)-6,10-Dimethyl-5,9-dodecadien-2-one	–				–			–	
2,3-Butanedione	3	20.1	25.6	28.7	8.29	7.56	<1	Buttery, sweet, cream, caramel	7, 10, 12, 13, 14, 15, 16, 20
3-Hydroxy-2-butanone	850				<1			Buttery, flower	1, 3
2-Hydroxy-3-pentanone	2500				<1			Fatty, truffle, earthy, nutty	5, 6, 10
Esters									
Methyl hexanoate	390				<1			Citrus, pineapple, ethereal	9, 16
Methyl 4-methyl pentanoate	–				–			Strawberry, roasted cocoa	21, 22
Ethyl butanoate	1	**4.32**	**27.0**	**53.8**	**78.3**	**62.8**	**288**	Fruity, apple, pineapple, banana, sweet, flower	1, 2, 3
Ethyl 2-methyl butanoate	–				–			Sweet, fruity	10, 13
Ethyl 3-methyl butanoate	0.1	<1	<1	<1	<1	**442**	**339**	Fruity, olive, sweet	2, 3
Ethyl pentanoate	8.7				<1			Fruity, sweet, acid, apple, pineapple, green, berry, tropical	5, 15, 18
Ethyl hexanoate	1	**18.7**	**183**	**343**	**453**	**480**	**216**	Fruity, apple, pineapple, banana, mouldy, flower	1, 2, 3
Ethyl hex-4-enoate	–				–			–	
Ethyl heptanoate	2.2	<1	<1	**2.17**	**2.62**	**1.75**	**15.4**	Fruity, pineapple, sweet, banana, berry, cognac and slightly green with a seedy nuance	5, 9, 18

(*continued on next page*)

Table 2 (*continued*)

Volatile compounds	OT[a]	OIR[b] values during ripening (days)						Described odour notes	References[c]
		1	7	14	30	60	120		
Ethyl octanoate	65	<1	<1	**1.10**	**1.85**	**1.55**	<1	Fruity, winey, pineapple, apricot, burned, earthy, flower	1, 2, 3
Ethyl nonanoate	377	<1						Cheesy, fruity	3
Ethyl decanoate	23	<1	<1	**19.9**	**43.6**	**3.82**	**13.9**	Fruity, winey, fatty, flower, humidity	2, 3
Ethyl dodecanoate	400	<1	<1	<1	<1	<1	**3.23**	Flower, vanilla	3
Propyl butanoate	124	<1						Fruity, sweet, pineapple, banana	1, 2
Propyl hexanoate	–	–						Fruity, pineapple, blackberry, fatty	1, 2
Propyl octanoate	–	–						Coconut	5
Butyl butanoate	100	<1						Fruity, pineapple, banana, sweet, fatty	1, 2
Butyl hexanoate	700	<1						Flower, fruity, pineapple, mouldy	2, 3
Pentyl butanoate	210	<1						Sweet, fruity, banana, pineapple, cherry, tropical	5
Hexyl hexanoate	6400	<1						Green, sweet, waxy, fruity with tropical and berry notes	5
1-Methylpropyl butanoate	–	–						Sweet, fruity, pineapple, rum, cherry, apple, overripe fruit	5
2-Methylpropyl hexanoate	–	–						Apple	1
2-Propenyl hexanoate	200	<1						Pineapple, fatty-fruity	1
3-Methylbutyl hexanoate	320	<1						Fruity, sweet, pineapple with a slightly pungent sour cheesy note	5
Hydrocarbons									
Heptane	950	<1						Solvent, sweet-ethereal, diffusive	1
1,3-Pentadiene	2500	<1						Plastic, paint, kerosene	23
t-3-Octene	–	–						Sharp, herbal, leather-like	2
1,3-Octadiene	5600	<1						Woody-moss	13
Toluene	1	**20.2**	**42.9**	**24.9**	**112**	**19.9**	**68.0**	Fruity, sweet-gassy, hydrocarbon	1
Sulphur compounds									
Dimethyl sulphide	1.2	**1.29**	<1	<1	**14.3**	<1	<1	Unpleasant wild radish, cabbage, sulphurous, pomegranate, corn, earthy, rancid	10, 12, 15, 20
Dimethyl sulphone	2.5	**10.0**	**3.22**	**2.96**	**24.5**	**3.10**	**2.71**	Sweet, flower, sulphurous, hot milk, burned	9, 10, 14
Terpenes									
D-Limonene	70	<1						Grass	3

[a] OT expressed as μg/L or μg/kg. Data are taken from the following: Abilleira et al. (2010b); Wang et al. (2021); Natrella et al. (2020); Sarhir et al. (2021); Majcher and Jeleń (2011); Kubícková and Grosch (1998); Attaie (2009); van Gemert (2011).

[b] OIR values calculated as mean relative abundance from 4 producers (A, B, C and D) at each ripening time (1, 7, 14, 30, 60, 120 days)/odour threshold. OIR with values higher than 1 are bold coloured.

[c] Described odour notes taken from: (1) Barron et al. (2005a); (2) Abilleira et al. (2010b); (3) Zabaleta et al. (2016); (4) Câmara et al. (2020); (5) The Good Scents Company Information System (2021); (6) Poveda et al. (2008); (7) Juric et al. (2003); (8) Jung et al. (2013); (9) Moio et al. (2000); (10) Curioni and Bosset (2002); (11) Zhang et al. (2022); (12) Natrella et al. (2020); (13) Sympoura et al. (2009); (14) Wang et al. (2020); (15) Karagul Yuceer et al. (2009); (16) Whetstine et al. (2005); (17) Chen et al. (2021b); (18) Qian and Reineccius (2002); (19) Fox et al. (2017); (20) Boscaini et al. (2003); (21) Campo et al. (2006); (22) Takeoka et al. (1995); (23) Horwood et al. (1981).

mainly methionine, by microorganisms (Fox et al., 2017; Le Quéré and Buchin, 2022), while hydrocarbons mainly originate from degradation of carotene (Povolo et al., 2007).

Subsequently, the dynamics of volatile compounds during ripening were analysed by multivariate analysis. Through an HCA, the volatile composition was divided into three stages: initial stage (1–14 days), middle stage (30–60 days) and advanced stage (120 days) (Fig. 1A). Acetic and heptanoic acids, methyl esters, most diketones, most straight-chain aldehydes and benzeneacetaldehyde, some alcohols (such as 2-methyl-3-pentanol or menthol), saturated hydrocarbons and terpenes characterized the initial stage. Most abundant acids, few ethyl esters, most methyl ketones and the rest of diketones, some primary and secondary alcohols, nonanal and benzaldehyde, unsaturated hydrocarbons and sulphur compounds characterized the middle stage. Finally, 3-hexenoic and nonanoic acids, most esters, the rest of primary and secondary alcohols, 3-methyl butanal and 8-nonen-2-one characterized the advanced stage. The characteristic presence of some volatile compounds at the initial and middle stage of ripening would explain why they have either not been identified or have been detected in smaller abundance in previous studies (Barron et al., 2005a, 2007; Abilleira et al., 2010b; Valdivielso et al., 2016). PCA approach confirmed the HCA results (Fig. 1B–C).

To study differences in the volatile composition among producers of the Idiazabal cheeses analysed, an OPLS-DA approach was applied. Despite its limitations, the model reported a clear distinction between producers, specifically, between A, B and C-D (Fig. 1D). Octanoic acid, ethyl octanoate or 2-octanone were some of the most important volatile compounds for such differentiation. The observed differentiation could be due to several reasons, such as the type of rennet used (Virto et al., 2003), as mentioned above; or the level of rennet lipase employed, since the higher the level, the greater FFAs release and sensory scores in Idiazabal cheese (Amores et al., 2021). Moreover, the sheep grazing system could also have affected the volatile composition of Idiazabal cheese, since clear differences have been reported in cheeses made from milk from extensive mountain grazing, compared to indoor feeding and part-time grazing sheep (Valdivielso et al., 2016). Finally, cheese making and ripening conditions (De Filippis et al., 2016) or microbial composition (Fox et al., 2017), have also been reported to affect the volatile composition of cheese, although it has not been studied in Idiazabal cheese.

3.2. OIR values

It is well known that not all volatile compounds contribute to cheese aroma, mainly due to their OTs (Starowicz, 2021). Therefore, OIR values were calculated to elucidate the key aromatic compounds of Idiazabal cheese (Table 2). Overall, esters and acids showed the highest OIR values during ripening and, consequently, were the most odour-active compounds. Esters OIR values increased as ripening progressed and all the identified odour-active esters were ethyl esters, predominating ethyl 3-methyl butanoate, ethyl butanoate and ethyl hexanoate (Table 2). Therefore, odour-active esters corresponded to predominant esters

(Table 1). The detected odour-active esters differ compared to other cheeses, although ethyl butanoate, ethyl hexanoate and ethyl octanoate are mainly reported (Qian and Reineccius, 2003; Taylor et al., 2013; Wang et al., 2020). Low OTs are reported for esters (Curioni and Bosset, 2002; Liu et al., 2004; Wang et al., 2021), hence they are described as potent odorants in several cheeses such as Grana Padano, providing sweet, floral and fruity odour notes and reducing the sharpness of FFAs (Curioni and Bosset, 2002; Liu et al., 2004; Bertuzzi et al., 2018). Fruity odour notes are related to ethyl esters (Moio et al., 2000; Curioni and Bosset, 2002; Liu et al., 2004) and specifically, apple, pineapple, olive or banana notes to the predominant odour-active esters (Table 2).

Volatile acids showed the highest OIR values at middle stage of ripening (Table 2), which is of special interest since excessive concentrations have been related to rancid or putrid odour notes in Idiazabal cheese (Barron et al., 2005b; Zabaleta et al., 2016). The predominant butanoic, hexanoic and octanoic acids (Table 1), together with pentanoic acid, were odour-active acids (Table 2). Therefore, a greater number of odour-active acids was observed compared to a previous study (Abilleira et al., 2010b). Volatile acids are notable aromatic compounds of various cheeses (Curioni and Bosset, 2002; Le Quéré and Buchin, 2022) and have been reported as key odorants in several cheeses, such as Cheddar, although there are differences among types of cheeses (Attaie, 2009; Taylor et al., 2013; Wang et al., 2020; Wang et al., 2021). It is noteworthy that there is no information on the OT of 3-hexenoic acid, which has been related to pungent or sweat odour notes (Table 2).

Some of the detected ketones presented high OIR values (Table 2), confirming their contribution to cheese aroma due to their low perception thresholds (Curioni and Bosset, 2002). The only odour-active diketone was 2,3-butanedione, which showed the highest OIR values at the initial stage of ripening and remained up to middle stage, providing buttery odour notes (Table 2). It has been described as an important odorant of many cheeses and fermented dairy products, such as Parmigiano Reggiano cheese (Qian and Reineccius, 2003; Attaie, 2009; Taylor et al., 2013). Within methyl ketones, the predominant 2-butanone (Table 1), together with 2-undecanone, showed high OIR values at middle stage of ripening and, therefore, less ripened Idiazabal cheeses would present sweet or fruity odour notes (Table 2). The 2-heptanone and 2-nonanone also showed high OIR values that increased until the end of the ripening, providing musty, blue cheese or floral odour notes. Methyl ketones are remarkable odorants of different cheeses, such as Cheddar, surface-ripened and blue-veined cheeses (Curioni and Bosset,

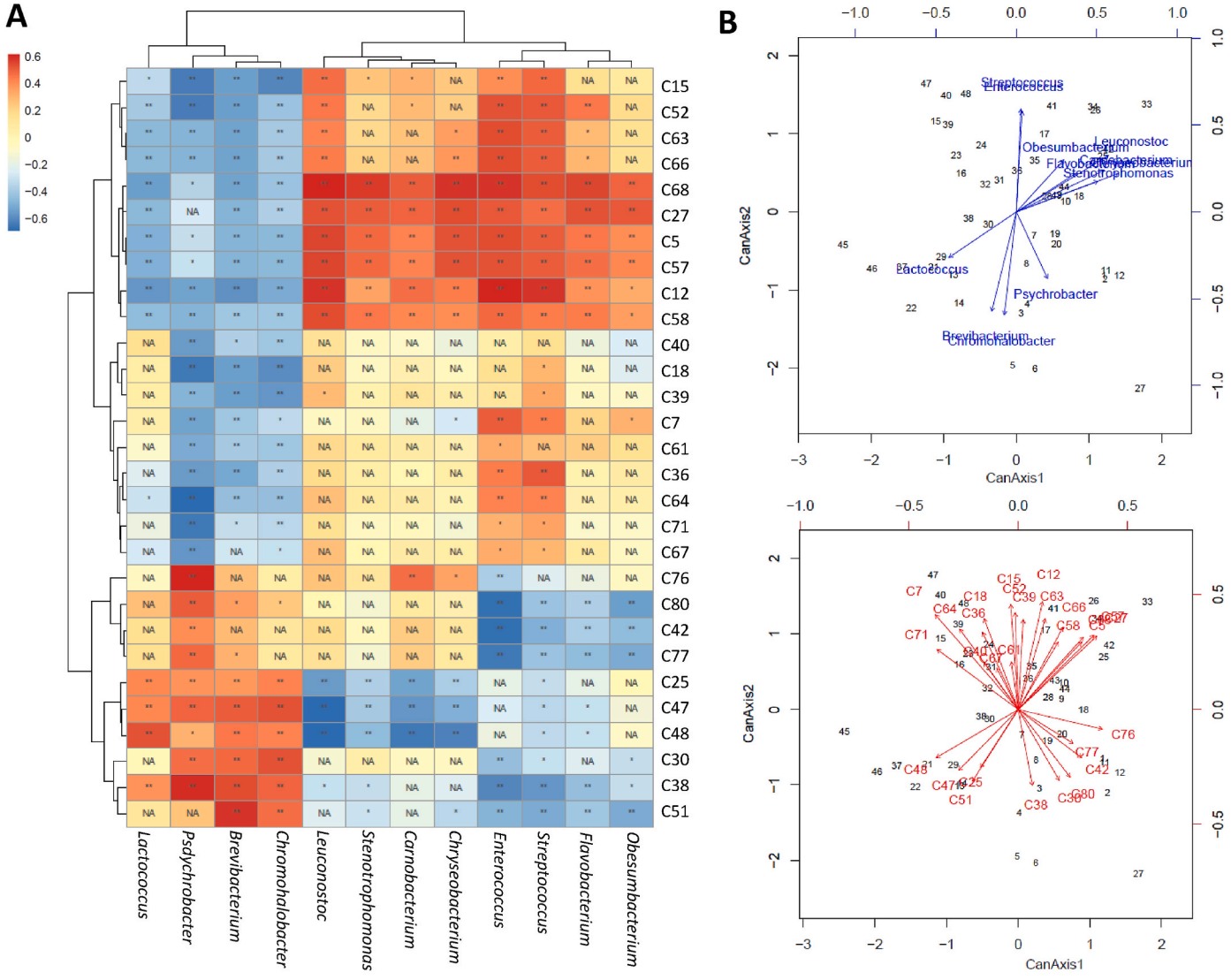


Fig. 2. "Correlation heatmap between key bacterial genera and volatile compounds (only those volatile compounds with at least one significant correlation greater than 0.500 are shown) (A) and verification of the resulted correlations through CCorA analysis (B). Volatile compounds are labeled according to the ID (Table 1). Significant correlations are represented by **P ≤ 0.01 and *P ≤ 0.05 and non-significant correlations (P > 0.05) by NA."

2002; Natrella et al., 2020; Wang et al., 2020; Wang et al., 2021). However, it is noteworthy that information on the odour impact of 2-butanone is scarce.

Aldehydes OIR values were in general low (<1) (Table 2). The 3-methyl butanal, which was the predominant aldehyde in the analysed Idiazabal cheeses (Table 1), was by far the most odour-active aldehyde, whose OIR values increased during ripening providing malt or chocolate notes (Table 2). Nonanal, which was also an abundant aldehyde (Table 1), presented lower OIR values, providing sweet or fatty-floral odour notes at initial and middle stages of ripening (Table 2). Within minor aldehydes, heptanal and benzeneacetaldehyde were also odour-active compounds and, although presenting low OIR values, they would contribute with fatty, fruity or floral odour notes at the initial stage of ripening (Table 2). These aldehydes have been previously reported as odour-active compounds in different cheeses (Natrella et al., 2020; Wang et al., 2020; Wang et al., 2021). Branched-chain aldehydes and, specifically 3-methyl butanal, are the most notorious odour-active aldehydes for cheese aroma, such as for Parmigiano Reggiano (Curioni and Bosset, 2002; Qian and Reineccius, 2003; Natrella et al., 2020). Straight-chain aldehydes are related to unpleasant green and herbaceous notes when exceed certain thresholds and nonanal is the most common odorant, remarkably for the aroma of Grana Padano cheese (Curioni and Bosset, 2002; Natrella et al., 2020; Wang et al., 2021). It is noteworthy that benzeneacetaldehyde has been reported as a remarkable odorant of Gruyère or Bovine Mozzarella cheeses (Curioni and Bosset, 2002; Wang et al., 2021).

Alcohols presented low OIR values (<1) and 2-butanol, which was one of the predominant alcohols (Table 1), was the only odour-active alcohol. It presented the highest OIR values between middle and advanced stages of ripening, providing winey or sweet odour notes (Table 2). 2-butanol is formed by reduction of 2-butanone (main ketone identified, as mentioned in section 3.1.), which, in turn, comes from the catabolism of butanoic acid (one of the predominant acids) and degradation of 2,3-butanediol (Urbach, 1993; Le Quéré and Buchin, 2022). Microbial degradation of 2,3-butanedione also leads to the production of 2-butanol (Urbach, 1993). However, information about its odour impact is scarce (Juric et al., 2003; Abilleira et al., 2010b). These results would confirm the small contribution of alcohols to cheese aroma, mainly due to their high OTs (Barlow et al., 1989).

Within the minor compounds identified (Table 1), hydrocarbons were minor odorants in cheese due to their high OTs, although they are precursors for other flavour compounds (Arora et al., 1995). The results obtained indicated toluene as the only odour-active hydrocarbon, which presented high OIR values throughout the entire ripening process, providing fruity, sweet-gassy or hydrocarbon odour notes (Table 2). Toluene originates from the degradation of carotene in milk (Villeneuve et al., 2013), although no information has been found in relation to its odour impact in cheese.

Both identified sulphur compounds presented high OIR values (Table 2). Dimethyl sulphide was an odour-active compound between initial and middle stage of ripening and dimethyl sulphone throughout the entire process. Therefore, sweet, flower or sulphurous odour notes would characterize the aroma of ripened Idiazabal cheese, but not unpleasant wild radish or cabbage (Table 2). Sulphur compounds are key odorants of many cheeses because of their low OTs (Curioni and Bosset, 2002; Le Quéré and Buchin, 2022), although they have not been reported for Idiazabal cheese. In other cheeses, such as Swiss, only dimethyl sulphide has been detected (Taylor et al., 2013; Natrella et al., 2020). Dimethyl sulphide is produced from sulphur-containing amino acids in the rumen (Villeneuve et al., 2013; Le Quéré and Buchin, 2022) and its oxidation results in the formation of dimethyl sulphone (Kilcawley et al., 2018).

The identified terpene (D-limonene) was not an odour-active compound (Table 2), as observed for Cheddar cheese (Wang et al., 2021). Information about the contribution of terpenes to the aroma of cheese is scarce (Curioni and Bosset, 2002). Terpenes are mainly attributed to the secondary metabolism of plants (Kilcawley et al., 2018), although, to a lesser extent, LAB can also synthetize them (Belviso et al., 2011). Consequently, they are mainly transferred from herbs or forages to milk fat (Viallon et al., 2000; Bertuzzi et al., 2018) and are potential pasture biomarkers (Abilleira et al., 2011; Kilcawley et al., 2018).

3.3. Correlation between bacterial communities and volatile compounds

According to a previous publication (Santamarina-García et al., 2022a), overall, the SLAB *Lactococcus* was the predominant genus during ripening of the analysed Idiazabal cheeses, although after 30 or 60 days of ripening its abundance decreased and the NSLAB began to proliferate. Specifically, the proliferation of *Lactobacillus* was promoted in cheeses collected from all producers (from a mean 0.0949% at 1 day of ripening to 8.96% at 120 days), while for the rest it depended on the producer. *Leuconostoc* proliferation was promoted in cheeses from producer A (from 4.48% at 1 day to 31.0% at 120 days), while in those from producers A and D, *Streptococcus* and *Enterococcus* proliferated (from 0.750% at 1 day to 4.52% at 120 days and from 0.675% at 1 day to 2.12% at 120 days, respectively). Additionally, the abundance of environmental or non-desirable bacteria decreased during ripening, although some genera remained abundant (>1%), namely *Obesumbacterium*, *Hafnia*, *Staphylococcus*, *Buttiauxella*, *Psychrobacter*, *Raoultella*, *Serratia, Brevibacterium* and *Erwinia*. Specifically, the abundance of *Buttiauxella* decreased during ripening, while that of *Staphylococcus* increased and the rest showed an increase at intermediate points of ripening (at 7, 14 or 30 days). Detailed data can be found in Santamarina-García et al. (2022a).

To elucidate the contribution of each bacterial genera to the volatile composition of Idiazabal cheese, an O2PLS approach with Spearman's correlations was applied (Fig. 2A). The obtained correlations were further confirmed through a CCorA (Fig. 2B). In this sense, 12 bacterial genera were reported as key bacteria related to volatile compounds production (Fig. 2A), namely *Psychrobacter, Enterococcus, Brevibacterium, Streptococcus, Leuconostoc, Chromohalobacter, Chryseobacterium, Carnobacterium, Lactococcus, Obesumbacterium, Stenotrophomonas* and *Flavobacterium.* The fact that LAB are reported as key bacteria is not surprising, since their genetic and metabolic diversity is considered responsible for flavour diversification in fermented products (Singh et al., 2006). Associations between these LAB and volatile compounds have previously been reported, such as for fermented yak milk (Jiang et al., 2020; Li et al., 2022). Nonetheless, information on *Carnobacterium* is scarce. Recent studies have also highlighted the notable contribution of environmental and/or non-desirable bacteria to the volatile composition of fermented products, such as Kazak cheese (Zheng et al., 2018; Meng et al., 2021).

Among LAB, *Enterococcus* and *Streptococcus,* followed by *Leuconostoc,* showed the largest number of high positive correlations with volatile compounds. These LAB showed positive correlations with volatile acids, mainly with 3-hexenoic and octanoic acids. These results could confirm that lipases and esterases from LAB are important lipolytic agents in cheese (Thierry et al., 2017), as reported in our previous publication (Santamarina-García et al., 2022b), and would affect the aroma of Idiazabal cheese (Table 2). Association between these LAB and acids production have also been observed for other fermented products such as sour meat (Meng et al., 2021; Zhong et al., 2021), although there is no information on 3-hexenoic acid. It is noteworthy the low correlation of these LAB to other acids, like butanoic or hexanoic acids, indicating a greater impact of the pregastric lipase of the rennet used (Virto et al., 2003). *Enterococcus*, *Streptococcus* and *Leuconostoc* were also highly correlated to alcohols, mainly 1-butanol and 2-propen-1-ol. This would indicate the implication of LAB metabolism in alcohols biosynthesis in cheese (Zuljan et al., 2016), as have been observed for other fermented food, such as Kazak fermented cheese (Zheng et al., 2018; Meng et al., 2021; Zhong et al., 2021). High correlations were also observed between these LAB and esters, such as pentyl butanoate, ethyl 4-hexenoate or

ethyl butanoate, some of which were odour-active compounds (Table 2). This could be indicative of LAB esterase activity (Santamarina-García et al., 2022b) and its impact on the aroma of Idiazabal cheese (Liu et al., 2004). Correlations between these LAB and ethyl butanoate have already been reported (Meng et al., 2021; Zhong et al., 2021), but there is no information on the rest of the esters. *Streptococcus* also showed high correlation to benzaldehyde, whose biosynthesis pathway is not very well known (Le Quéré and Buchin, 2022). Nonetheless, benzaldehyde production from phenylalanine by *Lactobacillus* has been demonstrated (Groot and de Bont, 1998). Finally, *Carnobacterium* and *Lactococcus* showed fewer high positive correlations to volatile aroma compounds. *Lactococcus* was the only LAB positively correlated to ketones, specifically to 3-hydroxy-2-butanone, whose production by the starter *Lactococcus lactis* has been proven (Le Quéré and Buchin, 2022) and suggested for Idiazabal cheese (Barron et al., 2007).

On the other hand, environmental and/or non-desirable bacteria also presented positive correlations with volatile compounds. *Psychrobacter* showed the largest number of correlations, mainly with 2-propanone, *t*-3-octene and dimethyl sulphone; followed by *Brevibacterium* that was correlated to 2-propanone, 2,3-butanedione and methyl 4-methyl pentanoate; and *Chromohalobacter* that was correlated to 6-heptene-2,4-diol and 2,3-butanedione. *Chryseobacterium*, *Obesumbacterium*, *Flavobacterium* and *Stenotrophomonas* showed fewer correlations, mainly with 2-propen-1-ol and pentyl butanoate. Associations between these 7 genera and other volatile compounds production have been observed in fermented products, such as Beitang shrimp paste (Yao et al., 2021; Zhao et al., 2022). However, to the best of our knowledge, the production of the aforementioned compounds by these bacteria has not been reported so far. Lipase and/or protease activities have been described for the aforementioned genera, for example *Chryseobacterium* and *Stenotrophomonas* (Mukhia et al., 2021; Rathakrishnan and Gopalan, 2022), except for *Obesumbacterium*. These activities could lead to the release of FFAs or FAAs that are substrates for the production of several volatile compounds (Le Quéré and Buchin, 2022). Taking into account the OIR values (Table 2), an interesting contribution of these environmental and/or non-desirable bacteria to the aromatic composition of Idiazabal cheese could be deduced. The metabolic potential of other environmental and/or non-desirable bacteria has also been reported (Zhao et al., 2021; Xia et al., 2022). For instance, Li et al. (2022) have observed a strong correlation between *Pseudomonas* and linalool in fermented red sufu. Similarly, Yang et al. (2021) have reported a correlation between *Staphylococcus* and 3-methyl butanal in fermented sausages.

In addition, several negative correlations were observed between key bacterial genera and volatile compounds (Fig. 2A). NSLAB were mainly negatively correlated to ketones (such as 2-propanone and 2,3-butanedione) and *Enterococcus* also to hydrocarbons (such as 1,3-octadiene) and sulphur compounds (such as dimethyl sulphone). Within environmental and/or non-desirable bacteria, *Psychrobacter, Brevibacterium* and *Chromohalobacter* showed the greatest number of negative correlations, mostly with alcohols and esters (such as 1-butanol or ethyl butanoate). These correlations could be related to volatile compounds degradation abilities by the aforementioned bacteria, which have been little described so far (Fonseca et al., 2021). Therefore, taking into account the bacterial dynamics during ripening (Santamarina-García et al., 2022a), the volatile composition evolution and the aroma development could be partially understood. Moreover, it is noteworthy that *Psychrobacter, Brevibacterium* and *Chromohalobacter* were negatively related to those volatile compounds that NSLAB could have synthetized and *vice versa*. Considering the obtained OIR values (Table 2), *Psychrobacter, Brevibacterium* and *Chromohalobacter* could have a negative effect on the aroma development caused by NSLAB and conversely. Negative correlations between bacterial genera and volatile compounds of fermented products have already been reported (Zhong et al., 2021; Li et al., 2022) and Zheng et al. (2018) have classified *Lactobacillus* as a negative factor for flavour development in Kazak cheese. Moreover, the fact that *Lactococcus* was positively related to ketones production while the rest of LAB were negatively related, could corroborate the previously observed competitive inhibition between autochthonous LAB and those that made up the starter (Santamarina-García et al., 2022a).

In summary, the results obtained would indicate that bacterial communities would have a notable role in the evolution of the volatile and aromatic composition of Idiazabal cheese during ripening. Thus, the different microbial composition observed between producers (Santamarina-García et al., 2022a) and compared to other raw ewe milk-derived cheeses (Gaglio et al., 2019a; Dimov et al., 2021), would partially explain the observed differences in the volatile composition.

4. Conclusion

The results showed that mainly ripening time modulated the volatile composition of raw ewe milk-derived Idiazabal cheese, with a predominance of acids, followed by esters, ketones and alcohols. In terms of odour impact, esters and acids were the predominant odour-active families during ripening and individually, ethyl hexanoate, ethyl 3-methyl butanoate, ethyl butanoate, butanoic acid or 3-methyl butanal were some of the most important aromatic compounds, providing fruity, rancid, cheesy or malt odour notes. In terms of functional relationship, *Psychrobacter, Enterococcus, Brevibacterium, Streptococcus, Leuconostoc, Chromohalobacter, Chryseobacterium, Carnobacterium, Lactococcus, Obesumbacterium, Stenotrophomonas* and *Flavobacterium* were key bacteria for the volatile and aromatic composition of Idiazabal cheese. Overall, non-starter lactic acid bacteria (NSLAB) showed high positive correlations to certain acids, esters and alcohols, whereas the starter LAB (SLAB) was related to particular ketones formation and environmental and/or non-desirable bacteria to certain ketones, hydrocarbons and sulphur compounds production. It is noteworthy that the SLAB *Lactococcus* and *Psychrobacter, Brevibacterium* and *Chromohalobacter* were described as having a negative impact on aroma development caused by NSLAB and *vice versa*.

CRediT authorship contribution statement

Gorka Santamarina-García: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, and, Visualization. **Gustavo Amores:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, and, Supervision. **Igor Hernández:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – review & editing. **Lara Morán:** Methodology, Validation, and, Resources. **Luis Javier R. Barrón:** Resources, Writing – review & editing, and, Funding acquisition. **Mailo Virto:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, and, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank the Idiazabal PDO cheesemakers for collaborating with this study and the technical and human support provided by SGIker (UPV/EHU/ERDF, EU), especially the chemometric support of L. Bartolomé. Financial support was provided by the Basque Government (IT944-16 and IT1568-22). G. Santamarina-García thanks the University of the Basque Country (UPV/EHU) for the predoctoral fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.100425.

References

Abilleira, E., Virto, M., Nájera, A.I., Salmerón, J., Albisu, M., Pérez-Elortondo, F.J., Ruiz de Gordoa, J.C., de Renobales, M., Barron, L.J.R., 2010a. Effects of seasonal changes in feeding management under part-time grazing on the evolution of the composition and coagulation properties of raw milk from ewes. J. Dairy Sci. 93, 3902–3909. https://doi.org/10.3168/jds.2009-2983.

Abilleira, E., Schlichtherle-Cerny, H., Virto, M., de Renobales, M., Barron, L.J.R., 2010b. Volatile composition and aroma-active compounds of farmhouse Idiazabal cheese made in winter and spring. Int. Dairy J. 20, 537–544. https://doi.org/10.1016/j.idairyj.2010.02.012.

Abilleira, E., Virto, M., Nájera, A.I., Albisu, M., Pérez-Elortondo, F.J., Ruiz de Gordoa, J.C., de Renobales, M., Barron, L.J.R., 2011. Effects of seasonal changes in feeding management under part-time grazing on terpene concentrations of ewes' milk. J. Dairy Res. 78, 129–135. https://doi.org/10.1017/S0022029910000750.

Aldalur, A., Bustamante, M.Á., Salmerón, J., Barron, L.J.R., 2021. Relationships between cheese-processing conditions and curd and cheese properties to improve the yield of Idiazabal cheese made in small artisan dairies: a multivariate approach. J. Dairy Sci. 104, 253–269. https://doi.org/10.3168/JDS.2020-18926.

Amores, G., Pérez-Elortondo, F.J., Albisu, M., Barron, L.J.R., 2021. Short communication: to what extent do environmental or technological conditions affect the sensory differentiation of raw Ewe milk cheeses produced in valley or mountain farms? J. Dairy Sci. 104, 301–307. https://doi.org/10.3168/JDS.2020-18358.

Arora, G., Cormier, F., Lee, B., 1995. Analysis of odor-active volatiles in cheddar cheese headspace by multidimensional GC/MS/sniffing. J. Agric. Food Chem. 43, 748–752. https://doi.org/10.1021/jf00051a035.

Attaie, R., 2009. Quantification of volatile compounds in goat milk Jack cheese using static headspace gas chromatography. J. Dairy Sci. 92, 2435–2443. https://doi.org/10.3168/JDS.2008-1732.

Ballesteros, C., Poveda, J.M., González-Viñas, M.A., Cabezas, L., 2006. Microbiological, biochemical and sensory characteristics of artisanal and industrial Manchego cheeses. Food Control 17, 249–255. https://doi.org/10.1016/j.foodcont.2004.10.008.

Barlow, I., Lloyd, G., Ramshaw, E., Miller, A., McCabe, G., McCabe, L., 1989. Correlations and changes in flavours and chemical parameters of Cheddar cheeses during maturation. Aust. J. Dairy Technol. 5, 7–18.

Barron, L.J.R., Redondo, Y., Aramburu, M., Pérez-Elortondo, F.J., Albisu, M., Nájera, A.I., de Renobales, M., 2005a. Variations in volatile compounds and flavour in Idiazabal cheese manufactured from Ewe's milk in farmhouse and factory. J. Sci. Food Agric. 85, 1660–1671. https://doi.org/10.1002/jsfa.2175.

Barron, L.J.R., Redondo, Y., Flanagan, C.E., Pérez-Elortondo, F.J., Albisu, M., Nájera, A. I., de Renobales, M., Fernández-García, E., 2005b. Comparison of the volatile composition and sensory characteristics of Spanish PDO cheeses manufactured from ewes' raw milk and animal rennet. Int. Dairy J. 15, 371–382. https://doi.org/10.1016/j.idairyj.2004.08.005.

Barron, L.J.R., Redondo, Y., Aramburu, M., Gil, P., Pérez-Elortondo, F.J., Albisu, M., Nájera, A.I., de Renobales, M., Fernández-García, E., 2007. Volatile composition and sensory properties of industrially produced Idiazabal cheese. Int. Dairy J. 17, 1401–1414. https://doi.org/10.1016/j.idairyj.2007.04.001.

Belviso, S., Giordano, M., Dolci, P., Zeppa, G., 2011. Degradation and biosynthesis of terpenoids by lactic acid bacteria isolated from cheese: first evidence. Dairy Sci. Technol. 91, 227–236. https://doi.org/10.1007/s13594-011-0003-z.

Bertuzzi, A.S., McSweeney, P.L.H., Rea, M.C., Kilcawley, K.N., 2018. Detection of volatile compounds of cheese and their contribution to the flavor profile of surface-ripened cheese. Compr. Rev. Food Sci. Food Saf. 17, 371–390. https://doi.org/10.1111/1541-4337.12332.

Boletín Oficial del Estado, 1993. Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen "Idiazabal" y su Consejo Regulador. BOE 289, 34591–34596. https://www.mapa.gob.es/images/es/boe_289_031293_tcm30-523120.pdf.

Boscaini, E., Van Ruth, S., Biasioli, F., Gasperi, F., Märk, T.D., 2003. Gas Chromatography−Olfactometry (GC-O) and proton transfer Reaction−Mass spectrometry (PTR-MS) analysis of the flavor profile of Grana Padano, Parmigiano Reggiano, and Grana trentino cheeses. J. Agric. Food Chem. 51, 1782–1790. https://doi.org/10.1021/JF020922G.

Bouhaddani, S. el, Houwing-Duistermaat, J., Salo, P., Perola, M., Jongbloed, G., Uh, H.-W., 2016. Evaluation of O2PLS in Omics data integration. BMC Bioinf. 17, S11. https://doi.org/10.1186/S12859-015-0854-Z.

Câmara, J.S., Lourenço, S., Silva, C., Lopes, A., Andrade, C., Perestrelo, R., 2020. Exploring the potential of wine industry by-products as source of additives to improve the quality of aquafeed. Microchem. J. 155, 104758 https://doi.org/10.1016/J.MICROC.2020.104758.

Campo, E., Ferreira, V., López, R., Escudero, A., Cacho, J., 2006. Identification of three novel compounds in wine by means of a laboratory-constructed multidimensional gas chromatographic system. J. Chromatogr. A 1122, 202–208. https://doi.org/10.1016/J.CHROMA.2006.04.048.

Cardinali, F., Ferrocino, I., Milanović, V., Belleggia, L., Corvaglia, M.R., Garofalo, C., Foligni, R., Mannozzi, C., Mozzon, M., Cocolin, L., Osimani, A., Aquilanti, L., 2021. Microbial communities and volatile profile of Queijo de Azeitão PDO cheese, a traditional Mediterranean thistle-curdled cheese from Portugal. Food Res. Int. 147, 110537 https://doi.org/10.1016/J.FOODRES.2021.110537.

Chávarri, F., Santisteban, A., Virto, M., de Renobales, M., 1998. Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase activities in industrial Ewe's milk and cheese. J. Agric. Food Chem. 46, 2926–2932. https://doi.org/10.1021/JF970968N.

Chen, C., Huang, K., Yu, H., Tian, H., 2021a. The diversity of microbial communities in Chinese milk fan and their effects on volatile organic compound profiles. J. Dairy Sci. 104, 2581–2593. https://doi.org/10.3168/JDS.2020-19053.

Chen, C., Zhou, W., Yu, H., Yuan, J., Tian, H., 2021b. Characterization of major odor-active compounds responsible for nutty flavor in Cheddar cheese according to Chinese taste. Flavour Fragrance J. 36, 171–181. https://doi.org/10.1002/FFJ.3627.

Curioni, P.M.G., Bosset, J.O., 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12, 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3.

De Filippis, F., Genovese, A., Ferranti, P., Gilbert, J.A., Ercolini, D., 2016. Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. Sci. Rep. 6, 1–11. https://doi.org/10.1038/srep21871.

Delgado-Martínez, F.J., Carrapiso, A.I., Contador, R., Ramírez, M.R., 2019. Volatile compounds and sensory changes after high pressure processing of mature "Torta del Casar" (raw Ewe's milk cheese) during refrigerated storage. Innovat. Food Sci. Emerg. Technol. 52, 34–41. https://doi.org/10.1016/J.IFSET.2018.11.004.

Dimov, S.G., Gyurova, A., Zagorchev, L., Dimitrov, T., Georgieva-Miteva, D., Peykov, S., 2021. NGS-based metagenomic study of four traditional Bulgarian green cheeses from tcherni vit. LWT–Food Sci. Technol. 152, 112278 https://doi.org/10.1016/j.lwt.2021.112278.

Fernández-García, E., Gaya, P., Medina, M., Nuñez, M., 2004. Evolution of the volatile components of raw ewes' milk Castellano cheese: seasonal variation. Int. Dairy J. 14, 39–46. https://doi.org/10.1016/S0958-6946(03)00148-1.

Fonseca, H.C., Melo, D. de S., Ramos, C.L., Menezes, A.G.T., Dias, D.R., Schwan, R.F., 2021. Sensory and flavor-aroma profiles of passion fruit juice fermented by potentially probiotic *Lactiplantibacillus plantarum* CCMA 0743 strain. Food Res. Int. 152, 110710 https://doi.org/10.1016/J.FOODRES.2021.110710.

Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., 2017. In: Fundamentals of Cheese Science, second. Springer US, New York. https://doi.org/10.1007/978-1-4899-7681-9.

Gaglio, R., Cruciata, M., Scatassa, M.L., Tolone, M., Mancuso, I., Cardamone, C., Corona, O., Todaro, M., Settanni, L., 2019a. Influence of the early bacterial biofilms developed on vats made with seven wood types on PDO Vastedda della valle del Belìce cheese characteristics. Int. J. Food Microbiol. 291, 91–103. https://doi.org/10.1016/J.IJFOODMICRO.2018.11.017.

Gaglio, R., Todaro, M., Scatassa, M.L., Franciosi, E., Corona, O., Mancuso, I., Di Gerlando, R., Cardamone, C., Settanni, L., 2019b. Transformation of raw ewes' milk applying "Grana" type pressed cheese technology: development of extra-hard "Gran Ovino" cheese. Int. J. Food Microbiol. 307, 108277 https://doi.org/10.1016/J.IJFOODMICRO.2019.108277.

Galindo-Prieto, B., Eriksson, L., Trygg, J., 2014. Variable influence on projection (VIP) for orthogonal projections to latent structures (OPLS). J. Chemometr. 28, 623–632. https://doi.org/10.1002/CEM.2627.

Gezginc, Y., Karabekmez-Erdem, T., Tatar, H.D., Dağgeçen, E.C., Ayman, S., Akyol, İ., 2021. Metagenomics and volatile profile of Turkish artisanal Tulum cheese microbiota. Food Biosci. 45, 101497 https://doi.org/10.1016/j.fbio.2021.101497.

Groot, M.N.N., de Bont, J.A.M., 1998. Conversion of phenylalanine to benzaldehyde initiated by an aminotransferase in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64, 3009. /pmc/articles/PMC106807/.

Horwood, J.F., Lloyd, G.T., Ramshaw, H., Stark, W., 1981. An off-flavour associated with the use of sorbic acid during Feta cheese maturation. Aust. J. Dairy Technol. 36, 38–40.

Jiang, Y., Li, N., Wang, Q., Liu, Z., Lee, Y.K., Liu, X., Zhao, J., Zhang, H., Chen, W., 2020. Microbial diversity and volatile profile of traditional fermented yak milk. J. Dairy Sci. 103, 87–97. https://doi.org/10.3168/jds.2019-16753.

Jung, H.J., Ganesan, P., Lee, S.J., Kwak, H.S., 2013. Comparative study of flavor in cholesterol-removed Gouda cheese and Gouda cheese during ripening. J. Dairy Sci. 96, 1972–1983. https://doi.org/10.3168/JDS.2012-5644.

Juric, M., Bertelsen, G., Mortensen, G., Petersen, M.A., 2003. Light-induced colour and aroma changes in sliced, modified atmosphere packaged semi-hard cheeses. Int. Dairy J. 13, 239–249. https://doi.org/10.1016/S0958-6946(02)00156-5.

Karagul Yuceer, Y., Tuncel, B., Guneser, O., Engin, B., Isleten, M., Yasar, K., Mendes, M., 2009. Characterization of aroma-active compounds, sensory properties, and proteolysis in Ezine cheese. J. Dairy Sci. 92, 4146–4157. https://doi.org/10.3168/JDS.2009-2124.

Kilcawley, K.N., Faulkner, H., Clarke, H.J., O'Sullivan, M.G., Kerry, J.P., 2018. Factors influencing the flavour of bovine milk and cheese from grass based versus non-grass based milk production systems. Foods 7, 37. https://doi.org/10.3390/FOODS7030037.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, 1–11. https://doi.org/10.1093/nar/gks808.

Kolde, R., 2019. Package "pheatmap". Retrieved from. https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf. (Accessed 12 July 2021).

Kubícková, J., Grosch, W., 1998. Quantification of potent odorants in camembert cheese and calculation of their odour activity values. Int. Dairy J. 8, 17–23. https://doi.org/10.1016/S0958-6946(98)00014-4.

Le Quéré, J.-L., Buchin, S., 2022. Cheese flavor. In: McSweeney, P.L.H., McNamara, J.P. (Eds.), Encyclopedia of Dairy Sciences. Academic Press, Amsterdam, pp. 79–90. https://doi.org/10.1016/b978-0-12-818766-1.00091-x.

Li, K., Tang, J., Zhang, Z., Wu, Z., Zhong, A., Li, Z., Wang, Y., 2022. Correlation between flavor compounds and microorganisms of Chaling natural fermented red sufu. LWT–Food Sci. Technol. 154, 112873 https://doi.org/10.1016/J.LWT.2021.112873.

Liu, S.Q., Holland, R., Crow, V.L., 2004. Esters and their biosynthesis in fermented dairy products: a review. Int. Dairy J. 14, 923–945. https://doi.org/10.1016/J.IDAIRYJ.2004.02.010.

Majcher, M.A., Jeleń, H.H., 2011. Key odorants of Oscypek, a traditional Polish Ewe's milk cheese. J. Agric. Food Chem. 59, 4932–4937. https://doi.org/10.1021/jf2002602.

Meng, Y., Chen, X., Sun, Z., Li, Y., Chen, D., Fang, S., Chen, J., 2021. Exploring core microbiota responsible for the production of volatile flavor compounds during the traditional fermentation of Koumiss. LWT–Food Sci. Technol. 135, 110049 https://doi.org/10.1016/j.lwt.2020.110049.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, R.A., 2008. The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinf. 9, 386. https://doi.org/10.1186/1471-2105-9-386.

Moio, L., Piombino, P., Addeo, F., 2000. Odour-impact compounds of Gorgonzola cheese. J. Dairy Res. 67, 273–285. https://doi.org/10.1017/S0022029900004106.

Mukhia, S., Kumar, A., Kumar, R., 2021. Generation of antioxidant peptides from soy protein isolate through psychrotrophic *Chryseobacterium* sp. derived alkaline broad temperature active protease. LWT–Food Sci. Technol. 143, 111152 https://doi.org/10.1016/j.lwt.2021.111152.

Natrella, G., Faccia, M., Lorenzo, J.M., De Palo, P., Gambacorta, G., 2020. Short communication: sensory characteristics and volatile organic compound profile of high-moisture mozzarella made by traditional and direct acidification technology. J. Dairy Sci. 103, 2089–2097. https://doi.org/10.3168/JDS.2019-17059.

Official Journal of the European Communities, 1996. Commission Regulation (EC) No 1107/96 of 12 June 1996 on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) No 2081/92. Q. J. Electron. Commer. QJEC 148, 1–10. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31996R1107.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., O'hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H., 2020. Package "vegan. Retrieved from. https://cran.r-project.org/web/packages/vegan/vegan.pdf. (Accessed 12 July 2021).

Poveda, J.M., Sánchez-Palomo, E., Pérez-Coello, M.S., Cabezas, L., 2008. Volatile composition, olfactometry profile and sensory evaluation of semi-hard Spanish goat cheeses. Dairy Sci. Technol. 88, 355–367. https://doi.org/10.1051/dst:2007021.

Povolo, M., Contarini, G., Mele, M., Secchiari, P., 2007. Study on the influence of pasture on volatile fraction of ewes' dairy products by solid-phase microextraction and gas chromatography-mass spectrometry. J. Dairy Sci. 90, 556–569. https://doi.org/10.3168/JDS.S0022-0302(07)71539-4.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196. https://doi.org/10.1093/nar/gkm864.

Qian, M.C., Reineccius, G., 2002. Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J. Dairy Sci. 85, 1362–1369. https://doi.org/10.3168/jds.S0022-0302(02)74202-1.

Qian, M.C., Reineccius, G., 2003. Quantification of aroma compounds in Parmigiano Reggiano cheese by a dynamic headspace gas chromatography-mass spectrometry technique and calculation of odor activity value. J. Dairy Sci. 86, 770–776. https://doi.org/10.3168/JDS.S0022-0302(03)73658-3.

R Core Team, 2020. The R Project for statistical computing. https://www.r-project.org. (Accessed 12 July 2021).

Rathakrishnan, D., Gopalan, A.K., 2022. Isolation and characterization of halophilic isolates from Indian salterns and their screening for production of hydrolytic enzymes. Environmental Challenges 6, 100426. https://doi.org/10.1016/J.ENVC.2021.100426.

Santamarina-García, G., Hernández, I., Amores, G., Virto, M., 2022a. Characterization of microbial shifts during the production and ripening of raw Ewe milk-derived idiazabal cheese by high-throughput sequencing. Biology-Basel 11, 769. https://doi.org/10.3390/BIOLOGY11050769.

Santamarina-García, G., Amores, G., López de Armentia, E., Hernández, I., Virto, M., 2022b. Relationship between the dynamics of gross composition, free fatty acids and biogenic amines, and microbial shifts during the ripening of raw Ewe milk-derived idiazabal cheese. Animals 12, 3224. https://doi.org/10.3390/ani12223224.

Sarhir, S.T., Amanpour, A., Bouseta, A., Selli, S., 2021. Fingerprint of aroma-active compounds and odor activity values in a traditional Moroccan fermented butter "Smen" using GC–MS–Olfactometry. J. Food Compos. Anal. 96, 103761 https://doi.org/10.1016/j.jfca.2020.103761.

Singh, T.K., Cadwallader, K.R., Drake, M., 2006. Biochemical processes in the production of flavor in milk and milk products. In: Hui, Y.H. (Ed.), Handbook of Food Products Manufacturing. John Wiley & Sons, Inc., New Jersey, pp. 715–748. https://doi.org/10.1002/9780470113554.ch78.

Starowicz, M., 2021. Analysis of volatiles in food products. Separations 8, 157. https://doi.org/10.3390/SEPARATIONS8090157.

Sympoura, F., Cornu, A., Tournayre, P., Massouras, T., Berdagué, J.L., Martin, B., 2009. Odor compounds in cheese made from the milk of cows supplemented with extruded linseed and α-tocopherol. J. Dairy Sci. 92, 3040–3048. https://doi.org/10.3168/JDS.2008-1802.

Takeoka, G.R., Buttery, R.G., Turnbaugh, J.G., Benson, M., 1995. Odor thresholds of various branched esters. LWT–Food Sci. Technol. 28, 153–156. https://doi.org/10.1016/S0023-6438(95)80028-X.

Taylor, K., Wick, C., Castada, H., Kent, K., Harper, W.J., 2013. Discrimination of Swiss cheese from 5 different factories by high impact volatile organic compound profiles determined by odor activity value using selected ion flow tube mass spectrometry and odor threshold. J. Food Sci. 78, C1509–C1515. https://doi.org/10.1111/1750-3841.12249.

The Good Scents Company Information System, 2021. Providing information for the flavor, fragrance, food and cosmetic industries. https://www.thegoodscentscompany.com. (Accessed 17 November 2021).

Thierry, A., Collins, Y.F., Mukdsi, M.C.A., McSweeney, P.L.H., Wilkinson, M.G., Spinnler, H.E., 2017. Lipolysis and metabolism of fatty acids in cheese. In: McSweeney, P.L.H., Fox, P.J., Cotter, P.D., Everett, D.W. (Eds.), Cheese: Chemistry, Physics and Microbiology, fourth ed. Academic Press, London, pp. 423–444. https://doi.org/10.1016/B978-0-12-417012-4.00017-X.

