eman ta zabal zazu

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DOKTOREGO-TESIA

TESIS DOCTORAL

Technical factors affecting cheese properties, yield and whey generated from raw sheep milk during cheesemaking in small rural dairies

Landa-gaztandegi txikietan ardi-esne gordinarekin ekoiztutako gaztaren ezaugarri, errendimendu eta gatzurari eragiten dieten faktore teknologikoak

Factores tecnológicos que afectan a las propiedades del queso, rendimiento y lactosuero generado a partir de leche cruda de oveja durante la producción de queso en pequeñas queserías rurales

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Elikagaien Kalitatea eta Segurtasunean Doktore Gradua lortzeko memoria

Memoria para optar al Grado de Doctor en Calidad y Seguridad Alimentaria

Dirigida por: Luis Javier R. Barron y Mª Ángeles Bustamante

Ane Aldalur Soto

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Ama

Aita

eta Joni

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ABBREVIATIONS AND SYMBOLS

2D	two-dimension
3D	three-dimension
AAS	atomic absorption spectroscopy
ANOVA	analysis of variance
AOAC	Association of Official Agricultural Chemists
BOD	biochemical oxygen demand
Ca	calcium
CGS	curd grain size
CH	pressed cheese
CH1mo	1-month ripened cheese
CLSM	confocal laser scanning microscopy
COD	chemical oxygen demand
CS	cutting speed
CT	cooking temperature
CTS	cutting tip speed
CTY	circularity
C_u	coefficient of uniformity
D[4,3]	volume-weighted mean diameter
DM	dry matter
EU	European Union
F/C	fat to casein ratio
F/P	fat to protein ratio
FCG	fresh curd grains
FG	fat globule
G''	loss modulus
G'	storage modulus
GF	globular fat
GLM	general linear model
ICP-OES	inductively coupled plasma-optical emission spectroscopy
IDF	International Dairy Federation
IMCU	international milk coagulating units
ISO	International Organization for Standardization
I_u	uniformity index

KD	knife density
K_g	graphic kurtosis
Mg	magnesium
NCF	number of cutting frames
NGF	non-globular fat
NIR	near infrared spectroscopy
P	phosphorous
PC	principal component
PCA	principal component analysis
PDO	Protected Denomination of Origin
PLSR	partial least square regression
PSD	particle size distribution
RAR	elongation
RH	relative humidity
RTY	rectangularity
RU	rennet units
SCC	somatic cell count
SCG	stirred curd grains
cryo-SEM	cryo-scanning electron microscopy
SEM	standard error of the mean
S_g	graphic skewness
S_l	relative span
SNF	solids-not-fat
S_v	size range variation coefficient
T	temperature
TPA	texture profile analysis
UPV/EHU	University of the Basque Country
WFCG	whey after the cutting process
WSCG	whey after the cooking process
Y_A	actual cheese yield
Y_{CA}	composition-adjusted cheese yield
Y_{MA}	moisture-adjusted cheese yield
Y_T	theoretical cheese yield

UNITS

%	percentage
º	degree
ºC	degree Celsius
ºBé	degree Baumé
v	volume
w	weight
U	units
RU	rennet units
s	second
min	minute
h	hour
d	day
mo	month
mg	milligram
g	gram
kg	kilogram
rev	revolution
rpm	revolution *per* minute
nm	nanometre
µm	micrometre
mm	millimetre
cm	centimetre
m	metre
mm^2	square millimetre
cm^2	square centimetre
m^2	square metre
µL	microlitre
mL	millilitre
L	litre
mM	millimolar
M	molar
Pa	Pascal
V	volt
kV	kilovolt
mA	milliampere
Hz	Hertz

ABSTRACT

The present Ph. D. Thesis has been performed within the Lactiker Research Group at the University of the Basque Country (UPV/EHU), which is dedicated to performing multidisciplinary research in the field of Quality and Safety of Foods of Animal Origin. The work carried out is contextualised in small rural dairies that manufacture Idiazabal PDO sheep cheese in the Basque Country region of Northern Spain, and was conducted in close collaboration with the cheese-makers.

Idiazabal PDO cheese is a semi-hard cheese manufactured using raw milk from Latxa and Carranzana sheep, which has to be ripened for a minimum of 2 months before consumption. Most of Idiazabal dairies (85%) are small family farms that manage the whole production chain, from the flock management to the commercialization of the cheeses in local markets. Their technical facilities are usually limited and the role of the cheese-makers during the manufacture of the cheese is crucial. Due to the economic and technological limitations, the collection, or reutilization and conversion of the whey produced during the cheesemaking process into value-added products have a finite real world application in the small rural dairies. Therefore, most of the whey currently generated is discharged into the environment and this could cause serious environmental problems. The aim of this Ph. D. Thesis is to contribute to the sustainability of the cheese production by adjusting the technical settings to the facilities available in the small rural dairies, in order to improve cheese yield and reduce the organic load and amount of the whey produced without affecting the properties of the cheese.

The cheesemaking technical factors that affect cheese properties, yield and milk compound losses in the whey have been previously reviewed, although most research has been conducted using bovine milk on a laboratory scale. Studies carried out on this topic at a commercial scale, or in milks other than bovine are especially scarce, even though the coagulation properties and syneresis have been reported to clearly differ among milks from different species. The main objective of this Ph. D. Thesis is to assess the impact of

cheesemaking technical conditions on curd grain characteristics, whey composition, and cheese properties and yield during the cheese production season. Three different studies were carried out to accomplish the objectives set: a two-year observational study in eight small rural dairies that manufacture Idiazabal cheese, an interventional study in two small rural Idiazabal dairies, and a controlled experimental study on a laboratory scale.

The first specific objective was to assess the current cheesemaking practices carried out in small rural dairies that manufacture Idiazabal cheese, and the impact of the technical conditions on the composition and properties of curd, cheese and whey as well as yield during the cheese production season. The effect of the seasonal changes on the composition of raw sheep milk together with the technological settings used for cheesemaking on cheese yield and whey composition were also investigated. The present work reported that the technical parameters used for the manufacturing of Idiazabal cheese, especially those regarding cutting and cooking processes, largely varied among small rural dairies during the cheese production season. However, it was observed that the cheese-makers did not adjust the technical settings to the seasonal changes of milk composition, which was unexpected. The content of fat, protein, calcium, magnesium, phosphorous and total solids of the milk increased from late winter to early summer, but due to the similar animal feeding and flock management systems no significant differences were observed among dairies. The changes in the composition of milk and the technical settings used during the cheese manufacturing clearly affected the cheese yield and milk compound losses in the whey. Higher cheese yield values were obtained in early summer compared to late winter (20.0 *vs* 17.2 kg of cheese/100 kg of milk, respectively) for all the dairies, with differences of 2-3 kg/100 kg *per* cheese production among the dairies. Additionally, milk compound losses in the whey also increased during early summer, and the conditions used during cutting and cooking especially affected the fat losses in the whey. The relationships between the cutting and cooking technical settings, cheese yield and milk compound losses in the whey were studied using principal component and partial least square regression analyses. The results indicated that higher cheese yield values were obtained when lower cooking temperatures and shorter cutting and cooking times were employed during the cheesemaking. On the other hand, the results showed that high cutting and cooking speeds remarkably increased fat and casein losses in the whey. In particular, a short cutting duration together with a high cooking speed was observed to increment fat losses in the whey due to the shattering of the curd grains. However, the speed used during cooking was more determinant than the size of the curd grain after the cutting process, in regards to the losses in the whey. Accordingly, the chemical oxygen demand of the whey also increased when the fat, protein and mineral content of the whey was higher. The composition of cheese among small rural dairies also varied due to the cutting, cooking, pressing and ripening conditions employed in each dairy. Cooking temperature especially seemed to be the main parameter responsible for the changes in the cheese composition. The results obtained showed that the cheesemaking process currently carried out in small rural dairies that manufacture Idiazabal cheese has room for improvement and that the process should be adapted to the facilities of each dairy.

A detailed study using 2-dimensional image analysis was performed to characterize the size, shape and particle size distribution of curd grains in Idiazabal cheese productions. As mentioned above, the wide variation of the cutting and cooking parameters used in the small rural dairies during the cheese production season caused large variations in the size, shape and size distribution of the curd grains. The size of the curd grains after cutting ranged from 11.66 to 56.82 mm^2 among dairies, and after the cooking process, it varied from 8.20 to 27.23 mm^2. Linear relationships between the size of the curd grains after cutting and cooking and the technical settings used were established. The size distribution indicators of the curd grains showed that the particles generated during cheesemaking in the small dairies were highly heterogeneous regardless of the technical cutting and cooking settings used. Moreover, it was observed that the cooking process did not improve the homogeneity of the particle size distribution as expected and that, in some cases, the heterogeneity even increased after this process. This was caused by the presence of a small amount of big curd grains after cooking together with a general size reduction of the curd particles. The shape of the curd grains at the end of the cooking process was reported to be less elongated and more circular than the ones after cutting, due to the collisions between the curd grains as well as the curd grains and the equipment. The design and arrangement of the cutting tools used during the cutting process, and the duration of the cooking process, were the main parameters responsible for the final shape of the curd grains. In summary, the size, shape and particle size distribution together with the composition, affect the deformation degree of the curd grains and, consequently, their compaction during pressing, which seems to be related to the final properties of the cheese. Therefore, the 2-dimensional image analysis is a useful tool for supervising the cutting and cooking processes, although its implementation in small rural dairies as a routine control tool currently seems hardly feasible.

Confocal laser scanning microscopy was used to observe the changes in the microstructure of the raw sheep milk, curd and cheese during cheese manufacturing. The two-dimensional images captured using this technique were reconstructed into three-dimensional information, which allowed fat and protein to be visualised and quantified. Using this information, a detailed description of the microstructure of milk, curd and cheese samples of Idiazabal cheese manufactured in small rural dairies was carried out for the first time. Additionally, cryo scanning electron microscopy was used in the experimental study on a laboratory scale to observe in detail the microstructure of the cheeses. Moreover, the influence of the technical settings on the microstructure of the curd grains and cheese was studied in the interventional study. The intensity of the cutting did not have marked effects on the microstructure of the curd grains. A high intensity cooking process, however, increased the volume of non-globular fat and decreased the porosity and the amount of globular fat in the curd grains, and this was reflected in the cheese after pressing. The cooking temperature was considered the main technical parameter influencing the porosity and the structural arrangement of the fat droplets. These changes in the microstructure also had important effects on the textural properties of the cheese. When higher cooking temperatures were used, the hardness and chewiness of the cheeses increased, and additionally some correlations between microstructural and textural properties of pressed cheese were observed. Anyhow, further experimental research should be conducted to elucidate the interactions between cheese properties and technical settings.

The work carried out in this Ph. D. Thesis contributes useful information about the effects of cheesemaking technical settings on curd, cheese, and whey properties from raw sheep milk in commercial productions, which is particularly limited. The results obtained confirm that the cheese manufacturing process currently carried out in small rural Idiazabal dairies could be improved by adapting some of the technical parameters studied, without affecting the properties of the cheese. This information is especially useful for cheese-makers in order to adapt the technical settings to their particular facilities, which would contribute to the economic and environmental sustainability of small rural dairies.

LABURPENA

Doktorego-Tesi hau Animalia Jatorriko Elikagaien Kalitatea eta Segurtasuna ikertzea helburu duen Euskal Herriko Unibertsitateko (UPV/EHU) Lactiker jakintza arlo anitzeko iker-taldean burutu da. Egindako lana, landa-gaztandegi txikietan ekoiztutako Idiazabal babestutako jatorri-deitura duen gaztaren testuinguruan egin da, gaztagileekin elkarlanean.

Idiazabal gazta Latxa eta Karranzana ardi-esne gordinarekin ekoiztutako gazta da, eta gutxienez 2 hilabetez ondu behar da kontsumitu baino lehen. Idiazabal gaztandegien gehiengoa (%85) ustiategi familiarrak dira, ardien maneiutik merkatu lokaletako gazten merkaturatzeraino kate guztia kudeatzen dutenak. Orokorrean, haien instalazio teknikoak oso mugatuak dira eta gaztagileen rola ezinbestekoa da. Muga ekonomiko eta teknologiko hauengatik, gatzuraren biltze, berrerabilpen edota balio-erantsia duten produktuen ekoizpena oso mugatutako aplikazio erreala duten konponbideak dira landa-gaztandegi txikietan. Beraz, gaur egun gaztandegi hauetan sortzen den gatzuraren gehiengoa ingurumenera botatzen da, eta honek arazo larriak eragin ditzake baserri-inguruneetan. Doktorego-Tesi honen interesa gazta-ekoizpenaren jasangarritasunari laguntzea da, gazta ekoizpenean erabilitako baldintza teknikoak landa-gaztandegietako instalazioetara doituz. Honela, gaztaren errendimendua handitu eta gatzuraren kantitate eta karga organikoa txikiagotuko litzateke gaztaren ezaugarriak kaltetu gabe.

Gaztaren ezaugarri eta esne osagaien gatzuraren bidezko galerari eragiten dieten faktore teknologikoak aldez aurretik ikertuak izan dira. Hala ere, ikerlan hauen gehiengoa behi-esnea erabiliz eta laborategiko eskalan eginiko ikerketak izan dira. Bereziki urriak dira eskala komertzialean eta abere-espezie ezberdinekin gai honen harira argitaratutako ikerlanak. Dena den, frogatu da koagulazio- eta sineresi-ezaugarriak desberdinak direla abere-mota ezberdinen esneen artean. Doktorego-Tesi honen helburu nagusia gazta ekoizpenean erabilitako baldintza teknologikoek gatzatuaren granulu, gatzura eta gaztaren ezaugarri eta errendimenduan duten inpaktua aztertzea

da. Horretarako, hiru ikerketa burutu ziren: bi urteko behaketa estudio bat zortzi Idiazabal landa-gaztandegi txikietan, parte-hartze ikerlan bat bi Idiazabal landa-gaztandegietan eta azkenik, kontrolatutako ikerketa esperimental bat laborategiko eskalan.

Lehenengo helburu espezifikoa gaur egun landa-gaztandegi txikietan erabiltzen diren baldintza teknologikoak ebaluatzea, eta baldintza hauek gatzatuan, gatzuran eta gaztaren konposizio, ezaugarri eta errendimenduan duten inpaktua ebaluatzea izan zen. Halaber, gaztaren errendimendu eta gatzuraren konposizioan ardi-esne gordinaren urtaroko aldaketa eta honekin batera baldintza teknologikoen efektua ikertu zen. Lan honetan ikusi da Idiazabal gazta ekoizten duten landa-gaztandegietan erabilitako parametro teknologikoak oso ezberdinak direla gaztandegien artean, batez ere gatzatuaren mozketa eta beroketa prozesuetan. Hala ere, gaztagileek ez zituzten baldintza teknologiko hauek esnearen urtaroko konposizio-aldaketei doitu. Esnearen gantz, proteina, kaltzio, magnesio, fosforo eta solido totalen edukia negu amaieratik uda hasierara areagotu egin zen, baina ez zen gaztandegien arteko ezberdintasunik behatu ardien elikadura eta maneiua oso antzekoa baita haien artean. Urtaroko esne konposizioaren aldaketek eta gaztandegietan erabilitako baldintza teknologikoek gaztaren errendimenduan eta esne osagaien gatzuraren bidezko galeran eragin handia daukatela baieztatu zen. Gaztaren errendimendua gaztandegi guztietan areagotu egin zen udaren hasieran (17.2 *vs* 20.0 kg gazta/100 kg esne), eta baldintza teknologikoen eraginez 2-3 kg/100kg-ko desberdintasunak aztertu ziren gaztandegien artean. Gainera, udan esne osagaien galera ere handiagoa izan zen eta parametro teknologikoek eragin handia izan zuten, batez ere gantzaren gatzuraren bidezko galeran. Osagai nagusien analisi eta karratu txiki partzialen erregresioak erabili ziren mozketa eta berotze baldintza teknologikoen, gaztaren errendimenduaren eta esne-osagaien galeren arteko erlazioak behatzeko. Gazta ekoizpenean zehar berotze tenperatura baxuagoak eta bai mozketa bai berotze denbora motzagoak erabili zirenean, gaztaren errendimendua handiagoa izan zen. Bestalde, emaitzek erakutsi zuten mozketa eta berotze prozesuetan erabilitako abiadura altuagoek gantz-eta kaseina-galerak nabarmenki handitzen zituztela. Bereziki, mozte denbora motzek eta berotze abiadura handiek gantz-galerak areagotu zituzten gatzatu-granuluen xehatzeagatik. Hala ere, gantz galerei dagokionez berotze prozesuan erabilitako abiadurek eragin handiagoa zutela aztertu zen, mozketa prozesuaren ondorioz sortutako gatzatu-granuluen tamainak baino. Ondorioz, gatzuraren oxigeno-eskari kimikoa handiagotu egin zen gatzuraren gantz, proteina eta mineralen kantitatea areagotzean. Gaztaren konposizioa ere ezberdina izan zen gaztandegien arteko mozketa, berotze, prentsatze eta ontze baldintzen diferentziaren ondorioz. Dena den, berotze tenperatura izan zen bereziki aldaketa hauen arduradun nagusia. Lortutako emaitzek, Idiazabal gazta ekoizten duten landa-gaztandegietako gazta produkzio prozesua hobetu daitekeela eta prozesu hori gaztandegi bakoitzera moldatu behar dela adierazten dute.