Trygg, J., Wold, S., 2003. O2-PLS, a two-block (X-Y) latent variable regression (LVR) method with an integral OSC filter. J. Chemometr. 17, 53–64. https://doi.org/10.1002/cem.775.

Urbach, G., 1993. Relations between cheese flavour and chemical composition. Int. Dairy J. 3, 389–422. https://doi.org/10.1016/0958-6946(93)90025-U.

Valdivielso, I., Albisu, M., de Renobales, M., Barron, L.J.R., 2016. Changes in the volatile composition and sensory properties of cheeses made with milk from commercial sheep flocks managed indoors, part-time grazing in valley, and extensive mountain grazing. Int. Dairy J. 53, 29–36. https://doi.org/10.1016/j.idairyj.2015.09.007.

van Gemert, L.J., 2011. In: Odour Thresholds: Compilations of Odour Threshold Values in Air, Water and Other Media, Second. Oliemans Punter & Partners BV, Zeist.

Viallon, C., Martin, B., Verdier-Metz, I., Pradel, P., Garel, J.-P., Coulon, J.-B., Berdagué, J.-L., 2000. Transfer of monoterpenes and sesquiterpenes from forages into milk fat. Lait 80, 635–641. https://doi.org/10.1051/LAIT:2000150.

Villeneuve, M.P., Lebeuf, Y., Gervais, R., Tremblay, G.F., Vuillemard, J.C., Fortin, J., Chouinard, P.Y., 2013. Milk volatile organic compounds and fatty acid profile in cows fed timothy as hay, pasture, or silage. J. Dairy Sci. 96, 7181–7194. https://doi.org/10.3168/JDS.2013-6785.

Virto, M., Chávarri, F., Bustamante, M.A., Barron, L.J.R., Aramburu, M., Vicente, M.S., Pérez-Elortondo, F.J., Albisu, M., de Renobales, M., 2003. Lamb rennet paste in ovine cheese manufacture. Lipolysis and flavour. Int. Dairy J. 13, 391–399. https://doi.org/10.1016/S0958-6946(03)00007-4.

Wang, B., Wang, J., Xu, L.Y., Zhang, J.H., Ai, N.S., Cao, Y.P., 2020. Characterization of the key odorants in kurut with aroma recombination and omission studies. J. Dairy Sci. 103, 4164–4173. https://doi.org/10.3168/JDS.2019-17521.

Wang, J., Yang, Z.J., Xu, L.Y., Wang, B., Zhang, J.H., Li, B.Z., Cao, Y.P., Tan, L., 2021. Key aroma compounds identified in Cheddar cheese with different ripening times by aroma extract dilution analysis, odor activity value, aroma recombination, and omission. J. Dairy Sci. 104, 1576–1590. https://doi.org/10.3168/JDS.2020-18757.

Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., Venables, B., Galili, T., 2020. Package "gplots". Retrieved from. https://cran.r-project.org/web/packages/gplots/gplots.pdf. (Accessed 12 July 2021).

Whetstine, M.E.C., Cadwallader, K.R., Drake, M.A., 2005. Characterization of aroma compounds responsible for the rosy/floral flavor in Cheddar cheese. J. Agric. Food Chem. 53, 3126–3132. https://doi.org/10.1021/JF048278O.

Xia, Y., Yu, J., Liu, H., Feng, C., Shuang, Q., 2021. Novel insight into physicochemical and flavor formation in koumiss based on microbial metabolic network. Food Res. Int. 149, 110659 https://doi.org/10.1016/J.FOODRES.2021.110659.

Xia, Y., Oyunsuren, E., Yang, Y., Shuang, Q., 2022. Comparative metabolomics and microbial communities associated network analysis of black and white horse-sourced koumiss. Food Chem. 370, 130996 https://doi.org/10.1016/J.FOODCHEM.2021.130996.

Yang, P., Zhong, G., Yang, J., Zhao, L., Sun, D., Tian, Y., Li, R., Rong, L., 2021. Metagenomic and metabolomic profiling reveals the correlation between the microbiota and flavor compounds and nutrients in fermented sausages. Food Chem. 375, 131645 https://doi.org/10.1016/J.FOODCHEM.2021.131645.

Yao, Y., Zhou, X., Hadiatullah, H., Zhang, J., Zhao, G., 2021. Determination of microbial diversities and aroma characteristics of Beitang shrimp paste. Food Chem. 344, 128695 https://doi.org/10.1016/J.FOODCHEM.2020.128695.

Yeluri Jonnala, B.R., McSweeney, P.L.H., Sheehan, J.J., Cotter, P.D., 2018. Sequencing of the cheese microbiome and its relevance to industry. Front. Microbiol. 9, 1020. https://doi.org/10.3389/fmicb.2018.01020.

Yvon, M., Rijnen, L., 2001. Cheese flavour formation by amino acid catabolism. Int. Dairy J. 11, 185–201. https://doi.org/10.1016/S0958-6946(01)00049-8.

Zabaleta, L., Gourrat, K., Barron, L.J.R., Albisu, M., Guichard, E., 2016. Identification of odour-active compounds in ewes' raw milk commercial cheeses with sensory defects. Int. Dairy J. 58, 23–30. https://doi.org/10.1016/j.idairyj.2016.01.018.

Zhang, J., Li, M., Zhang, H., Pang, X., 2022. Comparative investigation on aroma profiles of five different mint (*Mentha*) species using a combined sensory, spectroscopic and chemometric study. Food Chem. 371, 131104 https://doi.org/10.1016/J.FOODCHEM.2021.131104.

Zhao, Y., Wang, Y., Li, C., Li, L., Yang, X., Wu, Y., Chen, S., Zhao, Y., 2021. Novel insight into physicochemical and flavor formation in naturally fermented tilapia sausage based on microbial metabolic network. Food Res. Int. 141, 110122 https://doi.org/10.1016/J.FOODRES.2021.110122.

Zhao, D., Hu, J., Chen, W., 2022. Analysis of the relationship between microorganisms and flavour development in dry-cured grass carp by high-throughput sequencing, volatile flavour analysis and metabolomics. Food Chem. 368, 130889 https://doi.org/10.1016/j.foodchem.2021.130889.

Zheng, X., Liu, F., Shi, X., Wang, B., Li, K., Li, B., Zhuge, B., 2018. Dynamic correlations between microbiota succession and flavor development involved in the ripening of Kazak artisanal cheese. Food Res. Int. 105, 733–742. https://doi.org/10.1016/j.foodres.2017.12.007.

Zhong, A., Chen, W., Duan, Y., Li, K., Tang, X., Tian, X., Wu, Z., Li, Z., Wang, Y., Wang, C., 2021. The potential correlation between microbial communities and flavors in traditional fermented sour meat. LWT–Food Sci. Technol. 149, 111873 https://doi.org/10.1016/j.lwt.2021.111873.

Zuljan, F.A., Mortera, P., Alarcón, S.H., Blancato, V.S., Espariz, M., Magni, C., 2016. Lactic acid bacteria decarboxylation reactions in cheese. Int. Dairy J. 62, 53–62. https://doi.org/10.1016/j.idairyj.2016.07.007.

This article was published in Current Research in Food Science, Vol 6, Gorka Santamarina-García, Gustavo Amores, Igor Hernández, Lara Morán, Luis Javier R. Barrón, Mailo Virto, Relationship between the dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese, 100425,

4 | Appendix IV
Manuscript IV

Dairy, 3(3), 541-564

Antibiotics in dairy production: where is the problem?

Mailo Virto[1,*], **Gorka Santamarina-García[1],** Gustavo Amores[1] and Igor Hernández[1]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[*] *Correspondence: mailo.virto@ehu.eus; Tel.: +34-945013099*

Preliminary results related to this manuscript have been presented at:

International Symposium of Animal Sciences, 2023. *Producers' knowledge and attitudes on antibiotics utilisation in artisanal dairies producing a raw ewe milk-derived cheese (Idiazabal).* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Oral presentation).

3rd Food Chemistry Conference: Shaping a Healthy and Sustainable Food Chain through knowledge, 2023. *Elucidating the prevalence of antibiotic residues throughout a raw milk cheese production chain by LC-MS/MS and commercial testing: a farm-to-fork study.* Santamarina-García, G., Amores, G. and Virto, M. (Poster presentation).

37th EFFoST (European Federation of Food Science and Technology) International Conference, 2023. *Antimicrobial-resistant lactic acid bacteria in sheep flocks and in their raw milk used for cheese making.* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Oral presentation).

ECM 2023 - 2nd International Electronic Conference on Microbiology, 2023. *Distribution of antimicrobial resistant LAB along the production of a raw ewe milk-derived cheese.* Santamarina-García, G., Amores, G., Azcona, L., Hernández, I. and Virto, M. (Oral presentation).

EAR LTC-Sarea-ENLIGHT CONGRESS – Strengthening Antibiotic Resistance Networks, 2023. *High Throughput Quantitative PCR to elucidate the occurrence of antimicrobial resistance genes along the production-chain of raw ewe milk-derived Idiazabal cheese.* Santamarina-García, G., Amores G., Llamazares D., Hernández, I. and Virto M. (Poster presentation).

Published in **Dairy** (Milk Processing section)
ISSN: 2624-862X
CiteScore (2022): **2.4**
Category: **Agricultural & Biological Sciences (Q2, 59/147), Veterinary (Q2, 10/20)**
Citations: 36 (updated: 04/2024)
doi.org/10.3390/dairy3030039

dairy

MDPI

Review

Antibiotics in Dairy Production: Where Is the Problem?

Mailo Virto *, Gorka Santamarina-García, Gustavo Amores and Igor Hernández

Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain; gorka.santamarina@ehu.eus (G.S.-G.); gustavo.amores@ehu.eus (G.A.); igor.hernandezo@ehu.eus (I.H.)
* Correspondence: mailo.virto@ehu.eus; Tel.: +34-945013099

Abstract: Antibiotics have long been used for the prevention and treatment of common diseases and for prophylactic purposes in dairy animals. However, in recent decades it has become a matter of concern due to the widespread belief that there has been an abuse or misuse of these drugs in animals and that this misuse has led to the presence of residues in derived foods, such as milk and dairy products. Therefore, this review aims to compile the scientific literature published to date on the presence of antibiotic residues in these products worldwide. The focus is on the reasons that lead to their presence in food, on the potential problems caused by residues in the characteristics of dairy products and in their manufacturing process, on the development and spread of antibiotic-resistant bacteria, and on the effects that both residues and resistant bacteria can cause on human and environmental health.

Keywords: dairy animals; antibiotics; antibiotic residues; milk; dairy products; antibiotic resistance; resistant bacteria

Citation: Virto, M.; Santamarina-García, G.; Amores, G.; Hernández, I. Antibiotics in Dairy Production: Where Is the Problem? *Dairy* **2022**, *3*, 541–564. https://doi.org/10.3390/dairy3030039

Academic Editor: Steve Flint

Received: 23 May 2022
Accepted: 22 July 2022
Published: 1 August 2022

1. Use of Antibiotics in Dairy Animals

Antibiotics are defined as naturally occurring, semi-synthetic or synthetic compounds with antimicrobial activity that can be applied parenterally, orally, or topically. Antibiotics have been used in livestock care for more than 60 years for the prevention and therapy of common pathologies (mastitis, respiratory and foot diseases, etc.) and for prophylactic purposes. The use of antibiotics in animals destined to food production has been estimated to account for 73% of global antibiotic use [1] and 80% in the United States [2]. In Europe, the last joint report from the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) [3] indicates that the overall antibiotic consumption was, for the first time, lower in food-producing animals than in humans during the period covered in the report (2016−2018). The report concludes that the measures taken in Europe, at the state-level, to reduce the use of antibiotics in food-producing animals are being effective.

Tiseo et al. [4] estimated the global trends in antibiotics use in food animals from 2017 to 2030. They concluded that sales are expected to rise 11.5% by 2030. However, this increase is lower than previous estimates (53%) [2] due to recent reports indicating a decline in antibiotic use, particularly in China, the world's largest consumer.

Milk and dairy products are food of great nutritional, social and economic importance, produced all around the world by very diverse production systems and technologies [5]. Mastitis is the most frequent infectious disease in dairy animals, causing important economic losses in the dairy industry, despite the introduction of mastitis control programs over the last 30 years [6]. In United States, 16% of all lactating dairy cows receive antibiotic therapy for clinical mastitis each year [7]. Antibiotics used for its treatment include β-lactams (penicillin, cefapirin, ceftiofur, amoxicillin, hetacillin, and cloxacillin), macrolides (erythromycin), coumarines (novobiocin), and lincosamides (pirlimycin). In addition, during the dry period, cows are treated for existing subclinical mastitis infections and for

prevention [8]. In United States, more than 75% of all dairy cows receive intramammary infusions of prophylactic doses of antibiotics following each lactation, primarily penicillins, cefalosporins, or other β-lactams [7]. Currently, however, due to the growing concerns about antibiotic resistance, selective preventive treatment is being studied and considered worldwide in herds with low levels of contagious mastitis problems [9].

Other infectious diseases common in dairy cows are respiratory and uterine infections and infectious foot disease. Antibiotics used to treat foot infections include sulfonamides, β-lactams, tetracyclines, and lincomycin. Respiratory diseases or metritis are commonly treated with ceftiofur and other β-lactams, tylosin, tilmicosin, florfenicol, tetracyclines, and sulfadimethoxine [10].

On the other hand, the overall increase in milk production by dairy sheep and goats has resulted in an increased use of antimicrobials to treat mastitis and other diseases in these animals as well [6,11,12]. However, the availability of drugs registered for the use in lactating dairy sheep and goats is quite limited, leading to off-label use of some antibiotics by veterinarians and farmers [13].

2. Antibiotic Residues in Milk and Dairy Products

2.1. Origin of Antibiotic Residues in Milk

Residues, as defined by the European Union (EU) and the Centre for Veterinary Medicine, an agency under the Food and Drug Administration (FDA/CVM) in the United States, are "pharmacologically active substances (whether active principles, recipients or degradation products) and their metabolites which remain in foodstuffs obtained from animals to which the drugs in question have been administered" [14]. To ensure food safety for consumers, several regulatory authorities around the world, including the EFSA, FDA, and Codex Alimentarius, established tolerance levels of antibiotic residues (Maximum Residual Limit, MRL) in milk and other foodstuffs for consumer protection [15]. Table 1 shows the antibiotics used in human medicine and/or dairy animals and their MRL values as established by the European regulation.

Numerous factors influence the concentration of residues in milk, including the characteristics and health of the animal, the amount and type of the administered antibiotic, the method of antibiotics administration, the quantity of milk produced, etc.

Antimicrobials should be applied under veterinary prescription using authorized products and respecting the dose, the routes of administration, and withdrawal periods recommended by the manufacturers [13]. Once administrated to the animal, a big part of the antibiotic is metabolized for the purpose of detoxification and excretion. In general, most of the parent product and its metabolites are excreted in urine and, to a lesser extent, via faeces. However, part of the drug may persist for a period in the animal and can be found in the milk and meat [16]. In addition, for treating mastitis, antibiotics are generally administered by the intramammary route. By this route, the active antibiotic reaches high concentrations at the infection site, being more effective at lower doses [17]. However, the administered drug can be easily transferred from the mammary gland to the milk and is, therefore, the main cause of the presence of residues in it [18]. With intramammary application, residues are found for a longer period and in higher concentrations in milk than in cases where antibiotics are applied parenterally [19]. Because of that, it is compulsory to respect the 'withdrawal period'.

Table 1. Antibiotics used in human medicine and/or dairy animals (adapted from [20]).

Chemical Class	Compound	MRL (μg kg^{-1}) *	Primary Use
Aminoglycosides	Gentamycin	100	All animals, humans
	Kanamycin	150	Dogs, pigs, cattle, horses
	Neomycin	1500	All animals
	Spectinomycin	500	Dogs, pigs, cattle, horses
	Streptomycin	200	Obsolete
Quinolones	Enrofloxacin	100	All animals
	Ciprofloxacin	100	Humans
	Difloxacin	Not for use in animals from which milk is produced for human consumption	
	Danofloxacin	30	
	Marbofloxacin	75	All animals
	Flumequine	50	Humans
	Oxolinic acid	Not for use in animals from which milk is produced for human consumption	
β-Lactams (penicillins)	Amoxicillin	4	All animals
	Ampicillin	4	All animals
	Benzylpenicillin (Pen G)	4	All animals
	Cloxacillin	30	Cattle
	Dicloxacillin	30	Cattle
	Nafcillin	30	Humans
	Oxacillin	30	Cattle
β-Lactams (Cefalosporins)	Cefalonium	20	Dogs, cats and cattle
	Cefazolin	50	Humans
	Cefoperazone	50	Humans, cattle
	Cefquinome	20	Cattle, pigs
	Cefapirin	60	Cattle, sheep, goat and pigs
	Ceftiofur	100	Cattle, pigs
	Desacetylcefapirin	60	Metabolite of cefapirin
	Desfuroylceftiofur	100	Metabolite of ceftiofur
Macrolides	Tylosin	50	Animals only
	Tilmicosin	50	Sheep, cattle
	Spiramycin	200	All animals
	Neospiramycin	20	Metabolite of spiramycin
	Erythromicyn	40	Humans, cattle, chicken
Sulfonamides	Sulfadiazine	100	Humans
	Sulfadimethoxine	100	Cattle, pigs, chicken
	Sulfadimidine	100	Cattle, sheep, chicken
	Sulfamerazine	100	Humans and animals
	Sulfamethoxazole	100	Human
	Sulfamonomethoxine	100	Humans
	Sulfatiazole	100	Humans
	Trimethoprim	100	In combination with sulfonamides
Tetracyclines	Chlorotetracycline	100	Cattle, pigs
	Oxytetracycline	100	Humans, cattle, sheep, pigs
	Tetracycline	100	Humans, cattle, sheep, pigs
	Doxyicycline	Not for use in animals from which milk is produced for human consumption	
Lincosamides	Lincomycin	150	Pigs, cats, dogs, cattle
Amphenicols	Tiamphenicol	50	All animals
	Florfenicol	Not for use in animals from which milk is produced for human consumption	
	Florfenicol amine	Not for use in animals from which milk is produced for human consumption	
Nitroimidazoles	Dimetridazole	Prohibited	
	Ronidazole	Prohibited	
	Metronidazole	Prohibited	

* According to European regulation REG. 37/2010/CE.

As defined by Article 4 of the European Regulation (EU) 2019/6 [21], 'withdrawal period' is the minimum period between the last administration of a veterinary medicinal product to an animal and the production of foodstuffs from that animal. Under normal conditions of use, this period is necessary to ensure that such foodstuffs do not contain residues in quantities harmful to public health. This period has been determined, for different antibiotics, using scientific data and has to be provided by the supplier in the summary of the drug characteristics. For milk-producing animals, withdrawal periods have been established, in most cases, for cattle. For other dairy animals, like sheep and goats, the period has been determined for the most commonly used antibiotics. For antibiotics for which it is not provided, the European Regulation, e.g., sets some criteria to be applied by the veterinarian to calculate the minimum withdrawal period to be set, taking into consideration, among others, the time established for other dairy animals [21].

2.2. Antibiotic Residues in Commercial Cow's Milk Worldwide

The presence of antibiotics in commercial cow's milk has been known for many years. In early studies [22], it was reported that approximately 12% of the United States' cow milk supply was adulterated with β-lactam antibiotics prior to 1962. In Britain, in 1963, 11% of cow milk samples tested were found to contain penicillin. In 1998, data estimated that 1% of animal products in the United States and Europe contained antibiotic residues at very low levels [22]. In addition, a study published in 2000 [23] indicated that between 1988–1990, milk commercialized in North America contained detectable levels of tetracyclines (up to 80% of analysed samples in some studies), sulfamethazine, and other antibiotics. Nevertheless, the results of these early studies showed considerable variability due, as speculated by Mitchell et al. [22], to regional differences in animal husbandry, treatment, and slaughter practices and reflect the different sampling and test methodologies used.

Sachi et al. [16] reviewed the scientific literature that had antibiotic residues in milk as a research topic in the period between 1960 and 2017. They found 224 articles where antibiotic residues were analysed, quantitatively and qualitatively, in cow milk samples. However, the majority of works (82.14%) were about detection methods in which few samples were analysed and, in the majority, milk was spiked with known amounts of antibiotics in order to optimize the method.

In the same way, Treiber et al. [1] reviewed the scientific literature (from 1999 to 2019) about residues of antibiotics in food from an animal origin, focusing on commercial products. They found 73 studies in which antimicrobial residues were analysed in animal products; among them, 27 studied antimicrobial residues in milk. In relation to the importance of the topic, the number of publications found was relatively small.

Table 2 shows a detailed analysis of the articles cited in the mentioned reviews [1,16] in which data on commercial samples were provided. This table also includes articles found in a new search in which the same criteria as the cited reviews have been applied for the years 2019 to the present. Although the table does not pretend to be an exhaustive compilation of all published works, it reflects quite well the imbalance that exists in relation to the territories for which there are data published in the scientific literature in the last twenty years.

As can be appreciated in Table 2, the territories included in these studies correspond, in a great proportion, to Africa, Central and South America, and Asia. Trebier et al. [1] speculated that it might be because in the EU, United States, and some Asian territories, the legal guidelines regarding antibiotic residues in food are relatively strict and are normally checked by state authorities.

Table 2. Published research works on the detection of antibiotic residues in milk.

Territory	Sample Type	No. of Samples	Year [1]	Detection Method	Antimicrobials	% Positive Samples [2]	Concentration	Ref.
Kenya	Raw cow milk	1600	2001–2002	Two-tube diffusion Penicillinase βL plate assay	βL	13%	>4 µg/kg	[24]
	Cow milk	229	2014–2015	Charm II Blue-Yellow HPLC-UV	SAM TC	31.4% 0%	66.14–8979.51 µg/ kg	[25]
	Cow milk	95	2015	IDEXX SNAP®		7.4% βL, 3.2% TC		[26]
	Cow milk	55	2015–2016	IDEXX SNAP®	βL, SMZ,TC, GM	24% in at least one antibiotic		[27]
	Cow milk	65	2020	HPLC	AO, CO,TC, SMX,TriM	10.8% above MRL 20% detectable residues	6.7, 53.3, 30.6, 5.0, 6.2 µg/L, respectively	[28]
Tanzania	Cow milk	982	1999–2000	Charm AIM	βL, TC, AMG, ML, SAM	36%		[29]
	Raw cow milk	91	2006	Delvotest®		4.5%		[30]
	Raw cow milk	128	2006	IDF Method and Delvotest SP®		7%		[31]
	Raw cow milk From dairy farms	98	2010–2011	Delvotest SP®		83%		[32]
Algeria	Cow milk	194	2013–2014	Delvotest SP-NT® LC-MS/MS	βL, ML, SAM, QN, TC	25% (Delvotest®) 65% detectable		[33]
	Cow milk Goats milk	117 33	2019	Delvotest SP® BetaStar® Combo	βL, TC	12.67% (Delvo) 2.5% (cow βL), 1.7% (cow TC) 6.1% (goat βL), 3.0% (goat TC)		[34]
Nigeria	Cow milk	192	2015	Delvotest T®		9.9%		[35]
Ghana	Cow raw milk	224	2007	Charm Blue-Yellow		3.1%		[36]
Brazil	Pasteurized cow milk	151	2005–2006	SNAP tests ELISA kits	TC, βL, GM, CHA, StM, NM	41.3% 4 positive in CHA	dStM 260 µg/kg NM 69.8–110.2 µg/kg CHA 0.157–0.402 µg/kg	[37]
	Pasteurized cow milk	260	2006–2007	SNAP tests Ridascreen	TC, βL, GM, CHA, StM-dStM, NM	TC 18.5%, βL 3.5% GM 2.3%, CHA 1.5%, StM-dStM 0.4%, NM 17.4%	0.16–9.23 µg/kg, 25.86 µg/kg 60.08–278.42 µg/kg	[38]
	Pasteurized cow milk	299	2009	ELISA kit and LC–APCI–MS/MS QToF	StM, dStM	2 samples (ELISA) 0 LC-MS	213 and 290 µg/kg	[39]
	Pasteurized cow milk	252	2010–2011	Delvotest SP-NT® HPLC-DAD	TC OTC	8% positive 10% dubious	107–2297 µg/L 125–2782 µg/L	[5]
	Pasteurized milk	100	2010–2013	HPLC-UV/Vis	OTC, TC, cTC, dOC	3%	Average, 42.3 µg/kg	[40]
	Raw cow milk	184	2020	LC-MS/MS	βL, SAM, TC, QN, fQN, PY	CO (1), PNG (3) TC (11)	464 µg/L, 0.2–4.0 µg/L 7.1–49.7 µg/L	[41]

Table 2. *Cont.*

Territory	Sample Type	No. of Samples	Year [1]	Detection Method	Antimicrobials	% Positive Samples [2]	Concentration	Ref.
Puerto Rico, Barbados, Jamaica	UHT cow milk	80	1996–1997	Delvotest-P® HPLC-UV	βL	Puerto Rico 0% Barbados 8% Jamaica 10%	APC 1.8–18.4 µg/L CF 15 µg/mL CO 61–358 µg/L PNG 6.6–11.8 µg/L	[42]
Paraguay	Cow milk	450	2015	4Sensor and Gentasensor	GM, βL, StM, CHA, TC	0		[43]
Peru	Cow milk	156	2013	Snap Duo™ Beta-Tetra test		0–4.2%		[44]
Iran	Cow milk	196	2008	Copan test kit	βL,TC, SAM, AMG, ML	40.8%		[45]
	Pasteurized/raw cow milk	251	2009–2013	Copan test kit	βL,TC, SAM, AMG, ML	24.8%		[46]
	Pasteurized cow milk	432	2011–2012	HPLC-UV	TC	1.62%	274–1270 µg/L	[47]
	Cow tank milk	79	2012	HPLC-UV	βL	32.9 %		[48]
	Commercial cow milk	187	2012	Eclipse 100-kit HPLC-UV-vis	TC	19.8%	197–2452 µg/kg	[49]
India	Cow milk	491	2016–2017	DPA test and Charm ROSA	βL, TC, NV, EM, SMA	0.6%, 0.8%, 3.5%, 2.4%, 1%		[50]
	Raw/pasteurized cow milk	128/45	2018–2019	HPLC-DAD	AO, TC	1.7%, 1.2%	67.9 ± 40.9, 11.3 ± 1.5 µg/kg	[51]
	Cow milk	168	2019	MaxSignal (ELISA)	EF, OTC, PNG, SMX	1.7%, 1.2%, 0.6%, 0%	87.9 ± 44.0, 70.7 ± 45.9, 2.2 ± 1.5 µg/mL, nd	[52]
Bangladesh	Local/commercial cow milk	200	2011–2012	MIT, TLC HPLC	AO, TC, CPF	AO 14%, 38% local/commer TC 11%, 23% CPF 8%, 17%	AO 9.84, 56.16 µg/mL	[53]
	Cow milk	100	2019	TLC UHPLC	AO, OTC, StM, GM, CTX	2%, 3.33%, 1.33%, 0.6% 0.6 %	AO 124 µg/mL OTC 61.3 µg/mL	[54]

Table 2. *Cont.*

Territory	Sample Type	No. of Samples	Year [1]	Detection Method	Antimicrobials	% Positive Samples [2]	Concentration	Ref.
Nepal	Cow milk	140	2018	Agar diffusion HPLC	PN, SAM	23%	PN 0–16 µg/kg (2 samples 128, 256 µg/kg) SAM 0–64 µg/kg (in 9 samples 128–256 mg/kg)	[55]
Indonesia	Goat milk	36	2018	Triple bio screening test	TC, ML	TC 2.8%, ML 3.6%		[56]
Turkey	UHT cow milk	60	2005	Ridascreen	CHA, StM, TC	46.8% (CHA 30%)	806, 360, 602 ng/L	[57]
Kosovo	Milk from collection points and farms	1734	2009–2010	Delvotest P® SNAP tests, HPLC	βL, TC, SAM	6.11%	AO, PNG, and CO between 2.1 and 1973 mg/kg	[58]
	Cow milk	1055	2015–2016	Delvotest SP, SNAP test		10%		[59]
Croatia	Cow milk	90	2009	ELISA	StM, TC		0–73.82, 0–4.26 µg/kg	[60]
	Raw cow milk	1259	2008–2010	Delvotest® SP-NT Immunoassay (EIA) HPLC-DAD	PN, CPh, TC, SAM, AMG, ML	0.69%	12 µg/kg PNG 19 µg/kg AO, 1671 µg/kg TC	[61]
Slovenia	Raw cow milk	286	1991–2000	GC-ECD	CHA	1 sample	4.6 µg/ kg	[62]
Spain	Ewes raw milk	2686	2004	Delvotest® SP		1.7% positive, 2.1% "doubtful"	βL or SAM n.d.	[63]
	Ewes raw milk	71,228	2004–2008	Eclipse 100ov		1.36% (2004)–0.30% (2008)		[64]
Poland	Fresh and UHT cow milk	36 and 48	2019	PN ELISA Ridascreen	PN TC	1.15% below MRL 28.57% below MRL	0.040 to 0.804 µg/L 0.450 to 2.520 µg/L	[65]

[1] Year of collection or publication (in case no collection year is given). [2] When a screening test is used, data refers to samples that have an antibiotic concentration above the MRL. When a method that allows quantifying the antibiotic concentration is used, data refers to the proportion of samples in which the antibiotics are in concentration above the detection limit. Antibiotic abbreviations: Aminoglycosides (AMG), amoxicillin (AO), ampicillin (APC), β-lactams (βL), ceftriaxone (CTX), cephalin (CF), cephalosporins (CPh), chloramphenicol (CHA), chlortetracycline (cTC), ciprofloxacin (CPF), cloxacillin (CO), dihydrostreptomycin (dStM), doxycycline (dOC), enrofloxacin (EF), erythromycin (EM), fluoroquinolone (fQN), gentamicin (GM), macrolides (ML), neomycin (NM), novobiocin (NV), oxytetracycline (OTC), penicillins (PN), penicillin G (PNG), pyrimidine (PY), quinolones (QN), streptomycin (StM), sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfonamides (SAM), tetracyclines (TC), trimethoprim (TriM). Other abbreviations: Diodo Array Detector (DAD), Electron Capture Detector (ECD), Microbial Inhibition test (MIT), Maximun Residue Level (MRL), not detected (n.d.), Thin Layer Chromatography (TLC).

In this way, the report elaborated by The National Milk Drug Residue Data Base for the FDA in 2020 [66] collected the results of 4,049,727 tests on cow milk samples for three different antibiotic families (β-lactams, sulfonamides and tetracyclines). In this survey, 539 samples (0.013%) were reported as positive (above MRL) for at least one drug residue. With the exception of five samples that were positive for sulfonamides, the rest were positive for β-lactams. Similar results were found in reports of previous years, indicating an improvement in comparison with the early studies.

In the EU, the last report corresponds to 2019 [67]. For the group of antibacterials (e.g., β-lactams, tetracyclines, macrolides, aminoglycosides, sulfonamides and quinolones), 9555 samples of cow milk were tested, and the number of non-compliant samples were 0.12% (one sample in eleven states). However, three positive samples in chloramphenicol were detected (one sample in three states), although the use of this antibiotic is prohibited for veterinary use. Overall, the percentage of non-compliant samples in 2019 was comparable to the previous 11 years.

Data found in scientific literature referring to European states is very scarce (Table 2). Worth mentioning are the data from Kosovo, which are substantially worse than in other European states. The reason may be the fact that Kosovo is not a member of the EU, so it is not subject to its regulations and controls.

In Asia, China has been one of the world's largest dairy consumers for the last few decades, and food safety issues in the dairy sector have increasingly gained the attention of the Chinese government and the public [4,68]. Lu et al. [69] recently have published a review of the studies carried out in this territory. Because of that, we have not included them in Table 2. The review collects 46 surveillance cross-sectional studies published between 1988 and 2020, providing antibiotic levels for 8788 milk samples. Penicillin, tetracycline, chloramphenicol, and streptomycin are the most frequently tested antibiotics in milk samples. The pooled analysis reveals that 165 of 1701 fresh milk samples (9.7%) and 58 of 1220 sterilized milk samples (4.8%) exceeded the MRL limits. Overall, of the 18 evaluated antibiotics in Lu's work, the three with the highest positive rates are sulfamethoxazole, chloramphenicol, and trimethoprim. Nevertheless, although the antibiotic levels in fresh and sterilized milk fluctuate, they have greatly declined in recent years [69].

Data of other Asiatic territories, published in the scientific literature, are very variable and depend on the territory and, in some cases, even on the region, as happens, for example, in the case of Iran (Table 2).

In Central and South America, most published works refer to the situation of Brazil, where it seems to have improved according to the most recent studies.

In Africa, no MRL values are established, but according to values established in the EU or United States, the proportion of non-compliant samples is high in general (Table 2). This is due, most probably, to the fact that in most African territories there is no control over the distribution of veterinary antibiotics because the access to veterinarian pharmaceuticals is still unregulated [1].

The presence of chloramphenicol residues in some samples all around the world is worth mentioning. Chloramphenicol is a very effective broad-spectrum antibiotic, active against a wide variety of pathogens. However, its clinical use in humans can cause fatal side effects such as bone marrow aplasia, Gray baby syndrome, and aplastic anemia. For this reason, the FAO/WHO Expert Committee for antibiotics proposed zero tolerance for its residues in food in 1969 [62]. In the EU, the use of this drug for animal use has been prohibited since 1994 (Directive 1430/94 (EC 1994) [70]). Even so, it is still detected in some milk samples both in Europe, as mentioned before, and in the rest of the world (Table 2).

2.3. Antibiotic Residues in Sheep and Goats Milk

As can be seen in Table 2, the majority of the published data refers to cow's milk. Very few studies analyze milk from other ruminants, such as goats [34,56] or sheep [63,64]. In Mediterranean states, such as Spain, France, Italy, and Greece, the production of sheep and goat milk is mainly intended for the production of dairy products, such as different type

of cheese and yogurt. For this reason, the milk from these animals is not included in the EFSA's surveillance reports. Nevertheless, some states, such as Spain, control the presence of antibiotic residues prior to its use by methods that detect, at least, β-lactams [13].

Thus, very few scientific works have been found where a screening of a large amount of sheep or goats milk samples was carried out. For example, Yamaki et al. [63] analysed a total of 2686 raw sheep milk samples (of Manchega flocks that supplied milk for PDO Manchego cheese, from Spain). They found 1.7% positive results, although the test used did not allow them to identify which were the antibiotics present in the samples. A later work also carried out in Spain [64] analysed 209 dairy sheep flocks of the Assaf breed over 5 years. They obtained 71,228 records and found that the occurrence of non-compliant samples drastically decreased from 2004 (1.36%) to 2008 (0.30%), probably as a result of effective educational programs.

The two studies where commercial goat milk was analysed [34,56] are from Africa and Asia, and, in both, antibiotic residues were found above the MRL in percentages higher than those of sheep milk (Table 2).

2.4. Transfer of Antibiotic Residues from Milk to Dairy Products

Information on the transfer of antibacterial drugs from milk to dairy products found in the scientific literature is based, in most cases, on experimental processes in which antibiotic-free milk is spiked with known amounts of drugs and the effect of different treatments is analysed. Using this type of approach, it has been proved that the transfer depends on the characteristics of the production process and the treatments it includes, as expected [71].

Besides milk, the most consumed dairy products are yogurt and cheese. Although the process to make yogurt can vary depending on the type, in almost all cases, the production includes a first step of pasteurizing the milk. In general, thermal treatment leads to the degradation of antibiotic residues and, consequently, to a reduction of the residue concentration or bioactivity in the food product [72]. However, the values reported in the literature vary widely depending, among others, on the antibiotic, the matrix, and the applied temperature and time. Regarding the matrix, some authors [73] pointed out that penicillins degrade more in water than in milk under thermal treatment. However, other authors concluded that the results for penicillins and tetracyclines are inconclusive [72]. In any case, the temperature and time of application are the main factors that affect the rate of degradation of antibiotics, and, although their effect varies between antibiotics, in general, the treatments applied to pasteurize milk seem to only slightly reduce the concentration of most antibiotics [74]. For this reason, yogurts made from contaminated milk generally show an equal or slightly lower concentration of antibiotics than the milk used for their production [73,75].

Skimming, carried out usually by centrifugation, is used for producing low-fat dairy products. Hakk et al. [76] showed that the distribution of antibiotics between the fractions of milk is, mainly, based on their lipophilicity. They studied the distribution of penicillin G, sulfadimethoxine, oxytetracycline, and erythromycin between milk fat and skim milk fractions of cow milk and found that more than 90% of these antibiotics remained in the skimmed milk. In other studies, it also was found that tetracyclines remained in the skimmed milk in percentages higher than 80% [75] and that sulfonamides were distributed mostly to the aqueous milk fraction [77]. To the contrary, chloramphenicol seems to be mainly retained in high-fat products, such as butter and sour cream, with lower concentrations in white cheese and whey samples [78].

Hundreds of types of cheese are produced in the world, varying in the origin (animal) of the milk and its treatment (raw or pasteurized, full-fat or skimmed . . .) and the technology applied to produce them. Nevertheless, the majority of the following treatments are applied in the production of a great variety of cheeses: warming, starter culture addition and acidification, rennet addition and coagulation, whey draining, curd pressing, and salting. Among all these steps of cheese production, curd production and whey draining

are crucial in the fate of antibiotic residues, and they are the most studied processes using the aforementioned approach. Thus, some authors have studied the distribution of different antibiotics between curd and whey after spiking them to cow [75,79–82], sheep [83,84], and goats' milk [18,74,85,86].

In general, all these studies conclude that the retention of antibiotics in the curd and in the cheese depends fundamentally on their solubility in water and on their ability to interact with the fat and/or protein fraction [76,85]. Thus, β-lactam antibiotics are mostly transferred to whey due to their water-soluble nature [79,80,85], and, due to that, they are found in very low concentration in experimental [7,81] and commercial cheeses [72]. This phenomenon is important since cheese-making by-products, such as whey, are currently recycled in foodstuff manufacturing and are also used for animal feeding [87].

In addition to β-lactams, tetracyclines are the most-studied antibiotics to this respect, it being demonstrated for experimental cow [75], sheep [83,84], and goats [74] cheese that they are mostly retained in the curd and cheese. Giraldo et al. [85] concluded that, in general, aminoglycosides, quinolones, and tetracyclines seem to have a higher susceptibility to be retained in the cheese curd, as they found a reduction of the antimicrobial activity in the whey, ranging from 84 to 100% for these classes of antibiotics. Tetracyclines were also detected in commercial cheeses from Nigeria [71], Indonesia [74], or Pakistan [75]. Quintanilla et al. [15] described discrepant results in soft cheese made from milk enriched with various antibiotics, which they relate to the high whey content in this type of cheese.

Few studies have been found that describe what happens to the residues retained in the cheese during ripening [74,83,84,86]. These studies showed that, in general, the concentration of residues decreases significantly over time. Thus, β-lactams and erythromycin residues were not detectable after 30 days of Tronchon cheese ripening [74]. Quinolones enrofloxacin and ciprofloxacin seem to be more stable, showing a lower reduction rate along maturation (30–45%) [15,86]. Stability data for oxytetracycline varies between studies. In Tronchon-ripened cheese, a 95% reduction in the content of oxytetracycline was measured [74], whereas in 60- and 90-day-old ripened sheep cheese, only a reduction of 15–19% was observed when compared to 1-day ripened cheeses [83,84]. The reduction in antibiotic content during maturation is most likely due to degradation of the molecule. This process may depend on the ripening conditions, which are different for different types of cheese, which, in turn, would explain the different results found among studies.

2.5. Antibiotic Residues in Commercial Dairy Products

Since MRL values are not established for cheese or other dairy products, the analysis of these products is not included in the surveillance reports. Moreover, in the scientific literature, few works have been found where commercial dairy products were analysed (Table 3). As in the case of milk, most studies are from Africa and Asia, and antibiotic residues were found also in a high proportion of the analysed samples.

Table 3. Published research works on the detection of antibiotic residues in commercial dairy products.

Territory	Sample Type	No. of Samples	Year [1]	Method Detection	Antibiotics	% Positive Samples [2]	Concentration	Ref.
Nigeria	cow milk, goat milk, butterfat, soft cheese, yoghurt	8 of each	2014	HPLC-fluorimeter	TC	All below MRL	3.2 ± 1.8, 4.0 ± 1.1, 2.0 ± 0.8 8.0 ± 3.4, 1.9 ± 0.8 µg/L, respectively	[88]
	fresh milk local cheese fermented milk	328 180 90	2016	Premi® test HPLC	PNG	40.8% 24.4% 62.3%	15.22 ± 0.61 µg/L 8.24 ± 0.50 µg/L 7.6 ± 0.60 µg/L	[89]
Burkina Faso	Raw milk Curd Pasteurized milk Yogurt	29 40 42 90	2014	Microbial test	βL, SAM, TC	59.7% of samples positive for some antibiotic		[90]
Indonesia	Imported cheese (Cheddar, Mozzarella)	51	2015	Ridascreen	TC	13.7%	2.47 µg/L to 11.99 µg/L	[91]
Pakistan	Cheese Yogurt	40, 18	2011	HPLC	PNG, StM, TC		6.2, 4.0, 2.3 µg/L 1.7, 1.4, 1.1 µg/L	[92]

[1] Year of collection or publication (in case no collection year is given). [2] When a screening test is used, data refers to samples that have an antibiotic concentration above the MRL. When a method that allows quantifying the antibiotic concentration is used, data refers to the proportion of samples in which the antibiotics are in concentration above the detection limit. Antibiotic abbreviations: β-lactams (βL), penicillin G (PNG), streptomycin (StM), sulfonamides (SAM), tetracyclines (TC). Other abbreviations: Maximum residue level (MRL).

3. Effect of Antibiotic Residues in Dairy Products Elaboration

The presence of antibiotic residues in milk destined to make fermented dairy products could influence the technological processes, causing decreases in the quality of the final products, and, therefore, could have economic consequences for the dairy sector. The problems that the presence of antibiotics can cause in dairy product elaboration were described long time ago [93] and were summarized as failures in the growth of starter cultures, in the curdling of milk, in cheese ripening, and in acid and flavour production.

Lactic acid bacteria (LAB) help dairy products to gain their own aroma, smell, and texture [94]. Many LAB are used as starter cultures for producing fermented dairy products. In addition, non-starter LAB, coming from the raw material and the environment, contribute to the normal development of dairy product characteristics. Thus, most of the problems caused by antibiotic residues are due to the fact that they inhibit the development of LAB, slightly or completely, and delay the acid production by these bacteria. Lowering the pH is very important, for example, in the cheese-making process because it increases the activity of enzymes and the speed of coagulation, which is important especially in hard and long-matured cheeses [95]. In addition, insufficient pH lowering can cause early fermentation, supported by clostridia or by yeasts, and defects in the sensory characteristics of yogurt [96] and cheese [95].

Marth and Ellickson [94] reviewed the susceptibilities of various cheese and yogurt starter cultures to various antibiotics. They compiled the data on concentrations of antibiotics needed for the partial or complete inhibition of activity of various pure or mixed starter cultures and found differences in susceptibilities among species and in the amounts of antibiotics needed to cause inhibitory effects. In the same way, Cogan [97] analysed the susceptibility to penicillin, cloxacillin, tetracycline, and streptomycin of eight single-strain lactic streptococci, three commercial cheese starters, and six lactic acid bacteria isolated from yogurt. They found that the ranges of the antibiotics causing 50% inhibition of the bacteria were (µg/L): penicillin, 9–200; cloxacillin, 240–2500; tetracycline, 90–600; and streptomycin, 350–13,000. The yogurt isolates were found to be more resistant to streptomycin and more susceptible to penicillin than the cheese starters. All values are well above the MRL established for milk (Table 1).

The consequences of the inhibitory effect on the elaboration process and on the characteristics of the final product were analysed through experimental approaches, in which antibiotic-free milk is spiked with known amounts of antibiotics, and the effect of different treatments is analysed, as described before.

Marth and Ellickson [94] collected the studies carried out up to 1959 and showed that the addition of penicillin to milk for cheddar cheese production caused a delay in acid production in a dose-dependent manner. In addition, from a certain dose (variable depending on the study), the ripened cheeses presented a high pH value and defective sensory characteristics, like pasty body and fermented or yeasty aroma.

Similar approaches were carried out in more recent years to study the effect of residues of different types of antibiotics in the production of cheese [74,83,84,86,95,98,99] and yogurt [100–103]. For instance, antibiotic-free goat milk was spiked individually with seven antibiotics (amoxicillin, benzylpenicillin, cloxacillin, erythromycin, ciprofloxacin, enrofloxacin, and oxytetracycline) at an equivalent concentration of the European Union MRL and was used to make Tronchón mature cheeses [74]. The cheese-making process was unaffected by the presence of most antibiotics evaluated. Only erythromycin and oxytetracycline significantly increased the time required for cheese production because the kinetic of acidification was considerably affected by the presence of these antibiotics, requiring additional time to reach the final pH with respect to the control cheeses. The quality characteristics of the Tronchón cheeses were only slightly affected by antibiotics, with few significant differences in free fatty acids (FFA), which were in lower concentration in cheeses with amoxicillin and cloxacillin, and in the colour and some textural properties of the cheeses. Similarly, Cabizza et al. [83,84] showed that oxytetracycline at the MRL level produced a delay in the acidification of sheep milk, with no effect, in general, on physico-chemical parameters and the gross composition of cheeses. Quintanilla et al. [98] also found that the presence of oxytetracycline in goat raw milk, even at a concentration of up to double the MRL, only slightly affected the pH and some parameters of the ripened cheeses (FFA concentration, luminosity, springiness, and chewiness), without being perceptible by the sensory panel. Similar results were found for β-lactams in Manchego cheese elaboration [99] and for lincomycin at concentration lower than the MRL in a bovine milk cheese-making simulation [95]. To the contrary, the quinolone enrofloxacin does not produce significant changes in any of the technological, compositional, texture, and colour characteristics of Tronchón cheese when compared to the cheeses made with antibiotic-free milk, with the only exception of some compounds of the volatile fraction [86].

Similar results were found in the case of yogurt elaboration. For instance, studies with sheep milk yogurt have observed that the levels of some β-lactams (ampicillin, cephalexin and ceftiofur) close to or below the MRL and penicillin G above MRL could delay coagulation by more than 40 min and cause variations in final composition [100–102]. However, no delays were observed with amoxicillin at any concentration [100]. Enrofloxacin added to goat milk for elaborating yogurt also did not significantly affect the coagulation time and most yogurt properties [103].

4. The Use of Antibiotics and the Emergence of Antibiotic Resistant Bacteria

The greatest threat of the use of antibiotics is the emergence and spread of antibiotic resistance (AR) in pathogenic bacteria. Acquired resistance to certain antimicrobials is widespread to such an extent that their efficacy in the treatment of certain life-threatening infections is already compromised [104]. The selective pressure imposed by the use of antimicrobial agents plays a key role in the emergence of resistant bacteria (ARB). In a bacterial population exposed to antimicrobial agents, some are likely to develop resistance to them and, under selective pressure, may pass on their resistance genes to other members of the population [105]. Thus, the presence of antibiotic residues throughout the food chain can cause the development of transferable AR not only in pathogens, but even in commensal bacteria, including LAB [106,107]. Several reports indicate that fermented foods, including dairy products, could be considered as reservoirs of ARB. It has been reported that LAB and *Staphylococcus* sp. were the main AR gene (ARG) carriers in dairy products [108]. LAB isolated from traditional dairy products belong to different genera, such as *Lactococcus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Leuconostoc* sp., and *Streptococcus* sp. Among LAB, enterococci have been the subject of many studies regarding AR due to

the importance of some of them as opportunistic pathogens involved in serious infectious diseases in humans. Some studies have reported the detection of AR and virulence factors of enterococci in foods, including cheeses [109–111].

In the last twenty years, an overwhelming number of articles and reviews have been published describing the antibiotic resistance profile of bacteria, especially LAB, isolated from traditional fermented food, including dairy products. Therefore, it is beyond the scope of this review to collect all the published information on this topic (for that purpose, see, e.g., [106,112–114]).

Surprisingly, the relationship between the use of antibiotics in livestock and the presence of ARB in food has only been established indirectly and remains a contentious subject for study, which does not yield conclusive results [115,116]. This may be due, in part, to the lack of adequate models to study this relationship and because there is a poor understanding of the complex processes that lead to the emergence and spread of AR [115].

Aarestrup [117] collected several experimental and epidemiological studies and ecological observations showing that there is a close association between the use of standard dosages of antimicrobials in livestock and the emergence of resistance to those drugs. Zeina et al. [118] treated cows experimentally with gentamicin and streptomycin and found residues of the antibiotics in a concentration below their MRL in milk after the withdrawal period. All the *Staphylococcus aureus, Escherichia coli*, and *Listeria monocytogenes* they isolated from the milk showed high resistance to gentamicin, and 95% of *S. aureus*, 60% of *E. coli*, and 58% of *L. monocytogenes* isolates were resistant to streptomycin. In milk from non-treated cows, microbial isolates showed, in general, lower levels of resistance. However, they did not perform any statistical analysis to show whether the differences were significant or not. Moreover, a report on seven European states found a strong association between the total use of specific antimicrobial drugs and the level of resistance towards these agents in commensal *E. coli* isolates in pigs, poultry, and cattle [119]. More recently, the third ECDC/EFSA/EMA joint report on the integrated analysis of antimicrobial consumption (AMC) and AR [3] found statistically significant associations between antimicrobial use in animals in EU states and resistant *E. coli* and *Campylobacter jejuni* in the gut microbiota of animals. For *E. coli*, a positive association between AMC and AR was observed in almost all antimicrobial classes. Positive associations between AMC and AR were frequently also found in *C. jejuni*, but not in *Salmonella*.