Idiazabal gaztaren ekoizpenean zehar gatzatu-granuluen tamaina, forma eta tamaina banaketa aztertu zen bi dimentsioetako irudi analisia erabiliz. Lehenago aipatu bezala, landa-gaztandegietan erabilitako baldintza teknologiko anitzek granuluen tamaina, forma eta tamaina banaketan eragin handia izan zuten. Mozketa prozesuaren ondoren, gatzatu-pikorren tamaina 11,66 eta 56,82 mm^2 artean ibili zen, eta berotze prozesua eta gero 8,20 eta 27,23 mm^2 artean. Erlazio linealak ezarri ziren mozte eta berotzeen parametro tekniko eta granuluen tamainaren artean. Tamainaren banaketaren adierazleek gaztaren ekoizpen prozesuan sortutako gatzatu-pikorrak oso heterogeneoak zirela adierazi zuten, baldintza teknikoen menpekotasunetik at. Gainera, berotze prozesuak banaketaren homogeneotasuna hobetzen ez zuela ikusi zen, espero ez bezala, eta kasu batzuetan heterogeneotasuna handiagotu ere egin zen. Hau tamaina handiko granulu kopuru txiki baten eraginez gertatu zen, gatzatu-pikorren tamaina erredukzio orokorrarekin batera. Bestalde, berotze prozesuaren ondoren granuluen forma zirkularragoa izan zen mozketa prozesuaren pikorrekin konparatuta, granuluen arteko talka eta pikor eta ekipamenduaren arteko kolpeen eraginez. Beraz, gatzatu-pikoren amaierako forma, bereziki, mozketarako erabilitako tresnen diseinu eta antolaketa, eta berotze prozesuko denboraren ondorio da.

Halaber, gatzatu-granuluen tamaina, forma eta tamainen banaketa, haien konposizioarekin batera, pikorren deformazio graduari eragiten diete eta honek, aldi berean, prentsatzean zeharreko zanpatze graduari eta gaztaren amaierako ezaugarriengan eragina du. Beraz, bi dimentsioetako irudi analisia mozketa eta beroketa prozesuak kontrolatzeko tresna erabilgarria da, nahiz eta gaur egun errutinazko erreminta bezala landa-gaztandegietan inplementatzea bideragarria ez izan.

Parte-hartze ikerketan eta ikerlan esperimentalean, ardi- esnearen, gatzatuaren eta gaztaren mikroegituraren aldaketak behatu ziren laser ekorketa bidezko mikroskopia konfokala erabiliz. Gantz eta proteina irudikatu eta kuantifikatzeko, bi dimentsioetako irudiak 3 dimentsioetako informazioan bilakatu ziren. Gainera, Idiazabal gazta ekoizte prozesuan sortutako esne-matrizeen mikroegituraren deskribapen xehea lehenengo aldiz egin zen. Bestalde, laborategiko eskalan eginiko ikerlan esperimentalean, ekorketa bidezko krio-mikroskopia elektronikoa gaztaren mikroegitura zehatza behatzeko ere erabili zen. Halaber, parte-hartze ikerketan gaztaren produkzioan erabilitako parametro teknikoek gatzatu-pikorren eta gaztaren mikroegituran duten eragina aztertu zen. Gatzatuaren mozketaren intentsitateak ez zuen granuluen mikroegituran eragin nabarmenik izan. Hala ere, berotze prozesuan intentsitatea handiagotu zenean, gantz ez globularraren bolumena areagotu egin zen, eta proteina-sarearen porositatea eta gantz globularraren kantitatea gutxiagotu egin zen gatzatu-pikorretan, eta hau prentsatu ondorengo gaztan islatu zen. Berotze tenperatura hartu zen proteina eta gantzaren egituraren aldaketaren faktore erabakigarritzat. Aldaketa hauek era berean gaztaren testuran eragina izan zuten. Ikerlan esperimentalean, berotze tenperatura handiagoek gaztaren gogortasuna eta mastekatasuna areagotzea eragin zuten, eta gainera, prentsatutako gaztaren mikroegitura eta testuraren arteko korrelazio batzuk ere ezarri ziren. Dena den, parametro teknologiko eta gaztaren ezaugarrien arteko interakzioei buruzko ikerketa gehiago beharrezkoak dira erlazio hauek argitzeko.

Doktoretza-Tesi honetan eginiko lanak informazio aipagarri berria gehitzen du landa-gaztandegietan gaztaren ekoizpenerako erabiltzen diren faktore teknologiko eta sortutako gatzatuaren, gaztaren eta gatzuraren propietateei buruz. Lortutako emaitzek egiaztatzen dute gaur egun Idiazabal landa-gaztandegietan egiten den gazta-ekoizte prozesua hobetu daitekeela, ikertutako parametro teknologikoak moldatuz eta gaztaren ezaugarriak aldatu gabe. Emandako informazioa bereziki erabilgarria da gaztagileek parametro teknologiko hauek haien instalazioetan moldatzeko, honela landa-gaztandegi txikien ingurumen eta ekonomia jasangarritasunari lagunduz.

RESUMEN

La presente Tesis Doctoral se ha realizado dentro del Grupo de Investigación multidisciplinar Lactiker, de la Universidad del País Vasco (UPV/EHU), cuya actividad está centrada en la Calidad y Seguridad de los Alimentos de Origen Animal. El trabajo realizado se contextualiza en las pequeñas queserías rurales que elaboran queso de oveja denominación de origen protegida (DOP) Idiazabal en el País Vasco, y se realizó en estrecha colaboración con las queseras.

El queso DOP Idiazabal es un queso que se elabora a partir de leche cruda de ovejas Latxa y Carranzana, y el cual tiene que ser madurado al menos 2 meses antes de su consumo. La mayoría de las queserías Idiazabal (85%) son pequeñas explotaciones familiares que gestionan toda la cadena de producción, desde el manejo de las ovejas hasta la comercialización de los quesos en mercados locales. Sus instalaciones técnicas son normalmente limitadas y el rol de las queseras durante la elaboración del queso es muy importante. Debido a las limitaciones económicas y tecnológicas, la recolección, reutilización o conversión del lactosuero producido en productos con valor añadido tienen una aplicación real muy limitada en las pequeñas queserías rurales. Por lo tanto, hoy en día la mayoría del lactosuero que se genera en estas queserías se desecha al medio ambiente, pudiendo ocasionar serios problemas en los entornos rurales. El interés de esta Tesis Doctoral es contribuir a la sostenibilidad de la producción de queso ajustando las condiciones técnicas a las instalaciones disponibles en las pequeñas queserías rurales, mejorando el rendimiento quesero y reduciendo la carga orgánica y la cantidad de lactosuero generado sin detrimento de las propiedades del queso.

Los factores tecnológicos que afectan a las propiedades y al rendimiento del queso, además de a las pérdidas de los componentes de la leche en el lactosuero, han sido previamente revisados. Sin embargo, la mayoría de estos estudios se han llevado a cabo utilizando leche de vaca y a escala de laboratorio. Los estudios realizados en relación a este tema a escala

comercial o con leche de otras especies animales diferentes a la de vaca son especialmente escasos. Sin embargo, se ha demostrado que las propiedades de coagulación y sinéresis difieren claramente entre la leche de diferentes especies. El objetivo principal de esta Tesis Doctoral es evaluar el impacto de las condiciones tecnológicas utilizadas durante la elaboración de queso en las características de los gránulos de cuajada, composición del lactosuero y las propiedades y rendimiento del queso a lo largo de la época de producción. Se realizaron tres estudios para lograr los objetivos fijados: un estudio observacional llevado a cabo durante 2 años en ocho queserías rurales que elaboran Idiazabal, un estudio de intervención en dos queserías rurales, y un estudio experimental controlado a escala de laboratorio.

El primer objetivo específico fue evaluar los parámetros tecnológicos actualmente utilizados en las pequeñas queserías rurales que elaboran Idiazabal, y analizar el impacto de estas condiciones en la composición y propiedades de la cuajada, queso y lactosuero, así como en el rendimiento del queso durante la época de producción. Asimismo, se investigó el efecto de los cambios estacionales de la leche cruda de oveja junto con los factores tecnológicos utilizados en el rendimiento quesero y la composición del lactosuero. En el presente trabajo se observó que los parámetros tecnológicos utilizados para la elaboración del queso Idiazabal, especialmente aquellos relacionados con el corte y el recalentamiento de la cuajada, fueron muy diferentes entre las pequeñas queserías rurales. Sin embargo, los elaboradores no ajustaron los parámetros tecnológicos a los cambios estacionales en la composición de la leche. El contenido de grasa, proteína, calcio, magnesio, fósforo y solidos totales de la leche se incrementó desde finales de invierno a principios de verano, pero no se observaron diferencias entre las queserías debido a una alimentación y manejo similar de las ovejas. Los cambios en la composición de la leche y los parámetros tecnológicos utilizados durante la elaboración del queso afectaron claramente al rendimiento quesero y a la pérdida de componentes de la leche en el lactosuero. El rendimiento quesero fue mayor a principios de verano (17.2 *vs* 20.0 kg de queso/100 kg de leche) para todas las queserías, mientras que las diferencias en las prácticas tecnológicas utilizadas entre queserías provocaron diferencias de 2-3 kg/100 kg por producción de queso. Además, las pérdidas de compuestos de la leche en el lactosuero también aumentaron en verano debido a una cantidad mayor en la leche de partida, y los parámetros tecnológicos afectaron especialmente a la pérdida de grasa en el lactosuero. Se estudiaron las relaciones entre los parámetros tecnológicos de corte y recalentamiento, rendimiento quesero y las pérdidas de componentes en el lactosuero utilizado el análisis de componentes principales y de regresión de mínimos cuadrados parciales. El rendimiento quesero fue mayor cuando las temperaturas de recalentamiento fueron más bajas y cuando se utilizaron tiempos, tanto de corte como de recalentamiento, más cortos. Por otro lado, los resultados mostraron que velocidades más elevadas de corte y recalentamiento aumentaban notablemente las pérdidas de grasa y caseína en el lactosuero. En particular, las pérdidas de grasa se incrementaron con tiempos de corte cortos junto con velocidades altas de recalentamiento debido al desmenuzamiento de los gránulos de cuajada. Sin embargo, la velocidad utilizada durante el recalentamiento fue más determinante que el tamaño de los gránulos tras el corte. Asimismo, la demanda química de oxígeno del lactosuero aumentó a mayor cantidad de grasa, proteína y minerales en el lactosuero. La composición del queso también varió entre las pequeñas queserías rurales debido a las diferentes prácticas de corte, recalentamiento, prensado y maduración llevadas a cabo. Particularmente, la temperatura de recalentamiento parece ser el parámetro responsable de los cambios en la composición del queso. Los resultados obtenidos mostraron que el proceso de elaboración de queso que se lleva a cabo actualmente en las pequeñas queserías rurales de Idiazabal DOP se puede mejorar y que el proceso debe adaptarse a las instalaciones de cada quesería.

El tamaño, la forma y la distribución de tamaño de los gránulos de cuajada durante la elaboración del queso Idiazabal se caracterizó utilizando el análisis de imagen en 2 dimensiones. Como se ha mencionado anteriormente, los parámetros tecnológicos utilizados en las pequeñas queserías rurales fueron muy diferentes entre ellas, lo cual causó grandes variaciones en los tamaños, las formas y la distribución de los gránulos de cuajada. El tamaño de los gránulos tras el corte entre queserías osciló entre 11,66 y 56,82 mm^2, y después del proceso de recalentamiento varió entre 8,20 y 27,23 mm^2. Se establecieron relaciones lineales entre algunos parámetros tecnológicos de corte y recalentamiento y el tamaño de los gránulos de cuajada. Los indicadores de la distribución de los tamaños de los gránulos mostraron que las partículas generadas durante la elaboración de queso fueron muy heterogéneas con independencia de los parámetros tecnológicos utilizados. Además, se observó que el proceso de recalentamiento no mejoraba la homogeneidad de la distribución como se esperaba, e incluso que en algunos casos esta heterogeneidad aumentó durante el recalentamiento. Esto ocurrió debido a la presencia de una pequeña cantidad de gránulos grandes después del recalentamiento junto con la reducción generalizada del tamaño de las partículas. Por otro lado, la forma de los gránulos tras el recalentamiento fue menos alargada y más circular que los gránulos tras el corte, debido a las colisiones entre los gránulos y entre los gránulos y el equipamiento. Se concluyó que la forma final de los gránulos de cuajada se deriva, especialmente, del diseño y disposición de los objetos de corte junto con la duración del recalentamiento. Asimismo, el tamaño, la forma y la distribución de los tamaños de los gránulos de cuajada, junto con su composición afectan al grado de deformación de los gránulos y, en consecuencia, al grado de compactación durante el prensado, lo cual parece tener relación con las propiedades finales del queso. Por lo tanto, el análisis de imagen en 2 dimensiones es una herramienta útil para supervisar los procesos de corte y recalentamiento, aunque su implementación hoy en día como una herramienta de control rutinario no es viable en las pequeñas queserías rurales.

En los estudios de intervención y experimentales a escala de laboratorio, se observaron mediante microscopía confocal laser de barrido los cambios en la microestructura de la leche, cuajada y queso de oveja durante la elaboración del mismo. Las imágenes bidimensionales obtenidas mediante esta técnica se reconstruyen en información tridimensional, lo cual es muy útil para visualizar y cuantificar la grasa y la proteína. Se realizó por primera vez una descripción detallada de la microestructura de las matrices lácteas durante el proceso de elaboración del queso Idiazabal. Además, en el estudio experimental a escala de laboratorio se utilizó la crio-microscopía electrónica de barrido para el estudio detallado de la microestructura de los quesos. Asimismo, durante el estudio de intervención se analizó la influencia de los parámetros tecnológicos en la microestructura de los gránulos de cuajada y del queso. La intensidad del corte no afectó de manera notable a la microestructura de los gránulos. Sin embargo, una intensidad elevada durante el recalentamiento, incrementó el volumen de grasa no globular, y redujo la porosidad de la red proteica y la cantidad de grasa globular de los gránulos, lo cual se reflejó también en el queso tras el prensado. Se consideró la temperatura de recalentamiento como el factor más determinante de los cambios estructurales de la porosidad de la matriz y los glóbulos grasos. Estos cambios también afectaron a las propiedades de textura del queso. En el estudio experimental, temperaturas más altas de recalentamiento aumentaron la dureza y masticabilidad del queso, y además se establecieron algunas correlaciones entre propiedades microestructurales y texturales de los quesos prensados. De todos modos, se deberían realizar más investigaciones para dilucidar las interacciones existentes entre las propiedades del queso y los parámetros tecnológicos utilizados.

El trabajo realizado en esta Tesis Doctoral contribuye con información relevante sobre los efectos de los procesos tecnológicos de elaboración del queso en las propiedades de la cuajada, queso y lactosuero producido en producciones comerciales que utilizan leche cruda de oveja. Los resultados obtenidos confirman que el proceso de elaboración que se realiza actualmente en las pequeñas queserías rurales de Idiazabal DOP podría mejorarse adaptando algunos de los parámetros tecnológicos estudiados y sin modificación de las propiedades del queso. La información aportada es especialmente útil para que los elaboradores puedan ajustar los parámetros tecnológicos en sus instalaciones, contribuyendo así a la sostenibilidad tanto económica como ambiental de las pequeñas queserías rurales.

Chapter I

General Framework

1. Idiazabal Protected Denomination of Origin cheese production

Idiazabal Protected Denomination of Origin (PDO) cheese is a traditional product of the Basque Country and Navarre made according to the procedure approved by the Regulatory Council of the Denomination of Origin Idiazabal cheese (Ministerio de Agricultura, Pesca y Alimentación, 1993). This semi-hard cheese manufactured using raw milk from Latxa or/and Carranzana sheep breeds has to be ripened for a minimum of 2 months before consumption, although the usual maturation period ranges between three to six months. Idiazabal PDO cheese is usually manufactured during a 7-month period from January to July according to the breeding calendar and lactation period of the sheep flocks.

The cheesemaking process begins by heating the milk to 20 ºC and adding a commercial starter culture that commonly contains *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Commercial liquid rennet and/or artisanal lamb rennet paste is added when the milk reaches around 30 ºC, and 20-45 minutes later the curd is cut into small pieces to assist syneresis. The curd grains and whey mixture are then heated to 36-38 ºC while they are stirred. The whey is removed by pre-pressing the curd grains in the vat and creating a continuous curd mass that is cut into blocks that are introduced into the cheese moulds. These are pressed for about 3-7 hours at around 20 ºC, and salted by immersion in brine at 10 ºC for 10-24 hours. Finally, the cheeses are ripened in chambers at 85-90% relative humidity and 8-12 ºC for at least two months.

2. Sustainability of small rural dairies

The socio-economic and environmental sustainability of the manufacture of cheese in small rural dairies relies mainly on their capacity to increase cheese yield, maintaining the typical properties and quality of the cheese, and to reduce the amount and organic load of the whey generated during cheesemaking. Cheese yield can be defined in several ways but one of the most common is the amount of cheese produced from 100 kg of milk (Walstra *et al.*, 2006; Banks, 2007). This index is known as actual cheese yield and it is particularly valuable to determine the efficiency of the cheesemaking process and the potential economic profitability of a dairy. Therefore, improving cheese yield is a continuous challenge for the dairy sector, since a small increase in yield may have a great economic impact on the dairy (Banks, 2007). On the other hand, increasing the weight of cheese produced *per* 100 kg of milk inevitably reduces the amount and organic load of the whey, since more components of milk (i.e. fat and proteins) are retained in the cheese.

During the manufacture of cheese, approximately 85-90% of the milk volume employed is turned into whey (Liu *et al.*, 2005). The composition of this by-product changes widely depending on the composition of milk and the technological conditions used during cheese production. Whey contains mainly water (90-94%), lactose (4.5-6.0%), proteins (0.6-1.6%), fat (0.1-1.5%) and minerals (0.8-1.0%) (Jaeggi *et al.*, 2005; Prazeres *et al.*, 2012). Due to the rich composition on these compounds, especially in lactose and proteins, and the large amount of whey produced in the dairies, the recycling and/or transformation of whey into other value-added products has been extensively studied. These encompass a wide variety of products, such as whey and proteins concentrates, lactose, chemicals (ethanol, butanol or methane), biofuels, food and drink ingredients, starter cultures, edible coatings or products for animal feeding, among others (Kosikowski, 1979; Koutinas *et al.*, 2009; Parra, 2009; Ramos *et al.* 2012; Boura *et al.*, 2017). However, the reutilization and conversion of whey into these products entails a considerable economic investment, qualified staff, large volumes of whey and high technological complexity. Thus, the transformation of whey into value-added products in small rural dairies is very limited.

In the Basque Country, the small rural dairies that manufacture Idiazabal PDO cheese, in addition to having limited facilities, are usually scattered over wide geographical areas, which makes connecting with other industrial or logistical infrastructures especially difficult. The total number of Idiazabal dairies is currently 122 and the vast majority of them, 120 in accordance with the Regulatory Council, are registered as small and medium sized producers (Idiazabal PDO, 2019). According to the data extracted from the Department of Economic Development and Infrastructure (Basque Government, 2019), the total amount of sheep milk produced in the Basque Country increased from 7.1 to 9.9 million litres in the last five-year period of available data (2013-2017), with an average annual growth of 8% (Figure I.1). Approximately 95% of the sheep milk produced was used for cheese manufacturing, where almost 60% of it was produced in small rural dairies. Additionally, the production of cheese in small rural dairies showed a positive upward trend in the last two years (Figure I.1). The production of Idiazabal cheese also grew by 1,100 to 1,400 tonnes *per* year for the period 2013-2017, and consequently, the whey produced in the small rural dairies increased proportionally.

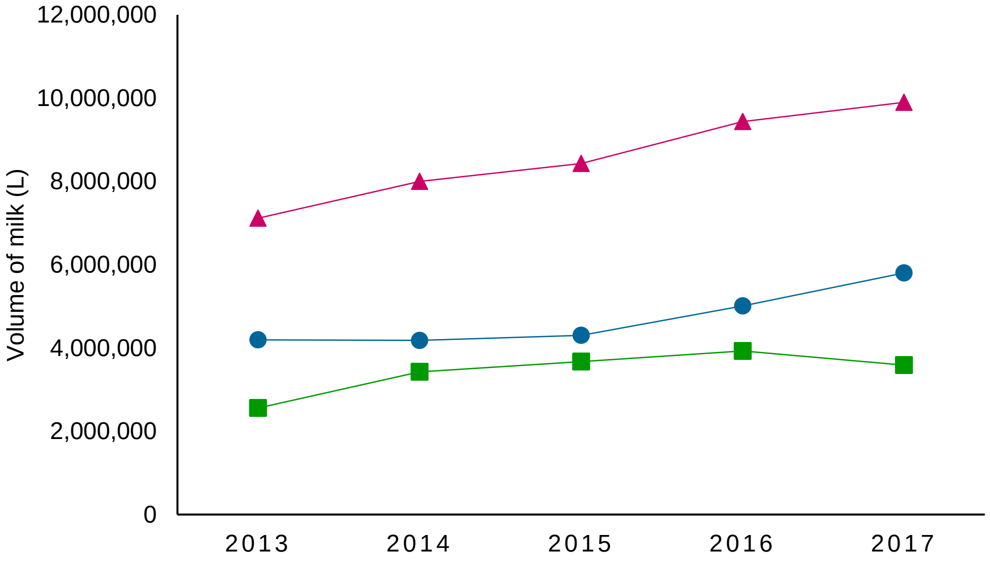

Figure I.1. Sheep milk produced in the Basque Country from 2013 to 2017. The total volume of sheep milk produced (▲) is divided into milk transformed into cheese in small rural dairies (●) and dairy plants (■).

In order to analyse the situation of the management of whey in the dairies of the Basque Country, several research projects at a local, regional and at European level have been carried out. According to the results from these projects, the amount of whey generated by all kind of dairies (approximately 150) was ~31 million litres *per* year, with nearly 60% of these dairies producing less than 50,000 L/year (Cebrián *et al.*, 2013). These projects suggested some possible solutions for the correct management of the whey produced in the dairies of the Basque Country. These solutions included the collection of around 90% of the whey produced in all dairies to a centralised whey processing plant. However, the collection routes proposed only considered half of the small and medium sized dairies due to their remoteness or the economic unviability of its collection. The reuse or transformation *in situ* of the whey generated in small rural dairies, using it as animal feed in its liquid state or producing new whey-based food products has been also considered, although these solutions would have a limited real world application and would involve a great predisposition and effort on behalf of the dairies. Therefore, most of the whey currently generated by small dairies is discharged into the environment.

The disposal of whey without appropriate treatment can cause serious environmental problems due to the large volumes generated and its high organic load (Rivas *et al.*, 2011). Due to the concern about water pollution, the discharge of whey, which is considered an industrial waste water, has been regulated by the European Union (EU) in the EU Water Framework Directive (European Commission, 2000), the Urban Wastewater Directive (European Commission, 1991), and the Animal by-products Regulation (European Commission, 2009). In these regulations, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) are used as contamination indicators. COD is defined as the amount of oxygen needed by chemical oxidation to consume the organic constituents in water, while BOD refers to the oxygen consumed by the biochemical oxidation of organic and inorganic matter (ISO, 2002; ISO, 2003). In general, the COD and BOD values of the whey generated from the production of cheese are between 50-102 g/L and 27-60 g/L, respectively (Prazeres *et al.*, 2012). The Urban Wastewater Directive states that industrial wastewaters should be subjected to pre-treatments before disposal to ensure that the wastewaters do not adversely affect collecting systems or the environment. However, currently, small rural dairies do not have the infrastructure to accomplish the pre-treatments required and to meet this directive. On the other hand, the Animal by-products Regulation contemplates the possibility of a controlled discharge of whey into the land, which could be a potential solution for the small rural dairies since it can be used as a fertilizer due to its rich composition (Ghaly *et al.*, 2007a). However, the excessive application of whey due to the uncertainty of the exact nutrient composition could also cause soil leaching, groundwater and air pollution, and eutrophication (Ghaly *et al.*, 2007b). The latter term is commonly used in aquatic ecosystems to describe the increase in the concentration of nutrients, especially nitrogen and phosphorous, and the subsequent biological effects produced, such as the quick growth of organisms, depletion of the concentration of oxygen in water, and changes in the aquatic plant and fauna biodiversity (Harper, 1992).

Therefore, the first step in solving this problem should be the reduction of the organic load and amount of whey during the manufacture of cheese. This would ease the subsequent treatment of the whey *in situ* in the small rural dairies, and the recycling or the ulterior controlled disposal into the environment, consequently enabling the dairies to meet the requirements of the current legislation. Additionally, the improvement of the cheesemaking process would increase cheese yield, which would enhance the economic benefit as well as the environmental sustainability of these dairies.

Chapter II

Literature Review

The factors affecting cheese yield and compound losses in whey are classified into two main groups: factors involved in the changes in milk composition and their relationship with cheese yield, and the effect of the cheesemaking technological conditions on whey losses and the final composition, structure, texture and yield of the cheese.

1. Factors that affect milk composition and quality

Milk is mainly composed of water, fat, protein, lactose, minerals and vitamins in variable amounts depending on several factors such as animal species, breed, genetic variants of proteins, season, lactation stage, feeding system, animal management or environmental conditions (Lucey & Kelly, 1994; Abilleira *et al.*, 2010; Abd El-Gawad & Ahmed, 2011). The composition and physico-chemical properties of the milk, in turn, have an influence on the functional properties, structure, sensory attributes and yield of the cheese (Chia *et al.*, 2017). Fat and protein, particularly casein, are the most important components of milk influencing cheese yield. Caseins are responsible for the formation of the protein network during the coagulation of the milk and this entraps the fat globules and water. Fat globules act as inner fillers in the casein network and this impedes syneresis, leading to a higher amount of moisture in the curd (Lopez *et al.*, 2007). The fat to protein ratio is also an important indicator related to the cheese manufacture efficiency, cheese yield and quality (Banks, 2007). Recently, Addis *et al.* (2018) showed that the standardization of milk greatly improved the cheesemaking efficiency in the production of Pecorino Romano cheese by resulting in higher fat recoveries in the cheese and composition-adjusted cheese yield. Therefore, milk is usually standardized to maximize cheese yield to a constant fat to protein ratio value that ranges between 1.05 and 1.45 depending on the type of cheese (Guinee *et al.*, 2007; Abilleira *et al.*, 2010). However, the standardization of some PDO cheeses, as for instance Idiazabal cheese, is not allowed by the Regulatory Boards.

Milk production is undoubtedly dominated by bovine milk, which accounts for 97% of the total milk produced in Europe. However, milk from other species is produced in relatively large quantities in countries such as Greece, Spain, France and Italy (Eurostat, 2017). The composition of milk among animal species largely varies as shown in Table II.1. In general, sheep milk contains a higher quantity of fat, protein, casein, lactose, minerals and hence, higher total solids compared to bovine and caprine milk (Park *et al.*, 2007; Chia *et al.*, 2017). Other physico-chemical properties such as fat globule size and casein micelle size also differ among animal species (Table II.1), and these have important implications for the rheological properties of milk and cheese yield (Logan *et al.*, 2014). For instance, Martini *et al.* (2008) showed that a higher percentage of fat globules with a diameter higher than 5 µm in sheep milk impaired the cheesemaking aptitude and cheese yield. Sheep milk is especially rich in β-casein and it has a high degree of calcium biding capacity, which enhances cross-linking and aggregation of the para-casein. These attributes make sheep milk particularly suitable for cheesemaking due to its improved physico-chemical and rheological properties (Park, 2007).

Table II.1. Composition and physico-chemical parameters of milk from different animal species.

Properties	**Ovine**	**Bovine**	**Caprine**
Water (%)	81.6	87.2	87.0
Fat (%)	6.1-12.6	3.4-5.5	3.4-4.5
Protein (%)	2.6-6.6	3.2-4.0	2.8-3.7
Casein (%)	4.2	2.6	2.4
αs1-casein (%)[a]	6.7	39.7	5.6
αs2-casein (%)[a]	22.8	10.3	19.2
β-casein (%)[a]	61.6	32.7	54.8
κ-casein (%)[a]	8.9	11.6	20.4
Lactose (%)	4.4-5.6	4.6-4.9	3.9-4.8
Ash (%)	0.8-1.0	0.7-0.8	0.7-0.8
Calcium (mg/100g)	193	122	134
Phosphorous (mg/100g)	158	119	121
Total solids (%)	12.1-20.7	11.5-14.5	11.5-14.5
pH	6.51-6.85	6.65-6.71	6.50-6.80
Fat globule diameter (µm)	3.4-5.3	4.0-4.6	3.2-3.5
Casein micelle diameter (µm)	193	180	260

Data from Chia *et al.* (2017), Park *et al.* (2007) and Balthazar *et al.* (2017).
[a] Percentage of total casein.

Milk composition is markedly affected by the concurrent influence of the stage of lactation, season and diet (Barron *et al.*, 2001). Sheep flocks are usually managed in seasonal lambing systems, and as a result, the composition of the milk changes widely from the start to the end of the milking period. The lactation stage of Latxa ewe flocks lasts usually 6 months from January to July (Nájera *et al.*, 2009). Feeding management also changes during the milk production season: during winter (early-lactation) indoor feeding is mainly used; from spring onwards (mid-lactation) flocks are fed by part-time grazing; and in summer (late-lactation) sheep graze in extensive systems (Abilleira *et al.*, 2010; de Renobales *et al.*, 2012). Although the amount of milk produced decreases during late lactation, the concentration of fat, protein, casein, calcium and total solids raises (Perea *et al.*, 2000; Barron *et al.*, 2001; Nájera *et al.*, 2009), increasing the cheese yield during summer (Barron *et al.*, 2001).

The milk somatic cell count (SCC) is another milk quality indicator that is also important as it can have large effects on the composition and properties of ewe milk. A high SCC content could cause compositional changes in the milk such as an increased amount of soluble proteins due to the hydrolysis of the casein and a lower concentration of lactose (Jaeggi *et al.*, 2003; Albenzio *et al.*, 2004; Revilla *et al.*, 2009). Due to these changes, the rheological properties of milk such as the coagulation time, curd firming rate and curd firmness deteriorate, and this could lead to a reduction in cheese yield and impair compound recovery (Pirisi *et al.*, 2000; Albenzio *et al.*, 2004; Kelly, 2007).

2. Cheesemaking technological factors that affect cheese yield and milk compound losses in the whey

The technological factors that affect cheese composition, yield and milk compound losses in whey encompass a wide variety of processes starting from the cold storage of the milk and finishing with the ripening of the cheese (Kammerlehner, 2009). These factors have been extensively reviewed, especially using bovine milk as a raw material, and on a laboratory scale. The conversion of milk into cheese involves concentrating to a greater or lesser extent the components of milk in the curd and ripening determines the commercial yield of the cheese depending on moisture loss (Banks, 2007). In the dairy industry, the technological conditions used during the whole cheese processing are automatically controlled (Walstra *et al.*, 2006). Small dairies, however, do not possess the infrastructure available in big industrial dairies, and therefore, the role of the cheese-maker is crucial during the entire process. In the following sections, cold storage of milk, milk pre-treatment, curd coagulation, curd cutting, curd cooking and stirring, curd pressing, cheese brining and cheese ripening will be reviewed.