On the other hand, antimicrobials at low dosages (i.e., residual levels in fed or food, sub-lethal or sub-therapeutic dosages) are also factors contributing to resistance because they promote genetic and phenotypic variability in exposed bacteria [115]. Thus, it has been demonstrated that the meat, meat products, and milk of cows treated with sub-therapeutic concentrations of antibiotics in South Africa had high counts of *Staphylococci* and *Enterobacteriaceae* resistant to streptomycin, methicillin, tetracycline, and gentamicin [120]. Other indirect studies demonstrating the relationship between the exposure to low antibiotic levels and the emergence of resistances are studies with milk, from cows receiving antimicrobial treatment, containing drug residues (waste milk, WM). Tempini et al. [121] found that 20% of *E. coli* isolated from WM showed multiple drug resistance, and only 40% of the isolates were sensitive to all antimicrobials tested. However, no significant association between the presence of drug residues in WM samples and AR in the *E. coli* isolates was found. Other studies, in which pre-weaned calves were fed WM containing very low concentration of antimicrobials, demonstrated that this practice led to increased faecal shedding of antimicrobial-resistant bacteria by calves [122–124].

Focusing on works that study the relationship between the presence of antibiotic residues in milk and dairy products and the appearance of ARB in these foods, the published data are very scarce. For example, in commercial samples, Brown et al. [26] quantified the prevalence of antibiotic residues in pasteurized and unpasteurized milk and ARB in milk sold in Kibera, Kenya. Among unpasteurized milk samples, 23% contained antibiotic residues and 66.7% contained detectable numbers of *E. coli* and, of these, 92.8% were positive for ampicillin and 50% for tetracycline-resistance. However, they did not find any

relationship between the presence of antibiotic residues and the presence of resistances. Zanella et al. [38] also did not find a significant relationship between the presence of antibiotic residues and antibiotic-resistant strains of coliform bacteria in pasteurized cow milk samples in Brazil. Similarly, El Zubeir [125] analysed milk samples for antibiotic residues and ARB in Khartoum, Sudan and found that 20% of samples were contaminated with antibiotics and that isolated bacteria from contaminated milk samples showed a wide range of multiple resistances. However, the authors did not analyse the relationship between the two parameters.

The inconsistency of the results and/or the lack of significant associations is most likely due to the complexity of the problem, as stated before. In all these studies it is impossible to separate AR originating from the pressure exerted by drug residues in the milk from other factors like, for example, those relating to on-farm or processing practices and the environment [120,121].

5. Other Aspects

5.1. Human Health

The presence of antibiotics residues in food, in general, and in milk and dairy products, in particular, may pose a serious threat to human health. The MRL values for antibiotics established by the corresponding authorities are based on the determination of the ADI (acceptable daily intake), which is the amount of a substance that can be ingested daily over a lifetime without appreciable health risk [126] (Figure 1a). Calculation of the toxicological ADI is based on an array of toxicological safety evaluation assays that take into account acute and long-term exposure to the drug and its potential impact on health [14]. Thus, health problems may arise when the MRL is exceeded or drug hypersensitivity reaction occurs.

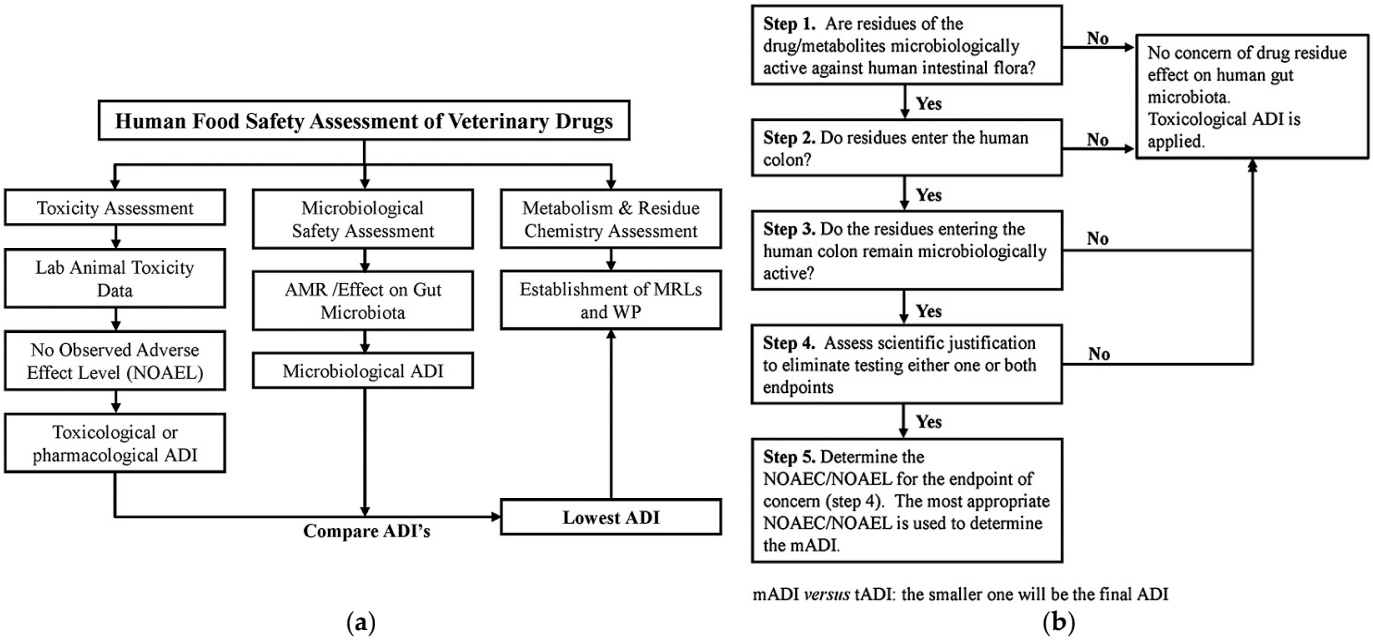


Figure 1. Food safety assessment of veterinary drugs (**a**). Stepwise determination of the need for a mADI (microbial acceptable daily intake) (**b**). ADI: acceptable daily intake; AMR: antimicrobial resistance; mADI: microbial ADI; MRL: maximum residue level; NOAEC: no observed adverse effect concentration; tADI: toxicological ADI; reproduced by permission from John Wiley and Sons [126].

Antibiotic residues may cause various toxic effects like allergy, immunopathological effects, carcinogenicity (sulfamethazine, oxytetracycline, furazolidone), mutagenicity, nephropathy (gentamicin), hepatotoxicity, reproductive disorders, bone marrow toxicity (chloramphenicol), and even anaphylactic shock in humans. All these effects have been

recently reviewed [14,127]. It must be noted that these reviews refer to the health effects of antibiotic residues in food in general. No reviews were found referring to milk or dairy products in particular.

In addition to their direct toxic effects, antibiotic residues can influence both gut microbiota composition and function. Antibiotics at therapeutic doses temporarily alter both the composition of the human gastrointestinal microbiota and the immune and metabolic health of the host [128]. However, the impact of residual concentrations, when ingested either via chronic or acute exposure events, remains very poorly understood [129], and, to the best of our knowledge, no study directed to analyse the impact of residues in specific food, such as milk or dairy products, on human microbiota has been conducted so far.

To establish the MRL values for antibiotics in food, a microbial ADI (mADI) is also estimated (Figure 1b) [126]. The assessment of the mADI for each antibiotic includes the evaluation of two possible effects. One is the capacity of the antimicrobial drug to disrupt the colonization barrier. The colonization barrier is a function of the intestinal microbiome that limits the colonization of the colon by exogenous microorganisms and the overgrowth of indigenous, potentially pathogenic, microorganisms. The second effect is the selection and emergence of AR, that is, the increase in populations of ARB in the gut. This effect may be due either to the acquisition of resistance by organisms which were previously sensitive, or to a relative increase in the proportion of bacteria that are already resistant [126]. Taking into account these two aspects, the no-observed-adverse-effect level (NOAEL) is estimated (Figure 1) in order to establish MRL values for each antibiotic. However, the challenge of this evaluation is to find appropriate methodologies to estimate these effects. To this respect, Piñeiro et al. [129] recently reviewed different aspects of the safety evaluation of veterinary drug residues in animal-derived foods and their effects on the human intestinal microbiome. They also discussed gaps in knowledge and methodology and reviewed the research and scientific approaches being carried out to fill those gaps. For instance, an early study on the subject [130] with murine models showed that the administration of sub-therapeutic antibiotic doses causes changes in the microbiome of young mice and in the copies of key genes involved in the metabolism of carbohydrates to short-chain fatty acids, increases colonic short-chain fatty acid levels, and alters the regulation of hepatic metabolism of lipids and cholesterol, highlighting the risk of feeding milk with antibiotic residues, especially early in life, during the lactation period.

One subject to which special attention has been paid is the role of the food chain in the transfer of ARB and ARG from food to human gut microbiota. As commented before, food contaminated with antibiotics, even at low levels, could be a reservoir of ARB. In addition, such bacteria may be commensal in animals but pathogenic in humans, or may be commensal in both but may later convey resistance to food-borne pathogens in the human gut [131]. However, demonstrating whether ARB in food could pass to the human gut microbiota has been challenging as a consequence of the complex transmission routes of resistances, which include animals, farms, food production facilities, food, and consumers (Figure 2). Nowadays, this study is more affordable thanks to the great development of molecular techniques that allow to detect the same gene in different samples, animals, humans, or food, even if they come from different species [132,133]. In this way, some of the ARG identified in food bacteria have also been detected in the human gut, providing indirect evidence for transfer by food handling and/or consumption. For instance, a study in the Netherlands reported increased levels of ESBL (extended spectrum β-lactamase) enzyme-producing bacteria with similar ARG in poultry meat and humans [134]. No similar work has been found reporting the transference of ARG from dairy product to the human gut microbiota.

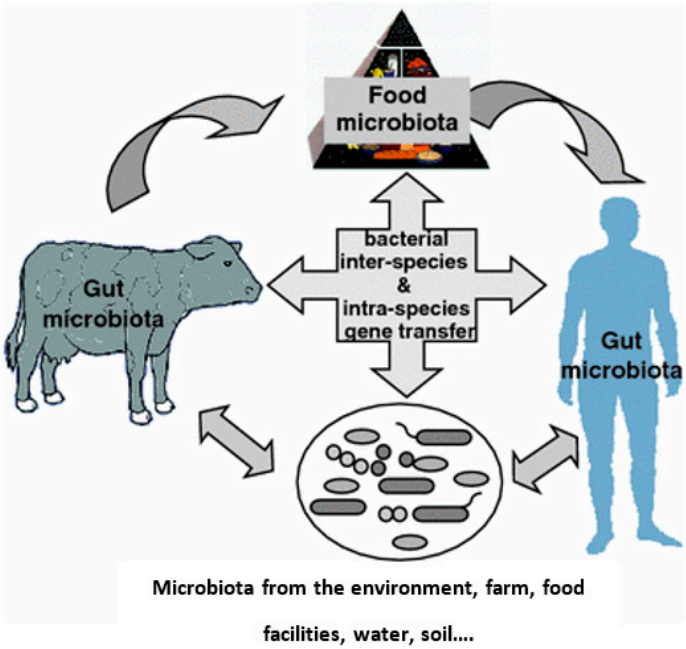


Figure 2. Graphic representation of transmission routes of resistant bacteria and resistance genes, which include animals, farms, food production facilities, food, and consumers (adapted from [112]).

However, several studies and reviews describing the resistance profile of LAB revealed the existence in their genome of mobile elements (plasmids, transposons, and integrons) and insertion sequences, which are responsible for intra- and inter-species transfer of genetic material [135,136]. These kinds of sequences have also been found in cheese [137,138]. In addition, the probiotic potential of many of these bacteria supports the idea of their potential ability to colonize the human gut and transfer ARG within the human gut microbiota, although this fact remains unproven to the best of our knowledge.

5.2. Dairy Farm Environment

Finally, antibiotic residues from dairy animal treatments also have environmental implications, as they can contaminate surface soil when eliminated through whey, urine, and/or faeces, which might affect the local microbiota and groundwater quality, having a big impact in the environment [20]. It has been estimated that 75% to 90% of antibiotics used in food animals are excreted, largely unmetabolized, into the environment and can be detected, for example, in the dust or the ground water of the farms [132]. Besides, by-products of dairies can be recycled. For example, the whey is used in the food manufacturing or animal feeding; manure can be used as fertilizer in vegetable cultivation and transfer residues to crops [139]. Thus, there are many ways by which dairy farming can contribute to the environmental spread of antibiotic residues (Figure 3).

Moreover, ARB present in the intestinal microbiota of farm animals are excreted in manure [140]. Thus, the application of manure in the land or the leaking of waste from storage tanks leads to the spread of ARG in the farm environment. In addition, ARG may be shared between animal, soil, and human bacteria via horizontal gene transfer [141] and, therefore, contribute to the worldwide problem of the increasing AR and multiresistance (Figure 3). However, the contribution of each link in the dairy chain, from farm to fork, to the global problem is still poorly understood [142], and its study is beyond the scope of this review. There are recent reviews and articles dealing with some aspects of this subject [141–143].

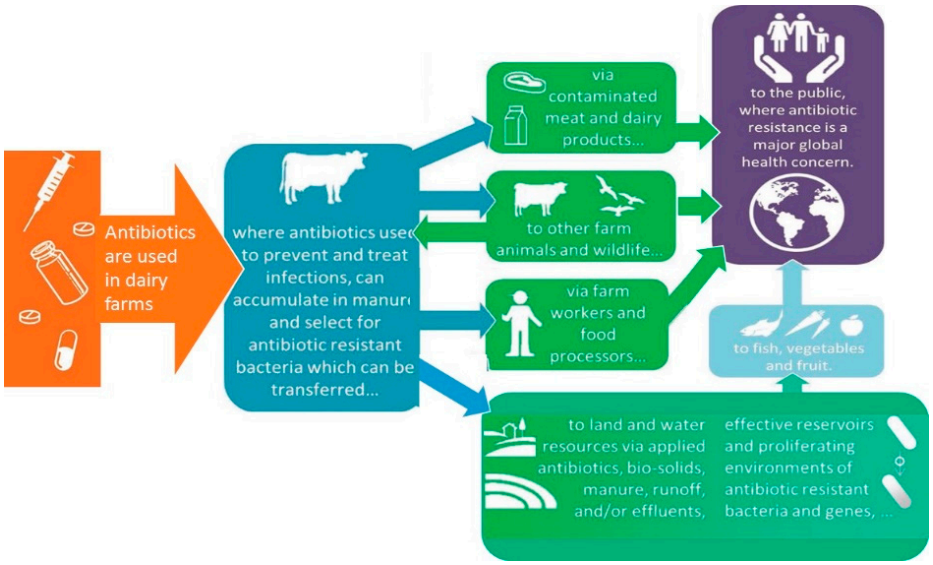


Figure 3. Potential routes of antibiotics and antibiotic resistance transmission, which have their origin in the use of antibiotics in the treatment of farm animals and can reach humans and the environment (adapted from [141] with the permission of Elsevier).

6. Conclusions: So, Where Is the Problem?

The presence of antibiotic residues is a problem with different levels. It is a systemic and widespread problem in developing territories, where regulation is lax or non-existent, constituting a major problem in their food chains. On the other hand, this is an episodic problem in states that have implemented regulation and monitoring systems for these compounds. These programs have reduced the proportion of contaminated food and companies related to its production, reinforcing the idea that its control is possible and desirable. In fact, the education of producers, the awareness of consumers, a guaranteed legal system, an adequate control system, and a "from farm to fork" strategy seem to be the pillars to reduce the concentration of these antibiotic residues throughout the food chain. Even after this strategy has been implemented, the occasional presence of food batches with traces of antibiotics on the market should force governments and institutions to continue supporting programs for their control.

As has been reviewed throughout the manuscript, milk and its derivatives may have antibiotic residues, but their evolution varies among products. The level of antibiotics in yogurts is similar to that present in the milk used for their elaboration. To the contrary, in the case of cheese, most antibiotics go with the whey in the elaboration process. Only aminoglycosides, quinolones, and tetracyclines seem to have a higher susceptibility to be retained in the cheese curd. Nevertheless, the remaining residues in the curd degrade throughout the ripening of cheese. Further research on new techniques/technologies for treating milk that remove antibiotics without affecting quality of dairy products, especially in the case of yogurt, could be the key to addressing this issue. In addition, it would be interesting to study the specific conditions of the production processes to unravel which parameters are important for the degradation or not of antibiotics.

Residues of antibiotics cause delays in the time needed to produce dairy products due to their inhibitory effects on LAB. However, only small changes in the characteristics of the final product have been described.

The development and spread of antibiotic resistant bacteria are the main concerns about the use of antibiotics in dairy animals. Resistant bacteria in the gut of dairy animals and in dairy products can transfer the resistance to pathogenic bacteria in the dairy product and in the human gut microbiota or contribute to its spread in the environment. However, the contribution of each link in the dairy production chain, from farm to fork, to the global problem is still poorly understood. Nevertheless, there is currently active research on the

different aspects of the subject, which gives hope that in the coming years the gaps in knowledge will gradually be filled.

Author Contributions: M.V.: writing—original draft preparation, G.S.-G., G.A. and I.H.: writing—review and editing, M.V.: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: The research group was funded by The University of the Basque Country (UPV/EHU) (grant COLAB20/14) and by the Basque Government, grant to Research Groups number IT944-16. G. Santamarina-García received a predoctoral grant from the University of the Basque Country.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the writing of the manuscript.

References

1. Treiber, F.M.; Beranek-Knauer, H. Antimicrobial Residues in Food from Animal Origin—A Review of the Literature Focusing on Products Collected in Stores and Markets Worldwide. *Antibiotics* **2021**, *10*, 534. [CrossRef] [PubMed]
2. Van Boeckel, T.P.; Brower, C.; Gilbert, M.; Grenfell, B.T.; Levin, S.A.; Robinson, T.P.; Teillant, A.; Laxminarayan, R. Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 5649–5654. [CrossRef]
3. European Centre for Disease Prevention and Control (ECDC); European Food Safety Authority (EFSA); European Medicines Agency (EMA). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA. *EFSA J.* **2021**, *19*, e06712. [CrossRef]
4. Tiseo, K.; Huber, L.; Gilbert, M.; Robinson, T.P.; Van Boeckel, T.P. Global Trends in Antimicrobial Use in Food Animals from 2017 to 2030. *Antibiotics* **2020**, *9*, 918. [CrossRef] [PubMed]
5. De Albuquerque Fernandes, S.A.; Magnavita, A.P.; Ferrao, S.P.; Gualberto, S.A.; Faleiro, A.S.; Figueiredo, A.J.; Matarazzo, S.V. Daily ingestion of tetracycline residue present in pasteurized milk: A public health problem. *Environ. Sci. Pollut. Res. Int.* **2014**, *21*, 3427–3434. Available online: https://link.springer.com/article/10.1007/s11356-013-2286-5 (accessed on 10 July 2021). [CrossRef]
6. Pengov, A.; Kirbis, A. Risks of antibiotic residues in milk following intramammary and intramuscular treatments in dairy sheep. *Anal. Chim. Acta* **2009**, *637*, 13–17. [CrossRef]
7. Landers, T.F.; Cohen, B.; Wittum, T.E.; Larson, E.L. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep.* **2012**, *127*, 4–22. Available online: https://journals.sagepub.com/doi/10.1177/003335491212700103 (accessed on 17 September 2021). [CrossRef] [PubMed]
8. McEwen, S.A.; Fedorka-Cray, P.J. Antimicrobial use and resistance in animals. *Clin. Infect. Dis.* **2002**, *34*, S93–S106. Available online: https://academic.oup.com/cid/article/34/Supplement_3/S93/293306?login=true (accessed on 19 April 2022). [CrossRef]
9. Rajala-Schultz, P.; Nødtvedt, A.; Halasa, T.; Persson Waller, K. Prudent Use of Antibiotics in Dairy Cows: The Nordic Approach to Udder Health. *Front. Vet. Sci.* **2021**, *8*, 623998. Available online: https://www.frontiersin.org/article/10.3389/fvets.2021.623998 (accessed on 17 September 2021). [CrossRef]
10. Pol, M.; Ruegg, P.L. Treatment Practices and Quantification of Antimicrobial Drug Usage in Conventional and Organic Dairy Farms in Wisconsin. *J. Dairy Sci.* **2007**, *90*, 249–261. [CrossRef]
11. Linage, B.; Gonzalo, C.; Carriedo, J.A.; Asensio, J.A.; Blanco, M.A.; De La Fuente, L.F.; San Primitivo, F. Performance of Blue-Yellow Screening Test for Antimicrobial Detection in Ovine Milk. *J. Dairy Sci.* **2007**, *90*, 5374–5379. [CrossRef] [PubMed]
12. Molina, A.; Molina, M.P.; Althaus, R.L.; Gallego, L. Residue Persistence in Sheep Milk Following Antibiotic Therapy. *Vet. J.* **2003**, *165*, 84–89. [CrossRef]
13. Beltrán, M.C.; Althaus, R.L.; Molina, A.; Berruga, M.I.; Molina, M.P. Analytical strategy for the detection of antibiotic residues in sheep and goat's milk. *Span. J. Agric. Res.* **2015**, *13*, e0501. Available online: https://revistas.inia.es/index.php/sjar/article/view/6522 (accessed on 2 December 2021). [CrossRef]
14. Beyene, T. Veterinary Drug Residues in Food-animal Products: Its Risk Factors and Potential Effects on Public Health. *J. Vet. Sci. Technol.* **2015**, *7*, 1–7. [CrossRef]
15. Quintanilla, P.; Doménech, E.; Escriche, I.; Beltrán, M.C.; Molina, M.P. Food Safety Margin Assessment of Antibiotics: Pasteurized Goat's Milk and Fresh Cheese. *J. Food Prot.* **2019**, *82*, 1553–1559. [CrossRef] [PubMed]
16. Sachi, S.; Ferdous, J.; Sikder, M.H.; Hussani, S.M.A.K. Antibiotic residues in milk: Past, present, and future. *J. Adv. Vet. Anim. Res.* **2019**, *6*, 315–332. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6760505/ (accessed on 9 September 2021). [CrossRef] [PubMed]
17. Pyörälä, S. Treatment of mastitis during lactation. *Ir. Vet. J.* **2009**, *62*, S4–S40. [CrossRef] [PubMed]
18. Rossi, R.; Saluti, G.; Moretti, S.; Diamanti, I.; Giusepponi, D.; Galarini, R. Multiclass methods for the analysis of antibiotic residues in milk by liquid chromatography coupled to mass spectrometry: A review. *Food Addit. Contam. Part A* **2018**, *35*, 241–257. [CrossRef] [PubMed]

19. Fejzic, N.; Begagic, M.; Šerić-Haračić, S.; Smajlovic, M. Beta lactam antibiotics residues in cow's milk: Comparison of efficacy of three screening tests used in Bosnia and Herzegovina. *Bosn. J. Basic Med. Sci.* **2014**, *14*, 155–159. Available online: https://www.bjbms.org/ojs/index.php/bjbms/article/view/155 (accessed on 19 April 2022). [CrossRef]
20. Kemper, N. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Ind.* **2008**, *8*, 1–13. [CrossRef]
21. Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on Veterinary Medicinal Products and Repealing Directive 2001/82/EC. Available online: http://data.europa.eu/eli/reg/2019/6/oj (accessed on 19 April 2022).
22. Mitchell, J.M.; Griffiths, M.W.; McEwen, S.A.; McNab, W.B.; Yee, A.J. Antimicrobial drug residues in milk and meat: Causes, concerns, prevalence, regulations, tests, and test performance. *J. Food Prot.* **1998**, *61*, 742–756. [CrossRef]
23. Katz, S.E.; Brady, M.S. Antibiotic residues in food and their significance. *Food Biotechnol.* **2000**, *14*, 147–171. [CrossRef]
24. Shitandi, A.; Sternesjö, A. Factors contributing to the occurrence of antimicrobial drug residues in Kenyan milk. *J. Food Prot.* **2004**, *67*, 399–402. [CrossRef] [PubMed]
25. Orwa, J.D.; Matofari, J.W.; Muliro, P.S.; Lamuka, P. Assessment of sulphonamides and tetracyclines antibiotic residue contaminants in rural and peri urban dairy value chains in Kenya. *Int. J. Food Contam.* **2017**, *4*, 5. [CrossRef]
26. Brown, K.; Mugoh, M.; Call, D.R.; Omulo, S. Antibiotic residues and antibiotic-resistant bacteria detected in milk marketed for human consumption in Kibera, Nairobi. *PLoS ONE* **2020**, *15*, e0233413. [CrossRef] [PubMed]
27. Kosgey, A.; Shitandi, A.; Marion, J.W. Antibiotic Residues in Milk from Three Popular Kenyan Milk Vending Machines. *Am. J. Trop. Med. Hyg.* **2018**, *98*, 1520–1522. Available online: https://www.ajtmh.org/view/journals/tpmd/98/5/article-p1520.xml (accessed on 14 December 2021). [CrossRef] [PubMed]
28. Ouma, J.; Gachanja, A.; Mugo, S.; Gikunju, J. Antibiotic Residues in Milk from Juja and Githurai Markets in Kenya by Liquid Chromatography-Tandem Mass Spectrometry. *Chem. Afr.* **2021**, *4*, 769–775. [CrossRef]
29. Kurwijila, L.R.; Omore, A.; Staal, S.; Mdoe, N.S. Investigation of the risk of exposure to antimicrobial residues present in marketed milk in Tanzania. *J. Food Prot.* **2006**, *69*, 2487–2492. [CrossRef]
30. Mdegela, R.H.; Ryoba, R.; Karimuribo, E.D.; Phiri, E.J.; Løken, T.; Reksen, O.; Mtengeti, E.; Urio, N.A. Prevalence of clinical and subclinical mastitis and quality of milk on smallholder dairy farms in Tanzania. *J. S. Afr. Vet. Assoc.* **2009**, *80*, 163–168. Available online: https://journals.jsava.aosis.co.za/index.php/jsava/article/view/195 (accessed on 23 December 2021). [CrossRef]
31. Kivaria, F.M.; Noordhuizen, J.P.; Kapaga, A.M. Evaluation of the hygienic quality and associated public health hazards of raw milk marketed by smallholder dairy producers in the Dar es Salaam region, Tanzania. *Trop. Anim. Health Prod.* **2006**, *38*, 185–194. Available online: https://link.springer.com/article/10.1007/s11250-006-4339-y (accessed on 23 December 2021). [CrossRef] [PubMed]
32. Gwandu, S.H.; Nonga, H.E.; Mdegela, R.H.; Katakweba, A.S.; Suleiman, T.S.; Ryoba, R. Assessment of Raw Cow Milk Quality in Smallholder Dairy Farms in Pemba Island Zanzibar, Tanzania. *Vet. Med. Int.* **2018**, *2018*, 1031726. [CrossRef] [PubMed]
33. Layada, S.; Benouareth, D.; Coucke, W.; Andjelkovic, M. Assessment of antibiotic residues in commercial and farm milk collected in the region of Guelma (Algeria). *Int. J. Food Contam.* **2016**, *3*, 19. [CrossRef]
34. Baazize-Ammi, D.; Dechicha, A.S.; Tassist, A.; Gharbi, I.; Hezil, N.; Kebbal, S.; Morsli, W.; Beldjoudi, S.; Saadaoui, M.R.; Guetarni, D. Screening and quantification of antibiotic residues in broiler chicken meat and milk in the central region of Algeria. *Rev. Sci. Tech.* **2019**, *38*, 863–877. [CrossRef] [PubMed]
35. Madougou, A.M.; Douny, C.; Moula, N.; Scippo, M.L.; Delcenserie, V.; Daube, G.; Hamani, M.; Korsak, N. Survey on the presence of antibiotic residues in raw milk samples from six sites of the dairy pool of Niamey, Niger. *Vet. World* **2019**, *12*, 1970–1974. [CrossRef] [PubMed]
36. Addo, K.K.; Mensah, G.I.; Aning, K.G.; Nartey, N.; Nipah, G.K.; Bonsu, C.; Akyeh, M.L.; Smits, H.L. Microbiological quality and antibiotic residues in informally marketed raw cow milk within the coastal savannah zone of Ghana. *Trop. Med. Int. Health* **2011**, *16*, 227–232. [CrossRef]
37. Bando, E.; Oliveira, R.C.; Ferreira, G.M.; Machinski, M., Jr. Occurrence of antimicrobial residues in pasteurized milk commercialized in the state of Paraná, Brazil. *J. Food Prot.* **2009**, *72*, 911–914. [CrossRef]
38. Zanella, G.N.; Mikcha, J.M.; Bando, E.; Siqueira, V.L.; Machinski, M., Jr. Occurrence and antibiotic resistance of coliform bacteria and antimicrobial residues in pasteurized cow's milk from Brazil. *J. Food Prot.* **2010**, *73*, 1684–1687. [CrossRef] [PubMed]
39. De Oliveira, R.C.; Paschoal, J.A.R.; Reyes, F.G.R. Streptomycin and dihydrostreptomycin residues in bovine milk from the Brazilian retail market. *Food Addit. Contam. Part B* **2010**, *3*, 156–162. [CrossRef]
40. Prado, C.K.; Ferreira, F.D.; Bando, E.; Machinski, M., Jr. Oxytetracycline, tetracycline, chlortetracycline and doxycycline in pasteurised cow's milk commercialised in Brazil. *Food Addit. Contam. Part B* **2015**, *8*, 81–84. [CrossRef]
41. Valença, L.M.; De Paiva, J.E.; Barbosa, S.B.P.; Pinheiro, I.O.; Batista, A.M.V.; Da Silva, M.J.F.V.; De Medeiros, E.S. Evaluation of residues of β-lactam, sulfonamide, tetracycline, quinolone, fluoroquinolone e pyrimidine in raw milk. *Food Sci. Technol.* **2021**, *41*, 603. Available online: http://old.scielo.br/scielo.php?script=sci_arttext&pid=S0101-20612021000300603&nrm=iso (accessed on 9 December 2021). [CrossRef]
42. Baynes, R.E.; Lyman, R.; Anderson, K.L.; Brownie, C.F. A Preliminary Survey of Antibiotic Residues and Viable Bacteria in Milk from Three Caribbean Basin Countries. *J. Food Prot.* **1999**, *62*, 177–180. [CrossRef] [PubMed]
43. De Arbo, L.M.; Céspedes, L.G.; Idoyaga, H.; Echeverría, P.; Caballero, E.G.; Arias, M.N.; Bernal, S.S.; Ulke, G.; Desvars, A.B.; Aguirre, F.P. Detección de residuos de antibióticos y micotoxinas en leche vacuna fluida pasteurizada comercializada en Paraguay. *Rev. Salud Publica Parag.* **2020**, *10*, 23–29. [CrossRef]

44. Redding, L.E.; Cubas-Delgado, F.; Sammel, M.D.; Smith, G.; Galligan, D.T.; Levy, M.Z.; Hennessy, S. Antibiotic residues in milk from small dairy farms in rural Peru. *Food Addit. Contam. Part A* **2014**, *31*, 1001–1008. [CrossRef] [PubMed]
45. Mohamadi Sani, A.M.; Nikpooyan, H.; Moshiri, R. Aflatoxin M1 contamination and antibiotic residue in milk in Khorasan province, Iran. *Food Chem. Toxicol.* **2010**, *48*, 2130–2132. [CrossRef] [PubMed]
46. Moghadam, M.M.; Amiri, M.; Riabi, H.R.; Riabi, H.R. Evaluation of Antibiotic Residues in Pasteurized and Raw Milk Distributed in the South of Khorasan-e Razavi Province, Iran. *J. Clin. Diagn. Res.* **2016**, *10*, FC31–FC35. [CrossRef] [PubMed]
47. Rassouli, A.; Zamani, Z.; Bahonar, A.; Shams, G.; Abdolmaleki, A. A trace analysis of oxytetracycline and tetracycline residues in pasteurized milk supplied in Tehran: A one-year study (April 2011–March 2012). *Iran. J. Vet. Med.* **2014**, *8*, 119–123. Available online: https://www.sid.ir/en/Journal/ViewPaper.aspx?ID=409506 (accessed on 11 February 2022).
48. Mokhtari, A.; Hosseini, B.; Panahi, P. β-Lactams and Tetracyclines Antibiotic Residue Detection in Bulk Tank Milk in Iran. *Iran. J. Public Health* **2013**, *42*, 447–448. Available online: https://ijph.tums.ac.ir/index.php/ijph/article/view/4558 (accessed on 11 February 2022).
49. Aalipour, F.; Mirlohi, M.; Jalali, M.; Azadbakht, L. Dietary exposure to tetracycline residues through milk consumption in Iran. *J. Environ. Health Sci. Eng.* **2015**, *13*, 80. [CrossRef] [PubMed]
50. Kumar, A.; Panda, A.K.; Sharma, N. Determination of antibiotic residues in bovine milk by HPLC-DAD and assessment of human health risks in Northwestern Himalayan region, India. *J. Food Sci. Technol.* **2022**, *59*, 95–104. [CrossRef] [PubMed]
51. Kumar, N.; Sharma, G.; Leahy, E.; Shome, B.R.; Bandyopadhyay, S.; Deka, R.P.; Shome, R.; Dey, T.K.; Lindahl, J.F. Understanding Antibiotic Usage on Small-Scale Dairy Farms in the Indian States of Assam and Haryana Using a Mixed-Methods Approach—Outcomes and Challenges. *Antibiotics* **2021**, *10*, 1124. [CrossRef] [PubMed]
52. Moudgil, P.; Bedi, J.S.; Aulakh, R.S.; Gill, J.P.S. Antibiotic residues and mycotoxins in raw milk in Punjab (India): A rising concern for food safety. *J. Food Sci. Technol.* **2019**, *56*, 5146–5151. [CrossRef] [PubMed]
53. Chowdhury, S.; Hassan, M.M.; Alam, M.; Sattar, S.; Bari, M.S.; Saifuddin, A.K.; Hoque, M.A. Antibiotic residues in milk and eggs of commercial and local farms at Chittagong, Bangladesh. *Vet. World* **2015**, *8*, 467–471. [CrossRef]
54. Rahman, M.S.; Hassan, M.M.; Chowdhury, S. Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh. *Heliyon* **2021**, *7*, e07739. [CrossRef]
55. Khanal, B.K.S.; Sadiq, M.B.; Singh, M.; Anal, A.K. Screening of antibiotic residues in fresh milk of Kathmandu Valley, Nepal. *J. Environ. Sci. Health. Part B* **2018**, *53*, 57–86. [CrossRef]
56. Wanniatie, V.; Sudarwanto, M.B.; Purnawarman, T.; Jayanegara, A. Chemical compositions, contaminants, and residues of organic and conventional goat milk in Bogor District, Indonesia. *Vet. World* **2019**, *12*, 1218–1224. Available online: http://www.veterinaryworld.org/Vol.12/August-2019/7.pdf (accessed on 16 February 2022). [CrossRef] [PubMed]
57. Unusan, N. Occurrence of chloramphenicol, streptomycin and tetracycline residues in ultra-heat-treatment milk marketed in Turkey. *Int. J Food Sci. Nutr.* **2009**, *60*, 359–364. [CrossRef] [PubMed]
58. Muji, S.; Mehmedi, B.; Rexhepi, A.; Ramadani, X. Antibiotics residue in raw milk samples from four regions of Kosovo. *Bulg. J. Agric. Sci.* **2018**, *24*, 871–874. Available online: https://www.agrojournal.org/24/05-21.pdf (accessed on 29 April 2022).
59. Rama, A.; Lucatello, L.; Benetti, C.; Galina, G.; Bajraktari, D. Assessment of antibacterial drug residues in milk for consumption in Kosovo. *J. Food Drug Anal.* **2017**, *25*, 525–532. [CrossRef] [PubMed]
60. Vragović, N.; Bažulić, D.; Njari, B. Risk assessment of streptomycin and tetracycline residues in meat and milk on Croatian market. *Food Chem. Toxicol.* **2011**, *49*, 352–355. [CrossRef]
61. Bilandžić, N.; Kolanović, B.S.; Varenina, I.; Scortichini, G.; Annunziata, L.; Brstilo, M.; Rudan, N. Veterinary drug residues determination in raw milk in Croatia. *Food Control* **2011**, *22*, 1941–1948. [CrossRef]
62. Cerkvenik, V. Analysis and monitoring of chloramphenicol residues in food of animal origin in Slovenia from 1991 to 2000. *Food Addit. Contam.* **2002**, *19*, 357–367. [CrossRef] [PubMed]
63. Yamaki, M.; Berruga, M.I.; Althaus, R.L.; Molina, M.P.; Molina, A. Occurrence of Antibiotic Residues in Milk from Manchega Ewe Dairy Farms. *J. Dairy Sci.* **2004**, *87*, 3132–3137. [CrossRef]
64. Gonzalo, C.; Carriedo, J.A.; García-Jimeno, M.C.; Pérez-Bilbao, M.; de la Fuente, L.F. Factors influencing variation of bulk milk antibiotic residue occurrence, somatic cell count, and total bacterial count in dairy sheep flocks. *J. Dairy Sci.* **2010**, *93*, 1587–1595. [CrossRef] [PubMed]
65. Buczkowska, M.; Gorski, M.; Garbicz, J.; Grajek, M.; Buczkowski, K.; Garbowska, D.; Klein, D.; Duda, S. Penicillin and tetracycline residues in selected fresh and UHT milk with different fat contents. *Int. Food Res. J.* **2021**, *28*, 780–787. Available online: http://ifrj.upm.edu.my/28%20(04)%202021/DONE%20-%2014%20-%20IFRJ20014.pdf (accessed on 15 February 2022). [CrossRef]
66. The National Milk Drug Residue Data Base. Available online: https://www.nmdrd.com/ (accessed on 26 April 2022).
67. European Food Safety Authority. Report for 2019 on the Results from the Monitoring of Veterinary Medicinal Product Residues and Other Substances in Live Animals and Animal Products. *EFSA Support. Publ.* **2021**, *18*, 1997E. [CrossRef]
68. Zheng, N.; Wang, J.; Han, R.; Xu, X.; Zhen, Y.; Qu, X.; Sun, P.; Li, S.; Yu, Z. Occurrence of several main antibiotic residues in raw milk in 10 provinces of China. *Food Addit. Contam. Part B* **2013**, *6*, 84–89. [CrossRef] [PubMed]
69. Lu, G.; Chen, Q.; Li, Y.; Liu, Y.; Zhang, Y.; Huang, Y.; Zhu, L. Status of antibiotic residues and detection techniques used in Chinese milk: A systematic review based on cross-sectional surveillance data. *Food Res. Int.* **2021**, *147*, 110450. [CrossRef] [PubMed]

70. Commission Regulation (EC) No 1430/94 of 22 June 1994 Amending Annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 Laying Down a Community Procedure for the Establishment of Maximum Residue Limits of Veterinary Medicinal Products in Foodstuffs of Animal Origin. Available online: http://data.europa.eu/eli/reg/1994/1430/oj (accessed on 7 September 2021).
71. Hassan, H.F.; Saidy, L.; Haddad, R.; Hosri, C.; Asmar, S.; Jammoul, A.; Jammoul, R.; Hassan, H.; Serhan, M. Investigation of the effects of some processing conditions on the fate of oxytetracycline and tylosin antibiotics in the making of commonly consumed cheeses from the East Mediterranean. *Vet. World* **2021**, *14*, 1644–1649. [CrossRef]
72. Tian, L.; Khalil, S.; Bayen, S. Effect of thermal treatments on the degradation of antibiotic residues in food. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3760–3770. [CrossRef]
73. Grunwald, L.; Petz, M. Food processing effects on residues: Penicillins in milk and yoghurt. *Anal. Chim. Acta* **2003**, *483*, 73–79. [CrossRef]
74. Quintanilla, P.; Beltrán, M.C.; Molina, A.; Escriche, I.; Molina, M.P. Characteristics of ripened Tronchón cheese from raw goat milk containing legally admissible amounts of antibiotics. *J. Dairy Sci.* **2019**, *102*, 2941–2953. [CrossRef]
75. Gajda, A.; Nowacka-Kozak, E.; Gbylik-Sikorska, M.; Posyniak, A. Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. *Food Addit. Contam. Part A* **2018**, *35*, 66–76. [CrossRef]
76. Hakk, H.; Shappell, N.W.; Lupton, S.J.; Shelver, W.L.; Fanaselle, W.; Oryang, D.; Yeung, C.Y.; Hoelzer, K.; Ma, Y.; Gaalswyk, D.; et al. Distribution of Animal Drugs between Skim Milk and Milk Fat Fractions in Spiked Whole Milk: Understanding the Potential Impact on Commercial Milk Products. *J. Agric. Food Chem.* **2016**, *64*, 326–335. Available online: https://pubs.acs.org/doi/abs/10.1021/acs.jafc.5b04726 (accessed on 25 February 2022). [CrossRef]
77. Ziv, G.; Rasmussen, F. Distribution of Labeled Antibiotics in Different Components of Milk Following Intramammary and Intramuscular Administrations®. *J. Dairy Sci.* **1975**, *58*, 938–946. [CrossRef]
78. Sniegocki, T.; Gbylik-Sikorska, M.; Posyniak, A. Transfer of chloramphenicol from milk to commercial dairy products—Experimental proof. *Food Control* **2015**, *57*, 411–418. [CrossRef]
79. Cayle, T.; Guth, J.H.; Hynes, J.T.; Kolen, E.P.; Stern, M.L. Penicillin Distribution during Cheese Manufacture and Membrane Treatment of Whey. *J. Food Prot.* **1986**, *49*, 796–798. [CrossRef] [PubMed]
80. Shappell, N.W.; Shelver, W.L.; Lupton, S.J.; Fanaselle, W.; Van Doren, J.M.; Hakk, H. Distribution of Animal Drugs among Curd, Whey, and Milk Protein Fractions in Spiked Skim Milk and Whey. *J. Agric. Food Chem.* **2017**, *65*, 938–949. [CrossRef] [PubMed]
81. Gbylik-Sikorska, M.; Gajda, A.; Nowacka-Kozak, E.; Posyniak, A. The "force" of cloxacillin residue will be with you in various dairy products—The last experimental evidence. *Food Control* **2021**, *121*, 107628. [CrossRef]
82. Lányi, K.; Darnay, L.; László, N.; Lehel, J.; Friedrich, L.; Győri, R.; Laczay, P. Transfer of certain beta-lactam antibiotics from cow's milk to fresh cheese and whey. *Food Addit. Contam. Part A* **2022**, *39*, 52–60. [CrossRef] [PubMed]
83. Cabizza, R.; Rubattu, N.; Salis, S.; Pes, M.; Comunian, R.; Paba, A.; Addis, M.; Testa, M.C.; Urgeghe, P.P. Transfer of oxytetracycline from ovine spiked milk to whey and cheese. *Int. Dairy J.* **2017**, *70*, 12–17. [CrossRef]
84. Cabizza, R.; Rubattu, N.; Salis, S.; Pes, M.; Comunian, R.; Paba, A.; Daga, E.; Addis, M.; Testa, M.C.; Urgeghe, P.P. Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications. *LWT Food Sci. Technol.* **2018**, *96*, 236–243. [CrossRef]
85. Giraldo, J.; Althaus, R.L.; Beltrán, M.C.; Molina, M.P. Antimicrobial activity in cheese whey as an indicator of antibiotic drug transfer from goat milk. *Int. Dairy J.* **2017**, *69*, 40–44. [CrossRef]
86. Quintanilla, P.; Beltrán, M.C.; Molina, M.P.; Escriche, I. Enrofloxacin treatment on dairy goats: Presence of antibiotic in milk and impact of residue on technological process and characteristics of mature cheese. *Food Control* **2021**, *123*, 107762. [CrossRef]
87. Castrica, M.; Rebucci, R.; Giromini, C.; Tretola, M.; Cattaneo, D.; Baldi, A. Total phenolic content and antioxidant capacity of agri-food waste and by-products. *Ital. J. Anim. Sci.* **2019**, *18*, 336–341. Available online: https://www.tandfonline.com/doi/full/10.1080/1828051X.2018.1529544 (accessed on 6 April 2022). [CrossRef]
88. Tona, G.O.; Olusola, A.D. Determination of tetracycline antibiotic residue in dairy products sold in Ogbomoso, south-western Nigeria. *Int. J. Food Agric. Vet. Sci.* **2014**, *4*, 136–140. Available online: https://www.cibtech.org/J-FOOD-AGRI-VETERINARY-SCIENCES/PUBLICATIONS/2014/Vol_4_No_1/JFAV-04-01-Contents.htm (accessed on 28 February 2022).
89. Olatoye, I.O.; Daniel, O.F.; Ishola, S.A. Screening of antibiotics and chemical analysis of penicillin residue in fresh milk and traditional dairy products in Oyo state, Nigeria. *Vet. World* **2016**, *9*, 948–954. [CrossRef]
90. Bagré, T.S.; Samandoulougou, S.; Traore, S.M.; Illy, D.; Bsadjo-Tchamba, G.; Bawa-Ibrahim, H.; Bouda, S.C.; Traore, A.S.; Barro, N. Détection biologique des résidus d'antibiotiques dans le lait et produits laitiers de vache consommés à Ouagadougou, Burkina Faso. *J. Appl. Biosci.* **2015**, *87*, 8105–8112. Available online: https://www.ajol.info/index.php/jab/article/view/116531 (accessed on 11 February 2022). [CrossRef]
91. Srimulyati, A.; Purnawarman, T.; Latif, H. Tetracycline residue detection on cheese imported trough Tanjung Priok seaport, Jakarta. *Glob. Vet.* **2015**, *14*, 819–823. Available online: https://www.idosi.org/gv/gv14(6)15/5.pdf (accessed on 21 February 2022).
92. Adetunji, V.O. Effects of processing on antibiotic residues (streptomycin, penicillin-G and tetracycline) in soft cheese and yoghurt processing lines. *Pak. J. Nutr.* **2011**, *10*, 792–795. [CrossRef]

93. Marth, E.H.; Ellickson, B.E. Problems Created by the Presence of Antibiotics in Milk—A Review. *J. Milk Food Technol.* **1959**, *22*, 266–272. [CrossRef]
94. Caplice, E.; Fitzgerald, G.F. Food fermentations: Role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* **1999**, *50*, 131–149. [CrossRef]
95. Chiesa, L.M.; DeCastelli, L.; Nobile, M.; Martucci, F.; Mosconi, G.; Fontana, M.; Castrica, M.; Arioli, F.; Panseri, S. Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. *LWT Food Sci. Technol.* **2020**, *131*, 109783. [CrossRef]
96. Berruga, M.I.; Molina, A.; Althaus, R.L.; Molina, M.P. Control and prevention of antibiotic residues and contaminants in sheep and goat's milk. *Small Rumin. Res.* **2016**, *142*, 38–43. [CrossRef]
97. Cogan, T.M. Susceptibility of Cheese and Yogurt Starter Bacteria to Antibiotics. *Appl. Microbiol.* **1972**, *23*, 960–965. [CrossRef] [PubMed]
98. Quintanilla, P.; Cornacchini, M.; Hernando, M.I.; Molina, M.P.; Escriche, I. Impact of the presence of oxytetracycline residues in milk destined for the elaboration of dairy products: The specific case of mature goat cheese. *Int. Dairy J.* **2020**, *101*, 104595. [CrossRef]
99. Berruga, I.; Battacone, G.; Molina, M.P.; Román, M.; Molina, A. Influence of β-lactams on Manchego cheese manufacture. In Proceedings of the 5th International Symposium on the Challenge to Sheep and Goats Milk Sectors, Alghero, Italy, 18–20 April 2007; Special Issue IDF 0801, Part 1/3. pp. 222–224.
100. Berruga, M.I.; Molina, M.P.; Noves, B.; Roman, M.; Molina, A. "In vitro" study about the effect of several penicillins during the fermentation of yogurt made from ewe's milk. *Milchwiss. Milk Sci. Int.* **2007**, *62*, 303–305.
101. Berruga, M.I.; Noves, B.; Molina, M.P.; Roman, M.; Molina, A. Influence of cephalosporins on the coagulation time of yogurt made from ewes' milk. *Int. J. Dairy Technol.* **2008**, *61*, 372–378. [CrossRef]
102. Novés, B.; Libran, C.; Licon, C.C.; Molina, M.P.; Molina, A.; Berruga, M.I. Technological failures caused by cephalexin in set-type sheep's milk yogurt. *CyTA-J. Food* **2015**, *13*, 408–414. [CrossRef]
103. Beltran, M.C.; Morari-Pirlog, A.; Quintanilla, P.; Escriche, I.; Molina, M.P. Influence of enrofloxacin on the coagulation time and the quality parameters of goat's milk yoghurt. *Int. J. Dairy Technol.* **2018**, *71*, 105–111. [CrossRef]
104. European Food Safety Authority (EFSA). Foodborne antimicrobial resistance as a biological hazard—Scientific Opinion of the Panel on Biological Hazards. *EFSA J.* **2008**, *765*, 4–87. [CrossRef]
105. Aarestrup, F.M. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *Int. J. Antimicrob. Agents* **1999**, *12*, 279–285. [CrossRef]
106. Mathur, S.; Singh, R. Antibiotic resistance in food lactic acid bacteria—A review. *Int. J. Food Microbiol.* **2005**, *105*, 281–295. [CrossRef] [PubMed]
107. Roca, I.; Akova, M.; Baquero, F.; Carlet, J.; Cavaleri, M.; Coenen, S.; Cohen, J.; Findlay, D.; Gyssens, I.; Heure, O.E.; et al. The global threat of antimicrobial resistance: Science for intervention. *New Microb. New Infect.* **2015**, *6*, 22–29. [CrossRef] [PubMed]
108. Wang, H.H.; Manuzon, M.; Lehman, M.; Wan, K.; Luo, H.; Wittum, T.E.; Yousef, A.; Bakaletz, L.O. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. *FEMS Microbiol. Lett.* **2006**, *254*, 226–231. [CrossRef] [PubMed]
109. Jamet, E.; Akary, E.; Poisson, M.; Chamba, J.; Bertrand, X.; Serror, P. Prevalence and characterization of antibiotic resistant Enterococcus faecalis in French cheeses. *Food Microbiol.* **2012**, *31*, 191–198. [CrossRef]
110. Hammad, A.M.; Hassan, H.A.; Shimamoto, T. Prevalence, antibiotic resistance and virulence of Enterococcus spp. in Egyptian fresh raw milk cheese. *Food Control* **2015**, *50*, 815–820. [CrossRef]
111. Výrostková, J.; Regecova, I.; Dudrikova, E.; Marcincak, S.; Vargova, M.; Kovacova, M.; Mal'ova, J. Antimicrobial Resistance of Enterococcus sp. Isolated from Sheep and Goat Cheeses. *Foods* **2021**, *10*, 1844. [CrossRef] [PubMed]
112. Devirgiliis, C.; Barile, S.; Perozzi, G. Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes Nutr.* **2011**, *6*, 275–284. [CrossRef] [PubMed]
113. Abriouel, H.; Knapp, C.W.; Gálvez, A.; Benomar, N. Chapter 29—Antibiotic Resistance Profile of Microbes from Traditional Fermented Foods. In *Fermented Foods in Health and Disease Prevention*; Frias, J., Martinez-Villaluenga, C., Peñas, E., Eds.; Academic Press: Boston, MA, USA, 2017; pp. 675–704, ISBN 9780128023099. [CrossRef]
114. Guo, H.; Pan, L.; Li, L.; Lu, J.; Kwok, L.; Menghe, B.; Zhang, H.; Zhang, W. Characterization of Antibiotic Resistance Genes from Lactobacillus Isolated from Traditional Dairy Products. *J. Food Sci.* **2017**, *82*, 724–730. [CrossRef] [PubMed]
115. Wall, B.A.; Mateus, A.; Marshall, L.; Pfeiffer, D.U.; Lubroth, J.; Ormel, H.J.; Otto, P.; Patriarchi, A. *Drivers, Dynamics and Epidemiology of Antimicrobial Resistance in Animal Production*; FAO: Rome, Italy, 2016; ISBN 978-92-5-109441-9. Available online: https://www.fao.org/publications/card/en/c/d5f6d40d-ef08-4fcc-866b-5e5a92a12dbf/ (accessed on 24 March 2022).
116. Firth, C.L.; Käsbohrer, A.; Pless, P.; Koeberl-Jelovcan, S.; Obritzhauser, W. Analysis of Antimicrobial Use and the Presence of Antimicrobial-Resistant Bacteria on Austrian Dairy Farms—A Pilot Study. *Antibiotics* **2022**, *11*, 124. [CrossRef]
117. Aarestrup, F.M.; Wegener, H.C.; Collignon, P. Resistance in bacteria of the food chain: Epidemiology and control strategies. *Expert Rev. Anti-Infect. Ther.* **2008**, *6*, 733–750. [CrossRef] [PubMed]
118. Zeina, K.; Pamela, A.K.; Fawwak, S. Quantification of Antibiotic Residues and Determination of Antimicrobial Resistance Profiles of Microorganisms Isolated from Bovine Milk in Lebanon. *Food Nutr. Sci.* **2013**, *4*, 9. Available online: www.scirp.org/journal/paperinformation.aspx?paperid=33574 (accessed on 24 March 2022). [CrossRef]