2.1. Cold storage of milk

The cold storage of milk in small rural dairies is a common practice mainly due to the need to gather a sufficient volume of milk in order to process it in the vat, which usually involves several milkings. In small rural dairies, the number of milkings needed for an appropriate milk volume oscillates between 2 to 6, depending on the season and number of animals in lactation. For Idiazabal PDO cheese production milk must be stored in refrigerated tanks at temperatures lower than 10 ºC (Ministerio de Agricultura, Pesca y Alimentación, 1993), and generally temperatures between 2 to 6 ºC are used. The storage time also fluctuates from a few hours to 2-3 days, and these conditions can cause several undesired changes in milk as explained below.

Firstly, the cold storage of raw milk hinders the growth of mesophilic microorganisms but increases the psychrotrophic bacteria population, mostly *Pseudomonas*, Enterobacteriaceae, *Flavobacterium* and *Acinetobacter* (Beresford, 2007). It is not only the initial amount of these bacteria that can have a negative consequence in milk properties (Hicks *et al.*, 1986; Guinot-Thomas *et al.*, 1995), but also the habitual presence of psychrotrophs in raw milk and their capability of growing at refrigeration temperatures (~4 ºC) makes them a constant threat. Psychrotrophic microorganisms produce proteolytic and lipolytic enzymes that can deteriorate milk fat globules and proteins (Fonseca *et al.*, 2012), increasing the compound losses during cheesemaking, especially with prolonged milk storage (up to 4 days) causing a high psychrotrophic bacteria population (Hicks *et al.*, 1986). Secondly, caseins and particularly β-caseins solubilize and dissociate increasing their vulnerability to hydrolysis mainly due to the enzymes produced by psychrotrophic bacteria (Guinee, 2007). This reduction of the micellar casein, and consequent increment of serum casein, impairs renneting properties, increasing the coagulation time of the milk (Maciel *et al.*, 2015).

Additionally, blending the various milkings in dairy farm storage tanks could also degrade milk proteins faster due to milk plasmin activity (Leitner *et al.*, 2008). Thirdly, fat globules could also be damaged during cold storage due to the activity of native milk lipase and the lipolytic enzymes produced by psychrotrophic bacteria, which hydrolyses triglycerides and reduces fat content (Gargouri *et al.*, 2013; Maciel *et al.*, 2015). Finally, cold storage could promote the solubilisation and dissociation of colloidal calcium phosphate, modifying the equilibrium between calcium, phosphorous and casein, and consequently hindering the rheological properties of milk (Malacarne *et al.*, 2013). The changes mentioned above are certainly dependent on the refrigeration temperature, with lower temperatures slowing down some of these processes (Malacarne *et al.*, 2013). All these changes due to refrigeration also have an effect on the pH of raw milk, which could increase or decrease during prolonged cold storage periods. These oscillations are attributed to two phenomena that occur simultaneously during the cooling of the milk; the acidification of milk due to the production of lactic acid generated by psychrotrophic bacteria, and the dissociation of colloidal calcium phosphate which increases pH values (Guinot-Thomas *et al.*, 1995; Malacarne *et al.*, 2013). However, the amount of soluble casein and calcium in ovine milk, unlike bovine and caprine milk, has been reported to remain unchanged during the first 48 h of refrigeration (~4 ºC), and therefore, no changes were observed in the rheological properties and syneresis rate of curd (Raynal & Remeuf, 2000). The apparent major stability during cold storage of ovine milk could be associated with the stronger bonds in the casein micelles and the higher calcium proportion. Consequently, ovine milk has better practical implications and advantages for cheesemaking in small rural dairies.

The study of the changes that occurred during cold storage is particularly interesting for cheeses such as Idiazabal cheese that during the cheesemaking process do not include a high heat treatment. Several authors have observed that thermal treatments reverse some of the effects caused by cold storage in milk, for instance, the reabsorption of soluble casein and restoration of the micellar structure (De la Fuente, 1998). However, severe heat treatments also have other side effects, such as the inclusion of whey proteins in the casein micelles and hence, the impairment of the rheological properties of the milk (Davies & Law, 1983; McSweeney, 2007). Taking into account all of the above mentioned, prolonged refrigeration times may have undesirable effects on milk properties and, consequently, on the cheese yield and milk compound losses due mainly to the potential degradation of protein and fat (Hicks *et al.*, 1986). Therefore, even though sheep milk has been shown to be more stable in refrigeration, Idiazabal PDO small rural dairies should try to avoid long periods in cold storage, especially longer than 48 hours, in order to maximize cheese yield and compound recovery.

2.2. Pre-treatment of milk

The milk used for cheesemaking usually undergoes several processes to improve its properties or to prevent possible defects that could occur during cheese production. Big industrial dairies especially carry out processes such as thermization, pasteurization, standardisation, homogenisation and addition of calcium chloride, which have important technological implications in cheese manufacturing, as shown below (Walstra *et al.*, 2006).

- Thermization, which is usually performed at 57-68 ºC during 15-20 seconds, is used to reduce detrimental bacteria and to prevent the generation of microbial proteinases and lipases, improving the microbiological quality of the milk (Sheehan, 2007).
- Pasteurization (62-65 ºC during 30 minutes) or higher heat treatments (72-75 ºC during 15-30 seconds) kill possible pathogenic bacteria, inactivate some enzymes, decrease the total level of lipolysis and cause denaturalization of whey proteins, which stick to caseins, increasing cheese yield. However, it could sometimes impair the rheological properties of milk and the final cheese quality (Dzurec & Zall, 1985; Chávarri *et al.*, 2000; McSweeney, 2007).
- Standardisation is used to adjust the fat to protein ratio in milk in order to obtain a more homogeneous cheesemaking process during the production season, improving cheese yield and reducing fat losses (Kelly, 2007; Addis *et al.*, 2018).
- Homogenisation is usually used to improve the yield in the production of soft cheeses since it increases moisture and fat retention. This occurs due to changes in the microstructure and the partial interaction of the casein

matrix and fat globules, although it has undesirable effects in the gel structure for semi-hard and hard cheese productions (Ong *et al.*, 2010; Abd El-Gawad & Ahmed, 2011).

- The addition of calcium chloride improves the rheological properties of milk, reduces fat losses in the whey and increases cheese yield in bovine milk (Walstra *et al.*, 2006; Ong *et al.*, 2015). However, some authors state that the addition of calcium chloride to sheep milk is not required for cheesemaking due to its intrinsic high calcium content (Bencini, 2002).

The Idiazabal PDO Regulation strictly forbids any of the pre-treatment processes described above and therefore, these practices are not carried out in the small rural Idiazabal dairies (Ministerio de Agricultura, Pesca y Alimentación, 1993).

2.3. Coagulation of milk

The coagulation process concentrates the components of milk by destabilising milk proteins through rennet addition, acidification or heat application. In this section, only the rennet-induced coagulation will be reviewed, since it is the mechanism responsible for the coagulation in Idiazabal cheese production.

Milk casein micelles are negatively charged sphere-like structures that are principally composed of different types of casein molecules and calcium phosphate nanoclusters (Horne, 2014). Due to the particular properties of each type of casein (α_{s1}, α_{s2}, β or κ), they arrange themselves in specific ways where the κ-casein, which has a 'hairy' hydrophilic C-terminal, forms a sterically stabilizing outer layer. Rennet addition causes the destabilisation of micelles in two concurrent phases: the enzymatic stage and the aggregation stage (Figure II.1). In the first stage, the enzymes of the rennet hydrolyse the C-terminal part of the micelle releasing the hydrophilic peptide, named caseinomacropeptide, into the whey generating an unstable para-κ-casein micelle (Figure II.1B). The enzymatic reaction in addition to reducing the steric repulsion, also decreases the electrostatic repulsion among para-κ-casein micelles causing their aggregation into small clusters (Figure II.1C). As the enzymatic reaction progresses, more caseins are aggregated and eventually the gel forms (Figure II.1D) (Horne & Banks, 2004; Walstra *et al.*, 2006; Lucey, 2014).

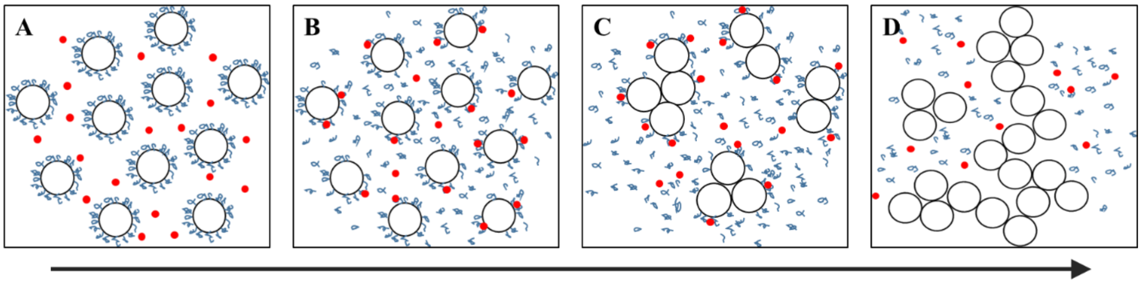

Figure II.1. Schematic representation of rennet-induced coagulation of milk. A, is the initial moment of the mixture of casein micelles (◌) and rennet enzymes (●); B, is the first enzymatic reaction stage where caseinomacropeptides (ɩ) are released; C, is the initial aggregation stage into small clusters; and D, is the further aggregation of caseins and gel formation stage.

The coagulation process and the characteristics of the resulting gel are influenced by many factors such as the pH level, temperature, casein concentration, enzyme content, the amount of calcium, ionic strength and many others. Some of the most important factors are shown below.

- *Enzyme concentration:* a higher enzyme concentration increases the reaction speed of the enzymatic stage, and although it has no effect on the aggregation stage, overall it reduces the coagulation time and increases gel firmness (Zoon *et al.*, 1988; Walstra *et al.*, 2006).
- *pH:* lowering the coagulation pH to a range between 5.2 and 7.0 causes the solubilisation of micellar calcium phosphate, the dissociation and reduction of the net charge of casein micelles, and an increase in the enzyme affinity, which as a result decreases coagulation time and increases gel firmness (Dalgleish & Law, 1988; 1989; Nájera *et al.*, 2003).
- *Temperature:* increasing the temperature during coagulation from 20 to 40 ºC especially affects the protein aggregation stage, which then reduces coagulation time and greatly increases gel firmness (Bencini, 2002; Nájera *et al.*, 2003; Panthi *et al.*, 2019).
- *Calcium concentration:* a higher calcium concentration increases calcium ion content and additionally decreases pH, reducing coagulation time and increasing gel firmness (Ong *et al.*, 2013).
- *Casein concentration:* increasing the casein concentration in milk generally improves the rheological properties of milk and increases gel firmness (Bencini, 2002).

- *Protein fraction concentration:* the proportion of the different casein types influences the coagulation properties of milk (Amalfitano *et al.*, 2019).

These factors can also interact with each other affecting coagulation parameters and gel firmness (Nájera *et al.*, 2003). The large differences between the composition and other physico-chemical properties between bovine and ovine milk (Table II.1) are also reflected in the coagulation properties (Bencini, 2002; Park, 2007). In the same conditions, coagulation in ovine milk occurs faster and forms a firmer curd than bovine milk. This occurs mainly due to the higher concentration of casein and colloidal calcium, and due to its higher sensitivity to rennet because of an increased β/α_s-casein proportion (Park, 2007). Additionally, sheep milk is not affected to the same extent by some of the factors mentioned above. Bencini (2002) reported that the curd firming rate in ovine milk, unlike bovine milk, was unaffected by changes in pH (6.10-6.65), calcium addition (0-2 mM), rennet concentration (12-20 µL rennet/mL milk) and temperature (30-38 ºC). However, coagulation time and gel firmness were affected in the same way as bovine milk but generally to a lesser extent. Calcium addition was an exception since the addition of up to 2 mM in sheep milk did not show differences in gel firmness (Balcones *et al.*, 1996; Bencini, 2002). However, these authors reported dissimilar results regarding the coagulation time: Bencini (2002) reported that calcium addition had no effect, whereas Balcones *et al.* (1996) showed that higher calcium concentrations resulted in reduced coagulation times.

For the coagulation process, Idiazabal dairies usually use artisanal lamb rennet paste or a combination of rennet paste and commercial bovine or ovine rennet (Bustamante, 2002). This type of rennet paste, in addition to causing the coagulation of milk, contributes to the characteristic flavour and aroma of Idiazabal cheeses, since during ripening free fatty acids are released due to its lipolytic activity (Virto *et al.*, 2003). However, the coagulant activity of these artisanal lamb rennet pastes is unknown for the cheese-makers and could oscillate between 155 and 363 U/g of tissue depending on the preparation of the rennet (Bustamante *et al.*, 2000). Bustamante (2002) carried out a thorough characterization of the artisanal lamb rennet pastes used for Idiazabal cheeses and showed that the percentage of enzymes varied from 70 to 85% for chymosin and 15 to 30% for pepsin. Other studies carried out in small rural Idiazabal dairies showed that the pH level of raw milk before adding the starter culture and rennet varied from 6.4 to 6.8 (Barron *et al.*, 2001; Nájera *et al.*, 2009; Abilleira *et al.*, 2010). This implies that the coagulation process is subjected to wide variations and that the control of this process is a difficult step in the small rural dairies.

2.4. Curd cutting

After milk coagulation, cheesemaking continues through the cutting, cooking and stirring processes in order to cause syneresis and consequently concentrate mainly casein, fat and minerals in the curd. The syneresis or expulsion of whey from the curd has been extensively reviewed due to its important implications for the dairy sector, since it is directly related to the final cheese properties and yield (Walstra *et al.*, 2006; Fagan *et al.*, 2017). Syneresis is a process in which the shrinkage of the gel causes the expulsion of whey out of the pores of the protein network, since whey is not chemically bound but only physically trapped (Lucey, 2011). The main mechanism of syneresis is the rearrangement of the para-casein network (Fagan *et al.*, 2017). During the coagulation of the rennet gel, casein micelles form junctions with the other 2-4 micelles but the majority of their surface does not touch other caseins, so they rearrange creating thicker protein strands (Figure II.2). During the hardening of the gel, especially when the network becomes more compact, forming new bonds is hampered as caseins are partially immobilized, but occasionally some strands are broken and microsyneresis occurs (Walstra *et al.*, 2006). However, under normal cheesemaking conditions, the curd does not show spontaneous syneresis and usually an external 'force' is needed to release the whey (Fagan *et al.*, 2017). Therefore, cheesemaking processes such as cutting, cooking, stirring and acid production will enhance the rearrangement of the casein strands and the syneresis of the curd.

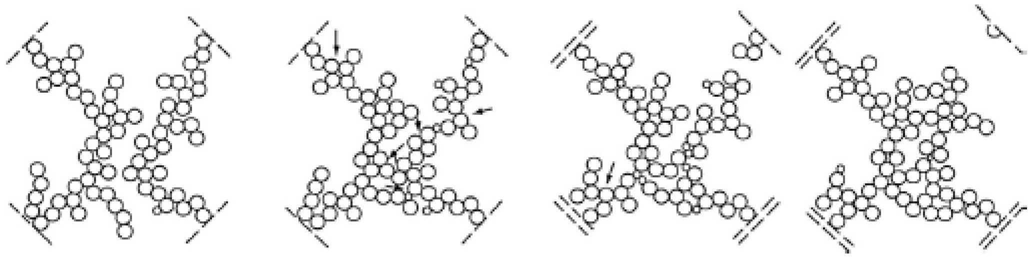

Figure II.2. Schematic representation of para-casein micelles strands creating new bonds, originating the breakage of protein strands and enhancing microsyneresis (From van Dijk., 1982).

The curd firmness at the cutting point is an important parameter that influences the syneresis of the curd and the migration of fat and protein to the whey. Therefore, the optimum firmness should be identified for each cheese variety (Kammerlehner, 2009). Gels with very low or very high curd firmness values will cause higher casein fines and fat globule losses in the whey (Riddell-Lawrence & Hicks, 1989; Walstra *et al.*, 2006; Abd El-Gawad & Ahmed, 2011). In general, for the manufacture of semi-hard or hard cheeses, the gel should be cut when it is still weak to favour the rearrangement of the network and consequently, syneresis (Kamerlehner, 2009; Lucey, 2011). In small rural Idiazabal dairies, the decision to start the cutting process is made according to the sensory observation of the curd consistency. Since, as explained before, the lack of control in the coagulant activity of the artisanal rennet paste, and the absence of specific equipment to measure the optimal cutting point, makes the automation of the process difficult.

The technical parameters and equipment used during cutting such as the rotation speed and cutting time, vat design, or the arrangement of the cutting equipment, have a considerable impact on the curd moisture and milk component losses in the whey (Lucey & Kelly, 1994; Mateo *et al.*, 2009). Cutting speed and time are some of the most relevant parameters that affect the physical breakage of the gel, the size of the curd grain obtained, the expulsion of the whey and resulting milk compound losses during the subsequent stirring. These losses could occur due to an excessive or an insufficient cutting process. A cutting procedure that is too intense damages the gel enhancing the generation of mini gel particles and increasing the fat and protein losses in the whey (Kammerlehner, 2009). Contrarily, a cutting procedure of insufficient intensity could cause the shattering of the curd particles during the stirring process, increasing compound losses in the whey (Johnston *et al.*, 1991; Everard *et al.*, 2008). The intensity of the cutting process will inevitably have an effect on the size and distribution of the curd grains, and these in turn will affect the syneresis rate of the curd, especially during the first 10 minutes after cutting (Unger Grundelius *et al.*, 2000). Bigger curd grains will have slower syneresis rates than smaller curd grains. This is explained using an oversimplification of the real situation assuming that one-dimensional syneresis occurs as explained by Darcy's law (Equation II.1).

$$v = \left(\frac{B}{\eta}\right)\left(\frac{p}{l}\right) \quad \text{(II.1)}$$

where *v* is the relative superficial velocity of the liquid being expelled, *B* the permeability coefficient, *η* the viscosity of the liquid, *p* the pressure acting on the liquid and *l* the distance over which the liquid must flow (Fagan *et al.*, 2017). Therefore, assuming that the viscosity, permeability and pressure conditions are the same during the first stage of cutting between two curd grains of different sizes, the one with a smaller diameter (smaller distance for the whey to be expelled) would show a higher syneresis rate. In this regard, obtaining a homogeneous particle size distribution after the cutting and cooking processes is also essential for producing cheeses with a uniform cheese moisture and texture (Whitehead & Harkness, 1954; Akkerman *et al.*, 1993; Iezzi *et al.*, 2012). Some previous studies have measured curd grain size and/or the distribution of the particles using the sieving method (Kosikowski, 1963; Johnston *et al.*, 1991; Akkerman, 1992; Johnston *et al.*, 1998), which consists of passing the curd particles through a set of different sized mesh sieves. This method has shown strong deviations, limitations and other difficulties due to the stickiness of the curd grains, lump formation and breakage of the curd during analysis (Jablonka & Munro, 1985; Johnston *et al.*, 1991; Igathinathane *et al.*, 2009). Additionally, the comparison of the effects caused by different cutting and stirring conditions is complicated due to the differences in the characteristics of the equipment used for the production of cheese. Iezzi *et al.* (2012) used a two-dimensional image analysis method for assessing the size and shape characteristics of the curd grains during the cheesemaking of some Italian cheeses. This method enables a more detailed measurement of curd particles and the option to carry out a complete study of the particle size distribution of curd grains, as has already been carried out in other foodstuffs (Du & Sun, 2004; Igathinathane *et al.*, 2009). Additionally, some authors have reported positive results using several image analysis techniques, such as threshold image processing, colour measurement or image texture analysis, for syneresis monitoring and curd moisture prediction in laboratory trials (Everard *et al.*, 2007; Fagan *et al.*, 2008; Mateo *et al.*, 2010).

In addition to the factors mentioned above, the temperature and pH conditions used during the cutting process also affect the syneresis and compound losses in the whey. Fagan *et al.* (2007) showed that cutting performed at high temperatures (~35 ºC) increased curd syneresis and fat losses in the whey due to a high permeability of the curd and a greater mobility of the fat at higher temperatures. On the other hand, lower pH values (up to 5.1) also increase the rate of syneresis, due to favourable casein rearrangement conditions (Pearse & Mackinlay, 1989; Walstra *et al.*, 2006). The differences between animal species during syneresis are also noteworthy. Some authors showed that the syneresis rate of curds made from ovine milk were generally slower than bovine milk, mainly due to the physico-chemical and compositional differences between the types of milk (Table II.1). However, temperature, pH, fat content or previous milk heat treatment affected in a similar way the syneresis of curds from different animal species (Calvo & Balcones, 2000).

The data available regarding the cutting conditions currently used in small rural Idiazabal dairies is scarce, especially the ones related to the rotation speed, duration of cutting, and the subsequent results on curd grains. However, the Regulation of Idiazabal PDO cheese (Ministerio de Agricultura, Pesca y Alimentación, 1993) states that the size of the curd grains after cutting must be between 5 and 10 mm in diameter, although no measuring method is suggested.

2.5. Curd cooking and stirring

The major expulsion of whey from the curd grains is caused by the cooking and stirring processes due to the increase in pressure, temperature and pH of the curd grains and whey mixture. Even if the effect of the temperature on syneresis has been shown to be determinant, some authors suggest that the effect exerted by stirring is much greater than increasing the temperature from 32 to 40 ºC (Lawrence, 1959a; 1959b; Geng *et al.*, 2011). In general, the stirring causes turbulent flow pressure and collisions between curd particles and between particles and equipment (stirrers and vat walls). The first one produces pressure differences occasioned by the velocity gradients as explained by Bernoulli’s law, and can generate pressures of up to 160 Pa (van den Bijgaart, 1988). Collisions between curd particles or between particles and equipment causes a brief compression of the curd grains, which also enhances liquid expulsion. In addition to the pressures generated during stirring, moving the curd particles and whey mass itself avoids the sedimentation of the curd grains, and thus, increases the surface area from which the whey can be expelled (Fagan *et al.*, 2017). Therefore, a higher stirring speed increases the probability of the particles colliding, which increments the average pressure and enhances syneresis (Patel *et al.*, 1972). However, a higher stirring speed causes in general, more fat and casein fine losses in the whey (Everard *et al.*, 2008). Several authors indicated that the technological conditions used during the cutting process were decisive for the effects caused during stirring on the curd grains, and that consequently there are combined cutting and stirring effects (Johnston *et al.*, 1991; Johnston *et al.*, 1998; Everard *et al.*, 2008). Although the pressures usually exerted during stirring are not enough to disrupt intact curd grains, any crack could ultimately break the curd particles (van den Bijgaart, 1988), which explains the increase of fat and protein losses in the whey.

The temperature is another well-known factor affecting the moisture expulsion from curd grains. At higher temperatures syneresis is enhanced, although a fast raise in temperature could impair the permeability and hinder moisture expulsion due to the creation of a shrunken outer layer in the curd particle (Fagan *et al*., 2007). In contrast, cooling the curd grains to refrigeration temperatures during cooking could even stop syneresis (Fagan *et al.*, 2017). Additionally, the temperature used during cooking greatly affects the properties of the fat globules and the spatial arrangement of other components in the curd, which eventually determines the structure of the cheese and its physico-chemical and sensory properties (Lopez *et al.*, 2006; Lamichhane *et al.*, 2018).

The temperature, pH and turbulent flow pressure are the main parameters affecting syneresis during the cooking and stirring of the curd grains. However, other factors such as curd grain size and milk composition, also influence the syneresis during cooking (Kern *et al.*, 2019; Panthi *et al.*, 2019). Therefore, all the conditions employed during curd syneresis will determine the extent of the syneresis and the moisture of the curd grains after cooking. These, in turn, relate to the moisture content of the cheese, highlighting the importance of syneresis for the final composition, structure and texture properties of the cheese (Yun *et al.*, 1993; Everard *et al.*, 2011).

2.6. Curd pressing

In Idiazabal cheese manufacturing, after the cooking process the curd grains are allowed to accumulate in the bottom of the cheese vat, while perforated plates are laid on top and on one side of the curd mass exerting pressure. This process enhances the drainage of the whey and generates a big mass of curd that is cut into cubic pieces that will be introduced into cylindrical moulds (15-20 cm diameter) covered by a linen cloth for shaping and pressing. All of this process is carried out manually in the small rural dairies. Afterwards, the moulds will be placed into hydraulic presses and pressed at room temperature (around 20 ºC) for several hours until the pH level of the cheese drops to 5.2-5.5.

The final purpose of the pressing is to favour the fusion of the curd grains, enhance whey expulsion, give the shape of the mould and form the rind of the fresh cheese (Walstra *et al.*, 2006). However, the inner processes that take place during shaping and pressing are rather complex, since many and mutually dependent parameters are involved (Akkerman, 1992). The deformation of curd grains is essential to achieve the desired shape and to promote the fusion of the curd grains (Fagan *et al.*, 2017). Fusion of the curd grains occurs as a result of the increase in the contact area over which they touch one another and the bonding of the networks of curd particles in contact, and will continue until the pores are closed and the permeability is reduced, impairing whey drainage (Akkerman *et al.*, 1993). Therefore, the factors that improve the deformability of the curd grains will also promote their fusion. The temperature, pH, pressure applied, pressing duration and curd grain composition are some of the main parameters affecting the fusion of the curd particles (Walstra *et al.*, 2006). In particular, higher temperatures, together with a higher water content of curd particles increase the deformability of curd grains, forming a firm and smooth cheese. Additionally, low pH values but higher than ~5.2 also increase the deformability of the curd grains, although pH values below 5.2 abruptly reduces it. If during cooking poorly deformable curd grains are generated (for example, very low pH, low water content and low temperature), the fusion of curd grains could be inadequate and a crumbly curd mass could be produced (Walstra *et al.*, 2006). However, some authors suggested that soft curd grains that deformed easily could also close the pores, impairing the release of whey during pressing (Scott Blair & Coppen, 1940).

Pressing enhances the formation of the rind, which will eventually hinder a major expulsion of whey and it is especially promoted when a cloth is placed between the cheese and the mould or when micro perforated cheese moulds are used. The expulsion of whey is affected by several factors such as, the temperature, pressure, size of the cheese loaf and duration of the process (Fagan *et al.*, 2017). A very high pressure in the early stage of pressing has been reported to close the pores near the edges of the cheese, impairing the expulsion of whey and increasing the moisture of the cheese (Reinbold *et al.*, 1994). Likewise, a drop in the temperature during pressing or a smaller cheese loaf (due to a faster cooling down) could also increase the moisture of the cheese (Walstra *et al.*, 2006). The existence of a high concentration of fat in the curd mass could also impair the total aggregation of casein and hinder the expulsion of whey during pressing (Lopez *et al.*, 2007). The distribution of the water content in cheese loaves has also been studied, and in general terms, the rind and the centre of the cheese showed the lowest water content (Geurts, 1978). This phenomenon has been attributed to the movement of water away from higher temperature and pressure zones (the centre of a cheese loaf maintains the temperature for longer time) (Everard *et al.*, 2011). The influence of the curd particle size and the particle size distribution of the curd grains generated after cooking also has a large implication during pressing (Kalab *et al*., 1982; Fagan *et al.*, 2017). A very heterogeneous particle distribution will produce an uneven distribution of moisture, and especially the formation of very small casein particles has been reported to impair the expulsion of whey, as these small particles tend to close the pores (Akkerman, 1992; Kammerlehner, 2009). Moreover, pressing greatly changes the microstructure of the curd and creates a compact casein network that embeds fat droplets (Ong *et al.*, 2012). Additionally, the fat conformation changes during pressing since fat globules are deformed and damaged due to an increase in the internal pressure, causing coalescence and higher amounts of free fat, and having future implications in the texture and flavour of the cheese (Everett & Auty, 2017).

2.7. Cheese salting

The salting process in Idiazabal cheese production is carried out after the pressing and usually by immersing the cheese loaves into a saturated sodium chloride brine. This step is essential for the formation of the cheese rind, the development of flavour and texture, and the preservation of cheese during ripening (Fox *et al.*, 2017). Salting also affects the cheese yield since it promotes the syneresis of the pressed curd, reducing the moisture content of the cheese (Walstra *et al.*, 2006). In addition to the syneresis of the pressed curd, this absorbs and diffuses salt inside the cheese loaf (Guinee & Fox, 2017). The amount of salt absorbed and moisture loss depends on the intrinsic characteristics of the pressed curd, more specifically the compaction, shape and composition (fat, protein, moisture and calcium content) of the pressed curd, the brine concentration and composition, and the temperature and duration of salting (Geurts, 1978; Turhan & Kalentuçn, 1992; Paulson *et al.*, 1998; McMahon *et al.*, 2009). It is widely accepted that a higher salt concentration and a larger duration of the brining process increases the salt and reduces the moisture content of cheese (Geurts *et al.*, 1980; Nájera *et al.*, 1994; Cuffia *et al.*, 2015; Akkerman *et al.*, 2017). Additionally, higher temperatures during brining can also contribute to a higher diffusion of the salt and the whey through cheese loaf (Turhan & Kalentuçn, 1992). It has also been suggested that the addition of small concentrations of calcium to the brine avoids the excessive moisture uptake by the outer layer of the cheese, preventing the soft rind defect (Geurts *et al.*, 1972). The composition and structure of the pressed curd has also a great influence on the diffusion of the salt and the whey through cheese loaf , which depends on the fat to solids-not-fat (SNF) and moisture to SNF ratios, and not only on the moisture content. The diffusion of the salt and the whey is determined by the concentration and arrangement of the fat and protein, increasing generally with a decrease in protein and an increase in fat volume fraction, due to a larger pore size in the protein matrix (Geurts *et al.*, 1974; Guinee & Fox, 2017). Therefore, the previous processes carried out during the cheesemaking have a marked effect on the brining of the cheese and this in turn, will determine the consequent ripening process.

2.8. Cheese ripening

After the brining process, Idiazabal cheese must be ripened for a minimum of two months to meet the food safety standards due to the usage of raw milk. During this ripening period Idiazabal cheeses achieve the minimum amount of dry matter, fat in dry matter and protein in dry matter of 55%, 45% and 25%, respectively, as stated in the Idiazabal PDO cheese Regulation (Ministerio de Agricultura, Pesca y Alimentación, 1993). During ripening, the development of texture, flavour, and appearance of the cheese occurs due to complex biological, biochemical, and chemical reactions (Fox *et al.*, 2017). These reactions encompass lipolysis, proteolysis, glycolysis, and many others (Chávarri *et al.*, 1999; Hernández *et al.*, 2009; Tekin & Güler, 2019). During ripening, the cheese gradually loses moisture, which depends on the relative humidity and temperature conditions of the ripening chamber (Abd El-Gawad & Ahmed, 2011). In general, the conditions used in Idiazabal small dairies range between 85 and 90% and 8 and 12 ºC of relative humidity and temperature, respectively. During ripening of Idiazabal cheeses, moisture drops around 15% from the 1-day ripened cheese to the 2-month ripened cheese. From then on, the loss of moisture occurs more slowly and reaches a total decrease of 24% after 8 months of ripening (Ibáñez *et al.*, 1995). The moisture reduction that occurs during the ripening process has a great impact on the economic profitability of the dairies since, regardless of the variations in the sale price of the cheese, the loss of moisture and in turn, the loss of weight has a direct implication on the commercial cheese yield.

Therefore, the study of the technological conditions used in small rural Idiazabal dairies and the effect of these settings in the cheese composition, microstructure, texture, yield and milk compound losses in the whey, would open a window of opportunity to improve the cheesemaking process carried out in these dairies. This, in turn, would contribute to develop the economic and environmental sustainability of small rural dairies that manufacture Idiazabal cheese by increasing cheese yield and reducing the amount and organic load of the whey.

Chapter III

Objectives

The present Ph. D. Thesis has been carried out within the Lactiker Research Group at the University of the Basque Country (UPV/EHU), which is dedicated to performing multidisciplinary research in the field of Quality and Safety of Foods of Animal Origin.

The current work was based on the following hypothesis: The improvement of the cheesemaking process and controlling *in situ* the most relevant technological conditions used in small dairies, will contribute to increase the cheese yield and reduce the organic load of the whey produced. The adjustment of the technological settings that the cheese-maker can control in accordance to the seasonal and lactation changes that are reflected in milk composition will assist to maximize cheese yield during the whole production season. Likewise, a reduction in the organic load of the whey, especially in the fat and the protein content, would greatly facilitate the subsequent processing for its use in agriculture or livestock farming, or the recycling of whey in the dairy. This Ph. D. Thesis is contextualised in small rural dairies that manufacture Idiazabal PDO cheese, and it has been carried out in close collaboration with the cheese-makers.

The overall aim of this Ph. D. Thesis is to contribute to the sustainability of cheese production by adjusting the technical settings to the facilities available in small dairies, improving cheese yield and reducing the organic load of the whey produced without affecting the properties of the cheese.