119. Chantziaras, I.; Boyen, F.; Callens, B.; Dewulf, J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: A report on seven countries. *J. Antimicrob. Chemother.* **2013**, *69*, 827–834. [CrossRef] [PubMed]
120. Manie, T.; Brözel, V.S.; Veith, W.J.; Gouws, P.A. Antimicrobial resistance of bacterial flora associated with bovine products in South Africa. *J. Food Prot.* **1999**, *62*, 615–618. [CrossRef]
121. Tempini, P.N.; Aly, S.S.; Karle, B.M.; Pereira, R.V. Multidrug residues and antimicrobial resistance patterns in waste milk from dairy farms in Central California. *J. Dairy Sci.* **2018**, *101*, 8110–8122. [CrossRef] [PubMed]
122. Pereira, V.V.R.; Lima, S.; Siler, J.D.; Foditsch, C.; Warnick, L.D.; Bicalho, R.C. Ingestion of Milk Containing Very Low Concentration of Antimicrobials: Longitudinal Effect on Fecal Microbiota Composition in Preweaned Calves. *PLoS ONE* **2016**, *11*, e0147525. [CrossRef]
123. Maynou, G.; Migura-Garcia, L.; Chester-Jones, H.; Ziegler, D.; Bach, A.; Terré, M. Effects of feeding pasteurized waste milk to dairy calves on phenotypes and genotypes of antimicrobial resistance in fecal Escherichia coli isolates before and after weaning. *J. Dairy Sci.* **2017**, *100*, 7967–7979. [CrossRef] [PubMed]
124. EFSA Panel on Biological Hazards (BIOHAZ); Ricci, A.; Allende, A.; Bolton, D.; Chemaly, M.; Davies, R.; Fernández Escámez, P.S.; Girones, R.; Koutsoumanis, K.; Lindqvist, R.; et al. Risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics. *EFSA J.* **2017**, *15*, e04665. [CrossRef] [PubMed]
125. El Zubeir, I.E.; El Owni, O. Antimicrobial resistance of bacteria associated with raw milk contaminated by chemical preservatives. *World J. Dairy Food Sci.* **2009**, *4*, 65–69. Available online: https://idosi.org/wjdfs/wjdfs4(1)/11.pdf (accessed on 24 March 2022).
126. Cerniglia, C.E.; Pineiro, S.A.; Kotarski, S.F. An update discussion on the current assessment of the safety of veterinary antimicrobial drug residues in food with regard to their impact on the human intestinal microbiome. *Drug Test. Anal.* **2016**, *8*, 539–548. [CrossRef]
127. Bacanlı, M.; Başaran, N. Importance of antibiotic residues in animal food. *Food Chem. Toxicol.* **2019**, *125*, 462–466. [CrossRef] [PubMed]
128. Francino, M.P. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Front. Microbiol.* **2016**, *6*, 1543. Available online: https://www.frontiersin.org/article/10.3389/fmicb.2015.01543 (accessed on 31 March 2022). [CrossRef] [PubMed]
129. Piñeiro, S.A.; Cerniglia, C.E. Antimicrobial drug residues in animal-derived foods: Potential impact on the human intestinal microbiome. *J. Vet. Pharmacol. Ther.* **2021**, *44*, 215–222. [CrossRef] [PubMed]
130. Cho, I.; Yamanishi, S.; Cox, L.; Methé, B.A.; Zavadil, J.; Li, K.; Gao, Z.; Mahana, D.; Raju, K.; Teitler, I.; et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* **2012**, *488*, 621–626. [CrossRef] [PubMed]
131. Singer, R.S.; Finch, R.; Wegener, H.C.; Bywater, R.; Walters, J.; Lipsitch, M. Antibiotic resistance—The interplay between antibiotic use in animals and human beings. *Lancet Infect. Dis.* **2003**, *3*, 47–51. [CrossRef]
132. Marshall, B.M.; Levy, S.B. Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.* **2011**, *24*, 718–733. [CrossRef] [PubMed]
133. Bengtsson-Palme, J. Antibiotic resistance in the food supply chain: Where can sequencing and metagenomics aid risk assessment? *Curr. Opin. Food Sci.* **2017**, *14*, 66–71. [CrossRef]
134. Overdevest, I.; Willemsen, I.; Rijnsburger, M.; Eustace, A.; Xu, L.; Hawkey, P.; Heck, M.; Savelkoul, P.; Vandenbroucke-Grauls, C.; Van der Zwaluw, K.; et al. Extended-Spectrum β-Lactamase Genes of *Escherichia coli* in Chicken Meat and Humans, the Netherlands. *Emerg. Infect. Dis.* **2011**, *17*, 1216. Available online: https://wwwnc.cdc.gov/eid/article/17/7/11-0209_article (accessed on 27 April 2022). [CrossRef] [PubMed]
135. Toomey, N.; Monaghan, Á.; Fanning, S.; Bolton, D.J. Assessment of Antimicrobial Resistance Transfer between Lactic Acid Bacteria and Potential Foodborne Pathogens Using In Vitro Methods and Mating in a Food Matrix. *Foodborne Pathog. Dis.* **2009**, *6*, 925–933. [CrossRef] [PubMed]
136. Van Reenen, C.A.; Dicks, L.M.T. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: What are the possibilities? A review. *Arch. Microbiol.* **2011**, *193*, 157–168. [CrossRef]
137. De Paula, A.C.L.; Medeiros, J.D.; De Azevedo, A.C.; De Assis Chagas, J.M.; Da Silva, V.L.; Diniz, C.G. Antibiotic Resistance Genetic Markers and Integrons in White Soft Cheese: Aspects of Clinical Resistome and Potentiality of Horizontal Gene Transfer. *Genes* **2018**, *9*, 106. [CrossRef] [PubMed]
138. Yao, J.; Gao, J.; Guo, J.; Wang, H.; Zhang, E.N.; Lin, Y.; Chen, Z.; Li, S.; Tao, S. Characterization of Bacteria and Antibiotic Resistance in Commercially Produced Cheeses Sold in China. *J. Food Prot.* **2022**, *85*, 484–493. [CrossRef]
139. Dolliver, H.; Kumar, K.; Gupta, S. Sulfamethazine uptake by plants from manure-amended soil. *J. Environ. Qual.* **2007**, *36*, 1224–1230. [CrossRef] [PubMed]
140. Kyselková, M.; Jirout, J.; Vrchotová, N.; Schmitt, H.; Elhottová, D. Spread of tetracycline resistance genes at a conventional dairy farm. *Front. Microbiol.* **2015**, *6*, 536. Available online: https://www.frontiersin.org/article/10.3389/fmicb.2015.00536 (accessed on 24 March 2022). [CrossRef] [PubMed]
141. Oliver, J.P.; Gooch, C.A.; Lansing, S.; Schueler, J.; Hurst, J.J.; Sassoubre, L.; Crossette, E.M.; Aga, D.S. Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. *J. Dairy Sci.* **2020**, *103*, 1051–1071. [CrossRef] [PubMed]

142. Guo, X.; Akram, S.; Stedtfeld, R.; Johnson, M.; Chabrelie, A.; Yin, D.; Mitchell, J. Distribution of antimicrobial resistance across the overall environment of dairy farms—A case study. *Sci. Total Environ.* **2021**, *788*, 147489. [CrossRef] [PubMed]
143. Pitta, D.W.; Dou, Z.; Kumar, S.; Indugu, N.; Toth, J.D.; Vecchiarelli, B.; Bhukya, B. Metagenomic Evidence of the Prevalence and Distribution Patterns of Antimicrobial Resistance Genes in Dairy Agroecosystems. *Foodborne Pathog. Dis.* **2016**, *13*, 296–302. [CrossRef] [PubMed]

This article was published in Dairy, Vol 3, Mailo Virto, Gorka Santamarina-García, Gustavo Amores and Igor Hernández, Antibiotics in Dairy Production: Where Is the Problem?, 541-564, Copyright MDPI (2024)

5 | Appendix V
Manuscript V

Food Chemistry, 442, 138445

Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain

Gorka Santamarina-García[1,2,3], Gustavo Amores[1,2,3], Nagore Gandarias[4], Igor Hernández[1,2,3], and Mailo Virto[1,2,3]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[2] *Bioaraba Health Research Institute-Prevention, Promotion and Health Care, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[3] *Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[4] *Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

* *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented at:

International Symposium of Animal Sciences, 2023. *Producers' knowledge and attitudes on antibiotics utilisation in artisanal dairies producing a raw ewe milk-derived cheese (Idiazabal).* Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Oral presentation).

3rd Food Chemistry Conference: Shaping a Healthy and Sustainable Food Chain through Knowledge, 2023. *Elucidating the prevalence of antibiotic residues throughout a raw milk cheese production chain by LC-MS/MS and commercial testing: a farm-to-fork study.* Santamarina-García, G., Amores, G. and Virto, M. (Poster presentation).

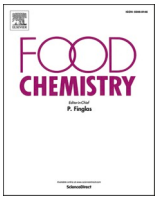
Published in **Food Chemistry**
ISSN: 0308-8146
IF (2022): **8.8**
Category: **Chemistry, Applied (D1, 5/73), Food Science & Technology (D1, 9/142), Nutrition & Dietetics (D1, 5/88)**
Citations: 0 (updated: 04/2024)
doi.org/10.1016/j.foodchem.2024.138445

Food Chemistry 442 (2024) 138445

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain

Gorka Santamarina-García [a,b,c,*], Gustavo Amores [a,b,c], Nagore Gandarias [d], Igor Hernández [a,b,c], Mailo Virto [a,b,c]

[a] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[b] Bioaraba Health Research Institute, Prevention, Promotion and Health Care Area, Lactiker-Nutrition and Health Line, Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[c] Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

ARTICLE INFO

Keywords:
Antibiotic Residues
Raw Milk Cheese
Charm Kidney Inhibition Swab Test
Eclipse Farm^{3G} Test
Ultra-High-Performance Liquid Chromatography/Triple Quadrupole Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS)
Liquid Chromatography/Triple Quadrupole Tandem Mass Spectrometry (LC-QqQ-MS/MS)

ABSTRACT

This study investigated antibiotic utilization in artisanal dairies and residue occurrence throughout the raw milk cheese production chain using commercial testing (Charm KIS and Eclipse Farm^{3G}) and UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS. The cross-sectional survey results revealed gaps in the producers' knowledge of antibiotic use. Commercial testing detected antibiotic levels close to the LOD in 12.5 % of the samples, mainly in raw milk and whey, with 10.0 % testing positive, specifically in fresh and ripened cheeses, indicating that antibiotics are concentrated during cheese-making. Chromatographically, several antibiotics were identified in the faeces of healthy animals, with chlortetracycline (15.7 ± 34.5 μg/kg) and sulfamethazine (7.69 ± 16.5 μg/kg) predominating. However, only tylosin was identified in raw milk (3.28 ± 7.44 μg/kg) and whey (2.91 ± 6.55 μg/kg), and none were found in fresh or ripened cheeses. The discrepancy between commercial and analytical approaches is attributed to compounds or metabolites not covered chromatographically.

1. Introduction

Antibiotics are natural or synthetic compounds with antimicrobial activities that impact essential bacterial physiology and biochemistry, leading to either cell death (bactericide) or growth cessation (bacteriostatic) (Rossi et al., 2017; Virto et al., 2022). These compounds have been utilized for over 60 years to prevent and treat infections in livestock, such as mastitis. It is estimated that 73 % of global antibiotic use is dedicated to food-producing animals (Treiber & Beranek-Knauer, 2021). Antibiotics must be administered under veterinary prescription, employing authorized products and adhering to recommended doses, routes of administration, and withdrawal periods. Despite the fact that a significant portion of the product is eliminated through urine and faeces, residues can still be present in foodstuffs (Brocca & Salvatore, 2023; Virto et al., 2022).

Antibiotic residues are defined as pharmacologically active substances, including excipients or degradation products and their metabolites, that persist in food obtained from animals treated with the respective drugs (Brocca & Salvatore, 2023; Virto et al., 2022). The presence of antibiotic residues in food poses a public health threat due to the development of resistant bacteria and various toxicological effects, such as allergies, dysfunction of the intestinal microbiota, immunopathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity, and anaphylactic shock (Virto et al., 2022). Consequently, regulatory authorities worldwide have established maximum residual limits (MRLs) based on acceptable daily intake (ADI), representing the amount of substance that can be ingested daily throughout life without appreciable

* Corresponding author.
E-mail addresses: gorka.santamarina@ehu.eus (G. Santamarina-García), gustavo.amores@ehu.eus (G. Amores), nagore.gandarias@ehu.eus (N. Gandarias), igor.hernandezo@ehu.eus (I. Hernández), mailo.virto@ehu.eus (M. Virto).

https://doi.org/10.1016/j.foodchem.2024.138445
Received 23 October 2023; Received in revised form 13 December 2023; Accepted 11 January 2024
Available online 16 January 2024

health risks (Quintanilla et al., 2019a; Virto et al., 2022). Moreover, the United Nations pays special attention to the occurrence of antibiotic residues, classifying it as a factor contributing to the development of antimicrobial resistance. Consequently, it is an important target for the Sustainable Development Goals, particularly for goal 3, which aims to "ensure healthy lives and promote well-being for all at all ages" (WHO, 2017). Notably, antibiotic residues not only negatively impact health but also have environmental consequences (Virto et al., 2022). Soil and groundwater contamination have been reported due to the excretion of antibiotics through urine and faeces, as well as crop contamination through manure (Marshall & Levy, 2011; Virto et al., 2022). Farmers' lack of knowledge about antibiotic use and awareness of their potential impacts have been linked to poor practices, resulting in the presence of residues in both foodstuffs and the environment (Phares et al., 2020; Visschers et al., 2014). Therefore, concerted efforts from all stakeholders are necessary to ensure the proper use of antibiotics and prevent the spread of residues (Phares et al., 2020; Virto et al., 2022; Visschers et al., 2014).

Screening for the presence of antibiotic residues in food-producing animals is essential for ensuring food safety (Brocca & Salvatore, 2023). Different analytical methods have been developed worldwide to detect and quantify antibiotics. These methods include capillary electrophoresis, gas chromatography, and liquid or ultra-high-performance liquid chromatography (LC) (Sta Ana et al., 2021; Virto et al., 2022). Among these techniques, LC predominates because the analytes are polar, non-volatile, and thermally unstable compounds and do not require derivatization. LC can be coupled with different detectors, such as Ultraviolet (UV), Diode Array (DAD), Fluorescence Detector (FLD), mass spectrometer (MS), and tandem MS (MS/MS). However, a highly sensitive detector, such as MS/MS, is required since antibiotics are often found at low concentrations (Chiesa et al., 2020; Giraldo et al., 2022; Sta Ana et al., 2021; Virto et al., 2022). In addition, not all antibiotics absorb light; consequently, UV or FLD detectors are not appropriate without derivatization (Sta Ana et al., 2021). Ultra-high-performance liquid chromatography (UHPLC), also known as ultra-performance liquid chromatography (UPLC), represents a significant improvement owing to substantial enhancements in speed, resolution, and sensitivity (Wang, 2009). In addition to analytical methods, various qualitative techniques are available to food chain stakeholders for the rapid detection of antibiotic residues above legal limits in foodstuffs (Brocca & Salvatore, 2023; Chiesa et al., 2020; Virto et al., 2022; Wang, 2009), such as enzyme-linked immunosorbent assays (ELISA), surface plasmon resonance, biosensor technology, and microbial inhibition tests (Richards et al., 2022; Sullivan et al., 2022; Virto et al., 2022; Wang, 2009). Although microbial inhibition tests are easy to use, inexpensive, and have a high throughput, they lack specificity and sometimes show a high false-positive rate. Overall, screening tests based on microbial, enzymatic, or immunological receptor assays are faster and more selective than other methods (Wang, 2009).

Milk and dairy products are of great nutritional, social, and economic importance and are produced worldwide using various systems and technologies (Virto et al., 2022). Several studies have reported antibiotic residues in dairy products of animal origin (Rossi et al., 2017; Chiesa et al., 2020; Virto et al., 2022). However, MRLs have only been established for milk, and there are no limits for other dairy products, such as whey or cheese (Virto et al., 2022), in which these compounds can be concentrated (Quintanilla et al., 2019b). Moreover, antibiotic residues affect dairy production systems, such as the cheese-making process, due to problems with the growth of starter cultures, acidification, milk curdling, and ripening (Chiesa et al., 2020; Quintanilla et al., 2019b; Virto et al., 2022). To the best of our knowledge, there is no information in the literature on the prevalence of antibiotics along the entire dairy production chain because of livestock treatment, i.e., from animals to ripened cheese. The majority of published studies only analyse a single product, such as milk, or consist of intentionally spiked products to analyse the effect of different processes, such as sterilization (Cabizza et al., 2017; Giraldo et al., 2022; Quintanilla et al., 2019a; Quintanilla et al., 2019b).

Therefore, taking into account the need for information in this regard, this study aimed to achieve four primary objectives: (1) conduct a comprehensive survey on the knowledge, practices, and attitudes regarding antibiotic usage in artisanal dairies; (2) analyse the natural occurrence of antibiotic residues in healthy sheep herds through screening tests (Charm Kidney Inhibition Swab and Eclipse Farm^{3G} tests) and chromatographic techniques (UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS); (3) ascertain the extent to which antimicrobials may be transferred to raw milk; and (4) investigate the impact of the cheese-making process. This represents the first case study adopting a farm-to-fork strategy, examining the knowledge and practices of artisanal dairies, the natural presence of antibiotics in food-producing animals, and their dissemination throughout the entire dairy production chain.

2. Materials and methods

2.1. Area of study

To assess the current status of antibiotic use in small artisanal dairies and to determine the presence of antibiotic residues throughout the production chain of raw milk cheese, the European Protected Designation of Origin (PDO) Idiazabal cheese was selected for this study. The focus on Idiazabal PDO cheese stems from the fact that its production is primarily carried out by small family artisanal dairies. These dairies manage the entire process, from herd management to cheese-making and sales. Idiazabal cheese is a semi-hard or hard cheese produced from the raw milk of Latxa and/or Carranzana sheep breeds. The geographical area for livestock management and milk production suitable for cheese production is located in the Southern Basque Country, a region spanning 17,213.06 km^2 in southwestern Europe (43° 27′ − 41° 54′ N and 1° 5 ' − 3° 37′ W). This area corresponds to the natural habitats of diffusion of the sheep breeds. Flock management involves indoor forage and feed feeding in winter, with semi-extensive or extensive grazing in spring. Milk production, and the consequent elaboration of cheese primarily occurs between January and June, following the traditional seasonal method determined by the biological rhythms of the sheep (BOE, 1993).

2.2. Cross-sectional survey

A cross-sectional survey was conducted to evaluate the knowledge, practices, and attitudes of artisanal producers toward antibiotic use. A five-page questionnaire was designed based on previous studies reported in the literature (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020; Visschers et al., 2014). The questionnaire was validated for ethical suitability, comprehension, and technical aspects by the regulatory council of the Idiazabal PDO and a specialist veterinarian. The questionnaire, written in Spanish, was divided into sections covering sociodemographic characteristics of the producers and dairies (e.g., gender or education level) and knowledge and practices related to antibiotic utilization (such as the type of antibiotics used or frequency). Producers were informed about various aspects of the study, including objectives and data protection, during a PDO meeting. Surveys were conducted anonymously, and participants had the option to withdraw from the study at any time. Verbal consent was obtained from each participant before responding to the survey.

2.3. Sampling

Four producers, designated as A, B, C, and D, affiliated with the PDO, were chosen for sampling, each representing a specific geographical production area (Alava, Biscay, Gipuzkoa, or Navarre). The sampling period extended from March to July 2022, covering the annual production cycle. All producers adhered to uniform flock management and

cheese-making conditions in accordance with the specifications outlined by the Idiazabal PDO regulatory board (BOE, 1993). Each flock comprised approximately 350–400 Latxa breed sheep, managed from indoor feeding in winter to semi-extensive or extensive grazing in spring. Milking was automated, and milk was refrigerated (3–4 °C) until cheese-making. For cheese manufacturing, the milk was initially tempered to 25 °C, and the commercial mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, DuPont NHIB Ibérica S.L., Barcelona, Spain) was used. Milk coagulation occurred at 28–32 °C for 20–45 min, employing artisanal rennet (extracted from the stomachs of Latxa or Carranzana lambs, obtained during the first month of lactation, cleaned, dried, salted, and ground) or commercial NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). The cheeses were ripened in chambers maintained at 80–95 % relative humidity and temperatures of 8–14 °C for 60 days.

Samples, including faecal, raw milk, whey, fresh cheese, and 2-month-old ripened cheese, were collected from each producer. All samples were obtained from healthy flocks, excluding animals that had undergone veterinary treatment. Aseptic collection was performed in quadruplicate, with each set of samples corresponding to the same batch. Producers conducted the sampling, eliminating the need for approval from the Ethics Committee for Animal Experimentation. Informed verbal consent was obtained from dairies during samples collection. Samples were transported under refrigeration (3 ± 1 °C) to the laboratory and stored in a freezer (−80 ± 1 °C) for subsequent analysis. Cheese samples were defrosted at 5 ± 1 °C for 24 h and allowed to reach room temperature for 1 h before analysis.

2.4. Reagents and materials

Eclipse Farm^{3G} inhibition test was acquired from Zeulab S.L. (Zaragoza, Spain). Charm Sciences Inc. (Lawrence, Massachusetts, United States) supplied the Charm Kidney Inhibition (KIS) test and Feed Extraction Buffer (FEB). Chemicals including acetonitrile (≥99.9 %, LC-grade), methanol (≥99.9 %, LC- and UHPLC-grades), anhydrous citric acid (≥99.5 %), and sodium phosphate (≥98.0 %) were sourced from Scharlab (Barcelona, Spain). UHPLC-grade acetonitrile (≥99.9 %) was provided by Avantor Performance Materials (Gliwice, Silesia, Poland). Citrate buffer (≥99.0 %) was purchased from Honeywell Fluka (Charlotte, North Carolina, United States). Ultrapure water was obtained using a Milli-Q system (Millipore Corp., Billerica, Massachusetts, United States). Oasis HLB cartridges (60 mg, 3 mL; 200 mg, 6 mL; and 500 mg, 6 mL) were acquired from Waters Chromatography Division (Milli-Q water purification system, model 185, <0.05 μS/cm, Millipore, Bedford, Massachusetts, United States). Chemicals such as anhydrous sodium sulphate (≥99.0 %) and oxalic acid (≥99.9 %) were obtained from Merck (Darmstadt, Hesse, Germany). Sodium chloride (≥99.0 %), sodium hydroxide (reagent grade), and UHPLC-grade formic acid (≥98.0 %) were sourced from PanReac AppliChem (Castellar del Vallés, Catalonia, Spain). Clarify-PP Polypropylene filters (0.22 μm) were obtained from Phenomenex (Torrance, California, United States). LC-grade formic acid (≥98.0 %), trisodium citrate (≥99.0 %), polyvinylidene fluoride filter (0.45 μm) and antibiotics (analytical grade), namely ansamicines (rifaximin), β-lactams penicillins (amoxicillin, ampicillin, penicillin G, cloxacillin, dicloxacillin, nafcillin, and oxacillin), β-lactams cephalosporins (cephapirin + desacetylcephapirin, cefoperazone, cephalexin, cefquinome, cephalonium, ceftiofur + desfuroylceftiofur, cefazolin, cefacetril, and cefuroxime), lincosamides (lincomycin), macrolides (tylosin, erythromycin, spiramycin, and tilmicosin), sulfonamides (sulfadiazine, sulfathiazole, sulfapyridine, sulfamethazine, sulfameracin, sulfadimethoxine, sulfaquinoxaline, sulfamethizole, and sulfachloropyridazine), tetracyclines (tetracycline, oxytetracycline, chlortetracycline, and doxycycline), and quinolones (enrofloxacin + ciprofloxacin, danofloxacin, sarafloxacin, flumequine, marbofloxacin, and oxolinic acid) were purchased from Sigma-Aldrich (Madrid, Spain).

2.5. Screening analysis

Two commercially available tests were used for the screening of antibiotic residues throughout the Idiazabal raw milk cheese production chain: the Charm Kidney Inhibition Swab (KIS) test for faecal samples and the Eclipse Farm^{3G} test for dairy samples. Sample preparation and analysis were performed according to the manufacturer's instructions, with slight modifications, as described below.

2.5.1. Charm Kidney Inhibition Swab (KIS) test

In the preparation of faeces samples, 1 g of previously ground faeces was combined with 30 mL of FEB and allowed to sit for 5 min. The mixture was then vigorously shaken for 1 min, followed by a 1-min settling period for the solids before conducting the test. Subsequently, the Charm Kidney Inhibition Swab (KIS) test swab was immersed in the stool extract for 10 s to facilitate absorption.

The Charm KIS test was executed following the manufacturer's instructions. This test relies on *Geobacillus stearothermophilus* cultured on agar with a purple pH indicator. In the absence of antibiotics, the bacteria proliferated during incubation, producing an acid that changed the medium's colour to yellow. Conversely, in the presence of antibiotics above the limit of detection (LOD), growth was inhibited, and the medium retained its blue/purple colour. Consequently, the swab containing the sample extract was introduced into the device and incubated at 64 ± 2 °C (Biometra TB1 Thermoblock, Gottingen, Germany) for the time required for a known antibiotic-negative sample to transition to yellow (approximately 3 h, depending on the lot). Results were interpreted through visual assessment of the colour change in the culture medium after incubation. Samples were categorized as positive (blue or purple) or negative (yellow). The LODs of the Charm KIS test are detailed in Supplementary Table 1

2.5.2. Eclipse Farm^{3G} test

The dairy samples underwent preparation and analysis in accordance with the manufacturer's instructions, with minor adjustments. In the case of raw milk samples, a 100 μL aliquot was dispensed into an Eclipse Farm^{3G} test tube using disposable pipettes supplied with the kit. The tubes were subsequently sealed and allowed to diffuse for 1 h at room temperature. Following incubation, 2–3 washes with deionised water was conducted, and the tube surfaces were dried and resealed with adhesive foil. The procedure for whey samples mirrored that of raw milk, with the caveat that the pH of the whey was maintained within the range of 6.5–7.0 (Diserens, 2014). For cheese samples, preparation followed the guidelines outlined in Bulletin N° 471/2014 of the International Dairy Federation (Diserens, 2014). In summary, 30 g of cheese was combined with 70 mL of antibiotic-free milk, pre-warmed to 45 °C, and homogenized for 4 min in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany). The resulting suspension underwent centrifugation at 3800 × g for 10 min, and the aqueous extract was retained. The pH was adjusted to 6.5–7.0 with 2 N sodium hydroxide. A 100 μL aliquot of this solution underwent processing in the same manner as explained earlier for milk and whey samples.

The Eclipse Farm^{3G} test was executed according to the manufacturer's specifications for the analysis of sheep milk. The Eclipse Farm^{3G} is designed to identify more than 50 antibiotics, encompassing beta-lactams, tetracyclines, sulfonamides, macrolides, lincosamides, and ansamycins. This assay relies on inhibiting microbial growth. The Eclipse Farm^{3G} tubes were equipped with a specific culture medium containing *Geobacillus stearothermophilus* spores and a pH indicator. In the absence of antibiotics (negative), incubation led to spore germination and multiplication, resulting in the production of acidic compounds that reduced the pH and changed the colour of the medium. Conversely, if the sample contained an antibiotic concentration surpassing the limit of detection (positive), microbial growth was hindered. Subsequently,

the tubes, following 2–3 washes with deionized water post-incubation at room temperature for 1 h, underwent further incubation at 65 ± 1 °C (Biometra TB1 Thermoblock, Gottingen, Germany). The duration of incubation was the time required for a known antibiotic-negative sample to transition to yellow (ranging from 2 h 15 min to 3 h, depending on lot and sample type). Test results were interpreted through visual assessment of the colour change in the medium after incubation, classifying samples as positive (blue-purple), negative (yellow), or close to the LOD (green–blue, indicating the presence of antibiotics at a concentration near the LOD). The LODs for the Eclipse Farm^{3G} test are provided in Supplementary Table 2

2.6. Chromatographic analysis of antibiotic residues

2.6.1. Solid-phase extraction (SPE) coupled to ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS)

The extraction and analysis of antibiotic residues in the faecal samples followed established and validated protocols at the Department of Analytical Chemistry and the Research Centre for Experimental Marine Biology and Biotechnology (PIE) of the University of the Basque Country (Vergara-Luis et al., 2023). Briefly, 2 g of homogenized faeces samples underwent extraction with 760 μL of Milli-Q water, 5 mL of acetonitrile, 2 g of anhydrous sodium sulphate, 0.5 g sodium chloride, 0.25 g anhydrous citric acid, and 0.025 g sodium phosphate. A ceramic homogeniser was added to the mixture, manually shaken and degasified until no gas was released. Samples were vortexed at 500 × g for 8 min at 10–15 °C, centrifuged at 2100 × g for 5 min at 10–15 °C, and 2 mL of the extract were diluted to 40 mL with 0.05 M citrate buffer (pH 4). Subsequently, using Solid-Phase Extraction (SPE), the diluted extract was loaded onto a 500 mg Oasis HLB cartridge, previously conditioned with 10 mL of acetonitrile, 10 mL of Milli-Q water, and 10 mL of citrate buffer. Cartridges were washed with 5 mL water, dried under vacuum, and eluted with acetonitrile (9 mL). The extracts were evaporated to 1 mL under a nitrogen flow (TurboVap LV evaporator, aliper Life Sciences, Hopkinton, MA, United States), and 125 μL of the resulting extract were reconstituted in 250 μL of 50:50 (v:v) 0.01 M acetonitrile:oxalic acid (pH 2) and filtered through 0.22 μm polypropylene filters.

Antibiotic residues were analysed using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Madrid, Spain) equipped with a degassing system, binary pump, and automatic injector, coupled to an Agilent 6430 Triple Quad tandem mass-spectrometer (QqQ) (Agilent Technologies), as previously described (Vergara-Luis et al., 2023). Antibiotic compounds were separated on a Kinetex C18 polar column (100 Å pore size, 2.6 μm particle size, 2.1 mm i.d. × 50 mm length) (Phenomenex, Alcobendas, Spain). Both column and pre-column (Kinetex C18 polar, 100 Å pore size, 2.6 μm particle size, 2.1 mm i.d. × 5 mm length) were maintained at 35 °C, and two solvents were used: (A) 0.1 % (v/v) UHPLC quality water in formic acid and (B) 0.1 % (v/v) UHPLC quality methanol in formic acid. The injection volume was fixed at 3 μL, the temperature at 35 °C, and the flow rate was established at 0.3 mL/min.

The chromatographic data obtained were analysed using the Agilent MassHunter Workstation software (Quantitative Analysis for QqQ, 10.0 version, Agilent Technologies). Antibiotic compounds were identified by MS/MS detector, and quantification was performed in dynamic multiple reaction monitoring (DMRM) acquisition mode, using nitrogen (99.999 %, Air Liquide, Paris, Île-de-France, France) as nebulizer and drying and collision gas (99.999 %, Messer, Bad Soden am Taunus, Hessen, Germany). The electrospray ionization source operated in positive ion mode (ESI +) for all analytes. The gas temperature was maintained at 300 °C, with a drying flow rate of 8 L/min, the capillary voltage was set at 3 kV, and the nebulizer pressure at 50 psi. Parameters related to mass spectrometry (fragmentor voltage, collision energy, or collision cell accelerator) were optimised by a standard containing all target compounds at a concentration level of 2.5 μg/mL through the specific Agilent MassHunter Optimizer software (10.0 version), considering both target analytes and surrogates.

2.6.2. Solid-phase extraction (SPE) coupled to liquid chromatography-triple quadrupole mass spectrometry (LC-QqQ-MS/MS)

The extraction and analysis of antibiotic compounds in dairy samples were conducted in accordance with established and validated protocols at the Instituto Lactológico de Lekunberri (Lekunberri, Navarre), as described by Quintanilla et al. (2019b), with slight modifications. In summary, 10 g or mL, depending on the sample type, were combined with 20 g trisodium citrate (20 % wt./wt.) and homogenized for 3 min at 40 °C twice in a stomacher. The resulting mixture underwent centrifugation at 9000 × g for 10 min at room temperature, and 2 g of the supernatant were extracted by SPE using an Oasis HLB cartridge preconditioned with 1 mL of methanol and 1 mL of ultrapure water. The cartridge was rinsed with 2 mL water, eluted with 2 mL methanol, and dried under vacuum. Subsequently, 500 μL of formic acid was added and homogenized for 5 min in an ultrasonic bath. The resulting extracts were filtered using 0.45 μm polyvinylidene fluoride filters.

Antibiotics were analysed using an Alliance 2695 Liquid Chromatography system equipped with a diode-array detector and a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (QqQ) (Waters Chromatography Division), as previously described with minor modifications (Quintanilla et al., 2019b). Antibiotics were separated on an XBridge C18 column (100 mm length, 34.6 mm, 2.1 mm i.d., 3.5 μm particle size, Waters Chromatography Division) using two solvents: (A) 0.1 % (v/v) formic acid in water and (B) 0.1 % (v/v) formic acid in acetonitrile. The gradient program was as follows: 0–8 min, 95 % A and 5 % B; 8–14 min, 25 % A and 75 % B; 14–15 min, 5 % A and 95 % B; and 15–20 min, 95 % A and 5 % B. For oxytetracycline, the gradient program consisted of: 0–6 min, 85 % A and 15 % B; 6–8 min, 82 % A and 18 % B; 8–15 min, 50 % A and 50 % B; and 15–20 min, 85 % A and 15 % B. The injection volume was established at 20 μL, and the flow rate was set at 0.2 mL/min.

The chromatographic data obtained were analysed using Mass-Lynx software (version 4.0; Waters Chromatography Division). Antibiotics were identified using an MS/MS detector and electrospray ionization in positive ion mode (ESI +). The source temperature was maintained at 140 °C, the needle voltage was set at 3.0 kV, lens voltage at 0.2 V, desolvation and cone gas (nitrogen) flow were set at 750 and 50 L/h, respectively; and desolvation temperature was maintained at 450 °C. Typical recoveries were approximately 85 to 100 % for the β-lactams and tetracyclines, 80 to 95 % for the macrolides, and 90 to 110 % for the quinolones. Calibration curves were generated for each antibiotic.

2.7. Food safety margin (FSM) calculation

To assess the risk to consumer health arising from the consumption of dairy products (milk, whey, or cheese) containing antibiotic residues, the Food Safety Margin (FSM) indicator was employed. The FSM value is calculated, as proposed by Quintanilla et al. (2019a), as follows:

$$FSM = \begin{cases} 0 & if\ \ HQ_i > 1 \\ 1 - HQ_i & if\ \ HQ_i \leq 1 \end{cases}$$

where

$$HQ_1 = \frac{(H_i \times C)/W}{ADI_i}$$

Where H_i is the concentration of the detected antibiotic (μg/kg), C corresponds to the daily intake (kg/person/day), W represents the mean body weight (kg) according to the age, and ADI_i is the acceptable daily intake. The numerator corresponds to the calculation of the estimated daily intake (EDI_i).

2.8. Statistical analysis

The data treatment and analysis involved the utilization of various packages and software. Data preparation and analysis were conducted utilizing IBM SPSS statistical package version 28.0 (IBM SPSS Inc., Chicago, IL, United States, 2019). Descriptive statistics, encompassing means and standard deviations, were employed for data summarization. Survey data analysis employed Pearson's chi-squared test to analyse the association between variables (dairies and producers' sociodemographic characteristics, practices, and knowledge related to antibiotic administration) at a 95 % confidence interval. The degree of association was assessed using Cramer's V test. Kruskal–Wallis one-way analysis of variance (ANOVA) with Bonferroni correction was executed using the SPSS package to analyse the significance ($P \leq 0.05$) of variations in screening test results and antibiotic concentrations (n.d. values were substituted with zeros) among samples based on the production chain and producer factors. Permutational multivariate analysis of variance (PERMANOVA) was carried out in RStudio version 1.4.1717 and R version 4.1.1 (R Core Team, Vienna, Austria, 2021) with the "vegan" package (https://github.com/vegandevs/vegan) to investigate the overall impact of production chain and producer factors on screening test results and antibiotic concentrations. Hierarchical Clustering Analysis (HCA) was employed to analyse groupings and trends of screening test results according to the production chain and producer factors. The analysis was performed with Unit Variance (UV) scaled data and presented in a heat-map using the "gplots" package (https://github.com/cran/gplots) in R. Principal Component Analysis (PCA) was conducted using SIMCA software version 17.0.2.34594 (Umetrics AB, Umeå, Sweden) to assess antibiotic concentration trends based on the production chain and producer factors. The number of principal components (PC) was determined through eigenvalues and cross validations. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was also executed in SIMCA to investigate potential differences in antibiotic concentrations among samples based on production chain and producer factors. Production chain and producer factors were utilized as Y-variables, while antibiotic concentrations served as X-variables. Model validation involved R2 and Q2 values, permutation tests, and Inner Relation plots. The significance of each antibiotic in the model was evaluated using Variable Influence on Projection (VIP) values and loading weights.

3. Results and discussion

3.1. Cross-sectional survey

3.1.1. Sociodemographic characteristics of dairies

The cross-sectional surveys or questionnaires employed in this study represent the most widely utilized methods for gathering information on antibiotic utilization or knowledge (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020). Supplementary Table 3 presents the sociodemographic characteristics of the Idiazabal PDO dairies participating in this investigation. Overall, a well-balanced distribution in terms of producers' gender was observed, with a slight predominance of females (53.3 %). All producers had received education, primarily through vocational training (66.7 %). However, the majority of their studies were unrelated to their professional activities (66.7 %), and all indicated participation in courses. A large proportion of producers had Internet access (86.7 %) and occasionally referred to articles on livestock production and health (76.9 %). Professional experience exceeding 10 years was common among producers (60 %), and 86.7 % indicated membership in livestock associations. The majority of dairies were situated outside urban cores (73.3 %), with an average annual production of 47,000 litters of raw ewe milk and 3,744 kg of cheese. Concerning flock management, producers reported an average barn confinement of 3.04 $\pm$ 0.782 months, with an average barn area of 643 $\pm$ 155 m^2. Grazing primarily occurred around the dairy (66.7 %), with an area exceeding 5 ha in most cases (86.7 %). Additionally, 66.7 % of producers mentioned grazing in mountainous areas. Mechanical milking systems predominated (86.7 %), and all producers exclusively utilized milk from their own herds for cheese production. To the best of our knowledge, there is a paucity of survey studies on antibiotic utilization in dairy sheep (Lee et al., 2022). Published studies on dairy cattle primarily concentrate on cattle (Casseri et al., 2022), while among food-producing animals, pigs and poultry predominate (Bedekelabou et al., 2022). Overall, the sociodemographic characteristics of dairy farms exhibit notable differences based on cattle type and geographical location (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020).

3.1.2. Antibiotics utilization and knowledge of producers

Table 1 presents participants' knowledge and attitudes regarding antibiotic use. The majority of producers lacked formal or informal training in antibiotics, although 60.0 % were able to accurately define antibiotics. Concerning antibiotic effectiveness, a significant proportion expressed uncertainty about whether antibiotics were currently less effective than in the past (60.0 %), although discrepancies were observed. Nevertheless, the majority expressed concerns regarding antibiotic resistance (93.3 %). The lack of knowledge about antibiotics and their use, coupled with disparities in current use, effectiveness, and concerns about antimicrobial resistance, aligns with findings from other published studies (Casseri et al., 2022; Lee et al., 2022; Moreno, 2014; Phares et al., 2020; Visschers et al., 2014).

Of the producers, 93.3 % indicated having administered antibiotics to their flock at least once, usually less than once a month, in the previous year (64.3 %). The antibiotics administered included β-lactams, such as penicillin G or amoxicillin, and tetracyclines, such as tetracycline and oxytetracycline. The use of antibiotics in farms is a widespread practice (Bedekelabou et al., 2022; Lee et al., 2022; Phares et al., 2020). Penicillin and tetracycline are the most commonly used veterinary antibiotics (Casseri et al., 2022; Dyar et al., 2020), although the compounds and doses vary between farms and territories (Bedekelabou et al., 2022; Casseri et al., 2022; Visschers et al., 2014). A significant portion of producers (53.3 %) mentioned the ease of obtaining antibiotics, always through veterinary services, unlike other studies where obtaining antibiotics without a prescription has been observed (Dyar et al., 2020; Lee et al., 2022; Phares et al., 2020). Most producers reported using veterinary services for treating livestock diseases once or twice a year (46.7 %), with fewer indicating more than twice a year (40.0 %). The use of veterinary services varies significantly by territory, with developing countries having the least use (Bedekelabou et al., 2022; Dyar et al., 2020; Phares et al., 2020). Lee et al. (2022) reported the effects of farm size, with small producers having the highest utilization. Visschers et al. (2014) suggested that the use of these services promotes the correct use of antibiotics. Veterinarians were responsible for administering antibiotics in only 13.3 % of cases, with the majority administered by the dairy owner or some employees (35.7 % and 28.6 %, respectively). The 92.9 % of the producers indicated using antibiotics only when they were certain about a bacterial infection, whereas the 7.14 % indicated also giving antibiotics when the animal appears to be sick. Additionally, 14.3 % used antibiotics for prevention, apart from curative purposes. In all cases, the recommended dose was reported to be respected, and the route of administration was parenteral. Farm workers or owners administering antibiotics, along with improper use and incorrect concentrations, have been reported in other studies (Bedekelabou et al., 2022; Dyar et al., 2020; Moreno, 2014; Phares et al., 2020), emphasizing that producer education is a crucial factor (Bedekelabou et al., 2022). Bedekelabou et al. (2022) have reported that producers in Togo mainly used antibiotics for preventive purposes, whereas Moreno (2014) indicated that Spanish producers did not clearly distinguish between curative and preventive purposes. In this study, antibiotics were not administered through feed and water, unlike in other studies (Dyar et al., 2020; Lee et al., 2022), which has been associated with antibiotic overuse and the spread of antimicrobial

Table 1
Antibiotics utilization and knowledge of producers.

Item	Percentage (%)
Training (formal or informal) on antibiotics	
Yes	6.67
No	86.7
No answer/don't know	6.67
Correct description of what antibiotics are	
Yes	53.3
No	40.0
No answer/do not know	6.67
In your experience, antibiotics are now less effective than in the past:	
I strongly disagree	0.00
I do not agree	13.3
Not sure	66.7
I agree	0.00
I strongly agree	6.67
No answer/don't know	13.3
Are you worried about antibiotic resistance?	
Yes	93.3
No	0.00
I do not know what antibiotic resistance is	0.00
No answer/don't know	6.67
Ever given antibiotics to animals	
Yes	93.3
No	6.7
Ease or difficulty of obtaining antibiotics	
Easy	53.3
Difficult	33.3
According to the veterinary staff	6.7
No answer/do not know	6.7
Antibiotics used	
Amoxicillin	5.26
Penicillin G	36.8
Dihydrostreptomycin	26.3
Oxytetracycline	15.8
Polymyxin b	10.5
Tetracycline	5.3
Antibiotics adquisition	
Veterinary staff	80.0
Veterinary staff of external company	13.3
Association	6.7
Availability of veterinary staff	
Always	33.3
Sometimes	53.3
Never	0.0
No answer/do not know	13.3
Veterinary services's use for the treatment of livestock diseases	
Never	6.67
Once a month	13.3
1 or 2 times a year	46.7
More than 2 times a year	40.0
Person administering antibiotics	
Veterinary staff	14.3
Employee	28.6
Owner	35.7
Veterinary staff and/or owner	21.4
Administered dose	
Dosage recommended by the veterinarian	100
Dose given based on your experience	0.0
Dose administered based on the recommendation of another farmer	0.0
When antibiotics are given to animal?	
When I am sure that the animal has a bacterial infection	92.9
When I am sure that the animal has a bacterial infection, whenever the animal is sick and/or whenever the animal appears ill	7.14
Why are antibiotics used?	
Prevent and cure (treat infections)	14.3
Prevent	0.0
Cure (treat infections)	85.7
Frequency of antibiotic administration during the last 12 months	
More than once a week	0.0
Once a week	0.0
More than once a month	7.14
Once a month	0.0
Less than once a month	64.3
According to the need	21.4
No answer/do not know	7.14
Method of antibiotics administration	
Along with the feed	0.0
Along with the drinking water	0.0
Parenterally (injected)	100
Herd management during antibiotic administration	
Antibiotics are given always or often along with feed or drinking water to keep animals healthy and prevent disease.	0.0
Antibiotics are given to all animals in the herd, when some of the animals are sick.	0.0
Antibiotics are given only to sick animals	100
How long does it take for the antibiotic to disappear from the milk?	
Same day of treatment	0.00
3 days after treatment	0.00
One week after treatment	0.00
According to the antibiotic	92.9
I do not know.	7.14
Has milk or cheese ever been tested for the presence and/or concentration of antibiotics?	
Yes, routine within dairy control	53.3
Yes, once within dairy control	6.67
Yes, once within dairy control and sometime of my own free will	6.67
Yes, routine within dairy control and sometime of my own free will	6.67
Never	26.7
Method used to examine the presence and/or concentration of antibiotics in milk or cheese	
Inhibitor analysis	13.3
No answer/don't know	86.7

resistance (Treiber & Beranek-Knauer, 2021). The 92.9 % of the producers indicated that the withdrawal period of an antibiotic depended on the compound. However, only 53.3 % of producers routinely examined the presence and concentration of antibiotics in milk and cheese, with some doing so voluntarily. Literature addressing adherence to withdrawal times and the analysis of residues is limited (Phares et al., 2020). Ensuring compliance with the withdrawal period is crucial to guarantee the absence of the compound in both animal organisms and derived products, such as milk (Virto et al., 2022).

Regarding the association between the sociodemographic characteristics of the dairies and producers and the practices and knowledge related to antibiotic use, it was observed that producers with lower education levels (primary education) were the only ones with training in relation to antibiotics ($P < 0.01$). Moreover, it was observed that those producers who used antibiotics to cure and prevent infections were only those whose training was related to the professional field ($P < 0.05$), indicating a lack of knowledge on antibiotic utilization (Moreno, 2014). Overall, fewer associations were observed compared with other studies (Dyar et al., 2020; Lee et al., 2022; Phares et al., 2020). For instance, Bedekelabou et al. (2022) have observed that knowledge about antibiotics differs according to the producer's gender, and producers with lower education are more likely to misuse antibiotics. Phares et al. (2020) found an association between farm location, income status, and antibiotic use in Ghana.

Notably, several associations were observed between the producers' knowledge and practices regarding antibiotic use. The results revealed that producers who received training on antibiotics analysed the presence of residues ($P < 0.01$). A large number of producers used antibiotics only when they were sure that the animal had a bacterial infection and routinely analysed the antibiotic residues ($P < 0.01$). The use of antibiotics and the reasons for their use were also significantly associated ($P < 0.05$). Most producers who used antibiotics when they were sure that the animal had a bacterial infection only used it to cure the animal, whereas those producers who administered antibiotics without being sure that it was a bacterial infection used them to prevent infection. A

relationship was also observed between the administration of antibiotics and opinions on their effectiveness ($P < 0.01$). Producers who had never administered antibiotics indicated that antibiotics were less effective today than in the past, whereas most producers who administered antibiotics indicated that they were not sure and, to a lesser extent, disagreed with the current lower effectiveness. In addition, the frequency of antibiotic use was associated with opinions on the effectiveness of antibiotics ($P < 0.05$). Most producers who were unsure of the current effectiveness of antibiotics had administered antibiotics to the flock less than once per month, while those who did not agree that antibiotics are less effective today indicated that there was no frequency of annual or monthly use since it corresponds to the need. Overall, these associations confirm that the formation of producers in terms of antibiotic knowledge and practices is an important factor in ensuring the correct utilization of these compounds and avoiding their presence in the food production chain (Bedekelabou et al., 2022; Dyar et al., 2020).

3.2. Antibiotics residues determination throughout cheese production chain by screening tests

Fig. 1 depicts the outcomes of the Charm KIS and Eclipse Farm^{3G} antibiotic screening tests. A noteworthy 76.3 % of the samples collected across the production chain yielded negative results, whereas 12.5 % (10/80) approached the LOD, and 10.0 % (8/80) yielded positive results (Fig. 1A). Although differences among producers were observed, they did not attain statistical significance ($P > 0.05$) (Fig. 1A). Across the production chain, statistically significant differences among sample types (faeces, raw milk, whey, fresh cheese, and ripened cheese) were observed ($P \leq 0.01$) (Fig. 1B-C). Employing a multivariate approach, PERMANOVA confirmed the impact of sample type ($P \leq 0.01$) and the non-significant impact of the producer factor ($P > 0.05$). Differentiation of samples based on antibiotic screening test results was further confirmed using HCA (Fig. 1A and 1D).

All faecal samples, irrespective of the producer ($P > 0.05$), tested negative in the Charm KIS test (Fig. 1), indicating the absence of antibiotic residues above the LOD. These findings are particularly

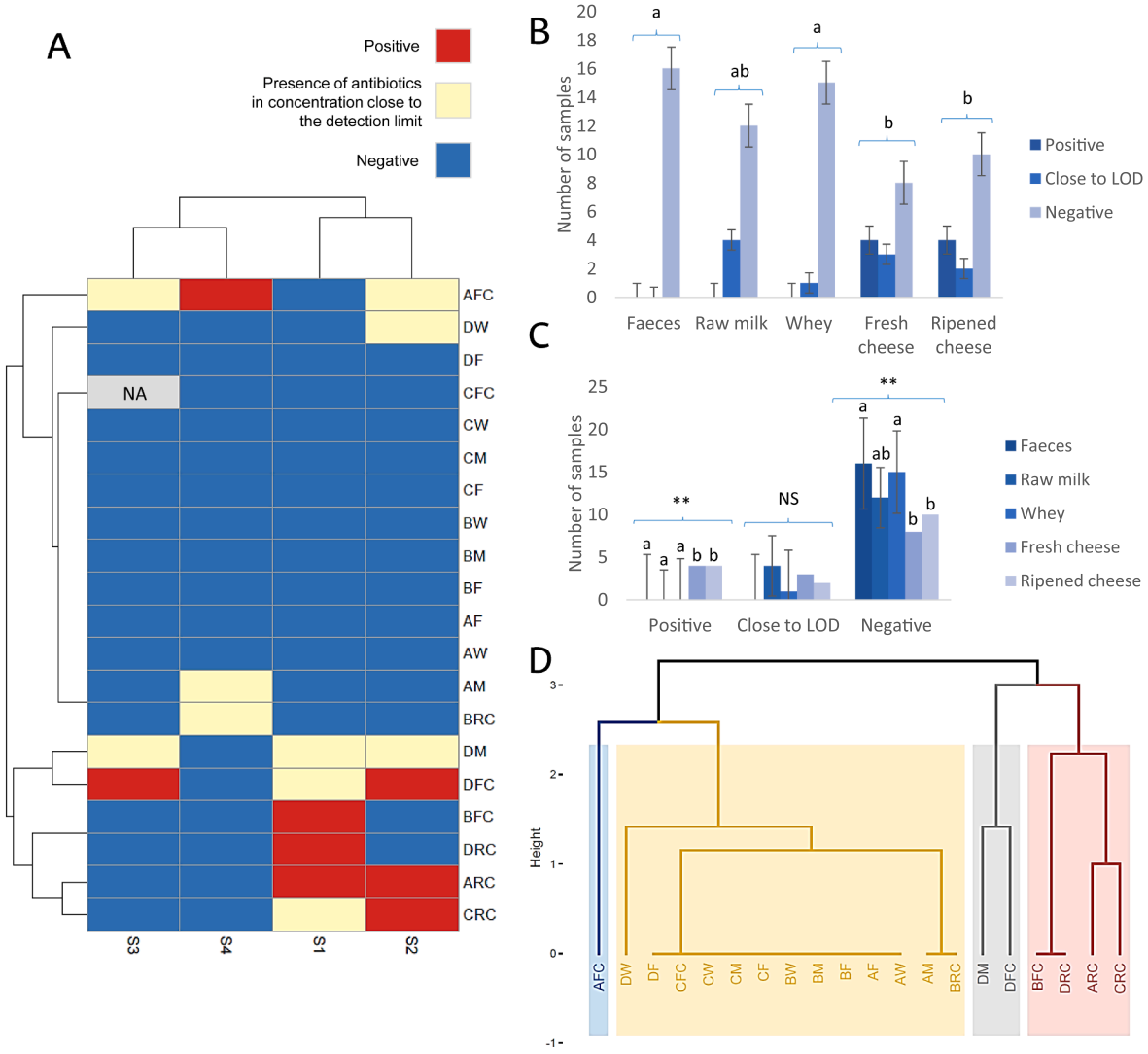


Fig. 1. HCA heat map (A), bar plots (B and C) and HCA dendogram based on the screening test (Charm KIS and Eclipse Farm^{3G}) results. The different letters within the group of bars indicate a significant difference at $P \leq 0.01$. Abbreviations: NA: not possible to analyse; S1: first sampling week; S2: second sampling week; S3: third sampling week; S4: fourth sampling week; AF, BF, CF, DF: faeces samples from producers A, B, C and D, respectively; AM, BM, CM, DM: raw milk samples from producers A, B, C and D, respectively; AW, BW, CW, DW: whey samples from producers A, B, C and D, respectively; AFC, BFC, CFC, DFC: fresh cheese samples from producers A, B, C and D, respectively; ARC, BRC, CRC, DRC: ripened cheese samples from producers A, B, C and D, respectively; **: $P \leq 0.01$; NS: non-significant, $P > 0.05$.

noteworthy given reports from various studies highlighting the potential transfer of manure antibiotics to the environment, with implications for soil microbiota, groundwater quality (Virto et al., 2022), and crops, posing a risk to human health (Zhang et al., 2022). However, no screening tests for faecal samples have been reported to date.

Similarly, the Eclipse Farm^{3G} test revealed that none of the raw milk samples collected tested positive for antibiotics (Fig. 1). Nevertheless, 25.0 % (4/16) of the samples exhibited concentrations close to the LOD, with no significant variation according to the producer ($P > 0.05$). The presence of residues in raw milk as opposed to animal faeces is meaningless (Fig. 1), since the administered compounds are primarily excreted through urine and faeces (Virto et al., 2022). The observed incongruity may be attributed to variations in the LODs of each test. Notably, the Charm KIS test features LODs 10^2–10^3 times higher than those of the Eclipse Farm^{3G} (Supplementary Tables 1 and 3). Consequently, higher concentrations are necessary for a positive result. For instance, a positive result for oxytetracycline in faeces would require 10^5 μg/L, while only 150 μg/L would be needed for milk. Based on the established MRLs, all raw milk samples can be classified as compliant (Brocca & Salvatore, 2023). These results agree with the latest results reported by the European Food Safety Authority (EFSA) for the monitoring of antibiotics in milk, which reported only 0.23 % (47/20407) of non-compliant samples, maintaining the low rate trend observed in the last decade (0.09–0.44 %) (Brocca & Salvatore, 2023). The Food and Drug Administration Agency of the United States (FDA) has also reported a low percentage of non-compliant raw milk samples in the United States over the last year (0.009 %) and the past decade (0.02–0.008 %) (FDA, 2023). However, these studies primarily focus on bovine milk. Although several studies have addressed the detection of antibiotic residues in raw milk through screening tests, most pertain to bovine milk, and information on milk obtained from small ruminants such as sheep is limited (Virto et al., 2022). Yamaki et al. (2006) identified a 2.6 % positivity rate in raw milk samples from the Assaf breed, while Gonzalo et al. (2010) reported a 0.6 % rate for raw milk of the Manchega breed. These rates imply higher noncompliance compared to the findings of this study.

Throughout the cheese-making process, none of the collected whey samples yielded a positive result, regardless of the producer ($P > 0.05$), with 6.25 % (1/16) of the samples yielding a result close to the LOD and predominating in a negative result (93.8 %) (Fig. 1). Concerning the fresh cheese samples, 18.8 % (3/16) yielded results close to the LOD, and 25.0 % (4/16) yielded a positive result. Nevertheless, some false positives or false close to the LOD results for fresh cheeses were observed, because the milk samples used to make the cheeses yielded negative results, which constituted 3.75 % (3/80) of the total samples. False-positive results for screening tests have been linked to substances that inhibit bacterial growth, which in this case could be related to lysozyme or free fatty acid content (Richards et al., 2022). However, this false-result rate cannot be compared with the literature because no results have been published on antibiotic screening tests in cheese, substantiating the novelty of these results. It is essential to improve the sensitivity and precision of inhibition tests to ensure food safety and avoid economic losses to producers due to the elimination of samples incorrectly classified as containing antibiotics and the unnecessary performance of confirmatory analytical tests (Chiesa et al., 2020). Without considering false results, 20.0 % (3/16) of the fresh cheese samples were positive, and 6.67 % (1/16) gave results close to the LOD. Few studies have analysed the effect of the cheese-making process on antibiotic concentrations; however, the retention of antibiotics in curd or elimination by whey during the cheese-making process depends on the compound and its characteristics (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b; Virto et al., 2022). These results indicate that the Idiazabal cheese-making process leads to a change in the screening test results from close to the LOD in milk to positive in fresh cheese; consequently, the antibiotics present in milk could be transferred and concentrated, mainly in the cheese. Notwithstanding the lack of MRLs for whey or cheese, as these products are transformed from raw milk and considering the MRLs, all whey samples were classified as compliant, whereas 20.0 % (3/16) of the fresh cheeses were possibly non-compliant (Diserens, 2014).

During ripening, a similar trend was observed, with 25.0 % (4/16) of the ripened cheese samples yielding a positive result and 12.5 % (2/16) being close to the LOD. However, false positives or false close to the LOD accounted for 6.25 % (5/80) of the total samples, which could be related to higher concentrations of these compounds inhibiting bacterial growth (Richards et al., 2022; Santamarina-García et al., 2022), as mentioned earlier. Thus, aside from false results, no ripened cheese close to the LOD was observed, and 6.25 % (1/16) were positive, and consequently, possibly non-compliant (Diserens, 2014). It is noteworthy that 100 % (3/3) of the possibly non-compliant fresh cheeses became compliant after ripening, confirming the degradative effect of the ripening process, although it has been little studied (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b; Virto et al., 2022). However, concerning the unique positive ripened cheese sample observed, its corresponding fresh cheese sample yielded a result close to the LOD (Fig. 1), suggesting a concentration effect during ripening, which has been little studied so far (Quintanilla et al., 2019b). Considering that national and international food safety authorities, such as the EFSA and FDA, do not monitor the presence of antibiotic residues in dairy products (Brocca & Salvatore, 2023; FDA, 2023), these results are of special interest because they indicate that, in most cases, the ripening process reduces the risk to consumers' health (Virto et al., 2022). Nonetheless, in some cases, residues could be maintained up to the final cheese; therefore, more research should be conducted to identify cheese-making or ripening conditions that could concentrate residues.

3.3. *UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS analysis of antibiotic residues' occurrence throughout cheese production chain*

Different national and international regulations stipulate that samples yielding non-compliant results through screening tests must undergo analysis using confirmatory methods to identify and quantify antibiotic compounds (Brocca & Salvatore, 2023; Diserens, 2014). Consequently, the concordance between screening tests and chromatographic analysis was assessed. Table 2 presents the average concentrations of antibiotic compounds identified throughout the Idiazabal cheese production chain. The results revealed that 26.3 % (21/80) of the samples were above the LOQ. However, only 13.0 % of the tested antibiotics were identified, specifically a macrolide (tylosin), a quinolone (enrofloxacin), two sulfonamides (sulfadiazine and sulfamethazine), and two tetracyclines (oxytetracycline and chlortetracycline) (Table 2). Notably, chlortetracycline was the most abundant compound, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg). It is worth mentioning that, in the cross-sectional survey (Table 1), only tetracyclines were reported among these compounds, possibly indicating a lack of producers' awareness of the provided antibiotics (section 3.1). Ansamicine, β-lactam (penicillin and cephalosporin), or lincosamide class antibiotics were not detected, despite being mentioned in the survey (Table 1), suggesting potential lack of recent use. Overall, these results affirm the efficacy and precision of LC coupled with MS/MS for detecting and quantifying antibiotics at low concentrations (Sta Ana et al., 2021).

According to the detected compounds, tetracyclines are natural broad-spectrum compounds produced by *Streptomyces aureofaciens* and *Streptomyces rimosus*, acting against gram-positive and gram-negative bacteria such as *Chlamydia*, *Mycoplasma*, *Rickettsiae*, and protozoan parasites (Alcock et al., 2023). However, recent years have seen high resistance rates reported in various bacteria (Virto et al., 2022). The European Medicines Association (EMA) categorizes these compounds as category D, designating them as first-line treatments and recommending cautious use only when medically necessary (CVMP and CHMP, 2020). The detected macrolide, tylosin, is also a natural compound produced by

Table 2
Concentration (mean ± standard deviation) of the antibiotic compounds identified throughout production chain of Idiazabal cheese (faeces, raw milk, whey, fresh cheese and ripened cheese) in four producers (A, B, C and D).

Antibiotics Class	Compound	Antibiotic concentration (μg/kg)[1]																				P-value[2]	
		Faeces Producer				Raw milk Producer				Whey Producer				Fresh cheese Producer				Ripened cheese Producer				C	P
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D		
Ansamycin	Rifaximin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (penicillin)	Amoxicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ampicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Penicillin G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Dicloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Nafcillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (cephalosporin)	Cephapirin + Desacetylcephapirin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefoperazone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalexin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefquinome	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalonium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ceftiofur + Desfuroylceftiofur	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefazolin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefacetril	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefuroxime	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Lincosamide	Lincomycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Macrolide	Tylosin	n.d.	n.d.	n.d.	n.d.	**6.15 ± 12.3**	n.d.	**6.98 ± 8.24**	n.d.	**5.35 ± 10.7**	n.d.	**6.28 ± 7.39**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	0.052
	Erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Spiramycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Tilmicosin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Quinolone	Enrofloxacin + Ciprofloxacin	n.d.	**2.05 ± 2.90**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.092	0.392
	Flumequine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sarafloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000

(*continued on next page*)

Table 2 (*continued*)

Antibiotics Class	Compound	Antibiotic concentration (μg/kg)[1] Faeces Producer A	B	C	D	Raw milk Producer A	B	C	D	Whey Producer A	B	C	D	Fresh cheese Producer A	B	C	D	Ripened cheese Producer A	B	C	D	P-value[2] C	P
	Danofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Marbofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxolinic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Sulfonamide	Sulfadiazine	n.d.	**9.10 ± 5.52**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.108
	Sulfathiazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfapyridine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamerazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamethazine	**45.0 ± 2.20**	n.d.	**2.30 ± 0.850**	**2.10 ± 0.28**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.540
	Sulfachloropyridazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfaquinoxaline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfametizole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Tetracycline	Oxytetracycline	**9.30 ± 0.60**	n.d.	**1.75 ± 2.47**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.278
	Tetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Chlortetracycline	n.d.	**3.00 ± 4.24**	n.d.	**52.0 ± 58.4**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.278
	Doxycycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000

[1] The concentration of each compound is expressed as the mean ± standard deviation. Values above the LOQ are bold colored. n.d.: not detected.

[2] C: Production chain factor effect; P: Producer factor effect.

S. fradiae and acts by inhibiting protein synthesis through interaction with the 50S subunit of the bacterial ribosome. It is effective against gram-positive bacteria, some gram-negative bacteria (*Helicobacter pylori*, *Haemophilus* spp., *Pasteurella* spp., *Legionella* spp.), spirochetes, *Cryptosporidium parvum*, *Chlamydia*, and *Mycoplasma*. However, certain enteric bacteria, such as *Escherichia coli* and *Salmonella* spp., are intrinsically resistant (Alcock et al., 2023). The EMA classifies tylosin into category C, indicating that it is an antibiotic to be used with caution, considering the availability of alternatives in human medicine. It should only be considered for veterinary treatment when there are no alternatives to Category D drugs that may be clinically effective (CVMP and CHMP, 2020). Sulfonamides are broad-spectrum synthetic compounds that act by inhibiting dihydropteroate synthase, which catalyses the conversion of *p*-aminobenzoic acid to dihydropteroic acid as part of the tetrahydrofolic acid biosynthetic pathway. This acid is essential for the synthesis of folate, a precursor of nucleotides and amino acids (Alcock et al., 2023). Sulfonamides are commonly used in veterinary medicine as antibacterial compounds to treat livestock diseases, such as gastrointestinal and respiratory tract infections (Ovung & Bhattacharyya, 2021). They are classified as category D (CVMP and CHMP, 2020). Finally, the quinolone enrofloxacin is a broad-spectrum compound that acts against gram-positive and gram-negative bacteria by interacting with topoisomerase II (DNA gyrase) to disrupt bacterial DNA replication, damage DNA, and cause cell death (Alcock et al., 2023). It is used to control bacterial infections in the respiratory and gastrointestinal tracts and mastitis in cattle. The EMA classifies it into category B; consequently, it is an antimicrobial of transcendental importance in human medicine, and its use in animals must be limited to mitigate the risk to public health. This should only be considered if there are no Category C or D alternatives (CVMP and CHMP, 2020).

The prevalence of antibiotics varied significantly according to the sample type, with faeces exhibiting the highest concentrations (Table 2). Chlortetracycline dominated, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg) and, in lower concentrations, oxytetracycline (1.75 ± 2.47 to 9.30 ± 0.60 μg/kg), sulfadiazine (9.10 ± 5.52 μg/kg), and enrofloxacin (2.05 ± 2.90 μg/kg). These concentrations were found to be below the LODs of the Charm KIS test (Supplementary Table 1), potentially explaining the observed discrepancy between the commercial and analytical methods (Table 2 and Fig. 1). Developing specific screening tests for faecal samples could enhance stakeholders' ability to determine when an animal no longer harbours a particular compound, thereby preventing its transfer to the environment and the food chain (Virto et al., 2022; Zhang et al., 2022). The identified compounds have been previously identified in livestock faeces (Berendsen et al., 2015; Virto et al., 2022), including sheep (Peng et al., 2022), confirming the excretion of certain antibiotics through faeces in food-producing animals (Virto et al., 2022). Approximately 70–90 % of administered compounds, whether in their unmetabolized form or as active metabolites, have been reported to be present in animals' faeces and urine (Phares et al., 2020). These findings are particularly significant since the faecal samples obtained originated from healthy herds that had not undergone antibiotic treatment. Consequently, the transfer of antibiotics from manure to the food chain poses potential risks to human health (Zhang et al., 2022). Tetracyclines, quinolones, sulfonamides, and macrolides have been frequently detected in manure (Kuppusamy et al., 2018; Virto et al., 2022).

Notably, there were large differences in the antibiotics detected in the faeces among the producers, although this difference was not statistically significant ($P > 0.05$) (Table 2). For example, sulfamethazine was detected in all samples except those from producer B, whereas chlortetracycline was detected in faeces from producers B and D, oxytetracycline was detected in faeces from producers A and C, and sulfadiazine and enrofloxacin were only detected in the faeces of producer B. Moreover, a clear distinction was also noted in the concentrations of the detected compounds. For instance, sulfamethazine was about 45.0 ± 2.20 μg/kg in samples from producer A, while it was considerably lower in samples from producer C (2.30 ± 0.850 μg/kg) and D (2.10 ± 0.283 μg/kg). The concentration of chlortetracycline was markedly higher in the samples of producer D (52.0 ± 58.4 μg/kg) than in those of producer B (3.00 ± 4.24 μg/kg). This would be indicative of the different herd management practices and, specifically, the use of antibiotics between farms, as reflected in the cross-sectional survey (Section 3.1). These results agree with those of previous studies. For example, Peng et al. (2022) have reported on the abundance of tetracyclines, sulfonamides, quinolones, and macrolides in faecal samples of different sheep herds from China.

It is essential to analyse the presence of antibiotic residues in raw milk to avoid negative effects on consumer health, both direct effects due to the toxicology of the compounds and indirect effects due to the development of resistant microorganisms (Virto et al., 2022). In the analysed raw milk samples, the results revealed that of all the antibiotics tested by LC-MS/MS, only tylosin was detected at a concentration that varied from 6.15 ± 12.3 to 6.98 ± 8.24 μg/kg (Table 2). However, in all samples, the concentrations were below the MRLs (Brocca & Salvatore, 2023), indicating no risk to consumer health. Nonetheless, several studies have shown that antibiotics, even at concentrations below the MRLs, can favour the development of resistant bacteria (Virto et al., 2022). Furthermore, concentrations below MRLs have also been reported as affecting the activity of starter cultures; for example, a 50 % reduction in the activity of *Streptococcus* sp. (Quintanilla et al., 2019b). Therefore, antibiotic residues can affect cheese microbiota (Quintanilla et al., 2019b), and considering their importance for cheese quality and safety (Santamarina-García et al., 2022), they can compromise the final characteristics of cheese, such as the aroma or presence of pathogenic bacteria (Quintanilla et al., 2019b; Santamarina-García et al., 2022). Information on the occurrence of tylosin in raw ewe milk is scarce, but it has been previously detected in milk derived from the Awassi and Merino breeds (Richards et al., 2022). The penetration of macrolides into tissues, milk, and blood is relatively fast, with high systemic availability. Thus, they show good penetration and distribution in the udder (Richards et al., 2022), which explains the presence of tylosin in milk (Table 2).

The raw milk samples revealed the presence of tylosin exclusively in samples obtained from producers A and C. Small variations in the concentration of this antimicrobial were noted between samples from both producers, as outlined in Table 2. These findings suggest disparities in herd management and antibiotic administration practices across farms, as outlined in the accompanying survey (Table 1). It is noteworthy that the existing literature lacks information regarding distinctions or similarities in the occurrence of antibiotic residues in dairy products among producers sharing the same PDO, thus underscoring the novelty of these results.

The LC-MS/MS results (Table 2) partially agreed with those of the Eclipse Farm^{3G} test (Fig. 1A). In the raw milk sample S4 from producer A, tylosin was detected at a concentration of 24.6 μg/kg, while in the samples S3 and S4 from producer C the concentration was lower (16.1 μg/kg and 11.8 μg/kg, respectively). Considering that the LOD of this test for tylosin is 25 μg/kg, the result of close to the LOD for producer A and not for producer C would be explained. However, the 75.0 % (3/4) of the samples from producer D also yielded results close to the LOD, in which no antibiotics were detected. Similar results have been reported in the literature as false positives (Chiesa et al., 2020), which have been attributed to different substances present in milk, such as bacteriocins or lysozymes, which inhibit the growth of *Geobacillus stearothermophilus* (Yamaki et al., 2006). Nevertheless, unlike chromatographic methods, screening tests also detect metabolites or degradation compounds of antibiotics, which are essential because they maintain antimicrobial activity (Serrano et al., 2022). Consequently, the obtained results could be related to antibiotics or metabolites of antibiotics not analysed by LC-MS/MS, which have not been reported so far for dairy products.

Antibiotic residues are concentrated during the cheese-making process, although this depends on the compound and its characteristics

(Quintanilla et al., 2019b). In this sense, throughout Idiazabal cheese-making process, tylosin was the only compound detected by LC-MS/MS in whey samples, although at a slightly lower average concentration than in milk (from 5.35 ± 10.7 to 6.28 ± 7.39 μg/kg) (Table 2). In contrast, no compounds were detected in the fresh cheese samples, including the sulfonamides, quinolones, and tetracyclines identified in the faeces (Table 2). The manufacturing process of producer A led to the elimination of 3.2 μg/kg of tylosin, while for producer C it was only 1.4 μg/kg. That is, the tylosin concentration was reduced from milk to whey by approximately 14 % in the case of producer A and by approximately 29 % in case of producer C [The large differences in the cheese making settings observed in producers A and C (Supplementary Table 4), which will be discussed a bit later, must have played a role in obtaining these quite different values]. Notably, the distribution of antibiotics during cheese-making has been reported to be dose-independent (Giraldo et al., 2022), which indicates the extrapolation potential of these results to other similar cheese-making processes. The partitioning from milk to cheese or whey depends not only on the solubility of the compound but also on its ability to interact with the protein and/or fat fraction (Quintanilla et al., 2019a) and other factors to be studied yet (Giraldo et al., 2022). These results agree with what has been reported by Giraldo et al. (2022), who observed during the elaboration of a goat cheese that 75.3 % of the tylosin present in milk was eliminated through the whey, while the remaining 24.7 % was retained in the cheese. Therefore, the concentrations retained in the fresh cheese samples analysed in this study were low and undetectable by the LC-QqQ-MS/MS method (<LOD). Overall, further studies are required to elucidate the effects of cheese-making settings on antibiotic concentrations.

Although the transfer of antibiotics from milk to whey is considered beneficial for cheese safety (Giraldo et al., 2022), it is also important to consider whey safety. Dairy whey has various applications, such as fertiliser in agriculture and human and animal food because of its nutritional value (Virto et al., 2022). Sheep milk whey has a higher total nitrogen/dry matter ratio than bovine whey, doubling the soluble protein content (Carvalho et al., 2013). Whey protein processing has also been used for therapeutic purposes to obtain bioactive peptides with antioxidant, antihypertensive, antithrombotic, antimicrobial, and antiviral activities (Carvalho et al., 2013). However, there is no information on the effect of whey treatment on antibiotic concentrations.

Throughout the cheese-making process, clear differences were also observed among producers, detecting tylosin only in whey samples from producers A and C and below the MRL limits (Table 2). However, this difference was not statistically significant ($P > 0.05$). The cheese-making process and its conditions affect the concentration of antibiotics and their transfer to curds or whey (Quintanilla et al., 2019a). In case of producers A and C, large differences were observed in the cheese-making settings (Supplementary Table 4), with significant differences in the temperatures used or in the duration of treatments, such as pressing ($P \leq 0.05$). Consequently, these parameters could have affected antibiotic concentrations (Quintanilla et al., 2019a). Specifically, heat treatments that take place during milk processing, such as pasteurisation or sterilisation, have been related to the degradation of the antibiotics present in milk (Roca et al., 2011), even though degradation depends on the treatment conditions (lower temperature and time, less degradation) and the compound (for example, macrolides such as erythromycin are quite thermosensitive, whereas quinolones are very thermostable) (Quintanilla et al., 2019a). In this regard, Quintanilla et al. (2019a) have reported a maximum of 30 % degradation of antibiotics in spiked goat milk, and Gajda et al. (2017) have informed a reduction below 19 % in tetracyclines in cow milk. Milk pasteurisation does not occur in raw milk cheeses, including Idiazabal cheese (Santamarina-García et al., 2022) and there is no information in the literature on the extent to which the different stages of the cheese-making process could affect antibiotic concentration, which could be useful information for producers.

Comparing the results of the Eclipse Farm^{3G} test and the LC-MS/MS method throughout the cheese-making process, both methods were in accordance with the whey samples. The whey sample from producer A that gave a result close to the LOD by means of the screening test, had a concentration of 21.4 μg/kg of tylosin by LC-QqQ-MS/MS, being close to the LOD of the test (25 μg/kg). However, the samples from producer C presented lower concentrations (10.8 and 14.3 μg/kg) and therefore yielded negative results. For fresh cheeses, a discrepancy was observed because no compound was detected by LC-QqQ-MS/MS in the samples that gave a positive or close to LOD result. This discrepancy could be related to antibiotics not being analysed, metabolites of antibiotics not detected by the LC-QqQ-MS/MS technique used, as aforementioned (Serrano et al., 2022), or to the above-mentioned false positives due to free fatty acids, bacteriocins, and lysozyme in a minority of cheese samples produced from negative milk samples.

During cheese ripening, no antibiotics were detected by LC-MS/MS (Table 2). Cheese ripening has been reported to degrade antibiotics that may appear in fresh cheese, although this depends on the compound and its characteristics (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b). For example, Quintanilla et al. (2019b) have observed a 95 % of oxytetracycline reduction during ripening and of only 30–45 % for quinolones, attributed to their stability at refrigeration temperatures. However, contradictory results have been reported in the literature. For example, Cabizza et al. (2017) have only observed a 17 % reduction in oxytetracycline concentration. This has been attributed to different types of milk (sheep and goat) and ripening conditions (such as acidification, ripening time, and ripening conditions) (Quintanilla et al., 2019b). As for fresh cheese samples, LC-MS/MS results did not agree with the screening test (Fig. 1), which could not only be related to false positives but also highlights the need to improve the confirmatory methods used, as stated before for meat samples (Serrano et al., 2022).

Using multivariate analysis, PERMANOVA confirmed the effect of the production chain or sample type on the occurrence of antibiotics ($P \leq 0.01$). Through PCA (Fig. 2A-B) and OPLS-DA models (Fig. 2C-D), despite its limitations, the distinction among sample types was confirmed, with faeces related to most antibiotic compounds, raw milk and whey related to tylosin, and fresh and ripened cheese samples without relation to any compound. Differences among samples, according to the manufacturer, were also observed.

3.4. Health risk assessment

To determine the health risks posed by the presence of antibiotic residues to human health, the FSM value was calculated in samples where antibiotic residues were found (Fig. 3). The FSM makes it possible to assess whether the margin between the exposure at an estimated daily intake (EDI) and the safety threshold as an acceptable daily intake (ADI) for the food hazard in question is sufficient (Quintanilla et al., 2019a). According to the results obtained, an average FSM value of 1.00 ± 0.00143 was obtained for tylosin in raw milk and whey, with the lowest value found for children (0.999 ± 0.00143). For the remaining compounds, whether concentrations similar to those found in faeces would have been maintained in raw milk and whey, the lowest FSM values would correspond to 0.945 for chlortetracycline and 0.983 for enrofloxacin in children. Nevertheless, differences in the FSM values for all antibiotics among the age categories (adults, teenagers, and children) were scarce and, consequently, were not significant ($P > 0.05$). According to the producer, differences were observed in the FSM values in milk and whey ($P \leq 0.001$) owing to the occurrence of different antibiotics along the production chain. Multivariable analysis confirmed the effect of the producer ($P \leq 0.001$), but not that of the age category ($P > 0.05$). PCA and OPLS-DA models confirmed these results (Supplementary Fig. 1). Therefore, in general, the FSM of all samples were close to 1, indicating that the consumption of raw milk or whey would not pose a significant health risk. These results agree with those reported by Quintanilla et al. (2019a), who observed that children were the most sensitive group, followed by adolescents and adults. This was mainly due to the EDI formulation, as exposure is inversely proportional to body

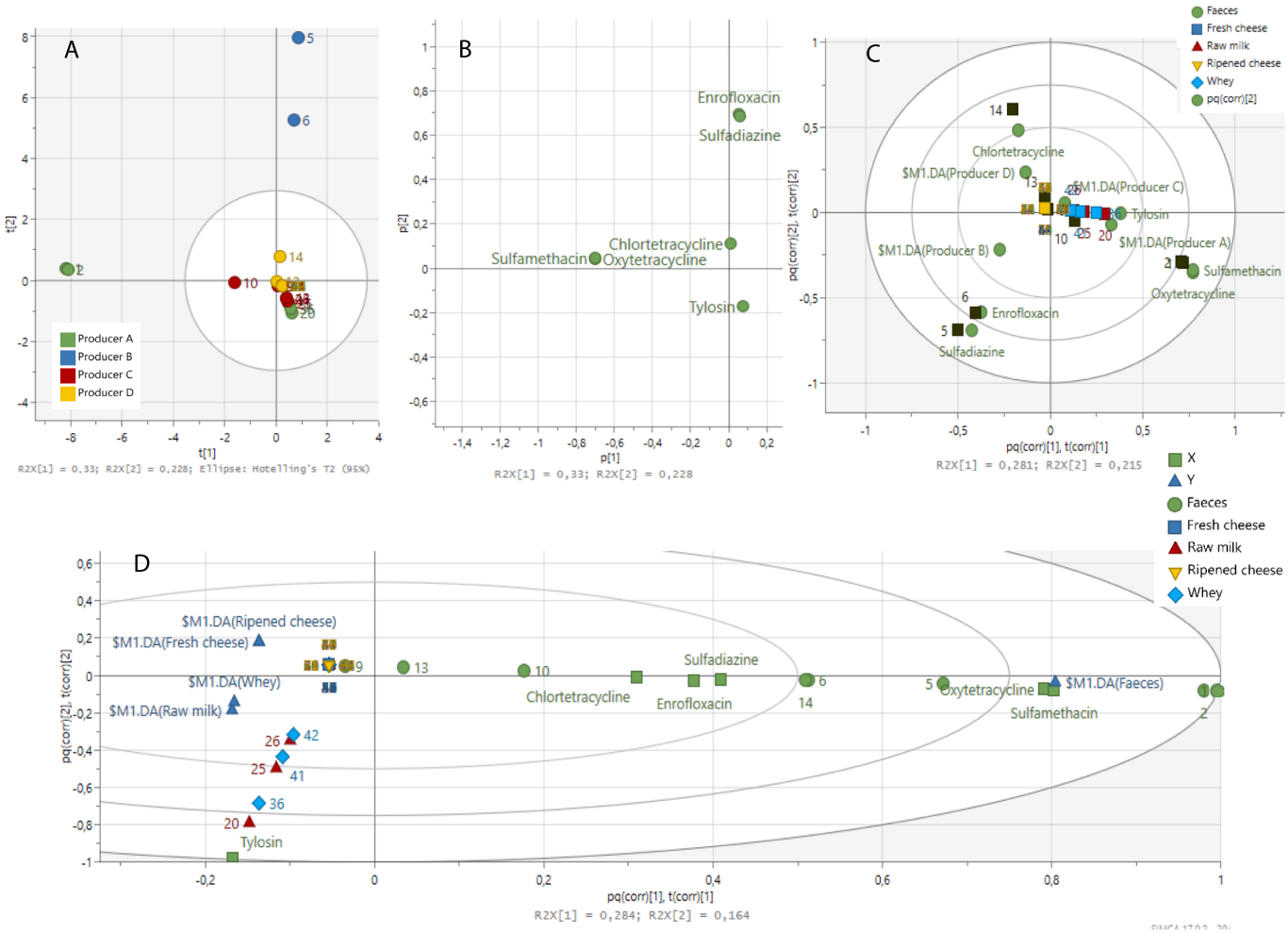


Fig. 2. PCA (A and B, scores and loadings, respectively) and OPLS-DA models (C and D, scores and loadings biplots based on the producer and production chain factors, respectively) based on the UHPLC-QQQ-MS/MS and LC-QQQ-MS/MS results.

weight (Quintanilla et al., 2019a). Quintanilla et al. (2019a) have observed for pasteurised goat's milk and cheese that macrolides, together with quinolones, have the lowest safety margins and present a greater probability of exceeding the safety margin, while β-lactams or tetracyclines present the highest safety margin. This discrepancy in the results is mainly due to the concentration of antibiotics (Virto et al., 2022).

4. Conclusion

This study elucidates antibiotic utilization in artisanal PDO dairies and the presence of residues throughout the raw milk cheese production chain. The results expose a lack of producers awareness regarding antibiotic utilization, specifically noting inconsistencies between declared and detected antibiotics. Screening tests identified samples, primarily raw milk and whey, near the LOD, with positive samples concentrated in fresh and ripened cheeses, suggesting a concentration effect during cheese-making. Chromatographically, diverse compounds were identified in faeces, including chlortetracycline and sulfamethazine. The low concentrations and high LODs in the screening test accounted for negative results. However, only tylosin was detected in milk and whey, while no antibiotics were found in fresh or ripened cheese. Positive or near-LOD results in screening tests, contrasted with non-detectable (n.d.) results by chromatography, were attributed to compounds or degradation metabolites not covered by chromatography. This underscores the potential of screening tests for stakeholders but also emphasizes their limitations, particularly when results are influenced by free fatty acids, bacteriocins, or lysozyme, for example. The study underscores the imperative for producer training, enhanced screening and analytical techniques, and the establishment of legal limits for all dairy products to prevent residue dissemination and safeguard consumer health. Furthermore, addressing the challenge of measurement techniques improvement is crucial, necessitating the development of screening tests with lower false positive rates. This entails devising more accurate tests for detecting antibiotic residues, unaffected by other compounds in milk or cheese, or implementing broad-spectrum analytical techniques for the swift identification of all antibiotic compounds and metabolites.

Funding

This research was performed with financial support provided by the University of the Basque Country (grant COLAB20/14) and the Basque Government (grant IT1568-22). Open Access funding provided by University of Basque Country.

CRediT authorship contribution statement

Gorka Santamarina-García: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. **Gustavo Amores:** Conceptualization, Data Curation, Formal Analysis, Investigation,

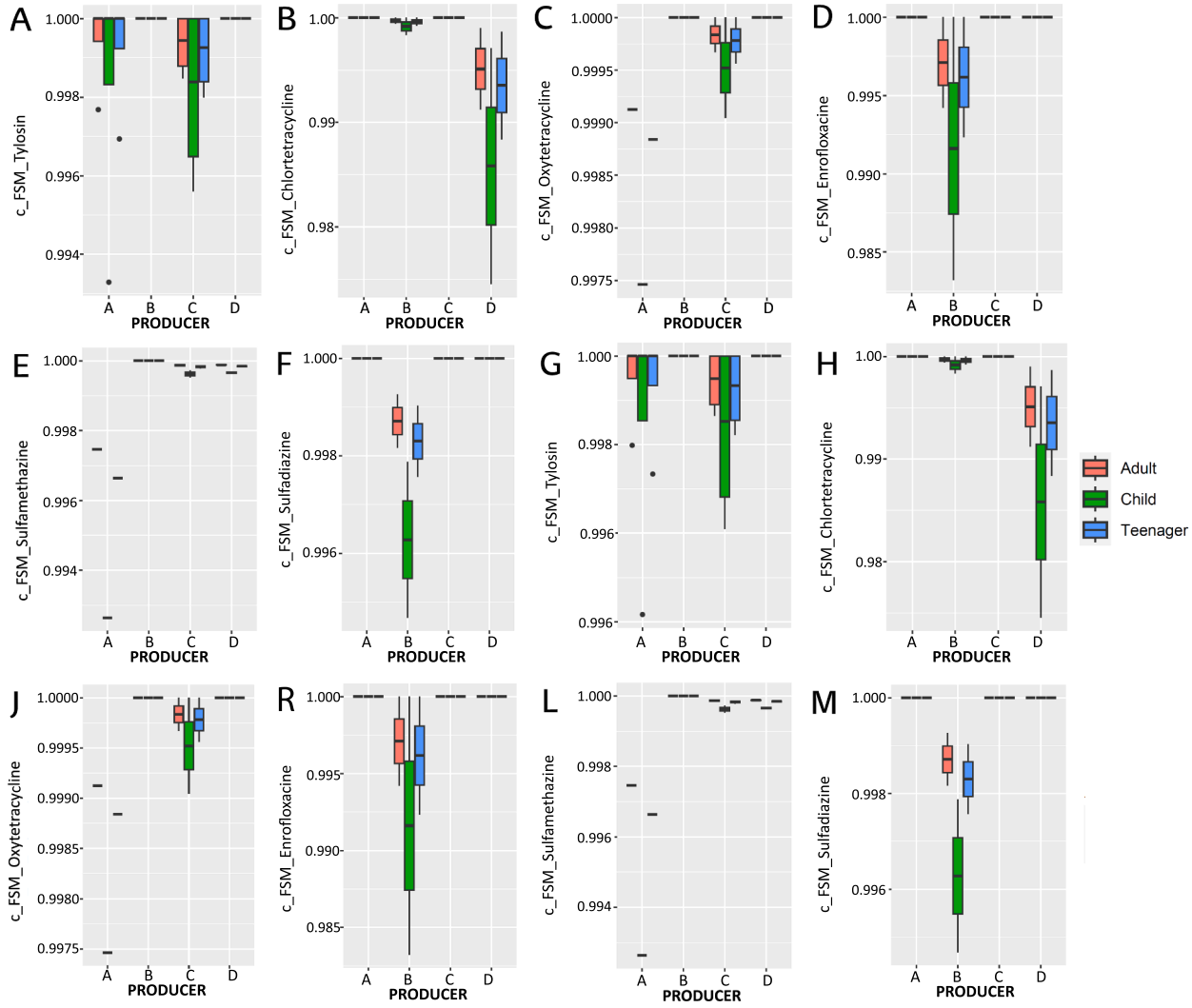


Fig. 3. Box plot representation of the FSM value calculated for adults, children and teenagers according to the analysed raw milk (A-F) and whey (G-M) samples.

Methodology, Resources, Supervision, Validation, Writing – review & editing. **Nagore Gandarias:** Data curation, Investigation. **Igor Hernández:** Conceptualization, Methodology, Resources, Writing – review & editing. **Mailo Virto:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

G. Santamarina-García thanks the University of the Basque Country (UPV/EHU) for a predoctoral fellowship. The authors are grateful for the collaboration of Idiazabal PDO producers and the technical support provided by the Instituto Lactológico de Lekunberri (Lekunberri, Navarre), the Department of Analytical Chemistry, and the Research Centre for Experimental Marine Biology and Biotechnology (PIE) of the University of the Basque Country (Plentzia, Biscay).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138445.

References

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., … McArthur, A. G. (2023). CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research, 51*(D1), D690–D699. https://doi.org/10.1093/NAR/GKAC920

Bedekelabou, A. P., Talaki, E., Dzogbema, K. F. X., Dolou, M., Savadogo, M., Seko, M. O., & Alambedji, R. B. (2022). Assessing farm biosecurity and farmers' knowledge and practices concerning antibiotics and antibiotic resistance in poultry and pig farms in Southern Togo. *Veterinary World, 15*(7), 1727–1737. https://doi.org/10.14202/VETWORLD.2022.1727-1737

Berendsen, B. J. A., Wegh, R. S., Memelink, J., Zuidema, T., & Stolker, L. A. M. (2015). The analysis of animal faeces as a tool to monitor antibiotic usage. *Talanta, 132*, 258–268. https://doi.org/10.1016/J.TALANTA.2014.09.022

Boletín Oficial del Estado (BOE). (1993). Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen «Idiazabal» y su Consejo Regulador. *BOE, 289*, 34591–34596. https://www.boe.es/boe/dias/1993/12/03/pdfs/A34591-34596.pdf.

Brocca, D., & Salvatore, S. (2023). Report for 2021 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. *EFSA Supporting Publications, 20*(2), 7886. https://doi.org/10.2903/sp.efsa.2023.en-7886

Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., Addis, M., Testa, M. C., & Urgeghe, P. P. (2017). Transfer of oxytetracycline from ovine spiked milk to whey and cheese. *International Dairy Journal, 70*, 12–17. https://doi.org/10.1016/J.IDAIRYJ.2016.12.002

Carvalho, F., Prazeres, A. R., & Rivas, J. (2013). Cheese whey wastewater: Characterization and treatment. *Science of The Total Environment, 445–446*, 385–396. https://doi.org/10.1016/J.SCITOTENV.2012.12.038

Casseri, E., Bulut, E., Llanos Soto, S., Wemette, M., Stout, A., Greiner Safi, A., Lynch, R., Moroni, P., & Ivanek, R. (2022). Understanding Antibiotic Resistance as a Perceived Threat towards Dairy Cattle through Beliefs and Practices: A Survey-Based Study of Dairy Farmers. *Antibiotics, 11*(8), 997. https://doi.org/10.3390/antibiotics11080997

Chiesa, L. M., DeCastelli, L., Nobile, M., Martucci, F., Mosconi, G., Fontana, M., Castrica, M., Arioli, F., & Panseri, S. (2020). Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. *LWT, 131*, 109783. https://doi.org/10.1016/J.LWT.2020.109783

Committee for Medicinal Products for Veterinary use (CVMP) and Committee for Medicinal Products for Human Use (CHMP) (2020). *Categorisation of antibiotics used in animals promotes responsible use to protect public and animal health*. Retrieved from https://www.ema.europa.eu/en/news/categorisation-antibiotics-used-animals-promotes-responsible-use-protect-public-animal-health. Accessed June 30, 2023.

Diserens, J.-M. (2014). *Bulletin of the International Dairy Federation (IDF) N° 471/2014: Detection of inhibitors and antimicrobial residues in milk and dairy products by screening methods - Guidance on preparation of the test portion. Bulletin of the IDF, N° 471/2014.* https://shop.fil-idf.org/products/detection-of-inhibitors-and-antimicrobial-residues-in-milk-and-dairy-products-by-screening-methods-guidance-on-preparation-of-the-test-portion.

Dyar, O. J., Zhang, T., Peng, Y., Sun, M., Sun, C., Yin, J., Ding, L., Sun, C., Wang, Y., Sun, Q., Greko, C., & Stålsby Lundborg, C. (2020). Knowledge, attitudes and practices relating to antibiotic use and antibiotic resistance among backyard pig farmers in rural Shandong province. *China. Preventive Veterinary Medicine, 175*, 104858. https://doi.org/10.1016/j.prevetmed.2019.104858

Gajda, A., Nowacka-Kozak, E., Gbylik-Sikorska, M., & Posyniak, A. (2017). Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. *Food Additives & Contaminants: Part A, 35*(1), 66–76. https://doi.org/10.1080/19440049.2017.1397773

Giraldo, J., Igualada, C., Cabizza, R., Althaus, R. L., & Beltrán, M. C. (2022). Transfer of antibiotics from goat's milk to rennet curd and whey fractions during cheese-making. *Food Chemistry, 392*, 133218. https://doi.org/10.1016/J.FOODCHEM.2022.133218

Gonzalo, C., Carriedo, J. A., García-Jimeno, M. C., Pérez-Bilbao, M., & de la Fuente, L. F. (2010). Factors influencing variation of bulk milk antibiotic residue occurrence, somatic cell count, and total bacterial count in dairy sheep flocks. *Journal of Dairy Science, 93*(4), 1587–1595. https://doi.org/10.3168/jds.2009-2838

Kuppusamy, S., Kakarla, D., Venkateswarlu, K., Megharaj, M., Yoon, Y. E., & Lee, Y. B. (2018). Veterinary antibiotics (VAs) contamination as a global agro-ecological issue: A critical view. *Agriculture, Ecosystems & Environment, 257*, 47–59. https://doi.org/10.1016/J.AGEE.2018.01.026

Lee, K., Pereira, R. V., Martínez-López, B., Busch, R. C., & Pires, A. F. A. (2022). Assessment of the knowledge and behavior of backyard and small-scale producers in California regarding disease prevention, biosecurity practices and antibiotics use. *PLOS ONE, 17*(11), e0277897. https://doi.org/10.1371/JOURNAL.PONE.0277897

Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews, 24*(4), 718–733. https://doi.org/10.1128/cmr.00002-11

Moreno, M. A. (2014). Opinions of Spanish pig producers on the role, the level and the risk to public health of antimicrobial use in pigs. *Research in Veterinary Science, 97*(1), 26–31. https://doi.org/10.1016/J.RVSC.2014.04.006

Ovung, A., & Bhattacharyya, J. (2021). Sulfonamide drugs: Structure, antibacterial property, toxicity, and biophysical interactions. *Biophysical Reviews, 13*(2), 259–272. https://doi.org/10.1007/S12551-021-00795-9

Peng, S., Zhang, H., Song, D., Chen, H., Lin, X., Wang, Y., & Ji, L. (2022). Distribution of antibiotic, heavy metals and antibiotic resistance genes in livestock and poultry feces from different scale of farms in Ningxia. *China. Journal of Hazardous Materials, 440*, 129719. https://doi.org/10.1016/J.JHAZMAT.2022.129719

Phares, C. A., Danquah, A., Atiah, K., Agyei, F. K., & Michael, O. T. (2020). Antibiotics utilization and farmers' knowledge of its effects on soil ecosystem in the coastal drylands of Ghana. *PLOS ONE, 15*(2), e0228777. https://doi.org/10.1371/journal.pone.0228777

Quintanilla, P., Beltrán, M. C., Molina, A., Escriche, I., & Molina, M. P. (2019b). Characteristics of ripened Tronchón cheese from raw goat milk containing legally admissible amounts of antibiotics. *Journal of Dairy Science, 102*(4), 2941–2953. https://doi.org/10.3168/jds.2018-15532

Quintanilla, P., Doménech, E., Escriche, I., Carmen Beltrán, M., & Molina, M. P. (2019a). Food Safety Margin Assessment of Antibiotics: Pasteurized Goat's Milk and Fresh Cheese. *Journal of Food Protection, 82*(9), 1553–1559. https://doi.org/10.4315/0362-028X.JFP-18-434

Richards, E. D., Pereira, R. V., Davis, J. L., Rowe, J. D., Clapham, M. O., Wetzlich, S. E., Rupchis, B. A., & Tell, L. A. (2022). Comparison of florfenicol depletion in dairy goat milk using ultra-performance liquid chromatography with tandem mass spectrometry and a commercial on-farm test. *Frontiers in Veterinary Science, 9*, 991772. https://doi.org/10.3389/fvets.2022.991772

Roca, M., Villegas, L., Kortabitarte, M. L., Althaus, R. L., & Molina, M. P. (2011). Effect of heat treatments on stability of β-lactams in milk. *Journal of Dairy Science, 94*(3), 1155–1164. https://doi.org/10.3168/jds.2010-3599

Rossi, R., Saluti, G., Moretti, S., Diamanti, I., Giusepponi, D., & Galarini, R. (2017). Multiclass methods for the analysis of antibiotic residues in milk by liquid chromatography coupled to mass spectrometry: A review. *Food Additives & Contaminants: Part A, 35*(2), 241–257. https://doi.org/10.1080/19440049.2017.1393107

Santamarina-García, G., Amores, G., López de Armentia, E., Hernández, I., & Virto, M. (2022). Relationship between the Dynamics of Gross Composition, Free Fatty Acids and Biogenic Amines, and Microbial Shifts during the Ripening of Raw Ewe Milk-Derived Idiazabal Cheese. *Animals, 12*(22), 3224. https://doi.org/10.3390/ani12223224

Serrano, M. J., Elorduy, J., Zabaleta, I., Istamboulie, G., González-Fandos, E., Bousquet-Mélou, A., Mata, L., Aymard, C., Martínez-Laorden, A., Da Silva-Guedes, J., Lacroix, M., García-Gonzalo, D., Condón, S., Abilleira, E., & Pagán, R. (2022). Antimicrobial residue assessment in 5,357 commercialized meat samples from the Spain-France cross-border area: A new approach for effective monitoring. *Food Control, 138*, 109033. https://doi.org/10.1016/j.foodcont.2022.109033

Sta Ana, K. M., Madriaga, J., & Espino, M. P. (2021). β-Lactam antibiotics and antibiotic resistance in Asian lakes and rivers: An overview of contamination, sources and detection methods. *Environmental Pollution, 275*, Article 116624. https://doi.org/10.1016/J.ENVPOL.2021.116624

Sullivan, M. V., Henderson, A., Hand, R. A., & Turner, N. W. (2022). A molecularly imprinted polymer nanoparticle-based surface plasmon resonance sensor platform for antibiotic detection in river water and milk. *Analytical and Bioanalytical Chemistry, 414*(12), 3687–3696. https://doi.org/10.1007/s00216-022-04012-8

Treiber, F. M., & Beranek-Knauer, H. (2021). Antimicrobial residues in food from animal origin—a review of the literature focusing on products collected in stores and markets worldwide. *Antibiotics, 10*(5), 534. https://doi.org/10.3390/antibiotics10050534

United States Food and Drug Administration Agency (FDA). (2023). *National Milk Drug Residue Database*. Retrieved from *National Milk Drug Residue Database Informational Web Page*. https://www.nmdrd.com/. Accessed June 29, 2023.