This Ph. D. Thesis has been performed following this main objective:

Assessment of the impact of cheesemaking technical conditions on curd grain characteristics, whey composition, cheese properties and yield during the cheese production season.

In order to accomplish this objective, the following specific goals were followed:

I. To determine the cheesemaking settings currently used in the production of Idiazabal PDO cheese in small rural dairies.

II. To characterize the size, shape and particle size distribution of curd grains during cheesemaking.

III. To analyse the relationships between the technical conditions, particularly those regarding the cutting and cooking processes, and the composition and properties of curd, cheese and whey as well as yield.

IV. To describe the changes in the microstructure of sheep milk, curd and cheese during cheese manufacturing.

V. To analyse the effect of the seasonal changes in the composition of raw sheep milk together with the technological settings used for cheesemaking on cheese yield and whey composition.

VI. To identify the optimal cheese processing conditions and to suggest a set of manufacturing guidelines, which would be highly beneficial to implement in the small rural dairies.

Chapter IV

Materials and Methods

1. Observational, interventional and experimental designs

1.1. Commercial studies in the small rural dairies

1.1.1. Observational and interventional studies

The observational study consisted of a general data collection carried out in a two-year period in eight small rural dairies that manufactured Idiazabal PDO cheese. These commercial dairies were located in the surroundings of Vitoria-Gasteiz (Araba, Spain) and each dairy was monitored four times, twice during late winter (mid lactation) and two more during early summer (late lactation) in consecutive years from May 2015 to March 2017. The production of Idiazabal cheese is extended from the end of January until July, and although flock management and feeding systems were similar among dairies, it changed during the cheese production season. This consisted of indoor feeding during winter, part-time grazing from spring onwards, and extensive grazing at the end of the lactation period, as explained before in Chapter II.

An interventional study was carried out selecting two out of the eight dairies that took part in the previous observational study. These dairies were selected for this study since the technological conditions employed for cheese manufacturing, as observed in the general observational study, were especially disparate. One of the dairies used moderate cutting and cooking conditions while the other employed intense cutting and cooking settings. Cheese productions were monitored three times for each dairy in three consecutive weeks from late May to early June of 2018. Both dairies were monitored in specific days of the same week and this schedule was repeated the two following weeks. The technological settings used for the cheese productions for the interventional study are specified in Chapter V (**Manuscript III**, Table 1).

1.1.2. Description of the facilities in the small rural dairies

The facilities in the small rural dairies (numbered from 1 to 8) included automatic milking machines, cold storage milk tanks, semi-automatic vats, hydraulic horizontal presses, and temperature and relative humidity controlled airing and ripening rooms. All the dairies used stainless-steel double-jacketed open vats with a volume capacity between 600 and 1,500 L, and included thermal sensors and potentiometers for regulating the temperature and rotation speed of cutting frames and stirrers. The design of the vat and the cutting and stirring devices, however, differed among some dairies, especially between dairy 1 and the rest of the dairies. Dairy 1 used an open double-O shaped vat with two fixed axes armed with vertical blade-cutters whose sharp knives were separated 6 cm from one another (Figure IV.1). The blade-cutters were converted into stirrers when the direction of rotation was inverted.

Figure IV.1. Double O-shaped vat with two fixed axes (Original photo: A. Aldalur).

The other dairies (dairies 2 to 8) used oval-shaped open vats with one or two axes of rotation where cutting frames and stirrers were inserted (Figures IV.2 and IV.3). The movement of the axes was circular and when two shafts worked together, the same or opposite rotating directions could be used. To avoid demixing of curd grains the axes also shifted from side to side of the vat while the circular rotation occurred.

Figure IV.2. Oval-shaped vat with two axes of rotation and cutting frames with vertical wires (Original photo: A. Aldalur).

Figure IV.3. Oval-shaped vat with one axis of rotation and cutting frames with separately arranged vertical and horizontal wires (Original photo: A. Aldalur).

Cutting frames were equipped with 32 to 42 metallic or nylon wires separated by 2 cm from one another. These wires were arranged in vertical alone or in separate vertical and horizontal disposition (Figure IV.4A). For the stirring of the curd grain and whey mixture while cooking, cheese-makers used stirring tools with diverse designs, such helix- or irregular-shaped stirrers (Figure IV.4B,C, respectively).

Figure IV.4. Cutting frame with vertically and horizontally arranged wires (A), helix-shaped (B) and irregular-shaped, (C) stirring tools used in small rural dairies (Original photos: A. Aldalur).

1.1.3. Idiazabal cheese manufacture

Idiazabal PDO cheese is a semi-hard or hard cheese from the Basque Country and Navarre and is produced with raw sheep milk of Latxa and Carranzana breeds. Idiazabal cheese characteristics and manufacture conditions are regulated by the Idiazabal PDO Regulation (Ministerio de Agricultura, Pesca y Alimentación, 1993; European Commission, 2015). Milk standardization or addition of any additive other than starter culture, lysozyme, rennet and salt is specifically prohibited.

The cheesemaking process carried out by the small rural dairies is detailed in Figure IV.5. The commercial cheeses were made with bulk raw milk from two to five milkings that was stored in cold storage tanks between 3 and 8 ºC for an average time of 34 h before starting the cheesemaking. Milk was pumped from the tanks to the vats and it was warmed up to 25 ºC with gentle agitation. A commercial homofermentative starter culture (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris,* Choozit, DuPont, Barcelona, Spain) was added and milk temperature progressively increased to around 30 ºC while stirring. An adequate amount of commercial rennet and/or artisanal lamb rennet paste was then added and agitated to ensure the correct blending of the mixture. The cheese-maker began the cutting process guided by her/his expertise and the sensory observation of the curd consistency, and therefore the coagulation time ranged between 30 and 50 min. Cutting was generally carried out at constant temperature (~ 30 ºC) and for a variable amount of time and speed of rotation depending on the dairy. Cutting frames were then changed with stirrers and temperature was increased to around 36 ºC during cooking. Cutting and cooking time and rotation speed are not specified in the Idiazabal PDO Regulation, so as previously mentioned they widely varied among small rural dairies. After cooking, stirrers were exchanged for holey metal panels and these were pressed against the curd grains inside the vat generating a continuous mass of curd. Whey was drained and the mass of curd was cut into cubic blocks that where placed inside plastic moulds covered with a piece of linen cloth. Moulds were pressed in hydraulic horizontal presses at room temperature during 3 to 7 h and with a pressure between 1.5 and 3.5 kg/cm^2. Pressed cheeses were then placed in saturated sodium chloride brine (11 – 20 ºBé) at around 10 ºC for 10 to 24 h. Some dairies included an airing process in temperature and relative humidity controlled chambers for an average of 10 d. Ripening was carried out generally at 8 to 12 ºC and 85% relative humidity (RH) for a minimum time of 60 d. Cheeses are then maintained at refrigeration temperatures until commercialization.

1.1.4. Sample collection

For the observational study, bulk milk, drained whey, curd grains after the cutting process (fresh curd grains, FCG), curd grains after cooking (stirred curd grains, SCG), and cheeses after pressing and 2-month ripened cheeses were collected (Figure IV.5). Before starting the cheesemaking process, a sample of 0.5 L of bulk raw milk was taken. Two whey samples (0.5 L each) were collected when the whey was being drained from the vat. For FCG and SCG samples, a round steel mesh sieve (22 cm diameter and 0.43 mm mesh openings) was submerged to a depth approximately halfway between the top and bottom of the vat right after the end of cutting (FCG) and cooking (SCG). The sampling took from 3 to 5 s and, after draining the excess of whey, curd grains for compositional analysis were introduced in 0.5 L containers. For the analysis of curd grains by image analysis, the extracted granules were scattered on a 210 x 297 mm^2 black plastic sheet and separated carefully into individual curd particles using a palette knife.

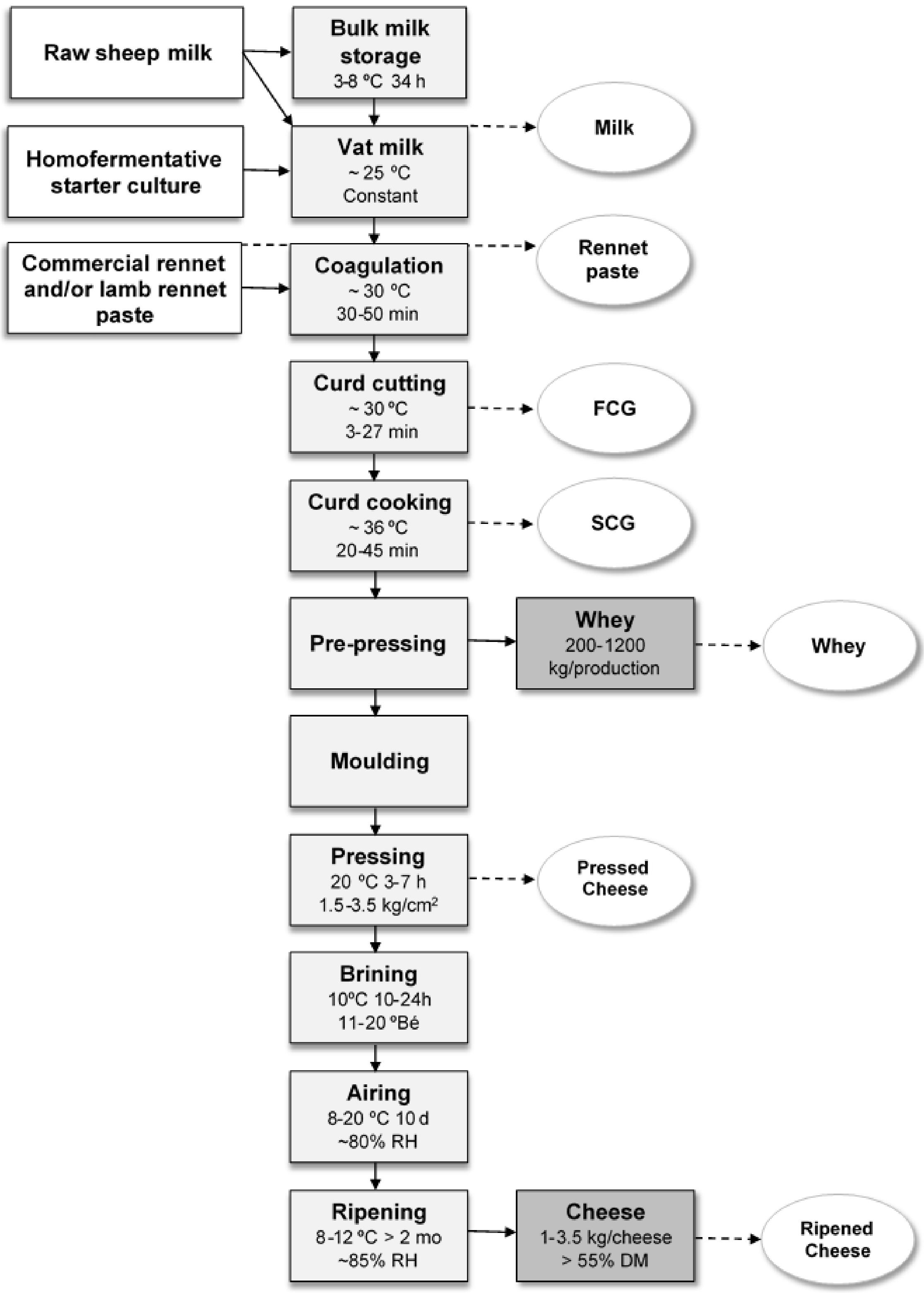

Figure IV.5. Flow chart of the cheesemaking process carried out for Idiazabal PDO cheeses in small dairies. White rectangles represent the raw materials used for the cheese manufacture; light grey rectangles depict the cheesemaking process; dark grey rectangles show the products obtained; white ellipses represent the samples collected for the observational studies. FCG, curd grains after cutting; SCG, curd grains after cooking; RH, relative humidity; DM, dry matter.

A whole cheese after pressing (~1.5 kg) and two cheeses after 2 months of ripening were collected for each trial. All the samples were transported to the laboratory in a portable cooler and divided into subsamples. Milk, whey, and curd grains were placed into 50 mL plastic containers, and each whole cheese was cut into six similar wedges, individually vacuum packed. All samples were stored at -80 ºC until analysis.

For the interventional study, samples of bulk milk, rennet paste, FCG, SCG, whey generated after cutting (WFCG), whey generated after cooking (WSCG), and drained whey, and two whole cheeses after pressing and two more after 1 month of ripening were collected (Figure IV.5). The same sampling methodology as described previously for the observational study was carried out in the selected dairies, except for rennet, FCG and SCG. Artisanal rennet paste (~50 g) was collected from each dairy, stored in plastic containers and kept in refrigeration (4 ºC) until analysis. FCG and SCG together with the whey generated during cutting and cooking processes, respectively, were collected submerging a 0.5 L plastic container to a depth approximately halfway between the top and bottom of the vat while stirring. In the laboratory, these samples were divided into drained FCG, WFCG, drained SCG and WSCG and stored in 50 mL plastic containers. For confocal laser scanning microscopy (CLSM) analysis, FCG and SCG samples were immersed in a formaldehyde solution at 0.5% (w/v) to prevent structure changes until analysis, while milk, pressed cheese and ripened cheese samples were kept in refrigeration (4 ºC). For the simulation of curd texture analysis, milk samples were kept in refrigeration for no longer than 5 h until analysis. For cheese texture analysis, one wedge of each 1-month ripened cheese was kept in refrigeration (4 ºC) for 3 days until analysis. For other physico-chemical analysis, samples were frozen at -80 ºC until analysis.

1.1.5. Technical data collection

For both observational and interventional studies, technical data during cheesemaking was collected *in situ* in the small dairies, with a special focus on cutting and cooking processes. Milk volume was measured for each cheese production using the flowmeters available in the dairies. The amount of whey drained after the cheesemaking process was estimated as the mass balance between the amount of bulk milk and cheese after pressing produced *per* vat. Time measurements during coagulation, cutting and cooking processes were measured with a digital chronometer (TFA, Dostmann, Wertheim, Germany), while temperatures were monitored with a mercury thermometer (Ludwig Schneider, Wertheim, Germany). Cooking temperature rate was calculated as the ratio between the difference of final and initial cooking temperature, and cooking time.

Rotation speed (rpm) during cutting and stirring was measured in triplicate as the time needed by cutting frames or stirrers, respectively, to take on a full 360º turn. Cutting, cooking or total revolutions used during the cheese manufacture were calculated multiplying the rotation speed and time of cutting, stirring or the sum of both cutting and cooking revolutions, respectively (Johnston *et al.*, 1991). The knife density (KD) of the cutting frames in each dairy was defined as the ratio between the number of wires or knives within the cutting frame *per* the area of idem. Cutting tip speed (CTS, m/min) was calculated as in Equation IV.1.

$$CTS = 2\pi r(NCF) \times (CS) \tag{IV.1}$$

where r was the radius (m) of cutting frame, NCF was the number of cutting frames operating in the vat and CS was the rotation speed during cutting (rpm). Other parameters related to pressing, brining, airing and ripening processes were also collected during each cheese production.

1.2. Experimental study on a laboratory scale

1.2.1. Experimental design in controlled conditions

In order to study the effects of cutting and cooking settings in cheese yield and milk compound losses in the whey in controlled conditions, a two-level full factorial experimental design was employed. Particularly, the effects of curd grain size (CGS) and cooking temperature (CT) were studied, while the other conditions remained fixed. Two levels of each factor were selected: big and small for CGS, and 36 and 45 ºC for CT. Bulk raw sheep milk was collected from a local farm (Meredith Dairy, Truganina, Australia) the day before to the first day of cheesemaking. Two experimental cheeses were made simultaneously each day for two consecutive days, obtaining at the end of the week four different experimental cheeses. On the first cheesemaking day, the big CGS factor level was selected and the CT was varied in the two identical vats. On the second cheesemaking day, the same procedure was followed but the CGS factor chosen was small. The four cheese productions were repeated the following week with a second collection of bulk milk.