Vergara-Luis, I., Bocayá, N., Irazola-Duñabeitia, M., Zuloaga, O., Lacuesta, M., Olivares, M., & Prieto, A. (2023). Multitarget and suspect screening of antimicrobials in soil and manure by means of QuEChERS — liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry, 1–20*. https://doi.org/10.1007/s00216-023-04905-2

Virto, M., Santamarina-García, G., Amores, G., & Hernández, I. (2022). Antibiotics in dairy production: Where is the problem? *Dairy, 3*(3), 541–564. https://doi.org/10.3390/dairy3030039

Visschers, V. H. M., Iten, D. M., Riklin, A., Hartmann, S., Sidler, X., & Siegrist, M. (2014). Swiss pig farmers' perception and usage of antibiotics during the fattening period. *Livestock Science, 162*, 223–232. https://doi.org/10.1016/j.livsci.2014.02.002

Wang, J. (2009). Chapter 38 - Analysis of Antibiotics in Milk and Its Products. In L. M. L. Nollet, & F. Toldrá (Eds.), *Handbook of Dairy Foods Analysis* (pp. 801–819). CRC Press. https://doi.org/10.1201/9781420046328.

World Health Organization (WHO). (2017). *Fact sheets on SDG: health targets and Antimicrobial Resistance*. Retrieved from https://www.who.int/europe/publications/i/item/WHO-EURO-2017-2375-42130-58025. Accessed May 15, 2023.

Yamaki, M., Berruga, M. I., Althaus, R. L., Molina, M. P., & Molina, A. (2006). Screening of antibiotic residues in ewes' milk destined to cheese by a commercial microbiological inhibition assay. *Food Additives and Contaminants, 23*(7), 660–667. https://doi.org/10.1080/02652030500428655

Zhang, Y., Cheng, D., Xie, J., Zhang, Y., Wan, Y., Zhang, Y., & Shi, X. (2022). Impacts of farmland application of antibiotic-contaminated manures on the occurrence of antibiotic residues and antibiotic resistance genes in soil: A meta-analysis study. *Chemosphere, 300*, 134529. https://doi.org/10.1016/J.CHEMOSPHERE.2022.134529

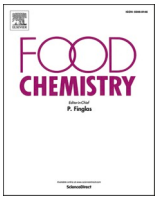
This article was published in Food Chemistry, Vol 442, Gorka Santamarina-García, Gustavo Amores, Nagore Gandarias, Igor Hernández, Mailo Virto, Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain, 138445,

Food Chemistry 445 (2024) 138888

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Corrigendum

Corrigendum to "Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain" [Food Chem. 442 (2024) 138445]

Gorka Santamarina-García [a,b,c,*], Gustavo Amores [a,b,c], Nagore Gandarias [d], Igor Hernández [a,b,c], Mailo Virto [a,b,c]

[a] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[b] Bioaraba Health Research Institute, Prevention, Promotion and Health Care Area, Lactiker-Nutrition and Health Line, Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[c] Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

The authors regret to inform that three modifications should be done to the published version to avoid any confusion. The changes are as follows:

1- In section 2.6.1 "The extraction and analysis of antibiotic residues in the faecal samples followed established and validated protocols at the Department of Analytical Chemistry and the Research Centre for Experimental Marine Biology and Biotechnology (PIE) of the University of the Basque Country (Vergara-Luis et al., 2023)." should be "The extraction and analysis of antibiotic residues in the faecal samples were conducted at the Department of Analytical Chemistry and the Research Centre for Experimental Marine Biology and Biotechnology (PIE) of the University of the Basque Country following established and validated protocols, as previously published (Vergara-Luis et al., 2023)". In other words, it should be indicated similarly to section 2.6.2 to avoid confusion.

2- In section 3.3 of the results "Chlortetracycline dominated, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg) and, in lower concentrations, oxytetracycline (1.75 ± 2.47 to 9.30 ± 0.60 μg/kg), sulfadiazine (9.10 ± 5.52 μg/kg), and enrofloxacin (2.05 ± 2.90 μg/kg)." should be "Chlortetracycline dominated, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg) and, in lower concentrations, oxytetracycline (1.75 ± 2.47 to 9.30 ± 0.60 μg/kg), sulfadiazine (9.10 ± 5.52 μg/kg), and enrofloxacin (2.05 ± 2.90 μg/kg), as previously published (Vergara-Luis et al., 2023)." This modification is to avoid confusion, as these results (only the chromatographic analysis of faeces, not the chromatographic analysis of the other samples (raw milk, whey, fresh cheese and ripened cheeses), nor the screening analysis for all samples (including faeces), or the cross-sectional survey—hence, only a minimal part of the results of interest in this study) appear in the previous publication (Vergara-Luis et al., 2023, indicated in the materials and methods). This is because these same samples were provided to develop and optimize the analytical method (Solid-phase extraction (SPE) coupled to ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS) for the simultaneous analysis of antibiotics in faeces, as well as other samples such as soil and compost. However, as clarified, both the analysis and interpretation of the results are entirely different. In this study, they are used to analyse the presence of antibiotic residues throughout the raw milk cheese production chain, while in the other publication, they are solely used for the development and optimization of the method.

Moreover, the main objective for which the samples were collected within the COLAB20/14 project (indicated in the funding section), was to analyse the presence of antibiotic residues throughout the raw milk cheese production chain, and, consequently, this paper.

3- In table 2 a footnote (indicating a 3 in the form of a superscript in the word "Faeces") is missing, indicating: "Data previously published within method development and validation (Vergara-Luis et al., 2023)." in relation to the aforementioned.

These changes are minor and do not alter the quality or content of the

DOI of original article: https://doi.org/10.1016/j.foodchem.2024.138445.
* Corresponding author.
E-mail addresses: gorka.santamarina@ehu.eus (G. Santamarina-García), gustavo.amores@ehu.eus (G. Amores), nagore.gandarias@ehu.eus (N. Gandarias), igor.hernandezo@ehu.eus (I. Hernández), mailo.virto@ehu.eus (M. Virto).

https://doi.org/10.1016/j.foodchem.2024.138888

Available online 2 March 2024

article in any way. However, as authors committed to rigorous and high-quality science, the authors find it appropriate to prevent any confusion for the reader. The authors would like to apologise for any inconvenience caused.

This article was published in Food Chemistry, Vol 445, Gorka Santamarina-García, Gustavo Amores, Nagore Gandarias, Igor Hernández, Mailo Virto, Corrigendum to “Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain” [Food Chem. 442 (2024) 138445], 138888, Copyright Elsevier Ltd. (2024)

6 | Appendix VI
Manuscript VI

Food Research International, 187, 114308

Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese

Gorka Santamarina-García[1,2,3], Gustavo Amores[1,2,3], Diego Llamazares[1], Igor Hernández[1,2,3], Luis Javier R. Barron[1,3] and Mailo Virto[1,2,3]

[1] *Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[2] *Bioaraba Health Research Institute-Prevention, Promotion and Health Care, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[3] *Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*

[*] *Correspondence: gorka.santamarina@ehu.eus; Tel.: +34-945013799*

Preliminary results related to this manuscript have been presented at:

37th EFFoST (European Federation of Food Science and Technology) International Conference, 2023. *Antimicrobial-resistant lactic acid bacteria in sheep flocks and in their raw milk used for cheese making*. Santamarina-García, G., Amores, G., Hernández, I. and Virto, M. (Oral presentation).

ECM 2023 - 2nd International Electronic Conference on Microbiology, 2023. *Distribution of antimicrobial resistant LAB along the production of a raw ewe milk-derived cheese.* Santamarina-García, G., Amores, G., Azcona, L., Hernández, I. and Virto, M. (Oral presentation).

EAR LTC-Sarea-ENLIGHT CONGRESS – Strengthening Antibiotic Resistance Networks, 2023. *High Throughput Quantitative PCR to elucidate the occurrence of antimicrobial resistance genes along the production-chain of raw ewe milk-derived Idiazabal cheese.* Santamarina-García, G., Amores G., Llamazares D., Hernández, I. and Virto M. (Poster presentation).

Published in **Food Research International**
ISSN: 0963-9969
IF (2022): **8.1**
Category: **Food Science & Technology (D1, 10/142)**
Citations: 0 (updated: 04/2024)
doi.org/10.1016/j.foodres.2024.114308

Food Research International 187 (2024) 114308

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese

Gorka Santamarina-García [a,c,d,*], Gustavo Amores [a,c,d], Diego Llamazares [a], Igor Hernández [a,c,d], Luis Javier R. Barron [b,d], Mailo Virto [a,c,d]

[a] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[b] Lactiker Research Group, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[c] Bioaraba Health Research Institute-Prevention, Promotion and Health Care, 01009 Vitoria-Gasteiz, Spain
[d] Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

ARTICLE INFO

Keywords:
Sheep
Raw ewe milk cheese
Antimicrobial resistance
Antibiotic resistance
Antimicrobial susceptibility testing
Broth microdilution method
High-throughput quantitative PCR
Resistance genes
Mobile genetic elements

ABSTRACT

Antimicrobial resistance (AMR) is a significant public health threat, with the food production chain, and, specifically, fermented products, as a potential vehicle for dissemination. However, information about dairy products, especially raw ewe milk cheeses, is limited. The present study analysed, for the first time, the occurrence of AMRs related to lactic acid bacteria (LAB) along a raw ewe milk cheese production chain for the most common antimicrobial agents used on farms (dihydrostreptomycin, benzylpenicillin, amoxicillin and polymyxin B). More than 200 LAB isolates were obtained and identified by Sanger sequencing (V1-V3 16S rRNA regions); these isolates included 8 LAB genera and 21 species. Significant differences in LAB composition were observed throughout the production chain ($P \leq 0.001$), with *Enterococcus* (e.g., *E. hirae* and *E. faecalis*) and *Bacillus* (e.g., *B. thuringiensis* and *B. cereus*) predominating in ovine faeces and raw ewe milk, respectively, along with *Lactococcus* (*L. lactis*) in whey and fresh cheeses, while *Lactobacillus* and *Lacticaseibacillus* species (e.g., *Lactobacillus* sp. and *L. paracasei*) prevailed in ripened cheeses. Phenotypically, by broth microdilution, *Lactococcus, Enterococcus* and *Bacillus* species presented the greatest resistance rates (on average, 78.2 %, 56.8 % and 53.4 %, respectively), specifically against polymyxin B, and were more susceptible to dihydrostreptomycin. Conversely, *Lacticaseibacillus* and *Lactobacillus* were more susceptible to all antimicrobials tested (31.4 % and 39.1 %, respectively). Thus, resistance patterns and multidrug resistance were reduced along the production chain ($P \leq 0.05$). Genotypically, through HT-qPCR, 31 antimicrobial resistance genes (ARGs) and 6 mobile genetic elements (MGEs) were detected, predominating *Str*, *Str*B and *aad*A-01, related to aminoglycoside resistance, and the transposons *tnp*A-02 and *tnp*A-01. In general, a significant reduction in ARGs and MGEs abundances was also observed throughout the production chain ($P \leq 0.001$). The current findings indicate that LAB dynamics throughout the raw ewe milk cheese production chain facilitated a reduction in AMRs, which has not been reported to date.

1. Introduction

Antibiotics are chemical compounds that attack essential bacterial physiology and biochemistry to cause cell death or growth cessation (Lade & Kim, 2021). For decades, antibiotics have been overused, both in human medicine and in animal production (Sobierajski et al., 2022), including the currently forbidden use of subtherapeutic doses as growth promoters (Patel et al., 2020). As a result, bacterial communities have been exposed to antibiotics and have developed the ability to withstand or resist the action of one or more antimicrobial agents, which is called

* Corresponding author at: Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain.
E-mail address: gorka.santamarina@ehu.eus (G. Santamarina-García).

https://doi.org/10.1016/j.foodres.2024.114308
Received 17 January 2024; Received in revised form 27 March 2024; Accepted 16 April 2024
Available online 18 April 2024

antimicrobial resistance (AMR) (Konopka et al., 2022; Virto et al., 2022). Bacteria can be intrinsically resistant to certain antimicrobial groups or agents, mediated by chromosomal genes and linked to physiological or anatomical characteristics. Nonetheless, acquired resistance also occurs due to horizontal transmission between bacteria by means of mobile genetic elements (MGEs), which can carry one or more resistance genes (Iskandar et al., 2022; Nunziata et al., 2022), or due to generational genetic transmission by point mutations in genes that give rise to resistance or increased expression of resistance mechanisms (vertical transmission) (Iskandar et al., 2022; Wall et al., 2016).

The antibiotics utilized in human medicine belong to the same pharmacological classes as those used in veterinary medicine (Devirgiliis et al., 2011); consequently, acquired resistance to certain antimicrobial agents is widespread to the point that effective treatment of certain fatal infections is already compromised (Virto et al., 2022). In fact, the proliferation of antimicrobial-resistant (AR) microorganisms has become one of the most important threats to human health (Wang et al., 2022) and is classified as one of the top 10 threats to global public health (WHO, 2022). It causes approximately 700,000 deaths worldwide per year and is projected to increase to 10 million each year by 2050 (IACG, 2019). Thus, AMR is of utmost importance and is included within the sustainable development goals (SDGs) set by the United Nations. Specifically, AMR affects SDG 3 on good health and well-being since it hinders the ability to control infectious diseases, increasing morbidity and mortality and resulting health care costs (United Nations, 2015).

The food and food production chain is classified as a possible vehicle for the dissemination of AR bacteria and genes (Caniça et al., 2019); and, specifically, fermented products are considered notable reservoirs (Wang et al., 2006; Yasir et al., 2022). In this regard, several studies have been developed recently (Zhao et al., 2022), for instance, on raw beef, sheep and lamb meat (Şanlıbaba, 2022) and dry-fermented sausages (Fraqueza, 2015). However, information about dairy products, especially raw milk cheeses, is limited, with most studies focused on raw cow milk cheeses (Dos Santos et al., 2022; Rola et al., 2016) and scarce information on raw sheep milk cheeses (Gaglio et al., 2016; Slyvka et al., 2022). Milk is an ideal growth medium for microorganisms due to its high nutrient content (Fusco et al., 2020). Consequently, the microbiota of raw ewe milk is diverse and is primarily composed of lactic acid bacteria (LAB), psychotropic bacteria and pathogens (Biçer et al., 2021; Santamarina-García et al., 2022a). Nonetheless, the cheese-making and ripening processes have a clear impact on bacterial communities, with a general predominance of LAB (Cardinali et al., 2021; Santamarina-García et al., 2022a). Several studies have highlighted the presence of resistant bacteria in raw ewe milk and cheese, including pathogenic *Escherichia coli* and *Staphylococcus aureus* (Imre et al., 2022; Karahutová & Bujňáková, 2023; Výrostková et al., 2020, 2021). Nonetheless, despite the predominance of LAB (Quigley et al., 2013; Santamarina-García et al., 2022a), there has been limited research on AMRs in LAB from raw ewe milk and derivate cheeses (Výrostková et al., 2020, 2021). In particular, species of the genus *Enterococcus,* such as *E. faecium* and *E. faecalis*, known as important opportunistic pathogens in nosocomial infections (Conde-Estévez et al., 2011), have been described as the most remarkable AR LAB (Výrostková et al., 2021). Addressing AMRs in LAB is essential since they can serve as potential reservoirs for the transfer of resistance genes to other bacteria, including pathogenic bacteria (Caniça et al., 2019).

Several studies have reported the preference of consumers for raw milk cheeses (Colonna et al., 2011; Meunier-Goddik & Waite-Cusic, 2019), based on their richer and more intense aromatic profiles than pasteurized milk cheeses (Barron et al., 2007; O'Sullivan & Cotter, 2017). Given the pressing need to minimize the development and dissemination of AR LAB to safeguard public health (Výrostková et al., 2021), the present study is focused on Idiazabal protected designation of origin (PDO) cheese. It is a semihard or hard cheese from the Basque Country (southwestern Europe) produced with raw milk from the Latxa and/or Carranzana autochthonous sheep breeds, and it has a minimum mandatory ripening period of 60 days (Official Journal of the European Communities, 1996). Thus, this study aimed to characterize the prevalence of AMRs in LAB from ovine faeces, raw ewe milk, whey, fresh cheeses and 2-month-old ripened cheeses by means of phenotypic and genotypic approaches. Moreover, the potential differences among producers producing the same kind of raw ewe milk cheese were also analysed. To our knowledge, no study has comprehensively analysed the prevalence of AMRs along the production chain of a raw ewe milk cheese.

2. Methods

2.1. Area of study

To evaluate the prevalence of AMRs in LAB along the production chain of artisanal raw ewe milk cheeses, this study was carried out within the European PDO Idiazabal cheese. This particular cheese was selected as a case study because its production is primarily carried out by small-scale artisanal dairies that oversee the entire process, from herd management to cheese-making. Idiazabal cheese is a semihard or hard cheese made from the raw milk of the autochthonous Latxa and/or Carranzana sheep breeds and has a mandatory minimum ripening time of 2 months. Herd management and milk production for cheese-making occur in the Basque Country, covering an area of 17,213.06 km^2 in southwestern Europe (43° 27′ − 41° 54′ N and 1° 5′ − 3° 37′ W). This region corresponds to the natural habitat of the sheep breeds (Official Journal of the European Communities, 1996). Herd management involves the use of indoor forage from October to March and semi-extensive or extensive grazing from March to October (Aldalur et al., 2019). Milk collection and cheese production mainly occur between January and June, following the traditional seasonal approach dictated by the biological rhythms of the sheep (Boletín Oficial del Estado, 1993).

2.2. Sampling

For sampling, four producers attached to the PDO Idiazabal cheese were chosen and identified as A, B, C, and D. Each producer came from one of the distinct geographical production areas (Alava, Biscay, Gipuzkoa, and Navarre). All the producers adhered to similar flock management and cheese-making practices in accordance with the specifications outlined by the Idiazabal PDO regulatory board (Boletín Oficial del Estado, 1993). The flocks consisted of approximately 350–400 Latxa breed sheep, following the management practices mentioned earlier. Milking was conducted automatically, and the milk was promptly refrigerated (3–4 °C) until cheese-making. For the cheese-making process, the milk was warmed to 25 °C, and the commercial mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added. Coagulation occurred at 28–32 °C for 20–45 min using artisanal rennet and/or the commercial NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). The resulting curds were cut into 5–10 mm diameter grains and heated to 36–38 °C. Cheeses were then moulded, pressed and salted in saturated brine, and subsequently ripened in chambers maintained at 80–95 % relative humidity and 8–14 °C for 2 months. Thus, ovine faeces, raw ewe milk, whey, fresh cheeses (1-day-old), and 2-month-old ripened cheese samples were obtained from each producer. Samples were collected aseptically in quadruplicate, with each set of samples corresponding to the same batch. The sampling was conducted by the producers, eliminating the need for approval from the Ethics Committee for Animal Experimentation. Verbal consent was obtained from dairies during samples collection. Samples were collected from healthy flocks, excluding animals that underwent antibiotic treatment. Samples were transported under refrigerated conditions (3 ± 1 °C) for analysis.

2.3. Reagents and materials

The peptone water was supplied by Panreac Química (Barcelona, Spain). De Man, Rogosa and Sharpe (MRS) agar, MRS broth medium, sodium citrate and sodium chloride were purchased from Scharlab (Barcelona, Spain). Tryptic soy broth (TSB) was obtained from Condalab (Madrid, Spain). Glycerol was obtained from Honeywell Fluka (Madrid, Spain). Amoxicillin was supplied by Sigma-Aldrich (Madrid, Spain). Dihydrostreptomycin and polymyxin B were purchased from Glentham Life Sciences (Corsham, United Kingdom). Benzylpenicillin was supplied by Tokyo Chemical Industry Co. (Tokyo, Japan). Mag-Bind Bacterial DNA 96 Kit was purchased from Omega Bio-Tek, Inc. (Norcross, United States). KAPA HiFi HotStart ReadyMix Kit was obtained from Roche Molecular Systems, Inc. (Branchburg, United States). CleanNGS and CleanDTR kits were obtained from CleanNA (Waddinxveen, The Netherlands). DNA 5 K Reagent Kit was obtained from PerkinElmer, Inc. (Waltham, United States). BigDye Terminator v3.1 Cycle Sequencing Kit and exonuclease I were purchased from Thermo Scientific (Waltham, United States). QIAamp® PowerFecal® Pro DNA Kit and QIAGEN® Multiplex PCR Kit were purchased from Qiagen (Valencia, United States). Petri dishes and 96-well plates were obtained from Deltalab (Barcelona, Spain). The Master Mix SsoFastTM EvaGreen® Supermix Kit with Low ROX was purchased from Bio-Rad Laboratories (Hercules, United States).

2.4. Phenotypic characterization of AMRs

2.4.1. LAB isolation and enumeration

For the faeces and cheese samples, 10 g was diluted in duplicate 1:10 in peptone water and homogenized for 30 s three times in a stomacher (Masticator Basic 400, IUL Instruments, Königswinter, Germany). Serial dilutions were made in peptone water and plated on MRS agar media. For the raw ewe milk and whey samples, 100 μL was taken directly and serially diluted. The plates were incubated at 37 ± 1 °C for 48 h. Three presumptive LAB isolates were randomly selected per sample based on colony morphology diversity. Then, the isolates were preenriched in TSB and subcultured onto MRS agar to ensure purity. All the cultures were stored at − 80 °C in 20 % (v/v) glycerol.

2.4.2. Identification of LAB isolates by Sanger sequencing

2.4.2.1. DNA extraction. Isolates were preenriched in TSB and subcultured onto MRS agar prior to DNA extraction to ensure purity and viability. Bacterial DNA was extracted using the Mag-Bind Bacterial DNA 96 Kit following the manufacturer's instructions for agar cultures, but the elution volume was reduced to 60 μL to improve the DNA yield. The quantity and quality of the DNA obtained were verified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA), in which the absorbance was measured at a wavelength of 260 nm and the 260/280 and 260/230 ratios were analysed. DNA extraction and subsequent Sanger sequencing were conducted in the Sequencing and Genotyping Unit of the Genomic Facility/SGIker (supported by UPV/EHU, MICINN, GV/EJ, FSE) of the University of the Basque Country.

2.4.2.2. Sanger sequencing. The V1–V3 regions of the 16S rRNA gene were amplified via PCR with the KAPA HiFi HotStart ReadyMix Kit using the forward primer 16S–V1–8F: 5′- AGAGTTTGSTCCTGGCTCAG-3′ and the reverse primer 16S–V3–534R: 5′- ATTACCGCGGCTGCTGG −3′. The PCR products were purified by means of the CleanNGS Kit following the manufacturer's instructions. Amplicon quantification was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and a LabChip GX Touch Nucleic Acid Analyser (PerkinElmer) with a DNA 5 K Reagent Kit. Sequencing of the purified product was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit following the manufacturer's protocol. The sequencing product was purified using a magnetic bead-based CleanDTR kit, and Sanger sequencing was performed on the SeqStudio platform (Thermo Fisher).

2.4.2.3. Bioinformatic analysis. Quality filtering and trimming of the raw reads were performed using SeqStudio Reporter software (Thermo Scientific). The sequences were visualized and edited by means of the BioEdit Sequence Alignment Editor software 7.2.5 (Hall et al., 2011). The resulting sequences were approximately 500 bp in length. Taxonomic classification was performed against the Nucleotide Basic Local Alignment Search Tool (NBLAST) 2.14.0+ (Zhang et al., 2000), with default parameters and taking into account the e-value, score, query cover and percentage of identification as quality indicators.

2.4.3. Antimicrobial susceptibility testing (AST) via the broth microdilution method

The minimum inhibitory concentration (MIC) of the LAB isolates was evaluated by the broth microdilution method for the most widely used antimicrobial agents on farms (namely, amoxicillin, dihydrostreptomycin, benzylpenicillin and polymyxin B) according to the updated International Organization for Standardization and International Dairy Federation Standards (ISO/IDF, 2010) and European Food Safety Authority guidance (Rychen et al., 2018), with minor modifications. Briefly, a 96-well plate was inoculated with MRS broth medium supplemented with serial (1:2) concentrations of antibiotics (amoxicillin: 0.0313–16 μg/mL; dihydrostreptomycin: 1–512 μg/mL; benzylpenicillin: 0.0313–32 μg/mL; and polymyxin B: 2–1024 μg/mL). The inocula of each isolate were prepared in saline solution (0.85 %, m/v) by picking up single colonies from previously subcultured isolates on MRS agar to obtain an optical density equivalent to 0.5 on the MacFarland scale. The inoculum was subsequently diluted 1:10 in antibiotic-free MRS broth, and 50 μL of the diluted suspension was added to each well and incubated at 37 ± 1 °C for 48 h. The inoculum in a well with MRS broth without antibiotics was used as a positive control, and an inoculum-free well was used as a negative control. The antimicrobial susceptibility or resistance was interpreted using the available microbiological cut-off values defined by the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Rychen et al., 2018) and employing the epidemiological cut-off values (ECOFFs) proposed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST; https://www.eucast.org).

2.5. Genotypic characterization of AMRs

2.5.1. DNA extraction

To analyse the presence of antimicrobial resistance genes (ARGs), DNA was extracted as previously described (Santamarina-García et al., 2022a), with some modifications. Briefly, for the faecal and cheese samples, 10 g was suspended in 90 mL of 2 % (w/v) sterile sodium citrate (pH 8.0) and homogenized six times (20 s ON and 10 s OFF) in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany). The resulting suspension was centrifuged at 6500 × g for 8 min at 4 °C, after which the fat-containing supernatant was discarded. The obtained pellet was washed with 50 mL of sodium citrate and centrifuged at 6500 × g for 8 min at 4 °C. The pellet was resuspended in 800 μL of sodium citrate and centrifuged three times at 6500 × g for 8 min at 4 °C. The DNA was extracted with a QIAamp® PowerFecal® Pro DNA Kit according to the manufacturer's protocol, but a double DNA elution step was carried out with 25 μL of C6 solution to improve DNA yields. To extract DNA from the milk and whey samples, 10 mL was processed as described above, but without the need for homogenization in the stomacher. The DNA was stored at − 80 °C until analysis.

2.5.2. High-throughput quantitative PCR (HT-qPCR)

The detection of ARGs was performed by means of HT-qPCR in a nanofluidic qPCR BioMarkTM HD system using 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm Corporation), as previously described (Jauregi et al., 2021). A total of 48 primer sets were used (Supplementary Table 1) to target the ARGs conferring resistance against the most commonly used antimicrobial agents on farms (12 ARGs encoding resistance to dihydrostreptomycin, 24 ARGs for benzylpenicillin and amoxicillin, 2 ARGs for polymyxin B and 2 multidrug ARGs conferring resistance to more than one of the aforementioned antimicrobial agents), MGE genes (5 genes encoding transposases and 2 genes encoding integrases) and the 16S rRNA gene as a reference gene. These genes were selected considering the CARD database for LAB (Alcock et al., 2023). The primers used for qPCR were previously validated (Gorecki et al., 2022; Hu et al., 2016). DNA samples were pre-amplified using the QIAGEN® Multiplex PCR Kit and a primer pool (final concentration for each primer pair = 50 nM), following the amplification program (at 95 °C for 15 min and 14 PCR cycles at 95 °C for 15 s, 60 °C for 4 min and a final extension step at 4 °C). Then, the samples were treated with exonuclease I (at 37 °C for 30 min for digestion, 80 °C for 15 min for inactivation of exonuclease I and kept at 4 °C). Subsequently, 1:10 dilutions of specific target amplification reactions were loaded onto the Dynamic Array IFCs following the Fluidigm's Fast Gene Expression Analysis—EvaGreen® Protocol (Fluidigm Corporation). For amplification, the Master Mix SsoFastTM EvaGreen® Supermix Kit with Low ROX was used, with a final concentration of primers of 500 nM, both forward and reverse. The program consisted of 1 min of denaturation at 95 °C, followed by 30 cycles of 95 °C for 5 s and 60 °C for 20 s, a melting curve at 60 °C for 3 s and a ramp rate of 1 °C/3 s up to 95 °C. Four replicates were included for each sample. Analyses were conducted at the Gene Expression Unit of The Genomics Facility/SGIker (supported by UPV/EHU, MICINN, GV/EJ, FSE) of the University of the Basque Country.

2.5.3. Bioinformatic analysis

Raw data were processed with Fluidigm Real-Time PCR Analysis Software (v.3.1.3, Fluidigm Corporation), with linear baseline correction and manual threshold settings. A cycle threshold (CT) value of 30 was chosen because the highest CT value obtained in this study was 29.0. The detection of an ARG or MGE gene was considered positive when 3 out of the 4 technical replicates for each sample were above the detection limit. The relative abundances of the ARGs were calculated on the basis of the comparative CT method (Jauregi et al., 2021), normalized to the abundance of the 16S rRNA control gene and expressed as the fold change (FC).

$$\Delta CT(per\ replicate) = CT(target\ gene) - CT(16S\ rRNA\ gene)$$

$$\Delta\Delta CT(per\ sample) = \overline{\Delta CT}$$

$$FC = 2^{-\Delta\Delta CT}$$

2.6. Statistical analysis

IBM SPSS statistical package version 26.0 (IBM SPSS, Inc., Chicago, IL, USA, 2019) was used for data preparation and analysis. Plot generation was performed in RStudio version 2023.03.1 and R version 4.3.0 (R Core Team, Vienna, Austria, 2023) with the "ggplot2" package (https://github.com/tidyverse/ggplot2) and in Microsoft Office Professional Plus 2016 Excel® version 16.0.5413 (Microsoft, Albuquerque, United States). Kruskal–Wallis one-way analysis of variance (ANOVA) with Bonferroni correction was performed with the SPSS package to determine the significance ($P \leq 0.05$) of the effects of producer and production chain (sample type) factors on the bacterial counts and abundances and phenotypic and genotypic results. Permutational multivariate analysis of variance (PERMANOVA) was carried out in R with the "vegan" package (https://github.com/vegandevs/vegan) to analyse the overall effect of producer and production chain factors. The data were log transformed when necessary and subjected to unit variance (UV) scaling, and a heatmap with hierarchical clustering analysis (HCA) was generated with the "pheatmap" package (https://github.com/raivokolde/pheatmap) to analyse the clustering of the phenotypic and genotypic results. Clustering of samples according to phenotypic and genotypic results was also performed by means of a dendrogram in R with the "factoextra" package (https://github.com/kassambara/factoextra). Trends in the bacterial counts and abundances and phenotypic and genotypic results according to producer and production chain factors were explored by means of principal component analysis (PCA), applied to log-transformed, when necessary, and UV-scaled data and performed in SIMCA software version 17.0.2.34594 (Umetrics AB, Umeå, Sweden). The number of principal components (PCs) was determined by the eigenvalues (greater than 1.0) and cross-validation. Similarly, orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed with SIMCA software to analyse whether the samples differed according to the producer and production chain factors. Variable influence on projection (VIP) values and loading weights were used to analyse the importance of each parameter in the model.

3. Results

3.1. LAB prevalence and distribution throughout the cheese production chain

Fig. 1A shows the prevalence of LAB throughout the Idiazabal cheese production chain. Overall, large differences were found according to the sample type ($P \leq 0.001$). Specifically, a mean LAB prevalence of 6.45 ± 0.451 log CFU/g was observed in the faeces. In the raw ewe milk, the LAB count was 3.73 ± 0.0659 log CFU/mL, which subsequently increased to 5.65 ± 0.0623 log CFU/mL in the whey and to 7.99 ± 0.172 log CFU/g in the fresh cheeses. However, during ripening, the LAB count slightly decreased to 7.75 ± 0.202 log CFU/g, although the difference was not significant (Fig. 1A). Moreover, among producers, significant differences were also observed for the whey ($P \leq 0.01$) and fresh cheese samples ($P \leq 0.05$), with producer A clearly differentiated from the rest due to the lower values. Using multivariate analysis, PERMANOVA confirmed the differences among sample types ($P \leq 0.001$) and, to a lesser extent, among producers ($P \leq 0.05$).

To identify the LAB communities, 203 isolates were obtained from the raw ewe milk Idiazabal cheese production chain. As expected, all the isolates belonged to the phylum Firmicutes and class Bacilli (Fig. 1B). Two orders were identified, predominantly Lactobacillales (69.0 %) and, to a lesser extent, Bacillales (31.0 %). All the isolates of the Bacillales order belonged to the *Bacillaceae* family and the *Bacillus* genus, identifying 4 different species, *B. cereus* (8.37 %), *B. thuringiensis* (6.40 %), *B. paramycoides* (2.96 %), and *B. anthracis* (0.99 %), in addition to other unidentified species (*Bacillus* sp., 12.3 %). The isolates of the order Lactobacillales belonged mainly to the *Enterococcaceae* (37.4 %) and *Lactobacillaceae* (22.2 %) families and, to a lesser extent, to the *Streptococcaceae* (9.36 %). All the *Enterococcaceae* isolates corresponded to the genus *Enterococcus*, identifying different species, such as *E. hirae* (19.2 %) and *E. faecalis* (13.3 %), and to a lesser extent, *E. faecium* (2.46 %), *E. mundtii* (1.48 %), *E. avium* (0.49 %) and *E. durans* (0.49 %). The *Lactobacillaceae* isolates belonged to the genus *Lactobacillus*, without being able to identify species (*Lactobacillus* sp., 7.88 %); *Lacticaseibacillus* (7.88 %), with the species *L. paracasei* (6.40 %) and *L. casei* (1.48 %); *Levilactobacillus* (3.94 %), namely, *L. brevis*; and *Lactiplantibacillus* (2.46 %), identified as *L. plantarum* (0.49 %) and *L. plantarum* subsp. *plantarum* (1.97 %). The *Streptococcaceae* isolates belonged to the genera *Lactococcus* (8.87 %), identifying *L. lactis* (4.43 %) and *L. lactis* subsp. *lactis* (3.45 %), in addition to unidentified strains (0.99 %), and *Streptococcus*, for which species could not be identified (*Streptococcus* sp.,

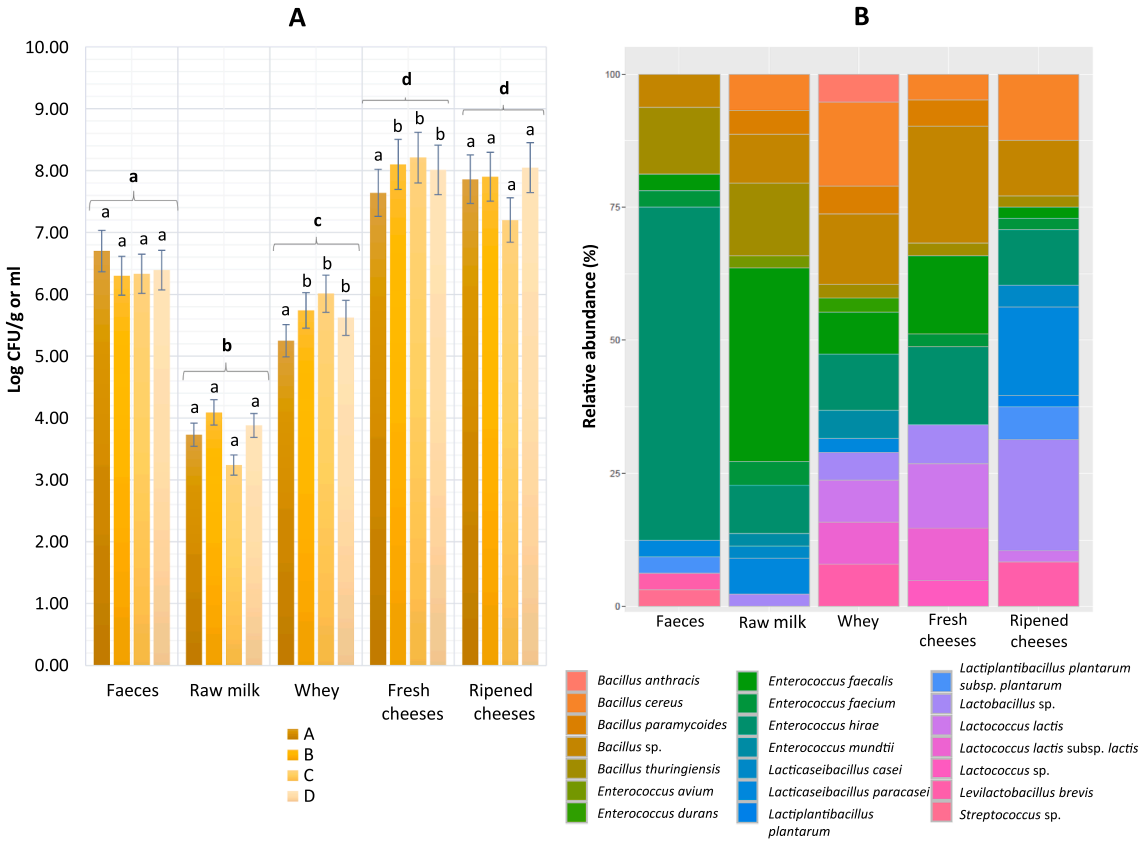


Fig. 1. Mean counts (log CFU/g or mL) and relative abundance (%) of lactic acid bacteria throughout the Idiazabal cheese production chain (faeces, raw milk, whey, fresh cheese and ripened cheese samples). The different lowercase letters for each type of sample indicate statistically significant differences.

0.49 %). Within all the samples, *E. hirae, E. faecalis, Bacillus* sp., *B. cereus* and *Lactobacillus* sp. were some of the most important species throughout the production chain of the Idiazabal cheese.

PERMANOVA confirmed the difference in LAB composition among the collected samples throughout the production chain ($P \leq 0.001$) (Fig. 1B). *E. hirae* clearly predominated in the faeces (62.5 %), followed by *B. thuringiensis* (12.5 %) and other unidentified species (*Bacillus* sp.) (6.25 %). In raw ewe milk, instead, *E. faecalis* (36.4 %) predominated, followed by *B. thuringiensis* (13.6 %), *Bacillus* sp. (9.09 %) and *E. hirae* (9.09 %). During cheese-making, *Bacillus* species, such as *B. cereus* (15.8 %) or *Bacillus* sp. (13.2 %), dominated the whey; together with *Enterococcus*, such as *E. hirae* (10.5 %) or *E. faecalis* (7.89 %); and *Lactococcus*, *L. lactis* (7.89 %) and *L. lactis* subsp. *lactis* (7.89 %), or *L. brevis* (7.89 %). In fresh cheeses, a similar trend was maintained, with a predominance of unidentified *Bacillus* species (22.0 %), along with *Enterococcus species*, such as *E. hirae* (14.6 %) and *E. faecalis* (14.6 %), and also *L. lactis* (12.2 %). However, after ripening, *Lactobacillus* species predominated (20.8 %), followed by *Lacticaseibacillus*, specifically *L. paracasei* (16.7 %). In general, the abundance of other species, such as *E. hirae* (10.4 %) and *Bacillus* sp. (10.4 %), decreased during ripening. The greatest differences among sample types along the production chain were mainly observed for *E. faecalis, L. paracasei, L. lactis* and *Lactobacillus* sp. ($P \leq 0.05$). PERMANOVA corroborated the lack of differentiation among producers ($P > 0.05$).

3.2. Phenotypic profile of antimicrobial resistance

Subsequently, antimicrobial susceptibility was tested by the broth microdilution method for more than 200 LAB isolates. The distributions of MICs are shown in Table 1. Clear differences were observed in the AMR phenotypes among the LAB communities ($P \leq 0.05$) (Table 1 and Fig. 2A), which was confirmed by an OPLS-DA model (Supplementary Fig. 1). Overall, *Lactococcus* and *Streptococcus* species had the greatest resistance rates (on average, 78.2 % and 75.0 % of resistance of all isolates to all antibiotics, respectively), followed by *Levilactobacillus, Enterococcus* and *Bacillus* (65.6 %, 56.8 % and 53.4 %, respectively). *Lactiplantibacillus, Lacticaseibacillus* and *Lactobacillus* species, instead, were the most susceptible bacteria (31.3 %, 31.4 % and 39.1 %, respectively).

In more detail, clear differences were observed in the AMR phenotypes among LAB species from the same genera and families ($P \leq 0.05$). Within the *Bacillaceae* and *Bacillus* genus, *B. anthracis* species clearly differed from the other species because of their low resistance (12.5 %), with the remaining species, *B. cereus*, *B. paramycoides*, *B. thuringiensis* and *Bacillus* sp., exhibiting greater resistance (69.1 %, 58.3 %, 71.2 % and 56.0 %, respectively). For the *Enterococcaceae* and *Enterococcus* isolates, differences were also detected among the species, with *E. durans* and *E. faecium* being the most resistant (100 % and 80.0 %, respectively); *E. hirae, E. mundtii* and *E. faecalis* presenting greater susceptibility (57.1 %, 58.3 % and 45.4 %, respectively); and *E. avium* isolates being sensitive to all the antibiotics tested. On the other hand, most *Lactobacillaceae* genera and species showed similar low resistance rates, including unidentified *Lactobacillus* species (39.1 %), *Lacticaseibacillus* species, namely, *L. paracasei* and *L. casei* (21.2 % and 41.7 %, respectively), and *Lactiplantibacillus* species, specifically *L. plantarum* and *L. plantarum* subsp. *plantarum* (25.0 % and 37.5 %, respectively). The *Levilactobacillus* genus and the *L. brevis* species were unique exceptions for their higher levels of resistance. Finally, *Streptococcaceae* isolates also showed low differences among genera and species. All *Lactococcus* species, including *L. lactis*, *L. lactis* subsp. *lactis* and other unidentified species (72.2 %, 75.0 % and 88.0 % on average, respectively), exhibited high resistance, similar to unidentified *Streptococcus* species (75.0 % on average). Thus, the HCA and dendrogram divided the LAB communities into two clusters (Fig. 2A and B). *E. avium, L. plantarum, L. plantarum* subsp. *plantarum* and

Table 1
Distribution of minimum inhibitory concentration (MIC) values for the 202 isolates obtained throughout the production chain (ovine faeces, raw ewe milk, whey, fresh cheese and 60-day-old ripened cheese) of raw ewe milk Idiazabal cheese.

Bacterial species	Antimicrobial compound	MIC (μg/kg)[1]																n	Resistance[2]						MIC_{50} (μg/kg)[3]	MIC_{90} (μg/kg)[4]
		< 0.032	0.032	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		Total	Faeces	Raw milk	Whey	Fresh cheese	Ripened cheese		
B. anthracis	Dihydrostreptomycin						1			1									2	0.00			0.00			< 1	4
	Polymyxin b							1				1							2	50.0	n.d.	n.d.	50.0	n.d.	n.d.	< 2	16
	Benzylpenicillin		2																2	0.00			0.00			0	0
	Amoxicillin	1	1																2	0.00			0.00			< 0.032	0
B. cereus	Dihydrostreptomycin						5	2	2	1	1		1			1	4		17	35.3		100	33.3	0.00	16.7	2	512
	Polymyxin b							1	1	2	2	1	1	2	2	3		2	17	88.2	n.d.	100	100	0.00	100	64	1024
	Benzylpenicillin	1			1		1		2		1	11							17	82.4		100	66.7	50.0	100.0	16	16
	Amoxicillin	5										12							17	70.6		66.7	83.3	0.00	83.3	16	16
B. paramycoides	Dihydrostreptomycin						2		1			1	1				1		6	50.0		50.0	50.0	50.0		2	512
	Polymyxin b							2		1		2				1			6	66.7	n.d.	50.0	50.0	100	n.d.	8	256
	Benzylpenicillin	1			1							4							6	66.7		50.0	50.0	100		16	16
	Amoxicillin	2			1			1				2							6	50.0		50.0	50.0	50.0		0.125	16
B. thuringiensis	Dihydrostreptomycin						1	1	1	1				1	1		6		13	61.5	25.0	66.7	100	100	100	128	512
	Polymyxin b							2		1		1	2	2	1	2		2	13	84.6	75.0	83.3	100	100	100	64	1024
	Benzylpenicillin		1	1	1			2		1		7							13	76.9	75.0	83.3	100	0.00	100	16	16
	Amoxicillin	4			1		1					7							13	61.5	100.0	66.7	0.00	0.00	0.00	16	16
Bacillus sp.	Dihydrostreptomycin						10	2	2	1	1						9		25	36.0	50.0	50.0	60.0	33.3	0.00	2	512
	Polymyxin b							8				1	1	1	4	4	1	5	25	68.0	50.0	75.0	100	33.3	100	128	1024
	Benzylpenicillin	5	1	1			1					17							25	68.0	50.0	50.0	100	44.4	100	16	16
	Amoxicillin	7	2		3							13							25	52.0	50.0	50.0	80.0	33.3	60.0	16	16
E. avium	Dihydrostreptomycin						1												1	0.00		0.00				< 1	< 1
	Polymyxin b							1											1	0.00	n.d.	0.00				2	2
	Benzylpenicillin	1																	1	0.00		0.00	n.d.	n.d.	n.d.	< 0.032	< 0.032
	Amoxicillin	1																	1	0.00		0.00				< 0.032	< 0.032
E. durans	Dihydrostreptomycin																1		1	100			100			512	512
	Polymyxin b														1				1	100	n.d.	n.d.	100	n.d.	n.d.	128	128
	Benzylpenicillin											1							1	100			100			16	16
	Amoxicillin											1							1	100			100			16	16
E. faecalis	Dihydrostreptomycin						3	3	2		1	2	2	1	2	1	10		27	40.7	0	43.8	33.3	50.0	0.00	64	512
	Polymyxin b							8	1		2	2	4	1	4			5	27	66.7	100	68.8	33.3	83.3	0.00	32	1024
	Benzylpenicillin	3	3	4		1	1	1	1	1	4	8							27	29.6	100	37.5	0.00	16.7	0.00	2	16
	Amoxicillin	9	1	1	1	1			1	1		12							27	44.4	0	62.5	33.3	16.7	0.00	2	16
E. faecium	Dihydrostreptomycin						1										4		5	80.0	0.00	100		100	100	512	512
	Polymyxin b							1				2				1		1	5	80.0	0.00	100	n.d.	100	100	16	1024
	Benzylpenicillin	1										4							5	80.0	0.00	100		100	100	16	16
	Amoxicillin		1									4							5	80.0	0.00	100		100	100	16	16
E. hirae	Dihydrostreptomycin						4	4	4	2	1	2	3	3	1	4	11		39	38.5	35.0	75.0	0.00	16.7	80.0	32	512
	Polymyxin b							4	4	3	2	10	4	1	3	3		5	39	79.5	85.0	100	25.0	66.7	100	16	1024
	Benzylpenicillin	1	1	8	2	2	2	1		1	1	20							39	51.3	50.0	75.0	25.0	33.3	80.0	16	16
	Amoxicillin	10	2	1	1	1				1		23							39	59.0	60.0	75.0	25.0	33.3	100	16	16
E. mundtii	Dihydrostreptomycin						1	1				1							3	0.00		0.00	0.00			1	16
	Polymyxin b							1					1		1				3	66.7	n.d.	100	50.0	n.d.	n.d.	32	128
	Benzylpenicillin				1							2							3	66.7		100	50.0			16	16
	Amoxicillin											3							3	100		100	100			16	16
Lactobacillus sp.	Dihydrostreptomycin							7		1	2	1	1			1	3		16	25.0		0.00	50.0	33.3	20.0	2	512
	Polymyxin b								6	1		1	2		1	2	1	2	16	56.3	n.d.	0.00	100	33.3	60.0	8	1024
	Benzylpenicillin	1	1			1	1	1	1	1	2		7						16	43.8		0.00	50.0	0.00	60.0	4	16
	Amoxicillin	9	1	1									5						16	31.3		0.00	50.0	66.7	20.0	< 0.032	16
L. casei	Dihydrostreptomycin							1						1			1		3	33.3		100			0.00	32	512
	Polymyxin b								1				1			1			3	66.7	n.d.	100	n.d.	n.d.	50.0	16	256
	Benzylpenicillin								1			1	1						3	33.3		0.00			50.0	8	16
	Amoxicillin	1			1								1						3	33.3		100			0.00	0.0625	16
L. paracasei	Dihydrostreptomycin							9				1			1		2		13	23.1	0.00	33	0.00		25.0	< 1	512
	Polymyxin b								9			1	1	1		1			13	30.8	100	33	100	n.d.	12.5	< 2	32
	Benzylpenicillin	7			1		2						3						13	23.1	0.00	0	100		25.0	< 0.032	16
	Amoxicillin	11				1							1						13	7.69	0.00	0	100		0.00	< 0.032	0.125
L. plantarum	Dihydrostreptomycin							1											1	0.00					0	< 1	< 1
	Polymyxin b								1										1	0.00					0	< 2	< 2
	Benzylpenicillin												1						1	100	n.d.	n.d.	n.d.	n.d.	100	16	16
	Amoxicillin	1																	1	0.00					0	< 0.032	< 0.032
L. plantarum subsp. *plantarum*	Dihydrostreptomycin							2		1							1		4	25.0	100				0	< 1	512
	Polymyxin b								1	1			1					1	4	50.0	100		n.d.	n.d.	33	2	1024
	Benzylpenicillin	1				1							2						4	50.0	100				33	0.125	16
	Amoxicillin	2						1					1						4	25.0	100				0	< 0.032	16
L. lactis	Dihydrostreptomycin							2	1	1							5		9	55.6			66.7	60.0	0	512	512
	Polymyxin b								1	1	1				1		1	4	9	77.8	n.d.	n.d.	100	80.0	0	512	1024
	Benzylpenicillin	1			1								7						9	77.8			100	60.0	100	16	16
	Amoxicillin	2											7						9	77.8			100	60.0	100	16	16
L. lactis subs. *lactis*	Dihydrostreptomycin							1		1		1					4		7	57.1			33.3	75.0		512	512
	Polymyxin b												1		1	1		4	7	100	n.d.	n.d.	100	100	n.d.	1024	1024
	Benzylpenicillin	1					1						5						7	71.4			66.7	75.0		16	16
	Amoxicillin	2											5						7	71.4			66.7	75.0		16	16
Lactococcus sp.	Dihydrostreptomycin																2		2	100				100		512	512
	Polymyxin b																	2	2	100	n.d.	n.d.	n.d.	100	n.d.	1024	1024
	Benzylpenicillin												2						2	100				100		16	16
	Amoxicillin	1											1						2	50.0				50.0		< 0.032	16
L. brevis	Dihydrostreptomycin							2				1		1	1		3		8	37.5	0.00		33.3		50.0	32	512
	Polymyxin b								2			1			2	1		2	8	75.0	100	n.d.	66.7	n.d.	75.0	64	1024
	Benzylpenicillin	1	1										6						8	75.0	100		66.7		50.0	16	16
	Amoxicillin	2											6						8	75.0	100		66.7		50.0	16	16
Streptococcus sp.	Dihydrostreptomycin											1							1	0.00	0.00					8	8
	Polymyxin b																1		1	100	100	n.d.	n.d.	n.d.	n.d.	512	512
	Benzylpenicillin												1						1	100	100					16	16
	Amoxicillin												1						1	100	100					16	16

[1]The range of dilutions tested for each antimicrobial agent is indicated in white. The vertical lines indicate the epidemiological cut-off (ECOFF) values according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or European Food Safety Authority (EFSA). MICs lower than the lowest concentration tested are indicated in the closest concentration of the grey range.
[2]n.d. = not detected.
[3] MIC_{50} (μg/kg) = MIC requeried for the inhibition of the growth of the 50% of the isolates.
[4] MIC_{90} (μg/kg) = MIC requeried for the inhibition of the growth of the 90% of the isolates.

B. anthracis were the most differentiated LAB, as they were the most sensitive bacteria to all the antibiotics tested (on average, 18.8 % of all the isolates were resistant to all the antibiotics) (cluster 1). On the other hand, the remaining LAB species exhibited higher resistance rates (cluster 2). *L. casei, L. paracasei, E. faecalis* and *Lactobacillus* sp. were closely related, as they showed higher but still lower resistance rates (on average, 36.8 %) (cluster 2.3). *E. durans, Lactococcus* sp., *E. mundtii* and *Streptococcus* sp. stood out (clusters 2.1 and 2.2), showing the highest resistance rates to all the antimicrobial agents tested (93.8 %). The resistance rate against dihydrostreptomycin was the main reason for the

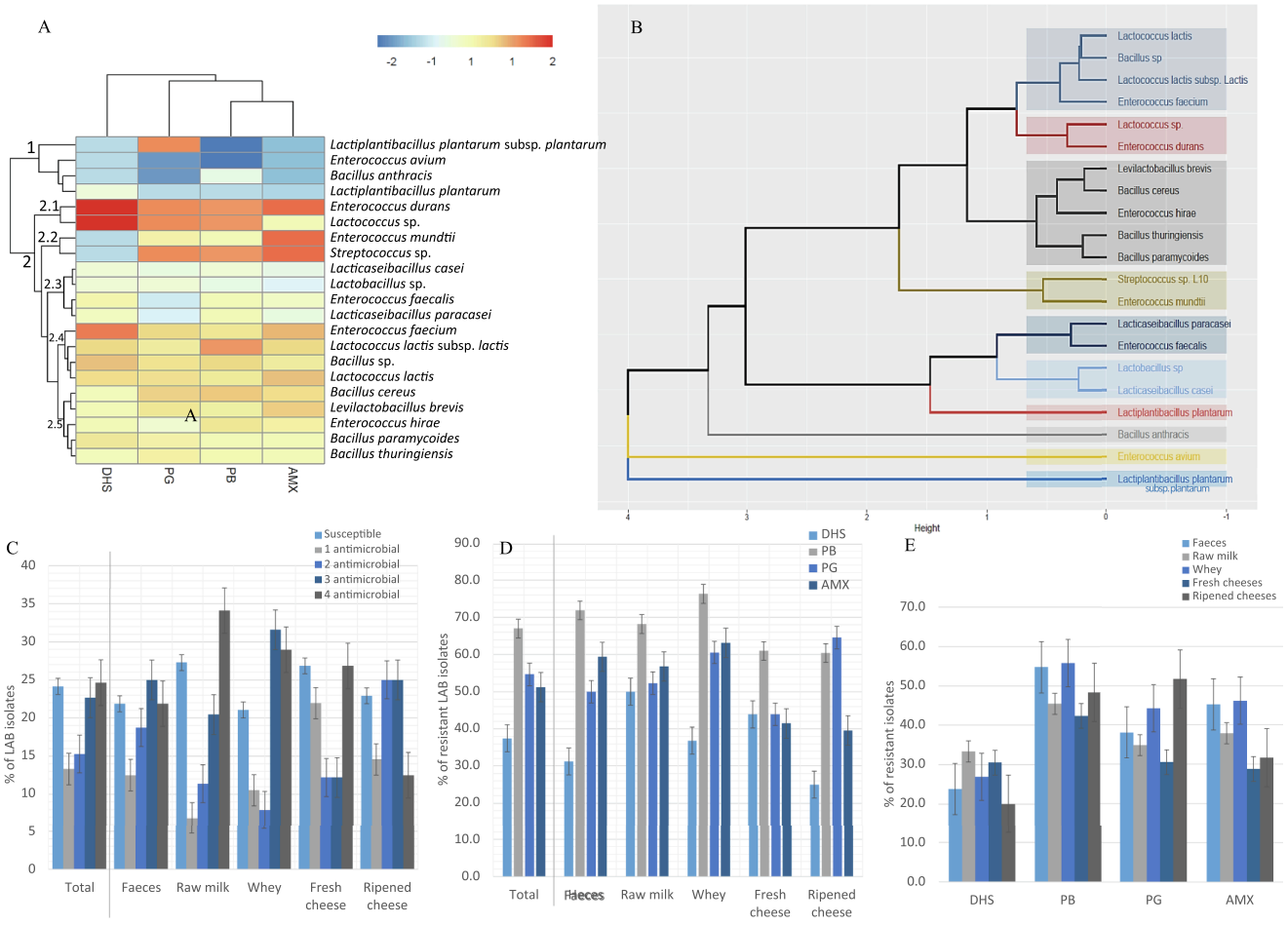


Fig. 2. HCA heatmap (A), dendogram clustering (B), box plot representations (C, D and E) based on antimicrobial susceptibility testing (AST) results according to the bacterial species (A and B), number of antimicrobials (C) and sample type along production chain (D and E). Abbreviations: DHS: dihydrostreptomycin; PB: polymyxin B; PG: benzylpenicillin; AMX: amoxicillin.

difference between these two clusters (2.1 and 2.2). The remaining bacterial species belonging to the *Bacillus, Enterococcus, Lactococcus* or *Levilactobacillus* genera exhibited similar high-intermediate resistance (66.7 % and 67.2 %, respectively) (clusters 2.4 and 2.5).

The MIC_{50} and MIC_{90}, defined as the MIC required for the inhibition of the growth of 50 % and 90 %, respectively, of the isolates confirmed the high resistance of some of the bacterial species (Table 1). However, some trends were observed along the production chain (Supplementary Table 2). *B. cereus, E. hirae, E. mundtti, L. lactis* subsp. *lactis* and *L. brevis* maintained similar MIC_{50} and MIC_{90} values throughout the production chain, while for *B. paramycoides*, *E. faecium*, *L. paracasei* and *Lactobacillus* sp. increased, indicating a greater prevalence of resistant bacteria throughout the production chain. Finally, the MIC_{50} and MIC_{90} of *B. thuringiensis*, *Bacillus* sp., *E. faecalis*, *L. casei*, *L. plantarum* subsp. *plantarum* and *L. lactis* decreased, which indicated an increase in the abundance of sensible bacteria during the cheese production process.

In general, 24.1 % (49/203) of the LAB isolates were susceptible to all the antimicrobial agents tested (Fig. 2C). Thus, 75.9 % (154/203) were resistant to at least one of the antibiotics tested, with resistance to 3 or 4 antimicrobial agents being the most common (46/203, 22.7 % and 50/203, 24.6 %, respectively) (Fig. 2C). However, differences were observed throughout the cheese production chain and were mainly related to differences in LAB composition ($P \leq 0.001$) (Fig. 2C). In faeces, LAB isolates resistant to 3 antimicrobial agents predominated (25.0 %), followed by those resistant to 4 antimicrobial agents (21.9 %), since the predominant *E. hirae* was mainly resistant to 2 or 3 antimicrobial agents. Other minor species, such as *L. brevis* or *Streptococcus* sp., were principally resistant to 3 or 4 individual compounds. In raw ewe milk, resistance to 4 antimicrobial agents predominated (34.1 %), followed by resistance to 3 antimicrobial agents (20.5 %), since the predominant species, *E. faecalis, B. thuringiensis, and E. hirae,* and most minor species were mainly resistant to 4 and, to a lesser extent, to 3 antimicrobial agents. In whey, a similar trend compared to milk was observed, although resistance to 3 antimicrobial agents predominated (31.6 %), followed by resistance to 4 antimicrobial agents (28.9 %). In this case, the predominant *B. cereus* was equally resistant to 3 or 4 antimicrobial agents, while *Bacillus* sp. was mainly resistant to 4 antimicrobial agents, *E. hirae* was resistant to 3 antimicrobial agents, and most minor species were resistant to 3 or 4 antimicrobial agents. In fresh cheeses, resistance to 4 antimicrobial agents was dominant (26.8 %), followed by resistance to 1 antimicrobial agent (22.0 %). The predominant *Bacillus* sp. species were equally resistant to 3 or 4 antimicrobial agents, while other dominant species such as *E. hirae* being mainly resistant to 1 antimicrobial, *E. faecalis* to 2 antimicrobial agents and *L. lactis* to 4 antimicrobial agents. A large proportion of the minor species were also resistant to 1 or 4 antimicrobial agents. Finally, in the ripened cheeses, resistance to 2 or 3 antimicrobial agents predominated (25.0 % in both cases), since the predominant *Lactobacillus* sp. and *L. paracasei* were resistant to 2 antimicrobial agents, and, to a lesser extent, other minor important species (*B. cereus* or *Bacillus* sp.) were resistant to 3 antimicrobial agents. Overall, the proportion of LAB resistant to 1 or 2 antimicrobial agents increased throughout the production chain, while the proportion of strains resistant to 3 or 4 antimicrobial agents decreased. No differences in terms of abundance were

observed for the susceptible bacteria throughout the production chain ($P > 0.05$), although the bacteria differed taxonomically. In faeces, raw ewe milk and whey, susceptible LAB belonged, mainly, to *E. hirae* and/or *E. faecalis* species, while in fresh cheese, they were, mainly, unidentified *Bacillus* species; and in ripened cheeses, they corresponded to *L. paracasei;* and, to a lesser extent, to *L. plantarum* subsp. *plantarum* and *Lactobacillus* sp.

Regarding individual compounds, the resistance against polymyxin B was the most common (67.0 %), followed by benzylpenicillin (54.7 %) and amoxicillin (51.2 %), and being clearly more sensitive to dihydrostreptomycin (37.4 %) (Table 1, Fig. 2D-E). The predominance of resistance to polymyxin B was observed in all the samples except for ripened cheeses, where a higher prevalence of LAB isolates resistant to benzylpenicillin was observed. Resistance to benzylpenicillin was primarily observed in ripened cheeses, while amoxicillin resistance was mainly detected in faeces and whey, and dihydrostreptomycin resistance was mainly detected in raw ewe milk and fresh cheeses (Fig. 2D-E). This differentiation along the production chain was related to the LAB composition of each sample type ($P \leq 0.05$) (Table 1). *E. durans* or *Lactococcus* sp., followed by *E. faecium,* were the most resistant to dihydrostreptomycin (Fig. 2A), and the most sensitive species were *B. anthracis, E. avium, E. mundtii*, *L. plantarum* or *Streptococcus* sp. In the case of benzylpenicillin, *L. plantarum* subsp. *plantarum, E. durans, Lactococcus* sp. and *Streptococcus* sp. were the most resistant, while *B. anthracis* or *E. avium* were the most sensitive. *E. durans, Lactococcus* sp., *L. lactis* subsp. *lactis* and *Streptococcus* sp. were the most resistant to polymyxin B, while *E. avium* and *L. plantarum* subsp. *plantarum* were the most susceptible. Finally, *E. durans, E. mundtii* or *Streptococcus* sp. were the most resistant to amoxicillin, and *B. anthracis, E. avium* or *L. plantarum* were the most sensitive.

Regarding the AMR profiles of LAB (Fig. 3A-B), which results from all possible combinations of all antibiotics tested, resistance to all antibiotics predominated (24.6 %), followed by polymyxin B-benzylpenicillin-amoxicillin (16.7 %), polymyxin B (6.90 %) and dihydrostreptomycin-polymyxin B (5.42 %). These patterns were mainly observed for *E. hirae* and, to a lesser extent, for *Bacillus* sp., *Bacillus cereus* and

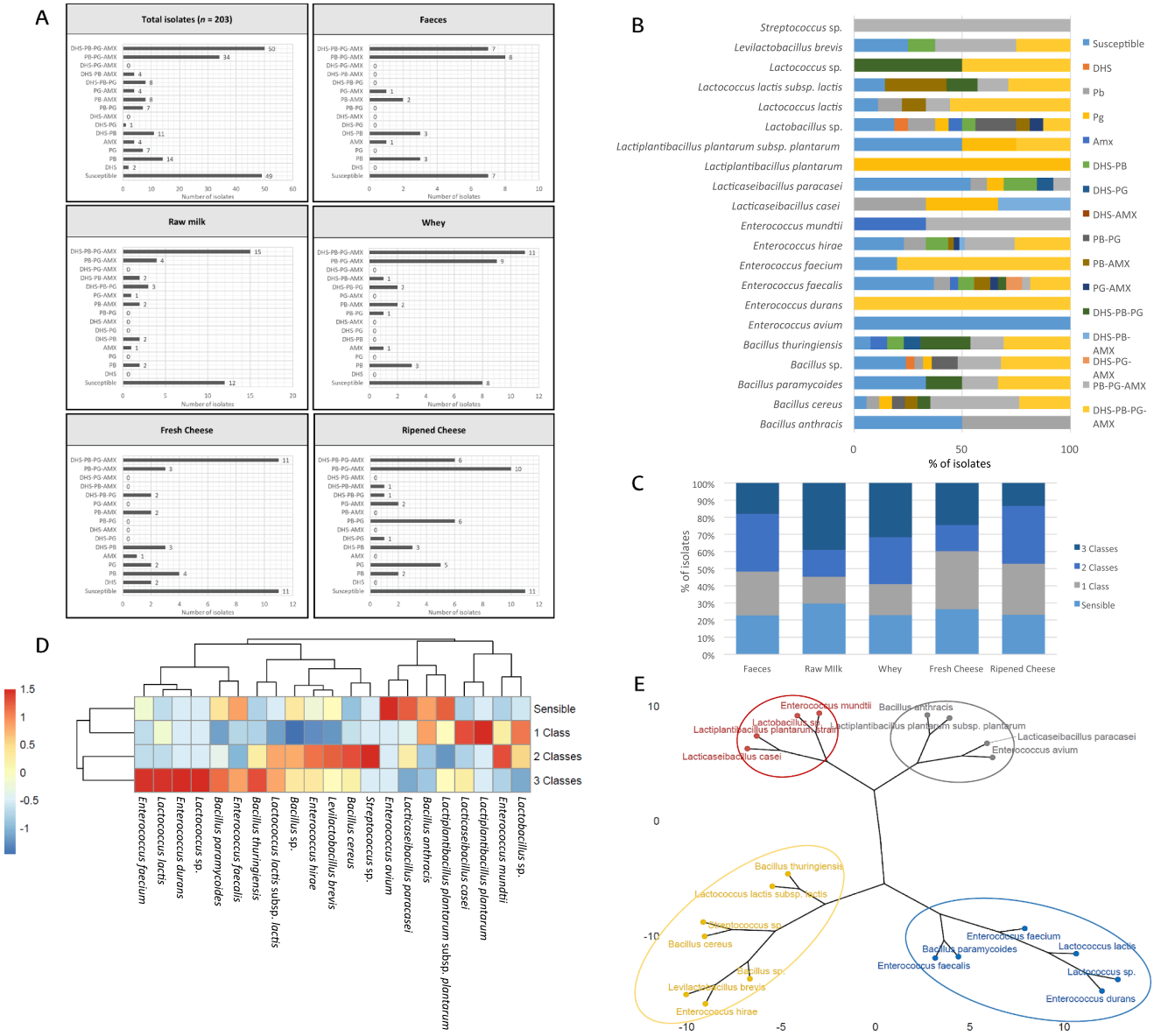


Fig. 3. Box plot representations based on the resistance patterns according to the sample type along production chain and bacterial species (A and B, respectively), and box plot representation (C), HCA heatmap (D) and dendogram clustering (E) based on the resistance against the antimicrobial classes tested according to the sample type along production chain (C) and bacterial species (D and E). Abbreviations: DHS: dihydrostreptomycin; PB: polymyxin B; PG: benzylpenicillin; AMX: amoxicillin.

E. faecalis. Resistance patterns were significantly related to the LAB communities ($P \leq 0.01$), consequently leading to differentiation in the cheese production chain. Resistance to all antibiotics was mainly observed for *E. hirae* and *Bacillus* sp., followed by *L. lactis* and *E. faecalis*, while the pattern of resistance to polymyxin B-benzylpenicillin-amoxicillin was most common for *E. hirae*, *B. cereus* and *Bacillus* sp. (Table 1, Supplementary Fig. 2). Resistance to polymyxin B was related to *E. hirae*, *E. faecalis* and *Lactobacillus* sp., and dihydrostreptomycin-polymyxin B combination was observed in *E. hirae*, *E. faecalis* and *L. paracasei* (Table 1, Supplementary Fig. 2). Thus, considering LAB communities throughout the production chain, resistance to polymyxin B-benzylpenicillin-amoxicillin (25.0 %) and to all antibiotics predominated (21.9 %) in faeces. In raw ewe milk, a similar trend was observed, although resistance to all antimicrobials was notably greater (34.1 %) than that to polymyxin B-benzylpenicillin-amoxicillin (9.09 %), which was maintained in whey (28.9 % and 23.7 %, respectively) and fresh cheeses (26.8 % and 7.32 %, respectively), albeit at different proportions. In the ripened cheeses, the resistance to all the antimicrobial agents was clearly lower (12.5 %), and the resistance to polymyxin B-benzylpenicillin and benzylpenicillin was also notable (12.5 % and 10.4 %, respectively). Notably, no isolate resistant to the combination of dihydrostreptomycin-amoxicillin or dihydrostreptomycin-benzylpeni cillin-amoxicillin was found in any type of sample.

In relation to multidrug resistance (MDR) (Fig. 3C-E), which is defined as resistance to 3 or more classes of antibiotics, it was observed in 30.5 % of the LAB isolates. The MDR differed throughout the production chain ($P \leq 0.05$). Specifically, the proportion of bacteria resistant to 1 class of antimicrobial did not differ throughout the production chain (12.9–29.0 %) ($P > 0.05$). However, the number of isolates resistant to 2 classes differed significantly according to the sample type ($P \leq 0.05$), with the highest prevalence found in ripened cheeses (32.8 %) and faeces (21.3 %) and the lowest in raw ewe milk and fresh cheeses (13.1 % in both cases) (Fig. 3C). The prevalence of multirresistant bacteria also differed throughout the production chain ($P \leq 0.01$), with the highest rate observed in raw ewe milk (32.3 %), which decreased throughout cheese-making and ripening processes (12.9 %) (Fig. 3C). These dynamics were related to the LAB communities ($P \leq 0.05$) (Supplementary Fig. 3), since MDR was mainly observed in *E. hirae* (17.2 %), *E. faecalis* (15.6 %), *B. thuringiensis* (15.6 %) and *Bacillus* sp. (15.6 %). Resistance to 2 classes was detected mainly in *E. hirae* (23.7 %), *B. cereus* (15.3 %) and *Bacillus* sp. isolates (13.6 %), and resistance to 1 class was more common in *Lactobacillus* sp. (19.4 %) and *E. hirae* (16.1 %). Moreover, the HCA and dendrogram divided LAB species into 4 clusters according to the predominant phenotype within each species. Thus, the MDR phenotype was predominant within *E. durans*, *Lactococcus* sp., *E. faecium, L. lactis, B. thuringiensis* and *B. paramycoides*.

3.3. Genotypic profile of antimicrobial resistance

Regarding the ARGs and MGEs, 37 out of the 47 genes studied were detected (Fig. 4A, Supplementary Table 3). Among all the samples, the predominant ARGs were *Str* (average relative abundance of 387), followed by *Str*B (39.3) and *aad*A-01 (19.3), while among the MGEs, *tnp*A-02 and *tnp*A-01 predominated (71.3 and 26.5, respectively). The ARGs *aph*, *aph*6ia, *bla*Z, *bla*TEM, *bla*GES, *bla*CTX-M−03, *bla*OKP and *pbp*2x and the MGEs intI1 and tnpA-03 were not detected. In general, aminoglycoside ARGs presented greater abundances than β-lactam, polymyxin and multidrug ARGs. Among the antimicrobial agents, the *Str* gene exhibited the highest relative abundance within aminoglycosides, followed by *Str*B and *aad*A-01, while *aac*A/*aph*D and *aad*A5-01 presented the lowest abundances. For β-lactams, *bla*-ACC-1, *pbp* and *bla*CMY2-01 dominated, while *amp*C, *bla*IMP-01 and *pbp*5 were minor ARGs. For polymyxins, *mcr*-2 presented the greatest abundance compared to *mcr-1*. Among the multidrug ARGs, two genes were also detected, namely, *tol*C-01 and *mex*D, the first presenting the greatest abundances. Among the MGEs, transposons presented the greatest abundance, especially *tnp*A-02 and *tnp*A-01, while *tnp*A-07 was the least abundant. A similar abundance was observed for integrons, namely, *int*I and IS613. Overall, predominant transposons presented higher abundances than predominant aminoglycoside ARGs, with the exception of *Str*, while lower abundances were observed for integrons, similar to β-lactams, polymyxins and multidrug ARGs.

PERMANOVA indicated that there were no differences among producers in terms of the detected ARGs or MGEs ($P > 0.05$). Nonetheless,

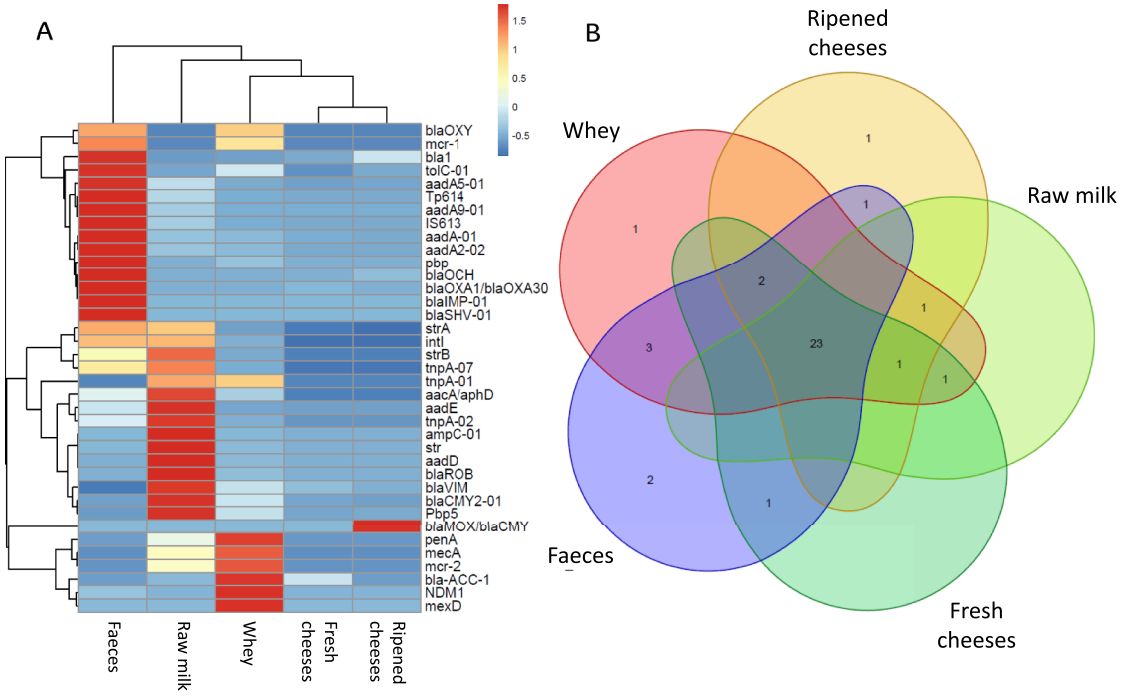


Fig. 4. HCA heatmap (A) and Venn diagram (B) showing the distribution of ARGs and MGEs along cheese production chain (faeces, raw milk, whey, fresh cheeses and ripened cheeses).

there was clear differentiation among the sample types along the production chain ($P \leq 0.001$), which was consistent with the phenotypic results. Specifically, significant differences were detected for all ARGs and MGEs among sample types, except for *aad*A5-01, *aad*D, *bla*IMP-01, *bla*MOX/*bla*CMY, *bla*OXA1/*bla*OXA30, *bla*ROB, *bla*VIM and *mex*D ($P > 0.05$) (Supplementary Table 3). As shown in Fig. 4B, the greatest number of ARGs and MGEs was observed in faeces and whey (32), followed by fresh and ripened cheeses (28 and 29, respectively) and, finally, raw ewe milk (26). However, considering their abundance, the raw ewe milk samples presented the greatest abundance of ARGs and MGEs, followed by the faeces and whey samples, while the fresh and ripened cheese samples presented the lowest abundances ($P \leq 0.001$) (Fig. 4A). Among the predominant ARGs, the greatest abundance of *Str* and *Str*B was observed in raw ewe milk, followed by whey ($P \leq 0.001$); and for *aad*A-01, the greatest abundance was observed in raw ewe milk, followed by whey and faeces ($P \leq 0.001$). For *bla*-ACC-1 and *pbp*, the greatest abundance was observed in raw ewe milk ($P \leq 0.01$), although it did not differ from that in fresh or ripened cheeses or whey. Similarly, *bla*CMY2-01 dominated in whey and fresh cheeses ($P \leq 0.001$). For *mcr*-2, the greatest abundance was observed in whey ($P \leq 0.05$), and for multidrug ARGs, the greatest abundance of *tol*C-01 was observed in faeces ($P \leq 0.05$). In terms of MGEs, the greatest abundance of *tnp*A-02 was observed in raw ewe milk and whey ($P \leq 0.001$), while for *tnp*A-01 and *int*I, it was observed in raw ewe milk ($P \leq 0.01$), and for IS613, it was observed in faeces ($P \leq 0.001$). Overall, the differences in the genotypic profiles of ARGs and MGEs among sample types and the lack of differentiation among producers were confirmed by PCA and OPLS-DA models (Supplementary Fig. 4).

As shown in Fig. 4B, a Venn diagram revealed that the detection of 14 ARGs and MGEs differed among sample types; these ARGs were mainly β-lactam ARGs (11) and, to a lesser extent, polymyxin ARGs (2) and multidrug ARGs (1). Specifically, *bla*IMP-01 and *NDM*1 were exclusively detected in faeces, *mex*D in whey and *bla*MOX/*bla*CMY in ripened cheese samples. The rest were detected in more than one sample type; for example, *mcr*-1, *bla*VIM and blaOXY were detected only in faeces and whey, while *bla*ROB was detected in milk, whey and fresh cheeses.

4. Discussion

Historically, studies on AMR have focused predominantly on pathogenic bacteria, primarily because of their direct implications for human health. However, recent studies have shifted their focus to non-pathogenic bacteria since they can act as AMR reservoirs that can be transferred to pathogens (Wolfe, 2023). In this context, the food and food production chain have been proposed as possible vehicles for the dissemination of AR bacteria and ARGs (Caniça et al., 2019; Wang et al., 2006; Yasir et al., 2022). Hence, the study of raw fermented foods holds particular significance due to their greater bacterial density, which could reach the gastrointestinal tract and interact with human microbiota transferring genes (Abriouel et al., 2015). Consequently, they may pose a significant risk, particularly whether ARGs are transferred to pathogens (Wolfe, 2023).

LAB predominate in fermented products, contributing to flavour and texture development, preventing the proliferation of pathogenic and spoilage bacteria (Fox et al., 2017; Santamarina-García et al., 2023) and exhibiting probiotic effects (Santamarina-García et al., 2020; Wolfe, 2023). However, studies on AMRs in LAB are limited to a certain product, such as milk or cheese (Výrostková et al., 2020, 2021), and to a certain LAB genera, such as *Lactobacillus* (Shi et al., 2023; Štšepetova et al., 2017). Therefore, this work aimed to shed light on the occurrence of AMR in LAB in ovine faeces, raw ewe milk, whey, fresh and ripened cheeses. To date, no information has been published in the literature in this regard.

The results revealed significant variations in LAB prevalence throughout the production chain. Higher LAB counts were observed in faeces than in raw ewe milk; which subsequently increased during whey production and, ultimately, in fresh and ripened cheeses. Although culture-dependent methods have been widely employed to characterize bacterial prevalence (Yap et al., 2022), to the best of our knowledge, no previous study has reported the evolution of LAB prevalence from animals, in this case, sheep, to the final transformed product, namely, ripened cheese. Previous research has focused primarily on the transition from raw milk to fresh and ripened cheeses, although information on raw ewe milk cheeses is scarce (Feutry et al., 2012; Navidghasemizad et al., 2009; Pérez-Elortondo et al., 1993, 1998, 1999). In fact, there are no results in the literature about the prevalence of LAB in ovine faeces and they are limited for whey (Gaglio et al., 2019; Blaiotta et al., 2021). Overall, based on the results obtained, there are notable variations in LAB counts in raw ewe milk or derived cheeses compared to those in other studies (Blaiotta et al., 2021; Khaldi et al., 2022; Rocha et al., 2023), due to factors such as breed, flock management and feeding, sources of microorganisms, for instance, diseases or the dairy environment, or practices followed by producers during milking or cheese-making, for example (Bokulich & Mills, 2013; Esteban-Blanco et al., 2020; Floridia et al., 2023; Fox et al., 2017; Jia et al., 2023; O'Sullivan & Cotter, 2017; Possas et al., 2021; Sun et al., 2019). The observed differences in LAB counts among the producers involved in this study were not statistically significant for all the samples, suggesting that they followed similar flock management and cheese-making practices, contrary to what has been reported in previous studies (Aldalur et al., 2019; Santamarina-García et al., 2022a). Nevertheless, there is no information in the literature on the similarities or differences in LAB counts among producers of the same type of cheese.

The LAB isolates obtained along the raw ewe milk cheese production chain were identified by sequencing the V1-V3 hypervariable region of the gene encoding 16S rRNA. This region was selected because of its great capability and accuracy in providing reliable taxonomic identification (Winand et al., 2019). All the LAB isolates belonged to the Firmicutes phylum, the class Bacilli and the orders Lactobacillales and Bacillales, as expected (Erkmen, 2022). Moreover, four LAB families were identified, namely, *Bacillaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Streptococcaceae*, and eight genera, *Bacillus, Enterococcus, Lactobacillus, Lacticaseibacillus, Lactiplantibacillus, Lactococcus, Levilactobacillus* and *Streptococcus*. The LAB composition along the production chain partially agrees with the findings of previous studies on Idiazabal cheese (Pérez-Elortondo et al., 1993, 1998, 1999) and other raw ewe milk cheeses (Blaiotta et al., 2021; Rocha et al., 2023), since there are differences in the identified LAB and their abundances. For example, *Leuconostoc* species have previously been identified along Idiazabal cheese and other raw ewe milk cheeses production chains, which have not been detected in this study (Pérez-Elortondo et al., 1993, 1998, 1999; Blaiotta et al., 2021; Rocha et al., 2023). Overall, the observed differences could be related to factors such as animal breed, flock management and feeding, sources of microorganisms or practices followed by producers during milking or cheese-making, as mentioned above (Abriouel et al., 2017; Bokulich & Mills, 2013; Esteban-Blanco et al., 2020; Floridia et al., 2023; Fox et al., 2017; Jia et al., 2023; O'Sullivan & Cotter, 2017; Possas et al., 2021; Sun et al., 2019). The discrepancy between these results and those of high-throughput sequencing (HTS) studies on Idiazabal cheese and other raw ewe milk cheeses should be highlighted (Cardinali et al., 2021; Dimov et al., 2021; Santamarina-García et al., 2022a). For instance, in our previous study, *Lactococcus* was identified as one of the dominant genera in raw milk from Latxa sheep and the dominant genus in Idiazabal cheese (Santamarina-García et al., 2022a). HTS techniques enable the detection of numerous bacteria, even those present at relatively low abundances (Abriouel et al., 2008; Michailidou et al., 2021; Santamarina-García et al., 2022a). However, the viability of these organisms cannot be determined, underscoring the necessity of complementing culture-dependent methods (Ferrocino et al., 2022).

The main antimicrobial agents used on sheep farms under the Idiazabal PDO include aminoglycosides, specifically dihydrostreptomycin; β-lactams, such as benzylpenicillin and amoxicillin; and polymyxins,

specifically polymyxin B; which have been previously reported as some of the most widely used antibiotics for livestock and, particularly, sheep (Virto et al., 2022). The presence of antimicrobial agents along the production chain exerts significant selective pressure, contributing significantly to the emergence of AR bacteria (Ammor et al., 2007; Virto et al., 2022; Wolfe, 2023). In fact, when bacterial populations, such as LAB, are exposed to these agents, they are prone to developing resistance (Virto et al., 2022). Subsequently, they can transfer their AMR genes to other bacteria, including pathogens that might pose a health risk (Abriouel et al., 2017; Nunziata et al., 2022; Virto et al., 2022). Hence, it is essential to investigate the resistance of microbial communities to the most commonly used antibiotics (Mathur & Singh, 2005; Wolfe, 2023). No study has analysed the antibiotic susceptibility of LAB in sheep and throughout the production of raw sheep milk cheeses. In fact, there are no data on the antibiotic susceptibility of LAB isolated from sheep, while information on raw ewe milk or raw ewe milk cheeses is scarce (Chen et al., 2020; Kmet' & Drugdová, 2012; Nalepa & Markiewicz, 2023; Rajput et al., 2022; Tsigkrimani et al., 2022), and there are also no data on the whey generated in the production of raw ewe milk cheese. In order to compare with LAB isolates obtained from other livestock or derived foods, the diverse approaches employed, as well as interpretations of the results, hamper comparing studies of the literature. Thus, this study followed international standardized methods to determine MICs (ISO/IDF, 2010; Rychen et al., 2018), namely, by the broth microdilution method and using ECOFFs established by either the EUCAST (https://www.eucast.org) or the EFSA (Rychen et al., 2018).

Out of the more than 200 LAB isolates collected during the Idiazabal cheese production chain, more than 75.0 % were resistant, primarily to polymyxin B, and were notably more susceptible to dihydrostreptomycin. Notably, there is no information on the resistance to polymyxin B or amoxicillin of LAB isolates obtained from faeces, raw milk, whey, curd and cheese from any sheep breed. Resistance against streptomycin has been proven for LAB isolates obtained from raw ewe milk and Feta and Kefalograviera cheeses, and even if the number of LAB isolates tested was lower, most of them were resistant, which supposes higher rates than those obtained in this study (Rajput et al., 2022; Tsigkrimani et al., 2022). For benzylpenicillin, Chen et al. (2020) have reported that all LAB isolates from sheep milk were susceptible, similar to the findings of Kmet' & Drugdová (2012) for ovine cheese isolates, which would not agree with these results. Compared to other studies (Nunziata et al., 2022; Wang et al., 2018; Zhou et al., 2005), D'Aimmo et al. (2007) have also reported a greater MIC for polymyxin B than for dihydrostreptomycin and, to a lesser extent, than for benzylpenicillin for LAB isolated from dairy products. Vidal & Collins-Thompson (1987) have reported a greater prevalence of LAB resistant to dihydrostreptomycin than to polymyxin B or benzylpenicillin, and Gad et al. (2014) have also reported a similar resistance of LAB to benzylpenicillin and amoxicillin, slightly predominating resistance to benzylpenicillin. The differences compared to previous studies are due to the different LAB obtained (D'Aimmo et al., 2007; Gad et al., 2014; Guan et al., 2017; Nunziata et al., 2022; Vidal & Collins-Thompson, 1987; Wang et al., 2018; Zhou et al., 2005). Nevertheless, while LAB have traditionally been considered resistant to aminoglycosides and susceptible to β-lactams, an increasing number of LAB isolates resistant to β-lactams have been obtained in recent years (Abriouel et al., 2015; Ammor et al., 2007; Devirgiliis et al., 2008; Nunziata et al., 2022), agreeing with the obtained results. This could be related to the selective pressure exerted by antibiotic utilization and the transfer of ARGs (Nunziata et al., 2022; Virto et al., 2022).

Taxonomically, *Lactococcus* species, such as *L. lactis* and *L. lactis* subsp. *lactis*, which play important roles in technological processes such as cheese production (Nunziata et al., 2022), were some of the most resistant LAB. No information on the resistance to polymyxin B or amoxicillin of *Lactococcus* species isolated from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses has been reported. Regarding benzylpenicillin, Chen et al. (2020) have reported that all *L. lactis* isolated from Hu sheep milk were sensitive, which do not agree with these results. For streptomycin, Tsigkrimani et al. (2022) have reported that all *L. lactis* isolates obtained from sheep milk and artisanal Feta and Kefalograviera cheeses were resistant, in line with these results. Overall, *Lactococcus* species are considered resistant to aminoglycosides, such as streptomycin (Ammor et al., 2007; Nunziata et al., 2022; Sharma et al., 2014), and polymyxins, specifically polymyxin B (Khemariya et al., 2013); however, they are reportedly sensitive to β-lactams, including benzylpenicillin and amoxicillin (Ammor et al., 2007; Nunziata et al., 2022; Sharma et al., 2014). However, recently, β-lactam-resistant *L. lactis* strains have been reported (Kazancıgil et al., 2019), which corroborates the resistance rates observed in this study. In addition, in the present study a distinction among species was observed, with *L. lactis* and *L. lactis* subsp. *lactis* being more sensitive to dihydrostreptomycin, while unidentified *Lactococcus* species were more susceptible to amoxicillin. No similar results have been reported to date (Nunziata et al., 2022).

The unidentified *Streptococcus* species also exhibited high resistance rates. Notably, information on the resistance of *Streptococcus* species isolated from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses to the tested antimicrobial agents is limited in the literature. In general, high but variable resistance to β-lactams, including benzylpenicillin, has been reported (Ammor et al., 2007; Flórez & Mayo, 2017; Morandi et al., 2015; Nunziata et al., 2022), in agreement with the obtained results. Information is also scarce for amoxicillin; although resistant strains of *S. agalactiae* isolated from raw goat milk have been identified (Shi et al., 2023), but other species, such as *S. dysgalactiae* or *S. uberis*, from dairy cows have been reported to be sensitive (Dyson et al., 2022). Regarding polymyxin B, there is little information on LAB isolated along the dairy production chain, but strains of *S. thermophilus* have also been reported to be resistant in the literature (Sozzi & Smiley, 1980), which would agree with the results of this study, although there are contradictory results (Rüegsegger et al., 2014). For aminoglycosides, moderate-high resistance has been reported, including streptomycin (Ammor et al., 2007; Nunziata et al., 2022), for which an upwards trend has been reported in recent years (Nunziata et al., 2022; Tosi et al., 2007), which was not observed in the present study.

Within the *Enterococcus* genus, several species presented high levels of resistance, namely, *E. durans* and *E. faecium* and, to a lesser extent, *E. hirae* and *E. mundtii*, which were, in general, mainly resistant to polymyxin B and amoxicillin and, to a lesser extent, to benzylpenicillin. Information on resistance to polymyxin B in *Enterococcus* species obtained from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses has not been reported to date, while for β-lactams and streptomycin is limited. Tsigkrimani et al. (2022) have reported that all *E. faecalis* isolates and more than 70 % of *E. faecium* isolates obtained from raw sheep milk and Artisanal Feta and Kefalograviera cheeses were resistant, similar to the results obtained for dihydrostreptomycin in the present study; whereas Rajput et al. (2022) have also reported an *E. hirae* isolate obtained from raw sheep milk to be resistant. Enterococci are part of the microbiota of many cheeses and contribute to modulating the microbiota, especially pathogenic and spoilage bacteria, due to their antimicrobial activity and to the development of aroma, flavour and texture (Dapkevicius et al., 2021; Franz et al., 2011; Santamarina-García et al., 2023; Santamarina-García et al., 2022b). However, these strains are not classified as qualified presumption of safety (QPS), as they present virulence genes and ARGs that cause them to commonly act as opportunistic pathogens (Dapkevicius et al., 2021; Franz et al., 2011). *Enterococcus* species have previously been described as resistant to β-lactams, including benzylpenicillin and amoxicillin, or to aminoglycosides, such as streptomycin (Dapkevicius et al., 2021; Franz et al., 2011). *E. faecium* and *E. faecalis* are considered the most important species in terms of AMRs (Dapkevicius et al., 2021), although there are notable differences in the literature (Dapkevicius et al., 2021; Franz et al., 2011; Gaglio et al., 2016; Gołaś-Prądzyńska et al., 2022; Juliano et al., 2022). Juliano et al. (2022) have reported similar resistance

against amoxicillin among *E. faecalis, E. faecium, E. hirae* and *E. mundtii,* and higher resistance against benzylpenicillin for *E. hirae* than for *E. faecalis* and *E. faecium* isolated from milk and dairy environments. Gołaś-Prądzyńska et al. (2022), instead, have not isolated benzylpenicillin or streptomycin-resistant *E. faecalis* or *E. faecium* from raw ewe milk and derived cheese. Information on the resistance of *Enterococcus* species to amoxicillin in livestock and dairy products is scarce, and only Bag et al. (2022) have reported that all *E. faecalis* isolates obtained from cow milk were sensitive. To the best of our knowledge, there is no information on polymyxin B.

Different species of the *Bacillus* genus have been studied for their potential use as probiotics, as their interest resides in the ability of spores to resist heat and gastric pH (Amoah et al., 2021; Santamarina-García et al., 2020). However, in addition to pathogenicity, AMRs should also be studied (Amoah et al., 2021; Sharma et al., 2014). In this study, most *Bacillus* species, such as *B. cereus, B. paramycoides, B. thuringiensis* or *Bacillus* sp., exhibited high resistance rates, specifically to polymyxin B, benzylpenicillin and amoxicillin and, to a lesser extent, to dihydrostreptomycin. Information on the resistance of *Bacillus* species isolated from ovine-derived faeces, raw ewe milk, whey or cheeses to the tested antimicrobial agents is scarce. In general, a high resistance of *Bacillus* species resistant to benzylpenicillin has been reported before in dairy environments and products (Bartoszewicz & Czyżewska, 2021; Gao et al., 2018; Owusu-Kwarteng et al., 2017; Zhai et al., 2023), albeit at different rates. For instance, Bartoszewicz & Czyżewska (2021) have reported that the 98.9 % of *B. cereus* isolates and 100 % of *B. thuringiensis* isolates obtained from raw cow milk and dairy environments were resistant, while Al-harbi et al. (2021) have found that only 52 % of the *Bacillus* sp. isolated from cow milk were resistant. For amoxicillin, information is scarce, but Owusu-Kwarteng et al. (2017) have reported that 100 % of *B. cereus* isolates obtained from dairy environments, milk and dairy products were resistant to amoxicillin. Information on susceptibility to streptomycin is also scarce, but most studies have reported *Bacillus* strains obtained from dairy cows, raw milk and dairy environments to be susceptible (Cui et al., 2016; Liu et al., 2022), which would corroborate the lowest resistance observed in this study compared to the other antimicrobial agents tested. Finally, a unique work has evaluated the polymyxin susceptibility of *Bacillus* species (Amoah et al., 2021), and, specifically, there is no information on dairy products. Amoah et al. (2021) isolated *B. tequilensis, B. velezensis* and *B. subtilis* species from the intestine of hybrid grouper, all of which were susceptible to polymyxin B.

The different species of the genus *Lactobacillus*, along with other species that have traditionally belonged to this genus but have recently been reclassified, specifically, *Lacticaseibacillus*, *Lactiplantibacillus* and *Levilactobacillus* (Zheng et al., 2020), presented lower resistance to antibiotics, except for *Levilactobacillus* isolates. Overall, no information has been reported regarding the resistance of *Lactobacillus* species isolated from ovine-derived faeces, raw ewe milk, whey or raw ewe milk cheeses to polymyxin B or β-lactams. For streptomycin, there is little information available, but Tsigkrimani et al. (2022) have recently reported that all *L. plantarum* isolates and more than 90 % of *L. brevis* isolates obtained from raw sheep milk and Artisanal Feta and Kefalograviera cheeses were resistant, which means higher resistance compared to the results of the present study. Traditionally, *Lactobacillus* species have exhibited low AMR, although greater variability has been reported in recent years (Abriouel et al., 2015; Ammor et al., 2007; Devirgiliis et al., 2008; Nunziata et al., 2022). In general, the number of currently reported lactobacilli-related infections is quite low, and there is no evidence of opportunistic infection by lactobacilli from fermented foods (Abriouel et al., 2015). Nonetheless, the acquired antimicrobial resistance of *Lactobacillus* to different antibiotics has been previously demonstrated (Abriouel et al., 2015; Mathur & Singh, 2005; Sharma et al., 2014). Furthermore, if *Lactobacillus* species act as reservoirs of AMRs, they could pose a threat to human health, especially if fermented foods containing antibiotic-resistant *Lactobacillus* are consumed in substantial quantities and if resistance genes are transferred to intestinal bacteria (Abriouel et al., 2015; Ammor et al., 2007; Nunziata et al., 2022). Moreover, different *Lactobacillus* species and strains have been widely used as probiotics, but previously must be classified as QPS, for which antimicrobial resistance profile is required (Sharma et al., 2014). Consequently, there are many studies in the literature on the AMRs of *Lactobacillus* species (Abriouel et al., 2015, 2017; Mathur & Singh, 2005). In the present study, the *Levilactobacillus* genus and the *L. brevis* species were the only of all those species previously classified as *Lactobacillus* that presented moderate-high resistance, especially to polymyxin B and β-lactams, and were more sensitive to the aminoglycoside dihydrostreptomycin, in agreement with previous works (Ammor et al., 2007; Nunziata et al., 2022). Nonetheless, great variability in the antimicrobial resistance profile among *Lactobacillus* species has been observed (Mathur & Singh, 2005; Nunziata et al., 2022), and there is no clear pattern of resistance according to species (Nunziata et al., 2022). In general, *Lactobacillus* species tend to be intrinsically resistant to aminoglycosides such as streptomycin, which is not observed in all *Lactobacillus* isolates obtained along the Idiazabal cheese production chain; however, for β-lactams, such as against benzylpenicillin and amoxicillin, are reportedly sensitive (Muñoz et al., 2014; Happel et al., 2020; Nunziata et al., 2022). Nonetheless, recent studies have highlighted a greater resistance to benzylpenicillin, for example, in *L. plantarum* or *L. casei* (Abriouel et al., 2015; Majhenič et al., 2007; Nunziata et al., 2022), in line with the results of this study. According to amoxicillin, although few isolates were resistant, particularly *Lactobacillus* sp. and *L. casei*, non-resistant *Lactobacillus* species have been reported to date in the literature (Muñoz et al., 2014; Happel et al., 2020). There is little information on the resistance of *Lactobacillus* species to polymyxin B (Ruiz-Moyano et al., 2019). Ruiz-Moyano et al. (2019) have recently reported that all *L. brevis, L. casei* and *L. paracasei* strains obtained from Serpa cheese were resistant to polymyxin B and more susceptible to benzylpenicillin, in line with the results of the present study.