1.2.2. Description of the pilot scale plant

The experimental study was carried out in a small cheesemaking facility located at The Bio21 Molecular Science and Biotechnology Institute (The University of Melbourne, Melbourne, Australia). Cheeses were made in cubic-shaped stainless-steel double-jacketed open vats with 20 L capacity (Figure IV.6A). Vats were connected by a circulating system to water baths were the temperature was controlled. Two cutting frames stretched with nylon wires were used for cutting the curd: one had 29 horizontal wires, and the other 23 vertical wires (Figure IV.6B). The spacing between the wires in both cutting frames was 1 cm. Stirrers were holey square metallic panels with slightly bended lower corners (Figure IV.6C) that were placed in an overhead automatic stirrer (RZR 2102 control, Heidolph, Schwabach, Germany) to control stirring speed. A metallic cheese mould with cylindrical shape (15 cm diameter) was used for shaping and pressing the curd, and the laterwas carried out in a horizontal hydraulic press.

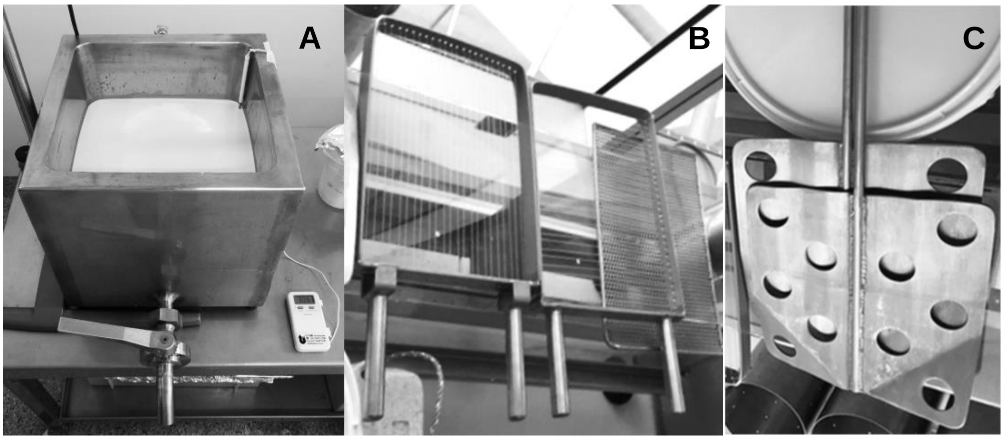

Figure IV.6. Cubic-shaped open vat (A), vertical and horizontal wired cutting frames (B) and stirrers (C) used for cheesemaking in controlled conditions on the laboratory scale study (Original photos: A. Aldalur).

1.2.3. Cheese manufacture

The cheesemaking process was similar to the one previously reported for Idiazabal cheese (Figure IV.5). The experimental cheeses were made using 10 kg of raw sheep milk, which had been stored for 1 or 2 d in refrigeration before cheese manufacture. Milk was warmed up to 25 ºC with constant agitation (30 rpm) for ~35 min and 0.02 g/kg of homofermentative mesophilic starter culture (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, Choozit, DuPont) was added. Temperature was raised to 30 ºC while stirring and 0.03 g/kg of commercial rennet with ~ 1,400 international milk-clotting units (IMCU) (Hansen Naturem, Chr. Hansen, Hørsholm, Denmark) was added. Milk was stirred for 2 min to ensure the correct blending of the rennet in the milk. The cutting process started 40 min after the rennet addition and it was performed manually. Two manual cutting frames with nylon wires in horizontal and vertical direction were inserted in the left side of the vat, moved simultaneously from left to right once, and removed. The two frames were then inserted again at the back of the vat and were pushed through the curds to the front of the vat. This entire movement was replied twice to obtain big curd grains, or six times to obtain small curd grains. After cutting, the temperature of the curd grain and whey mixture was increased from 30 ºC to either 36 or 45 ºC, depending on the cooking temperature selected and stirred at constant speed (40 rpm) for 50 min. After cooking, curd grains were pressed using a holey metal panel creating a continuous curd mass. Whey was drained from the vat and the curd mass was placed into a metallic cheese mould (15 cm diameter) covered with a wet cloth. The cheese was pressed at 4.2 kg/cm^2 in a horizontal hydraulic press for ~4-5.5 h at room temperature (~ 20 ºC) until the cheese pH dropped to ~5.50. Finally, the cheese was weighed (~ 2 kg) and stored vacuum packed in wedges without brining and ripening processes. The pH was monitored during the whole cheesemaking process using a pH meter (S220 SevenCompact, Mettler Toledo, Port Melbourne, Australia).

1.2.4. Sample collection

For the experimental design, samples of milk, drained whey, curd, FCG, SCG and cheese after pressing were collected. FCG and SCG samples were obtained submerging a round steel mesh sieve 13 cm in diameter to a depth approximately halfway between the top and the bottom of the vat right after cutting and cooking processes, respectively. For CLSM analysis, intermediate samples were stored at 5 ºC for no longer than 3 h, while cheeses were analysed within the next 24 h. For compositional analysis, all the samples were frozen at -30 ºC until analysis. For cheese texture analysis, cheese was kept in refrigeration at 5 ºC for no longer than 3 days. CLSM, fat globule size determination and rheological analysis of milk samples was carried out the day of milk collection.

2. Sample analysis

2.1. Milk

2.1.1. General composition

The general composition of milk samples was analysed in duplicate using different methods detailed below. Milk fat was determined in duplicate by Gerber (ISO/IDF, 2008a) and Babcock (AOAC, 2005a) methods. Totalnitrogen was measured by Kjeldhal (IDF/ISO, 2014) and combustion (ISO/IDF,2002) methods. For casein nitrogen analysis, acid precipitation was used for separating nitrogen fractions and these were analysed using Kjeldhal method (IDF/ISO, 2004a). Total nitrogen and casein nitrogen values were then multiplied by a factor of 6.38 to convert them into protein content. Fat, protein, lactose and dry matter content of the milk samples collected in the interventional study were analysed by near infrared spectroscopy (NIR) (SpetrAlyzer 2.0, Zeutec, Rendsburg, Germany) calibrated for sheep milk. Dry matter was measured by drying the samples overnight at 102 ºC (IDF/ISO, 2010). For calcium, magnesium and phosphorous determination, two different methods were used: microwave digestion followed by Flame Atomic Absorption Spectroscopy (AAS) for calcium and magnesium contents (de la Fuente *et al.*, 1997)and photometric method for phosphorous content (AOAC, 2005b); and on the other hand, Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Chen *et al.*, 2016).

2.1.2. Milk fat globule size

The milk fat globule size distribution was determined by light scattering using a particle size analyser (Mastersizer 3000; Malvern Instruments, Malvern, United Kingdom), as previously reported by Ong *et al.* (2010). The refractive indices of the milk fat and water were set at 1.460 and 1.334, respectively. Two hours before analysis sheep milk samples were diluted (1:1; v/v) in ethylenediaminetetraacetic acid (50 mM, pH 7), which was used to dissociate casein micelles and to obtain a clear separation of fat globules. Milk samples were poured into a circulating cell containing a mixture of water and sodium dodecyl sulphate (0.05%), which dissociated any aggregated fat globules. The volume-weighted mean diameter

(D[4,3]) was calculated by the particle analyser software. The milk samples were analysed in duplicate.

2.1.3. Confocal laser scanning microscopy and image analysis

Preparation of milk samples for CLSM was carried out as described previously by Ong *et al.* (2011). Milk samples were stained mixing Nile Red (0.02 mg/mL), fast green FCF (0.02 mg/mL), agarose solution (0.5%) and the milk sample in an Eppendorf tube. The stained sample was placed on a cavity slide and covered with a coverslip (0.17 mm thick) glued with nail polish.

Microstructural analysis were performed using inverted confocal microscopes (SP2 and SP8; Leica Microsystems, Heidelberg, Germany) located at the Faculty of Medicine and Dentistry (UPV/EHU, Leioa, Spain) and at The Bio21 Molecular Science and Biotechnology Institute (The University of Melbourne), respectively. The methodology used in both microscopes was identical. The excitation wavelengths were set to 488 nm for fat and 638 nm for protein, whereas the emission wavelengths were between 520-590 nm and 660-750 nm for fat and protein, respectively. The two-dimensional images acquired were 512 x 512 pixels in size.

Three-dimensional (3D) image analysis of milk CLSM micrographs was carried out using ImageJ software (National Institutes of Health, Bethesda, USA). A minimum of 30 adjacent pictures were obtained with a separation of 0.5 µm and converted into a 3D stack. Stacks were equalized using Stack Normalizer plug-in and the red and green channels were split to separate fat and protein, respectively. No analysis of the green channel (protein) was carried out for milk samples. For the red channel (fat), the background of the picture was adjusted to enhance the quality of the image and 3D Median filter was applied to smoothen the edges of fat globules. The red channel was then thresholded using IsoData thresholding method (Ridler & Calvard, 1978). Watershed binary function was used to separate fat globules that appeared coalesced in the pictures. The 3D Object Counter was used to quantify volume and surface area of the fat. Fat globule diameter, distribution parameters and sphericity index were determined for milk samples.

2.1.4. Rheological properties of milk

For assessing the coagulation properties of milk during the experimental design a Twin Drive rheometer (MCR702, Anton Paar, North Ryde, Australia) was used. The rheometer was equipped with a CC27 cup and bob accessory and temperature was maintained at 30 ºC during analysis. A sample of milk was previously warmed up to 30 ºC and immediately after adding 0.03 g/kg of rennet (IMCU ~1400, Hansen Naturem, Chr. Hansen) the mixture was loaded into the cup. The angular frequency and shear strain were set up to 0.8 Hz and 0.1%, respectively. Storage modulus (G'), loss modulus (G'') and loss factor tan (δ) were measured every 10 s for 60 min. Final curd firmness, curd firming rate and onset of gelation were calculated as reported by Logan *et al.* (2014). Final curd firmness was defined as the G' value at 60 min after the addition of rennet, curd firming rate was measured as the maximum slope over 12 consecutive points, and the onset of gelation was described as the time at which the G' first reached 0.05 Pa. Milk samples were analysed in duplicate.

2.1.5. Curd texture at cutting point

Two different methodologies were used to measure curd strength at cutting point. The first method was used in the experimental design on a laboratory scale carried out at The Bio21 Molecular Science and Biotechnology Institute (The University of Melbourne), while the second one was conducted in the Faculty of Pharmacy (UPV/EHU, Vitoria-Gasteiz, Spain).

The first method was carried out using a texture analyser (TA.HD plus; Stable Micro Systems, Godalming, United Kingdom) equipped with a 5 kg load cell and a cylindrical probe of 10 mm diameter. The test speed was set up to 1 mm/s and the penetration was established to 20 mm with a trigger force of 1 g. During cheesemaking, and right after the addition of rennet, two samples of 50 mL of milk were taken directly from the cheese vat, poured into a plastic container (42 mm diameter and 55 mm height) and incubated at coagulation temperature (30 ºC) for 40 min. Gel strength was defined as the maximum force (g) measured during penetration. Each curd sample was measured in duplicate and the mean value of gel strength was used for each vat.

The second method was based on the compression-extrusion test previously described by Nájera *et al.* (2009), using a texture analyser (TA-XT2i; Stable Micro Systems) that was armed with a 5 kg load cell and a cylindrical probe of 25 mm diameter. A test speed of 2 mm/s was used and the penetration was set to 10 mm. The curd coagulation conditions (pH of milk, temperature, rennet concentration and time until curd cutting point) of each production were simulated in the laboratory using the same milk and rennet paste collected during the morning from the dairy. Milk was poured into plastic containers (28 mm diameter) and the same concentration of rennet as the one used by the cheese-makers was added. Coagulation time was adjusted to the time used during the cheesemaking carried out in the dairy. Gel strength was defined as the area of the curve (g·s) measured during penetration. Eight measurements were performed for each milk sample that simulated the production conditions and the mean value was used for each production.

2.2. Rennet

2.2.1. Milk-clotting activity

The milk-clotting activity of rennet was determined as described by Bustamante (2002). This method is based on the visual determination of the appearance of the first floccules produced by the addition of a known amount of clotting enzyme in a known amount of standardized milk at constant conditions. For the preparation of the substrate, reconstituted skimmed milk powder at 12% (w/v) (Chr. Hansen) in calcium chloride (0.01 M) was used. The substrate was stirred for 30 min and left it for no more than 2 h at room temperature in the dark. For the preparation of the rennet extract, 1.0 g of rennet paste was weighed in a Potter homogenizer and a volume of ~7.0 mL of distilled water was added. The extraction of the enzymes from the rennet paste was carried out for ~10 min and the final extract was filtered and diluted to a concentration of 0.1 g/mL. This extract was diluted adding distilled water as required to obtain the optimum clotting conditions during the analysis.

For the milk-clotting analysis, reconstituted milk was warmed up to 30 ºC, 10 mL were poured into the test tube, and 1 mL of an adequate dilution of the rennet extract was added. The test tube was placed in a water bath at 30 ºC and was kept rotating at an angle of 30º while clotting occurred. The time between the addition of rennet extract and the appearance of the first floccules was measured. Rennet units (RU) were calculated as the quantity of enzyme required to clot 10 mL of standardized milk in 100 s in the analytical conditions used. Results were expressed as RU *per* g of rennet paste.

2.3. Whey

2.3.1. General composition

The general composition of whey samples was analysed in duplicate using the methods previously detailed for milk samples. Briefly, whey fat was analysed by Gerber (ISO/IDF, 2008a) and Babcock (AOAC, 2005a) methods. Total nitrogen was determined by Kjeldhal (IDF/ISO, 2014) and combustion (ISO/IDF, 2002) methods, and multiplied by 6.38 to obtain the total protein content. Dry matter was measured by drying the samples overnight at 102 ºC (IDF/ISO, 2010). Fat, protein, calcium, phosphorous, magnesium and dry matter content of whey samples collected in the interventional study were analysed by NIR. For the analysis of calcium, magnesium and phosphorous contents, AAS, photometric method and ICP-OES was used as previously detailed (de la Fuente *et al.*, 1997; AOAC, 2005b; Chen *et al.*, 2016).

2.3.2. Whey casein fines analysis

The casein fines content of the whey was measured using the method described by Everard *et al.* (2008). A 35 mL whey sample was centrifuged for 15 min at 1,500 g and at 5 ºC to ease the removal of the fat layer after centrifugation. Fat was removed using a spatula and liquid was poured without disturbing the pellet. Distilled water was added and centrifuged again for 15 min at 1,500 g. The remaining fat was removed and the supernatant phase was poured off again. Distilled water at 40 ºC was added to the pellet and filtered onto a Whatman nº 1 filter paper with a Buckner funnel in vacuum conditions. The centrifuge tube was then washed with warm distilled water and filtered two more times. The filter paper with casein fines was dried in an oven at 102 ºC for 1 h and weight. Results were expressed as mg of casein fines *per* kg of whey.

2.3.3. Chemical oxygen demand

The chemical oxygen demand (COD) of whey samples was determined by the dichromate method in small-scale sealed-tubes (ISO, 2002). This value can be considered an appraisal of the amount of oxygen consumed by the organic compounds present in the whey due to chemical oxidation. The diluted whey sample (1:80; v/v) was introduced in the sealed-tubes (HI94754C-25, Hannah Instruments, Woonsocket, United States of America), mixed and digested at 150 ºC for 2 h. Absorbance was measured using an spectrophotometer (Spectronic 20D, Milton Roy, Point-Saint-Pierre, France) and results were compared against a standard solution of potassium hydrogen phthalate. Results were expressed as mg of oxygen *per* litre of whey.

2.3.4. Calculation of milk compound losses in the whey

The estimation of fat, protein, calcium, magnesium and phosphorous loss in the whey on milk basis was calculated as in Equation IV.2 (Guinee *et al.*, 2006).

$$\%\,Loss = 100(W_W \times W_C)/(M_W \times M_C) \qquad \text{(IV.2)}$$

where W_W and M_W were the amount of drained whey and bulk milk in kg, respectively, and W_C and M_C were the amount of fat, protein, calcium, magnesium or phosphorous in whey and milk, respectively, depending on the compound being calculated.

2.4. Curd grains

2.4.1. General composition

The general composition of curd grains after cutting and after cooking, were analysed in duplicate using different methods detailed below. Fat content was determined by Van Gulik (ISO/IDF, 2008b) and Babcock (modification of AOAC (2005a) for solid samples) methods. Total nitrogen was measured by Kjeldhal (IDF/ISO, 2008c) and combustion (ISO/IDF, 2002) methods, and nitrogen values were then multiplied by a factor of 6.38 to convert them into total protein content. Dry matter was measured by drying the samples overnight at 105 ºC (IDF/ISO, 2004b). For the analysis of total calcium, magnesium and phosphorous content similar methods described above for milk and whey samples were carried out using AAS, photometric method or ICP-OES (de la Fuente *et al.*, 1997; AOAC, 2005b; Chen *et al.*, 2016).