It has previously been observed that the bacterial dynamics during the production of raw ewe milk cheeses determine the different quality and safety parameters of cheese, such as biogenic amines or volatile compounds (Santamarina-García et al., 2023; Santamarina-García et al., 2022b). In this sense, the resistance rates and trends observed in each sample along the production chain were also due to the different composition of LAB. Thus, the predominance of *Enterococcus* species, such as *E. hirae*, and *Bacillus* species, such as *B. cereus* or *Bacillus* sp., in ovine faeces and raw ewe milk, as well as *Lactococcus* species, specifically *L. lactis*, in whey and fresh cheese, contributed to the high resistance rates observed. However, the resistance rate of LAB in the final cheese decreased, mainly due to the predominance of *Lactobacillus* sp. and *Lacticaseibacillus* species. Similarly, MDR was also reduced along the production chain due to the inhibition of the main multidrug-resistant species, such as *E. hirae* or *Bacillus* sp. Similarly, the predominant patterns of resistance, namely, all antimicrobial agents and polymyxin B-benzylpenicillin-amoxicillin, were also reduced in ripened cheeses since they were mainly observed in *E. hirae, B. cereus* and *Bacillus* sp., while polymyxin B-benzylpenicillin and benzylpenicillin patterns gained importance related to the greater abundance of *Lactobacillus* species. Therefore, it can be concluded that the production chain modulates the prevalence of AMR in LAB and favours a greater abundance of susceptible LAB. This is of special interest in terms of cheese safety (Virto et al., 2022), considering that ripened cheese presented the highest number of CFU compared to the rest of the samples, specially ovine faeces, raw ewe milk and whey, and is indeed what is consumed and comes into contact with the intestinal microbiota (Wolfe, 2023). Overall, there are no results on resistance patters on MDR along production chain for other cheeses.

Several studies have highlighted the importance of LAB as reservoirs of ARGs, which can be transferred to the human microbiome (Nunziata et al., 2022). To analyse the ARGs that confer resistance to those antibiotics used in dairies, the most important genes for LAB were selected

using the CARD database (Alcock et al., 2023) and subsequently analysed by HT-qPCR (Jauregi et al., 2021). Compared to traditional qPCR, this technique has many advantages, such as greater speed and efficiency, in addition to the large number of genes that can be detected at the same time. Likewise, it has better detection limits than metagenomic sequencing approaches (Waseem et al., 2019). However, this approach also has several disadvantages, such as the inability to analyse unknown sequences since it requires the previous design of primers (Waseem et al., 2019). Notably, although this technique is one of the most appropriate methods for targeting ARGs, it has been applied mainly to environmental samples and not to dairy products (Wei et al., 2022; Yang et al., 2022), which highlights the novelty of this study.

In this study, clear differences were observed in the abundances of ARGs and MGEs throughout the production chain, with the greatest abundances observed in raw ewe milk, followed by ovine faeces and whey, while fresh and ripened cheeses presented the lowest abundances. Overall, these genotypic results would coincide with the phenotypic results obtained, indicating that microbial dynamics during the cheese-making process facilitate the reduction of ARGs and MGEs, which has not been reported so far. Overall, this would make sense since microbial dynamics have also been previously described as responsible for the evolution of other food safety concerns, such as biogenic amines (Santamarina-García et al., 2022b). In different studies, the presence of ARGs and MGEs in animal manure and dairy products, such as milk or cheese, is analysed via qPCR (Delannoy et al., 2023; Kang et al., 2022). However, there are no results using HT-qPCR. Anyway, to date, no study has analysed the prevalence of ARGs and MGEs throughout the dairy production chain using qPCR or HT-qPCR techniques.

The genes with the highest abundance were *Str*, *Str*B and *aad*A-01, which were also the predominant ARGs in terms of resistance against aminoglycosides. Few studies have analysed ARGs related to LAB using qPCR or HT-qPCR techniques (Guo et al., 2020), since most genotyping approaches are based on conventional PCR (Nalepa & Markiewicz, 2023; Obioha et al., 2023), which makes it impossible to determine which genes are the most abundant. The predominance of these genes throughout the production chain in terms of resistance to aminoglycosides is consistent with what has been reported in the literature for LAB obtained from dairy products, although there are differences (Gaglio et al., 2016; Nunziata et al., 2022; Obioha et al., 2023). For instance, Obioha et al. (2023) have detected only the *aad*E gene in LAB from a fermented dairy product. For β-lactams, different ARGs, such as *bla*Z, *amp*C or *mec*A, have been described as predominant (Nunziata et al., 2022; Rosander et al., 2008), most of which were detected in this study. However, there is no information on the predominant genes observed in this study, namely, *bla*-ACC-1, *pbp* and *bla*CMY2-01. On the other hand, the detected *mcr*-1 gene was the first ARG described in the literature for polymyxins (Nagy et al., 2021) and has been detected in livestock faeces and dairy products, although mainly from cows (Chen et al., 2017; Zheng et al., 2019). Up to 11 variants have been described because they are found in a plasmid; therefore, they are easily transmitted between bacteria (Nagy et al., 2021). However, *mcr* genes have been described only for *Enterobacteriaceae* (Chen et al., 2017; Nagy et al., 2021; Zheng et al., 2019), and there are no results related to LAB. Similarly, genes that confer multidrug resistance in LAB have also been described (Nunziata et al., 2022), within which the multidrug ARGs *mex*D and *tol*C were detected. Overall, few genes that confer multiresistance in LAB have been described, although the phenotypic profile has been widely observed, mainly in *Enterococcus*, as in this study (Gaglio et al., 2016; Obioha et al., 2023; Oguntoyinbo & Okueso, 2013). As previously mentioned, ARGs can be transmitted between bacteria by means of MGEs, within which, transposons and integrons predominate (Iskandar et al., 2022; Nunziata et al., 2022). In this study, 6 out of the 7 MGEs studied were detected, predominantly the transposons *tnp*A-02 and *tnp*A-01 and the integrons *int*I and IS613. Overall, information on these MGEs from LAB is scarce, but several integrons and transposons have been reported before (Nunziata et al., 2022).

5. Conclusions

This study provides new insights into the prevalence of AMRs related to LAB along a raw ewe milk cheese production chain (ovine faeces, raw ewe milk, whey, fresh cheese and 60-day-ripened cheese). Both phenotypic and genotypic results revealed a decrease in resistance rates, including patterns and MDR, as well as a reduction in the relative abundance of ARGs and MGEs along the production chain. This was related to the changes in the LAB composition throughout the production chain. Thus, the abundance of those LAB that presented high resistance in ovine faeces, raw ewe milk, whey and fresh cheeses, such as *Lactococcus, Enterococcus* and *Bacillus* species, was notably reduced in the ripened cheeses. In contrast, more sensitive LAB species, such as *Lacticaseibacillus* and *Lactobacillus*, became more abundant. These findings are of special interest, because they indicate the role of the production chain in minimizing AMRs, which has not been reported to date, and the greater susceptibility of those LAB that are consumed with cheese and that come into contact with the intestinal microbiota. However, further research is needed to elucidate the influence of different factors, such as herd management or cheese-making conditions, on the AMRs of LAB to identify the key factors involved in controlling resistant LAB.

CRediT authorship contribution statement

Gorka Santamarina-García: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gustavo Amores:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Diego Llamazares:** Investigation. **Igor Hernández:** Conceptualization, Methodology, Resources, Writing – review & editing. **Luis Javier R. Barron:** Funding acquisition, Project administration, Resources. **Mailo Virto:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the University of the Basque Country [grant number COLAB20/14], the Basque Government [grant number IT1568-22] and the MCIN/AEI/10.13039/501100011033 [grant number PID2020-113395RB-C21]. G. Santamarina-García thanks the University of the Basque Country (UPV/EHU) for a predoctoral fellowship. The authors are grateful for the collaboration of Idiazabal PDO producers, the technical and human support provided by SGIker (UPV/EHU/ERDF, EU) and the assistance of L. Azcona during sample preparation and analysis. Open Access funding provided by University of Basque Country.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114308.

References

Abriouel, H., Knapp, C. W., Gálvez, A., & Benomar, N. (2017). Antibiotic resistance profile of microbes from traditional fermented foods. In J. Frias, C. Martinez-Villaluenga, & E. Peñas (Eds.), *Fermented foods in health and disease prevention* (pp. 675–704). Academic Press.

Abriouel, H., Muñoz, M. C. C., Lerma, L. L., Montoro, B. P., Bockelmann, W., Pichner, R., Kabisch, J., Cho, G. S., Franz, C. M. A. P., Gálvez, A., & Benomar, N. (2015). New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. *Food Research International, 78*, 465–481. https://doi.org/10.1016/j.foodres.2015.09.016

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology, 127*, 200–208. https://doi.org/10.1016/j.ijfoodmicro.2008.07.004

Al-harbi, H., Ranjbar, S., Moore, R. J., & Alawneh, J. I. (2021). Bacteria isolated from milk of dairy cows with and without clinical mastitis in different regions of australia and their AMR profiles. *Frontiers in Veterinary Science, 8*, Article 743725. https://doi.org/10.3389/fvets.2021.743725

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., McCarthy, M. C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., Kwong, M., Liang, Z. C., Niu, K. C., Shan, P., Yang, J. Y. J., Gray, K. L., Hoad, G. R., Jia, B., Bhando, T., Carfrae, L. A., Farha, M. A., French, S., Gordzevich, R., Rachwalski, K., Tu, M. M., Bordeleau, E., Dooley, D., Griffiths, E., Zubyk, H. L., Brown, E. D., Maguire, F., Beiko, R. G., Hsiao, W. W. L., Brinkman, F. S. L., Van Domselaar, G., & McArthur, A. G. (2023). CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the comprehensive antibiotic resistance database. *Nucleic Acids Research, 51*, D690–D699. https://doi.org/10.1093/NAR/GKAC920

Aldalur, A., Bustamante, M.Á., & Barron, L. J. R. (2019). Effects of technological settings on yield, curd, whey, and cheese composition during the cheese-making process from raw sheep milk in small rural dairies: Emphasis on cutting and cooking conditions. *Journal of Dairy Science, 102*, 7813–7825. https://doi.org/10.3168/JDS.2019-16401

Ammor, M. S., Flórez, A. B., & Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology, 24*, 559–570. https://doi.org/10.1016/J.FM.2006.11.001

Amoah, K., Dong, X. H., Tan, B. P., Zhang, S., Kuebutornye, F. K. A., Chi, S. Y., Yang, Q. H., Liu, H. Y., Zhang, H. T., & Yang, Y. Z. (2021). In vitro assessment of the safety and potential probiotic characteristics of three bacillus strains isolated from the intestine of hybrid grouper (Epinephelus fuscoguttatus♀ × Epinephelus lanceolatus♂). *Frontiers in Veterinary Science, 8*, Article 675962. https://doi.org/10.3389/fvets.2021.675962

Bag, M. A. S., Arif, M., Riaz, S., Khan, M. S. R., Islam, M. S., Punom, S. A., Ali, M. W., Begum, F., Islam, M. S., Rahman, M. T., & Hassan, J. (2022). Antimicrobial resistance, virulence profiles, and public health significance of *Enterococcus faecalis* isolated from clinical mastitis of cattle in Bangladesh. *BioMed Research International, 2022,* Article 8101866. doi: 10.1155/2022/8101866.

Barron, L. J. R., Redondo, Y., Aramburu, M., Gil, P., Pérez-Elortondo, F. J., Albisu, M., Nájera, A. I., de Renobales, M., & Fernández-García, E. (2007). Volatile composition and sensory properties of industrially produced Idiazabal cheese. *International Dairy Journal, 17*, 1401–1414. https://doi.org/10.1016/j.idairyj.2007.04.001

Bartoszewicz, M., & Czyżewska, U. (2021). Comparison of the antibiotic resistance between genetically diverse and toxigenic *Bacillus cereus* sensu lato from milk, pepper and natural habitats. *Journal of Applied Microbiology, 130*, 370–381. https://doi.org/10.1111/jam.14792

Biçer, Y., Telli, A. E., Sönmez, G., Telli, N., & Uçar, G. (2021). Comparison of microbiota and volatile organic compounds in milk from different sheep breeds. *Journal of Dairy Science, 104*, 12303–12311. https://doi.org/10.3168/jds.2021-20911

Blaiotta, G., Marrone, R., Aponte, M., Peruzy, M. F., Smaldone, G., Vollano, L., & Murru, N. (2021). Characterisation of Conciato Romano: One of the oldest Italian cheeses. *International Dairy Journal, 120*, Article 105077. https://doi.org/10.1016/J.IDAIRYJ.2021.105077

Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied and Environmental Microbiology, 79*, 5214–5223. https://doi.org/10.1128/AEM.00934-13

Boletín Oficial del Estado. (1993). Orden del 30 de noviembre por la que se aprueba el Reglamento de la Denominación de Origen "Idiazabal" y su Consejo Regulador. *BOE, 289*, 34591–34596.

Caniça, M., Manageiro, V., Abriouel, H., Moran-Gilad, J., & Franz, C. M. A. P. (2019). Antibiotic resistance in foodborne bacteria. *Trends in Food Science & Technology, 84*, 41–44. https://doi.org/10.1016/J.TIFS.2018.08.001

Cardinali, F., Ferrocino, I., Milanović, V., Belleggia, L., Corvaglia, M. R., Garofalo, C., Foligni, R., Mannozzi, C., Mozzon, M., Cocolin, L., Osimani, A., & Aquilanti, L. (2021). Microbial communities and volatile profile of Queijo de Azeitão PDO cheese, a traditional Mediterranean thistle-curdled cheese from Portugal. *Food Research International, 147*, Article 110537. https://doi.org/10.1016/J.FOODRES.2021.110537

Chen, K., Chan, E. W. C., Xie, M., Ye, L., Dong, N., & Chen, S. (2017). Widespread distribution of *mcr*-1-bearing bacteria in the ecosystem, 2015 to 2016. *Eurosurveillance, 22*, Article 17–00206. https://doi.org/10.2807/1560-7917.ES.2017.22.39.17-00206

Chen, T., Wang, L., Li, Q., Long, Y., Lin, Y., Yin, J., Zeng, Y., Huang, L., Yao, T., Abbasi, M. N., Yang, H., Wang, Q., Tang, C., Khan, T. A., Liu, Q., Yin, J., Tu, Q., & Yin, Y. (2020). Functional probiotics of lactic acid bacteria from Hu sheep milk. *BMC Microbiology, 20*, 228. https://doi.org/10.1186/s12866-020-01920-6

Colonna, A., Durham, C., & Meunier-Goddik, L. (2011). Factors affecting consumers' preferences for and purchasing decisions regarding pasteurized and raw milk specialty cheeses. *Journal of Dairy Science, 94*, 5217–5226. https://doi.org/10.3168/JDS.2011-4456

Conde-Estévez, D., Grau, S., Albanell, J., Terradas, R., Salvadó, M., & Knobel, H. (2011). Clinical characteristics and outcomes of patients with vancomycin-susceptible *Enterococcus faecalis* and *Enterococcus faecium* bacteraemia in cancer patients. *European Journal of Clinical Microbiology and Infectious Diseases, 30*, 103–108. https://doi.org/10.1007/s10096-010-1029-5

Cui, Y., Liu, X., Dietrich, R., Märtlbauer, E., Cao, J., Ding, S., & Zhu, K. (2016). Characterization of *Bacillus cereus* isolates from local dairy farms in China. *FEMS Microbiology Letters, 363*, Article fnw096. https://doi.org/10.1093/femsle/fnw096

D'Aimmo, M. R., Modesto, M., & Biavati, B. (2007). Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology, 115*, 35–42. https://doi.org/10.1016/J.IJFOODMICRO.2006.10.003

Dapkevicius, M. L. E., Sgardioli, B., Câmara, S. P. A., Poeta, P., & Malcata, F. X. (2021). Current trends of enterococci in dairy products: A comprehensive review of their multiple roles. *Foods, 10*, Article 821. https://doi.org/10.3390/foods10040821

Delannoy, S., Hoffer, C., Tran, M. L., Madec, J. Y., Brisabois, A., Fach, P., & Haenni, M. (2023). High throughput qPCR analyses suggest that Enterobacterales of French sheep and cow cheese rarely carry genes conferring resistances to critically important antibiotics for human medicine. *International Journal of Food Microbiology, 403*, Article 110303. https://doi.org/10.1016/j.ijfoodmicro.2023.110303

Devirgiliis, C., Barile, S., & Perozzi, G. (2011). Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes & Nutrition, 6*, 275–284. https://doi.org/10.1007/s12263-011-0226-x

Devirgiliis, C., Caravelli, A., Coppola, D., Barile, S., & Perozzi, G. (2008). Antibiotic resistance and microbial composition along the manufacturing process of Mozzarella di Bufala Campana. *International Journal of Food Microbiology, 128*, 378–384. https://doi.org/10.1016/j.ijfoodmicro.2008.09.021

Dimov, S. G., Gyurova, A., Zagorchev, L., Dimitrov, T., Georgieva-Miteva, D., & Peykov, S. (2021). NGS-based metagenomic study of four traditional bulgarian green cheeses from tcherni vit. *LWT, 152*, Article 112278. https://doi.org/10.1016/j.lwt.2021.112278

Dos Santos, R. A., Araújo, G. B., Correia, E. F., & Costa Sobrinho, P. D. S. (2022). Minas artisanal cheese as potential source of multidrug-resistant *Escherichia coli*. *Foodborne Pathogens and Disease, 19*, 316–323. https://doi.org/10.1089/fpd.2021.0102

Dyson, R., Charman, N., Hodge, A., Rowe, S. M., & Taylor, L. F. (2022). A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds. *Journal of Dairy Science, 105*, 1504–1518. https://doi.org/10.3168/jds.2021-20955

Erkmen, O. (2022). Analysis of fermented foods. In O. Erkmen (Ed.), *Microbiological Analysis of Foods and Food Processing Environments* (pp. 361–379). Academic Press.

Esteban-Blanco, C., Gutiérrez-Gil, B., Puente-Sánchez, F., Marina, H., Tamames, J., Acedo, A., & Arranz, J. J. (2020). Microbiota characterization of sheep milk and its association with somatic cell count using 16s rRNA gene sequencing. *Journal of Animal Breeding and Genetics, 137*, 73–83. https://doi.org/10.1111/jbg.12446

Ferrocino, I., Rantsiou, K., & Cocolin, L. (2022). Investigating dairy microbiome: An opportunity to ensure quality, safety and typicity. *Current Opinion in Biotechnology, 73*, 164–170. https://doi.org/10.1016/J.COPBIO.2021.08.009

Feutry, F., Oneca, M., Berthier, F., & Torre, P. (2012). Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food Microbiology, 29*, 33–42. https://doi.org/10.1016/j.fm.2011.08.011

Flórez, A. B., & Mayo, B. (2017). Antibiotic resistance-susceptibility profiles of *Streptococcus thermophilus* isolated from raw milk and genome analysis of the genetic basis of acquired resistances. Article 02608 *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.02608.

Floridia, V., Giuffrè, L., Giosa, D., Arfuso, F., Aragona, F., Fazio, F., Chen, C., Song, C., Romeo, O., & D'Alessandro, E. (2023). Comparison of the faecal microbiota composition following a dairy by-product supplemented diet in nero siciliano and large white × landrace pig breeds. Article 2323 *Animals, 13*. https://doi.org/10.3390/ANI13142323.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017). *Fundamentals of cheese science* ((2nd ed.).). Springer.

Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology, 151*, 125–140. https://doi.org/10.1016/j.ijfoodmicro.2011.08.014

Fraqueza, M. J. (2015). Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. *International Journal of Food Microbiology, 212*, 76–88. https://doi.org/10.1016/J.IJFOODMICRO.2015.04.035

Fusco, V., Chieffi, D., Fanelli, F., Logrieco, A. F., Cho, G. S., Kabisch, J., Böhnlein, C., & Franz, C. M. A. P. (2020). Microbial quality and safety of milk and milk products in the 21st century. *Comprehensive Reviews in Food Science and Food Safety, 19*, 2013–2049. https://doi.org/10.1111/1541-4337.12568

Gad, G. F. M., Abdel-Hamid, A. M., & Farag, Z. S. H. (2014). Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. *Brazilian Journal of Microbiology, 45*, 25–33. https://doi.org/10.1590/S1517-83822014000100005

Gaglio, R., Couto, N., Marques, C., de Fatima Silva Lopes, M., Moschetti, G., Pomba, C., & Settanni, L. (2016). Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses.

International Journal of Food Microbiology, 236, 107–114. https://doi.org/10.1016/J.IJFOODMICRO.2016.07.020

Gaglio, R., Todaro, M., Scatassa, M. L., Franciosi, E., Corona, O., Mancuso, I., Di Gerlando, R., Cardamone, C., & Settanni, L. (2019). Transformation of raw ewes' milk applying "Grana" type pressed cheese technology: Development of extra-hard "Gran Ovino" cheese. *International Journal of Food Microbiology, 307*, Article 108277. https://doi.org/10.1016/J.IJFOODMICRO.2019.108277

Gao, T., Ding, Y., Wu, Q., Wang, J., Zhang, J., Yu, S., Yu, P., Liu, C., Kong, L., Feng, Z., Chen, M., Wu, S., Zeng, H., & Wu, H. (2018). Prevalence, virulence genes, antimicrobial susceptibility, and genetic diversity of *Bacillus cereus* isolated from pasteurized milk in China. *Frontiers in Microbiology, 9*, Article 00533. https://doi.org/10.3389/fmicb.2018.00533

Gołaś-Prądzyńska, M., Łuszczyńska, M., & Rola, J. G. (2022). Dairy products: a potential source of multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium* strains. *Foods, 11*, Article 4116. https://doi.org/10.3390/foods11244116

Gorecki, A., Musialowski, M., Wolacewicz, M., Decewicz, P., Ferreira, C., Vejmelkova, D., Grzesiuk, M., Manaia, C. M., Bartacek, J., & Dziewit, L. (2022). Development and validation of novel PCR primers for identification of plasmid-mediated colistin resistance (mcr) genes in various environmental settings. *Journal of Hazardous Materials, 425*, Article 127936. https://doi.org/10.1016/J.JHAZMAT.2021.127936

Guan, X., Xu, Q., Zheng, Y., Qian, L., & Lin, B. (2017). Screening and characterization of lactic acid bacterial strains that produce fermented milk and reduce cholesterol levels. *Brazilian Journal of Microbiology, 48*, 730–739. https://doi.org/10.1016/j.bjm.2017.02.011

Guo, J., Li, X., Li, B., Yang, J., Jin, D., & Li, K. (2020). Transcriptome analysis of *Lactobacillus paracasei* SMN-LBK under ethanol stress. *Journal of Dairy Science, 103*, 7813–7825. https://doi.org/10.3168/jds.2019-16955

Hall, T., Biosciences, I., & Carlsbad, C. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences, 2*, 60–61.

Happel, A. U., Kullin, B., Gamieldien, H., Wentzel, N., Zauchenberger, C. Z., Jaspan, H. B., Dabee, S., Barnabas, S. L., Jaumdally, S. Z., Dietrich, J., Gray, G., Bekker, L. G., Froissart, R., & Passmore, J. A. S. (2020). Exploring potential of vaginal *Lactobacillus* isolates from South African women for enhancing treatment for bacterial vaginosis. *PLoS Pathogens, 16*, Article e1008559. https://doi.org/10.1371/journal.ppat.1008559

Hu, H. W., Wang, J. T., Li, J., Li, J. J., Ma, Y. B., Chen, D., & He, J. Z. (2016). Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils. *Environmental Microbiology, 18*, 3896–3909. https://doi.org/10.1111/1462-2920.13370

Imre, K., Ban-Cucerzan, A., Herman, V., Sallam, K. I., Cristina, R. T., Abd-Elghany, S. M., Morar, D., Popa, S. A., Imre, M., & Morar, A. (2022). Occurrence, pathogenic potential and antimicrobial resistance of *Escherichia coli* isolated from raw milk cheese commercialized in Banat Region, Romania. *Antibiotics, 11*, Article 721. https://doi.org/10.3390/ANTIBIOTICS11060721

Interagency Coordination Group on Antimicrobial Resistance (IACG). (2019). *No Time To Wait: Infections From Drug-Resistant Securing the Future From Drug-Resistant Infections*. Retrieved from https://cdn.who.int/media/docs/default-source/antimicrobial-resistance/amr-gcp-tjs/iacg/summaries/iacg_final_summary_en.pdf?sfvrsn=f346e650_5. Accessed September 8, 2023.

Iskandar, K., Murugaiyan, J., Halat, D. H., Hage, S. El, Chibabhai, V., Adukkadukkam, S., Roques, C., Molinier, L., Salameh, P., & Van Dongen, M. (2022). Antibiotic discovery and resistance: the chase and the race. *Antibiotics, 11,* Article 182. doi: 10.3390/ANTIBIOTICS11020182.

International Standardisation Organisation (ISO) and International Dairy Federation (IDF). (2010). *ISO 10932:2010 - Milk and milk products - Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB)*. Geneva, Switzerland: ISO and IDF.

Jauregi, L., Epelde, L., Alkorta, I., & Garbisu, C. (2021). Antibiotic resistance in agricultural soil and crops associated to the application of cow manure-derived amendments from conventional and organic livestock farms. *Frontiers in Veterinary Science, 8*, Article 633858. https://doi.org/10.3389/FVETS.2021.633858/BIBTEX

Jia, X., He, Y., Kang, Z., Chen, S., Sun, W., Wang, J., & Lai, S. (2023). Comparison of fecal microbiota communities between primiparous and multiparous cows during non-pregnancy and pregnancy. *Animals, 13*, Article 869. https://doi.org/10.3390/ANI13050869

Juliano, L. C. B., Gouvêa, F. L. R., Latosinski, G. S., Fabri, F. H. H., Salvador, T. B., Guimaraes, F. F., Ribeiro, M. G., Langoni, H., Rall, V. L. M., Hernandes, R. T., Leite, D. S., & Pantoja, J. C. F. (2022). Species diversity and antimicrobial resistance patterns of *Enterococcus* spp. isolated from mastitis cases, milking machine and the environment of dairy cows. *Letters in Applied Microbiology, 75*, 924–932. https://doi.org/10.1111/lam.13768

Kang, J., Liu, Y., Chen, X., Xu, F., Xiong, W., & Li, X. (2022). Shifts of antibiotic resistomes in soil following amendments of antibiotics-contained dairy manure. *International Journal of Environmental Research and Public Health, 19*, 10804. https://doi.org/10.3390/ijerph191710804

Karahutová, L., & Bujňáková, D. (2023). Occurrence and molecular surveillance of pathogenesis risk-associated factors in *Staphylococcus aureus* recovered from raw sheep's milk cheese. *Small Ruminant Research, 222*, Article 106967. https://doi.org/10.1016/J.SMALLRUMRES.2023.106967

Kazancıgil, E., Demirci, T., Öztürk-Negiş, H.İ., & Akın, N. (2019). Isolation, technological characterization and in vitro probiotic evaluation of *Lactococcus* strains from traditional Turkish skin bag Tulum cheeses. *Annals of Microbiology, 69*, 1275–1287. https://doi.org/10.1007/s13213-019-01512-4

Khaldi, Z., Nafti, M., & Jilani, M. T. (2022). Small ruminants milk from Tunisian oasis: Physicochemical characteristics, mineral contents, and microbiological quality. *Tropical Animal Health and Production, 54*, 1–10. https://doi.org/10.1007/S11250-021-03000-X

Khemariya, P., Singh, S., Nath, G., & Gulati, A. K. (2013). Isolation, identification, and antibiotic susceptibility of nis+ lactococcus lactis from dairy and non-dairy sources. *Czech Journal of Food Sciences, 31*, 323–331. https://doi.org/10.17221/316/2012-cjfs

Kmet', V., & Drugdová, Z. (2012). Antimicrobial susceptibility of microflora from ovine cheese. *Folia Microbiologica, 57*, 291–293. https://doi.org/10.1007/S12223-012-0128-3

Konopka, J. K., Chatterjee, P., LaMontagne, C., & Brown, J. (2022). Environmental impacts of mass drug administration programs: Exposures, risks, and mitigation of antimicrobial resistance. *Infectious Diseases of Poverty, 11*, Article 78. https://doi.org/10.1186/S40249-022-01000-Z

Lade, H., & Kim, J. S. (2021). Bacterial targets of antibiotics in methicillin-resistant *Staphylococcus aureus*. *Antibiotics, 10*, Article 398. https://doi.org/10.3390/ANTIBIOTICS10040398

Liu, N., Wang, X., Shan, Q., Xu, L., Li, Y., Chu, B., Yang, L., Wang, J., & Zhu, Y. (2022). *Lactobacillus rhamnosus* ameliorates multi-drug-resistant *Bacillus cereus*-induced cell damage through inhibition of NLRP3 inflammasomes and apoptosis in bovine endometritis. *Microorganisms, 10*, Article 137. https://doi.org/10.3390/microorganisms10010137

Majhenič, A.Č., Lorberg, P. M., & Rogelj, I. (2007). Characterisation of the *Lactobacillus* community in traditional Karst ewe's cheese. *International Journal of Dairy Technology, 60*, 182–190. https://doi.org/10.1111/j.1471-0307.2007.00336.x

Mathur, S., & Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria—a review. *International Journal of Food Microbiology, 105*, 281–295. https://doi.org/10.1016/J.IJFOODMICRO.2005.03.008

Meunier-Goddik, L., & Waite-Cusic, J. (2019). Consumers acceptance of raw milk and its products. In I. L. A. Nero, & A. F. De Carvalho (Eds.), *Raw milk: balance between hazards and benefits* (pp. 311–350). Academic Press.

Michailidou, S., Pavlou, E., Pasentsis, K., Rhoades, J., Likotrafiti, E., & Argiriou, A. (2021). Microbial profiles of Greek PDO cheeses assessed with amplicon metabarcoding. *Food Microbiology, 99*, Article 103836. https://doi.org/10.1016/J.FM.2021.103836

Morandi, S., Silvetti, T., Miranda Lopez, J. M., & Brasca, M. (2015). Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk valtellina casera cheese. *Journal of Food Safety, 35*, 193–205. https://doi.org/10.1111/jfs.12171

Muñoz, M. C. C., Benomar, N., Lerma, L. L., Gálvez, A., & Abriouel, H. (2014). Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. *International Journal of Food Microbiology, 172*, 110–118. https://doi.org/10.1016/j.ijfoodmicro.2013.11.025

Nagy, Á., Székelyhidi, R., Hanczné Lakatos, E., & Kapcsándi, V. (2021). Review on the occurrence of the *mcr*-1 gene causing colistin resistance in cow's milk and dairy products. *Heliyon, 7*, Article e06800. https://doi.org/10.1016/j.heliyon.2021.e06800

Nalepa, B., & Markiewicz, L. H. (2023). Microbiological biodiversity of regional cow, goat and ewe milk cheeses produced in poland and antibiotic resistance of lactic acid bacteria isolated from them. *Animals, 13*, Article 168. https://doi.org/10.3390/ani13010168

Navidghasemizad, S., Hesari, J., Saris, P., & Nahaei, M. R. (2009). Isolation of lactic acid bacteria from Lighvan cheese, a semihard cheese made from raw sheep milk in Iran. *International Journal of Dairy Technology, 62*, 260–264. https://doi.org/10.1111/J.1471-0307.2009.00462.X

Nunziata, L., Brasca, M., Morandi, S., & Silvetti, T. (2022). Antibiotic resistance in wild and commercial non-enterococcal Lactic Acid Bacteria and Bifidobacteria strains of dairy origin: An update. *Food Microbiology, 104*, Article 103999. https://doi.org/10.1016/J.FM.2022.103999

O'Sullivan, O., & Cotter, P. D. (2017). Microbiota of raw milk and raw milk cheeses. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese: chemistry, physics and microbiology* (pp. 301–316). Academic Press.

Obioha, P. I., Anyogu, A., Awamaria, B., Ghoddusi, H. B., & Ouoba, L. I. I. (2023). Antimicrobial resistance of lactic acid bacteria from nono, a naturally fermented milk product. *Antibiotics, 12*, Article 843. https://doi.org/10.3390/antibiotics12050843

Official Journal of the European Communities. (1996). Commission Regulation (EC) No 1107/96 of 12 June 1996 on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) No 2081/92. OJEC, 148, 1–10. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31996R1107.

Oguntoyinbo, F. A., & Okueso, O. (2013). Prevalence, distribution and antibiotic resistance pattern among enterococci species in two traditional fermented dairy foods. *Annals of Microbiology, 63*, 755–761. https://doi.org/10.1007/s13213-012-0530-8

Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., & Jespersen, L. (2017). Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus* sensu lato isolated from dairy farms and traditional dairy products. *BMC Microbiology, 17*, Article 65. https://doi.org/10.1186/s12866-017-0975-9

Patel, S. J., Wellington, M., Shah, R. M., & Ferreira, M. J. (2020). Antibiotic stewardship in food-producing animals: challenges, progress, and opportunities. *Clinical Therapeutics, 42*, 1649–1658. https://doi.org/10.1016/J.CLINTHERA.2020.07.004

Pérez-Elortondo, F. J., Albisu, M., & Barcina, Y. (1993). Changes in the microflora of Idiazabal cheese with the addition of commercial lactic starters. *Australian Journal of Dairy Technology, 48*, 10–14.

Pérez-Elortondo, F. J., Aldámiz Echobarria, P., Albisu, M., & Barcina, Y. (1998). Indigenous lactic acid bacteria in Idiazabal ewes' milk cheese. *International Dairy Journal, 8*, 725–732. https://doi.org/10.1016/S0958-6946(98)00113-7

Pérez-Elortondo, F. J., Albisu, M., & Barcina, Y. (1999). Physicochemical properties and secondary microflora variability in the manufacture and ripening of Idiazabal cheese. *Lait, 79*, 281–290.

Pérez Elortondo, F. J., Albisu, M., & Barcina, Y. (1999). Brining time effect on physicochemical and microbiological parameters in Idiazabal cheese. *International Journal of Food Microbiology, 49*, 139–149. https://doi.org/10.1016/S0168-1605(99)00068-9

Possas, A., Bonilla-Luque, O. M., & Valero, A. (2021). From cheese-making to consumption: Exploring the microbial safety of cheeses through predictive microbiology models. *Foods, 10*, Article 355. https://doi.org/10.3390/foods10020355

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews, 37*, 664–698. https://doi.org/10.1111/1574-6976.12030

Rajput, K., Dubey, R. C., & Kumar, A. (2022). Probiotic potential and immunomodulatory properties in *Enterococcus faecium* GMB24 and *Enterococcus hirae* SMB16 isolated from goat and sheep milk. *Archives of Microbiology, 204*, 619. https://doi.org/10.1007/s00203-022-03217-w

Rocha, R., Couto, N., Pinto, R. P., Vaz-Velho, M., Fernandes, P., & Santos, J. (2023). Microbiological characterization of protected designation of origin serra da estrela cheese. *Foods*, *12*, Article 2008. doi: 10.3390/FOODS12102008.

Rola, J. G., Czubkowska, A., Korpysa-Dzirba, W., & Osek, J. (2016). Occurrence of *Staphylococcus aureus* on farms with small scale production of raw milk cheeses in poland. *Toxins, 8*, Article 62. https://doi.org/10.3390/TOXINS8030062

Rosander, A., Connolly, E., & Roos, S. (2008). Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Applied and Environmental Microbiology, 74*, 6032–6040. https://doi.org/10.1128/AEM.00991-08

Rüegsegger, F., Ruf, J., Tschuor, A., Sigrist, Y., Rosskopf, M., & Hässig, M. (2014). Antimicrobial susceptibility of mastitis pathogens of dairy cows in Switzerland. *Schweizer Archiv Fur Tierheilkunde, 156*, 483–488. https://doi.org/10.1024/0036-7281/a000635

Ruiz-Moyano, S., Gonçalves dos Santos, M. T. P., Galván, A. I., Merchán, A. V., González, E., de Córdoba, M. G., & Benito, M. J. (2019). Screening of autochthonous lactic acid bacteria strains from artisanal soft cheese: Probiotic characteristics and prebiotic metabolism. *LWT, 114*, Article 108388. https://doi.org/10.1016/j.lwt.2019.108388

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., Puente, S. L., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R. E., Wallace, R. J., Wester, P., Glandorf, B., Herman, L., Kärenlampi, S., Aguilera, J., Anguita, M., Brozzi, R., & Galobart, J. (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal, 16*, Article 5206.

Şanlıbaba, P. (2022). Prevalence, antibiotic resistance, and enterotoxin production of *Staphylococcus aureus* isolated from retail raw beef, sheep, and lamb meat in Turkey. *International Journal of Food Microbiology, 361*, Article 109461. https://doi.org/10.1016/J.IJFOODMICRO.2021.109461

Santamarina-García, G., Amores, G., Hernández, I., Morán, L., Barrón, L. J. R., & Virto, M. (2023). Relationship between the dynamics of volatile aroma compounds and microbial succession during the ripening of raw ewe milk-derived Idiazabal cheese. *Current Research in Food Science, 6*, Article 100425. https://doi.org/10.1016/J.CRFS.2022.100425

Santamarina-García, G., Amores, G., López de Armentia, E., Hernández, I., & Virto, M. (2022). Relationship between the dynamics of gross composition, free fatty acids and biogenic amines, and microbial shifts during the ripening of raw ewe milk-derived idiazabal cheese. Article 3224 *Animals, 12*. https://doi.org/10.3390/ani12223224.

Santamarina-García, G., Fresno, J. M., Virto, M., Amores, G., & Aranceta, J. (2020). Cheese microbiota and its functional importance. *Revista Espanola de Nutricion Comunitaria-Spanish Journal of Community Nutrition, 26*, 248–256.

Santamarina-García, G., Hernández, I., Amores, G., & Virto, M. (2022). Characterization of microbial shifts during the production and ripening of raw ewe milk-derived idiazabal cheese by high-throughput sequencing. *Biology, 11*, 769. https://doi.org/10.3390/BIOLOGY11050769

Sharma, P., Tomar, S. K., Goswami, P., Sangwan, V., & Singh, R. (2014). Antibiotic resistance among commercially available probiotics. *Food Research International, 57*, 176–195. https://doi.org/10.1016/j.foodres.2014.01.025

Shi, H., Zhou, M., Zhang, Z., Hu, Y., Song, S., Hui, R., Wang, L., Li, G., & Yao, L. (2023). Molecular epidemiology, drug resistance, and virulence gene analysis of *Streptococcus agalactiae* isolates from dairy goats in backyard farms in China. *Frontiers in Cellular and Infection Microbiology, 12*, Article 1049167. https://doi.org/10.3389/fcimb.2022.1049167

Slyvka, I., Tsisaryk, O., Musii, L., Kushnir, I., Koziorowski, M., & Koziorowska, A. (2022). Identification and Investigation of properties of strains *Enterococcus* spp. Isolated from artisanal Carpathian cheese. *Biocatalysis and Agricultural Biotechnology, 39*, Article 102259. https://doi.org/10.1016/J.BCAB.2021.102259

Sobierajski, T., Mazińska, B., Chajęcka-Wierzchowska, W., Śmiałek, M., & Hryniewicz, W. (2022). Antimicrobial and antibiotic resistance from the perspective of polish veterinary students: an inter-university study. *Antibiotics, 11*, Article 115. https://doi.org/10.3390/ANTIBIOTICS11010115

Sozzi, T., & Smiley, M. B. (1980). Antibiotic resistances of yogurt starter cultures. *Streptococcus thermophilus and Lactobacillus bulgaricus*. *Applied and Environmental Microbiology, 40*, 862–865. https://doi.org/10.1128/aem.40.5.862-865.1980

Štšepetova, J., Taelma, H., Smidt, I., Hütt, P., Lapp, E., Aotäht, E., & Mändar, R. (2017). Assessment of phenotypic and genotypic antibiotic susceptibility of vaginal *Lactobacillus* sp. *Journal of Applied Microbiology, 123*, 524–534. https://doi.org/10.1111/JAM.13497

Sun, G., Zhang, H., Wei, Q., Zhao, C., Yang, X., Wu, X., Xia, T., Liu, G., Zhang, L., Gao, Y., Sha, W., & Li, Y. (2019). Comparative analyses of fecal microbiota in European mouflon (*Ovis orientalis musimon*) and blue sheep (*Pseudois nayaur*) living at low or high altitudes. *Frontiers in Microbiology*, *10*, Article 1735. doi: 10.3389/fmicb.2019.01735.

Tosi, L., Berruti, G., Danielsen, M., Wind, A., Huys, G., & Morelli, L. (2007). Susceptibility of *Streptococcus thermophilus* to antibiotics. *Antonie van Leeuwenhoek, 92*, 21–28. https://doi.org/10.1007/s10482-006-9130-6

Tsigkrimani, M., Panagiotarea, K., Paramithiotis, S., Bosnea, L., Pappa, E., Drosinos, E. H., Skandamis, P. N., & Mataragas, M. (2022). Microbial Ecology of Sheep Milk, Artisanal Feta, and Kefalograviera Cheeses. Part II: Technological, Safety, and Probiotic Attributes of Lactic Acid Bacteria Isolates. *Foods*, *11*, Article 459. doi: 10.3390/foods11030459.

United Nations (UN), 2015. *The 2030 Agenda for Sustainable Development*. Retrieved from https://sdgs.un.org/publications/transforming-our-world-2030-agenda-sustainable-development-17981. Accesed June 25, 2023.

Vidal, C. A., & Collins-Thompson, D. L. (1987). Resistance and sensitivity of meat lactic acid bacteria to antibiotics. *Journal of Food Protection, 50*, 737–740. https://doi.org/10.4315/0362-028X-50.9.737

Virto, M., Santamarina-García, G., Amores, G., & Hernández, I. (2022). Antibiotics in dairy production: Where is the problem? *Dairy, 3*, 541–564. https://doi.org/10.3390/dairy3030039

Výrostková, J., Regecová, I., Dudriková, E., Marcinčák, S., Vargová, M., Kováčová, M., & Mal'ová, J. (2021). Antimicrobial resistance of *Enterococcus* sp. Isolated from sheep and goat cheeses. *Foods*, *10*, Article 1844. doi: 10.3390/foods10081844.

Výrostková, J., Regecová, I., Kováčová, M., Marcinčák, S., Mal'ová, J., & Dudriková, E. (2020). Antimicrobial resistance of *Lactobacillus johnsonii* and *Lactobacillus zeae* in raw milk. *Processes*, *8*, Article 1627. doi: 10.3390/PR8121627.

Wall, B.A., Mateus, A., Marshall, L., Pfeiffer, D., Lubroth, J., Ormel, H.J., Otto, P., Patriarchi, A., 2016. Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Retrieved from https://www.fao.org/documents/card/en?details=d5f6d40d-ef08-4fcc-866b-5e5a92a12dbf/. Accessed June 8, 2023.

Wang, H. H., Manuzon, M., Lehman, M., Wan, K., Luo, H., Wittum, T. E., Yousef, A., & Bakaletz, L. O. (2006). Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. *FEMS Microbiology Letters, 254*, 226–231. https://doi.org/10.1111/J.1574-6968.2005.00030.X

Wang, J., Li, M., Wang, J., Liu, M., Yang, K. U. N., Zhang, J. I. E., Fan, M., & Wei, X. (2018). Antibiotic resistance of coagulase-negative staphylococci and lactic acid bacteria isolated from naturally fermented chinese cured beef. *Journal of Food Protection, 81*, 2054–2063. https://doi.org/10.4315/0362-028X.JFP-18-195

Wang, Y., Xu, Y., Guo, X., Wang, L., Zeng, J., Qiu, H., Tan, Y., Chen, D., Zhao, H., & Gu, Y. (2022). Enhanced antimicrobial activity through the combination of antimicrobial photodynamic therapy and low-frequency ultrasonic irradiation. *Advanced Drug Delivery Reviews, 183*, Article 114168. https://doi.org/10.1016/J.ADDR.2022.114168

Waseem, H., Jameel, S., Ali, J., Ur Rehman, H. S., Tauseef, I., Farooq, U., Jamal, A., & Ali, M. I. (2019). Contributions and challenges of high throughput qPCR for determining antimicrobial resistance in the environment: A critical review. *Molecules, 24*, Article 163. https://doi.org/10.3390/molecules24010163

Wei, N., Lu, J., Dong, Y., & Li, S. (2022). Profiles of microbial community and antibiotic resistome in wild tick species. *MSystems, 7*, Article e00037–22. https://doi.org/10.1128/msystems.00037-22

Winand, R., Bogaerts, B., Hoffman, S., Lefevre, L., Delvoye, M., Van Braekel, J., Fu, Q., Roosens, N. H. C., De Keersmaecker, S. C. J., & Vanneste, K. (2019). Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (illumina) and third (oxford nanopore technologies) generation sequencing technologies. *International Journal of Molecular Sciences, 21*, Article 298. https://doi.org/10.3390/IJMS21010298

Wolfe, B. E. (2023). Are fermented foods an overlooked reservoir of antimicrobial resistance? *Current Opinion in Food Science, 51*, Article 101018. https://doi.org/10.1016/J.COFS.2023.101018

World Health Organization (WHO), 2022. Antimicrobial resistance. Retrieved from https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed February 18, 2023.

Yang, L. Y., Zhou, S. Y. D., Lin, C. S., Huang, X. R., Neilson, R., & Yang, X. R. (2022). Effects of biofertilizer on soil microbial diversity and antibiotic resistance genes. *Science of the Total Environment, 820*, Article 153170. https://doi.org/10.1016/j.scitotenv.2022.153170

Yap, M., Ercolini, D., Álvarez-Ordóñez, A., O'Toole, P. W., O'Sullivan, O., & Cotter, P. D. (2022). Next-generation food research: use of meta-omic approaches for characterizing microbial communities along the food chain. *Annual Review of Food Science and Technology, 13*, 361–384. https://doi.org/10.1146/ANNUREV-FOOD-052720-010751

Yasir, M., Al-Zahrani, I. A., Bibi, F., Abd El Ghany, M., & Azhar, E. I. (2022). New insights of bacterial communities in fermented vegetables from shotgun metagenomics and identification of antibiotic resistance genes and probiotic bacteria. *Food Research International, 157*, Article 111190. https://doi.org/10.1016/J.FOODRES.2022.111190

Zhai, Z., Cui, C., Li, X., Yan, J., Sun, E., Wang, C., Guo, H., & Hao, Y. (2023). Prevalence, antimicrobial susceptibility, and antibiotic resistance gene transfer of *Bacillus* strains isolated from pasteurized milk. *Journal of Dairy Science, 106*, 75–83. https://doi.org/10.3168/jds.2022-22199

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology, 7*, 203–214. https://doi.org/10.1089/10665270050081478

Zhao, J., Liu, R., Sun, Y., Yang, X., & Yao, J. (2022). Tracing enterococci persistence along a pork production chain from feed to food in China. *Animal Nutrition, 9*, 223–232. https://doi.org/10.1016/J.ANINU.2022.01.005

Zheng, B., Feng, C., Xu, H., Yu, X., Guo, L., Jiang, X., & Song, X. (2019). Detection and characterization of ESBL-producing *Escherichia coli* expressing *mcr*-1 from dairy cows in China. *Journal of Antimicrobial Chemotherapy, 74*, 321–325. https://doi.org/10.1093/jac/dky446

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology, 70*, 2782–2858. https://doi.org/10.1099/ijsem.0.004107

Zhou, J. S., Pillidge, C. J., Gopal, P. K., & Gill, H. S. (2005). Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology, 98*, 211–217. https://doi.org/10.1016/j.ijfoodmicro.2004.05.011

This article was published in Food Research International, Vol. 187, Gorka Santamarina-García, Gustavo Amores, Diego Llamazares, Igor Hernández, Luis Javier R. Barron, Mailo Virto, Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese, 114308, Copyright Elsevier Ltd. (2024)

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Doctoral thesis

Metagenomics of Idiazabal cheese: elucidating the microbiota, its impact on cheese quality and safety, and factors affecting its composition

Gorka Santamarina-García

OVERVIEW

The present doctoral thesis was conducted within the Lactiker Research Group (UPV/EHU), which focuses on the quality and safety of animal-origin foods, particularly the Protected Designation of Origin Idiazabal cheese. This cheese, produced in small artisanal dairies in the Basque Country from Latxa or Carranzana raw ewe milk, undergoes a minimum 60-day ripening period. Raw milk cheeses, compared to those produced from pasteurized milk, are known for their rich aroma due to microbial dynamics during production. However, limited information exists on the microbiota of raw ewe milk cheeses, including Idiazabal cheese, and its subsequent impact on cheese quality and safety. Therefore, this thesis aimed (1) to characterise the microbiota of Idiazabal cheese and how it evolves by amplicon sequencing, (2) to explore its impact on cheese quality (gross composition, free fatty acids, and volatile compounds) and safety parameters (biogenic amines and antimicrobial resistances) determined by several approaches, and (3) to investigate key production factors (including herd feed, teat skin, dairy surfaces, and processing ingredients) affecting the microbial composition of raw ewe milk and Idiazabal cheese, and its genetic potential in terms of quality and safety by shotgun sequencing. Results revealed significant differences in the microbiota among producers, which affected cheese quality and safety parameters, and were related to microbial niches in dairy environments. This research contributes valuable novel insights not only for the scientific community, as information on raw ewe milk cheeses is limited, but also for Idiazabal cheese producers, facilitating improvements in the quality and safety of the final product.

ABOUT THE AUTHOR

Gorka Santamarina García holds a bachelor's degree in Food Science and Technology (2018) from the University of the Basque Country (UPV/EHU) and a master's degree in Food Quality and Safety (2019), also from the UPV/EHU. Throughout the development of this international doctoral thesis, he served as a predoctoral researcher at the Lactiker Research Group – Quality and Safety of Foods from Animal Origin and at the Department of Biochemistry and Molecular Biology at the Faculty of Pharmacy of the UPV/EHU. His research focuses on characterizing the microbiota of fermented foods, particularly raw milk cheeses, using a range of technologies including culture-dependent and culture-independent next-generation sequencing (NGS) techniques, to assess through bioinformatics, chemical, biochemical and statistical analyses the functional and metabolic impact on food quality and safety. He has participated in several research projects, authored various scientific papers in prestigious journals, conference contributions, scientific divulgation articles and other scientific divulgation contributions like media interviews. Throughout this PhD project, he has also completed a research stay at Teagasc - Irish Agriculture and Food Development Authority and he has additionally collaborated as assistant professor in the Department of Biochemistry and Molecular Biology at the Faculty of Pharmacy of the UPV/EHU.

ORCID: 0000-0002-2495-3709

CITATION

Santamarina-García, G. (2024). *Metagenomics of Idiazabal cheese: elucidating the microbiota, its impact on cheese quality and safety, and factors affecting its composition* [Doctoral thesis, University of the Basque Country].