2.4.2. Curd grains properties

2.4.2.1. Image acquisition and analysis

Curd grains after cutting and cooking were sampled for two-dimensional (2D) image analysis as described above. The curd grains that had been carefully separated on a black plastic sheet were photographed within the first 5 minutes after extracting the curd grains from the vat to prevent size changes due to syneresis. A digital camera (D80, Nikon, Tokyo, Japan) was used to take the 2896 x 1944 pixel photographs. The scale of the image was set including a graph paper (model 81718, Unipapel, Tres Cantos, Spain) at the time the picture was taken. Image analysis was carried out using ImageJ software (National Institutes of Health). The brightness and contrast were adjusted to improve quality and enhance the edge of each curd grain, respectively. The image was then thresholded, dividing the picture into features of interest (curd grains) and background. Curd particles that were separate and clearly defined from one another with an area higher than 1 mm^2 were selected for the analysis.

2.4.2.2. Curd grain size and shape measurements

The size and shape of individual curd grains was determined using image analysis. For size parameters, area, perimeter and Feret's diameter were measured for each curd grain and defined as follows.

- Area (mm^2) was the projected area of a curd grain on a 2D plane.
- Perimeter (mm) was the length of the outside boundary of a curd grain.
- Feret's diameter (mm) was the longest distance between any two points along the particle boundary, also known as maximum caliper.

Regarding shape parameters, reciprocal aspect ratio or elongation (RAR), rectangularity (RTY) and circularity (CTY) were measured for each curd grain (Igathinathane *et al.*, 2008). These parameters were calculated and defined as follows:

- RAR was defined as the elongation of a particle and it was calculated as $RAR = B/A$, where A and B were the major and minor axes of the best fitting ellipse of the curd grain. Elongation values closer to zero indicated elongated shape, whereas values closer to 1 meant no flatness.
- RTY was the portion of the area of the curd grain that fitted the bounding rectangle of idem curd grain, and was calculated as $RTY = AREA/(H \times W)$', where H and W were the height and the width of the smallest rectangle enclosing the curd grain, respectively. Rectangularity values approaching 1 indicated a perfect rectangle shape.
- CTY was calculated as $CTY = 4\pi(area)/(perimeter\hat{}2)$' with a value of 1 indicating a perfect circle.

All the values used in these calculations, such as, the length of the major and minor axes of best fitting ellipses or the height and the width of the smallest rectangle were directly measured by the ImageJ software.

2.4.2.3. Particle size distribution analysis

Particle size distribution (PSD) analysis based on the area of FCG and SCG samples was carried out for each cheesemaking production. This analysis was performed in order to measure the homogeneity degree of the size of the curd grains at the end of cutting and cooking processes. Table IV.1 shows the PSD parameters measured together with the meaning of each parameter (Igathinathane *et al.*, 2009).

Table IV.1. Definitions of particle size distribution (PSD) parameters used for assessing the homogeneity degree of the size of curd grains after cutting and cooking processes in Idiazabal cheese productions.

PSD parameter		Formula	Meaning
Uniformity index	I_u	$\frac{D_5}{D_{90}} \times 100$	Values close to 100 indicate homogeneous distribution
Size range variation coefficient	S_v	$\frac{D_{84} - D_{16}}{2D_{50}} \times 100$	Values close to 0 indicate homogeneous distribution
Relative span	S_l	$\frac{D_{90} - D_{10}}{D_{50}} \times 100$	Values closer to 1 indicate homogeneous distribution
Coefficient of uniformity	C_u	$\frac{D_{60}}{D_{10}}$	Values closer to 1 indicate homogeneous distribution
Graphic skewness	S_g	$\left[\frac{D_{84} + D_{16} - 2D_{50}}{2(D_{84} - D_{16})}\right] + \left[\frac{D_{95} + D_5 - 2D_{50}}{2(D_{95} - D_5)}\right]$	Indicates the asymmetry degree of the distribution[1]
Graphic kurtosis	K_g	$\left[\frac{D_{95} - D_5}{2.44(D_{75} - D_{25})}\right]$	Indicates the peakiness degree of the distribution[1]

[1]Further description of this classification is provided in Folk & Ward (1957).
D_x is the x^{th} percentile value of the area distribution of each production.

2.4.3. Confocal laser scanning microscopy

Curd grain samples were previously stored in refrigeration in a fresh form for samples being analysed in less than 3 h, or in a formaldehyde solution (0.5%, w/v) for samples being analysed the following day. Before CLSM analysis, the later were washed in a phosphate buffered saline solution (pH 7.4) for at least 1 h. From there onwards, preparation of curd grain samples was carried out following the method described by Ong *et al.* (2011). Briefly, curd grains were cut into approximately 5 x 5 x 2 mm in size using a surgical blade, and stained at ~ 4 ºC using Nile Red (0.1 mg/mL) and fast green FCF (0.1 mg/mL) (both from Sigma-Aldrich, Madrid, Spain). The equipment and settings of the confocal microscopes were the same as the ones described above for milk samples. The micrographs obtained were also 512 x 512 pixels in size.

ImageJ software was used to analyse the CLSM 3D images of curd grains. A minimum of 20 adjacent planes were obtained with a separation of 0.5 µm and converted into a 3D stack. Stacks were equalized and their quality was enhanced, as previously described for milk samples, and the red and green channels were then split. For the red channel (fat), the background of the picture was adjusted to enhance the quality of the image and 3D Median filter was applied to smoothen the edges of fat globules. Fat and protein channels were then thresholded using IsoData thresholding method (Ridler & Calvard, 1978). Watershed binary function was used to separate fat globules that appeared coalesced in the pictures. The 3D Object Counter was used to quantify volume and surface area of fat and protein. Fat globule volume-weighted mean diameter, sphericity index and network porosity were determined. Fat globule size distribution was also measured and two groups were distinguished and quantified by image analysis: globular fat droplets, and non-globular or coalesced fat droplets. Those droplets with a diameter smaller than the volume-weighted mean diameter of the fat globule in milk were defined as globular fat droplets. On the other hand, droplets with a diameter higher than 10 µm were considered non-globular or coalesced fat droplets, since the areas of fat were most likely pools of free fat (Everett & Auty, 2008). The percentage of fat volume for each group was measured.

2.5. Cheese

2.5.1. General composition

The general composition of cheese samples was analysed in duplicate using the same methods described above for curd grains. Fat content was determined in duplicate by Van Gulik (ISO/IDF, 2008b) and Babcock (modification of AOAC (2005a) for solid samples) methods. Total nitrogen was measured by Kjeldhal (IDF/ISO, 2008c) and combustion (ISO/IDF, 2002) methods, and nitrogen values were then multiplied by a factor of 6.38 to convert them into total protein content. Dry matter was measured by drying the samples overnight at 105 ºC (IDF/ISO, 2004b). For the analysis of total calcium, magnesium and phosphorous content AAS, photometric method and ICP-OES were used (de la Fuente *et al.*, 1997; AOAC, 2005b; Chen *et al.*, 2016). Fat, protein and dry matter content of cheese samples collected in the interventional study were analysed by NIR (SpetrAlyzer 2.0, Zeutec) calibrated for ovine cheese up to 3 month of ripening.

2.5.2. Confocal laser scanning microscopy

Cheese sample preparation for CLSM and image analysis were carried out following the method described above for curd grains. Briefly, cheese samples were cut into 5 x 5 x 2 cm in size and were stained using Nile Red (0.1 mg/mL) and fast green FCF (0.1 mg/mL). CLSM micrographs (512 x 512 pixels) were obtained and these were analysed using ImageJ software. Several microstructure parameters defined above for curd grains such as, the number, diameter, distribution parameters and sphericity of fat globules, and porosity of the protein network were measured.

2.5.3. Cryo-scanning electron microscopy

In the experimental study on a laboratory scale, cryo-scanning electron microscopy (cryo-SEM) was also carried out for cheese samples as described by Ong *et al.* (2011). Cheese samples were cut into approximately 5 x 5 x 2 mm in size and placed on the sample holder. These were immersed into a liquid nitrogen slush (nitrogen at its freezing point, -210 ºC) for 10 s. The frozen samples were immediately transferred into the cryo preparation chamber, which was kept under high vacuum conditions (> 10^{-4} Pa) at -140 ºC. Samples were then fractured in horizontal disposition using a chilled scalpel blade and temperature was raised to -95 ºC for etching during 30 min. The temperature was decreased again to -140 ºC and samples were coated using a cold magnetron sputter coater (300 V) with 10 mA of sputtered gold/palladium alloy (60/40) for 120 s generating a layer ~ 6 nm thick. Samples were finally introduced into a nitrogen gas cooled module under vacuum at -140 ºC and observed using a field emission gun SEM (Quanta, Fei Company, Hillsboro, United States of America) at 15 kV. A solid-state backscattered electron detector was used for the observation.

2.5.4. Cheese texture

Two different texture analysis tests were carried out on cheese samples: texture profile analysis (TPA) and uniaxial compression at 50%. For the experimental study on a laboratory scale, the TPA method was based on the one previously published by Ong *et al.* (2012), while for the interventional study the uniaxial compression test at 50% was carried out. Different sample preparation and settings were used for both studies.

For the experimental study on a laboratory scale, a knife and a cheese corer were used to cut the centre of the cheese wedges into 2.5 cm in diameter and 2.5 cm in height cheese samples. Before analysis, these were tempered in a closed plastic container at ~20 ºC for 1 h. For TPA analysis, a texture analyser (TA.HD plus, Stable Micro Systems) equipped with a 25 kg cell and a 35 mm cylindrical flat probe was used for the double compression test at 50% at a constant crosshead speed of 2 mm/s. Three replicates were carried out for each cheese treatment.

For the interventional study, cheese wedges were maintained at ~25 ºC for 1 h. Each cheese wedge was cut in at least four slices of 1.2 cm in height, casting aside ~1 cm from the top and bottom pieces that corresponded to the rind of the cheese (Figure IV.7). Each slice in turn, was cut into five cubes of 1.2 cm in side as represented in Figure IV.7. For each cheese wedge, the cubes obtained from slices A and B were used for the uniaxial compression test using a texture analyser (TA-XT2i, Stable Micro Systems) equipped with a 25 kg cell and conducted with a 25 mm cylindrical probe at a constant speed of 1 mm/s.

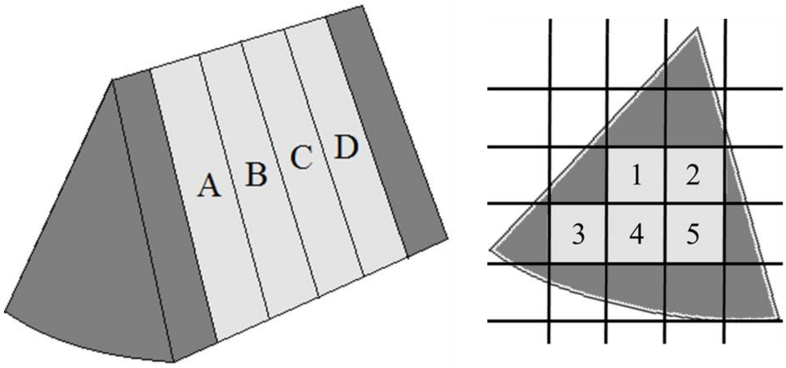

Figure IV.7. Sketch of the cheese sample preparation for the uniaxial compression test. On the left, drawing of a cheese wedge sliced in four (A-D) and pieces casted aside (dark grey). On the right, position of the five cubes (1-5) obtained from each cheese slice.

The compression test was replicated 10 times for each cheese sample, using the 10 cubes described in the sample preparation. The textural parameters measured were defined as follows and are represented in Figure IV.8:

1. Stiffness is the resistance to reversible deformation being this proportional to the force applied. The slope between the start and the maximum force is the stiffness measure (g/s).
2. Hardness and fracturability are the maximum force of compression, and the stress required to fracture the sample, respectively. In this case both measurements are at the same point of the curve (g).
3. Brittleness is considered the structural collapse period, where internal eyes and small cracks disappear. It was calculated as the slope between the maximum force and the next lower point (g/s).
4. Hardness work is indicative of the resistance to deformation over the time and it was measured as the total area under the compression curve (g·s).
5. Adhesiveness is the work necessary to overcome the attractive forces between the surface of the sample and the test probe. The adhesiveness measure is the area in the negative zone after the decompression of the sample (g·s).

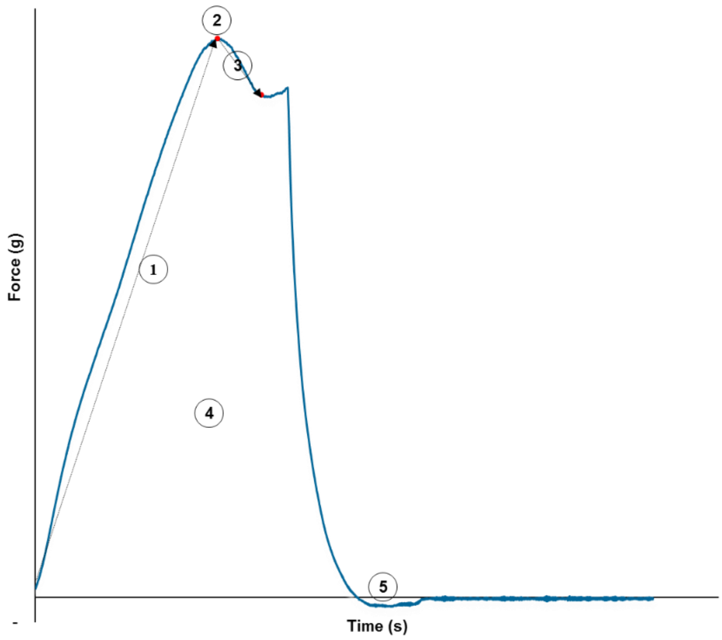

Figure IV.8. Standard curve obtained in the single uniaxial compression test. Numbers indicate the parameters defined above. 1, Stiffness; 2, Hardness and fracturability; 3, Brittleness; 4, Hardness work; 5, Adhesiveness.

2.5.5. Cheese yield calculation

Cheese yield was expressed in several formats, which have been previously described by Guinee *et al.* (2006), and defined as follows:

- **Actual cheese yield** (**Y_A**, kg of cheese/100 kg of milk) was the ratio between the total weight of cheese after pressing and after ripening, and the amount of milk used in the cheese production.

- **Moisture-adjusted cheese yield (Y_{MA})** was calculated as in Equation IV.3 in order to compare cheese yield of different moisture content cheeses:

$$Y_{MA} = Y_A \times ((100 - M_A)/(100 - M_R)) \quad \text{(IV.3)}$$

  where M_A and M_R were the actual and reference (45.0%) moisture contents of cheese, respectively.

- **Composition-adjusted cheese yield (Y_{CA})** was measured as in Equation IV.4 to eliminate the effect of changes in milk fat and protein content, and in cheese moisture, and assess only the effect of the technological conditions on cheese yield:

$$Y_{CA} = Y_A \times ((F_R + P_R)/(F_C + P_C)) \times ((100 - M_A)/(100 - M_R)) \quad \text{(IV.4)}$$

where F_R and P_R were the percentages of fat (6.75%) and protein (5.25%) in the reference milk; F_C and P_C were the actual fat and protein contents in the milk; and M_A and M_R were the actual and reference (42.6%) moisture contents of cheese, respectively.

- **Theoretical cheese yield (Y_T)** was calculated as in Equation IV.5 using the Van Slyke formula (Emmons & Modler, 2010):

$$Y_T = [(0.93F + C - 0.1) \times 1.09 \times 100]/(100 - M) \quad \text{(IV.5)}$$

where F and C were the fat and casein content of milk and M was the moisture content of cheese.

Finally, cheese yield efficiency was also calculated comparing the actual to the theoretical cheese yield estimated for each production (Barbano & Sherbon, 1984). This index provided useful information about the productivity and profitability of the cheesemaking process in the small rural dairies.

2.6. Statistical analysis

The IBM-SPSS version 25.0 (New York, United States of America) and Addinsoft XLSTAT version 2011.2.06 (New York, United States of America) softwares were used for statistical analysis. Statistical significance was declared at $P \leq 0.05$.

2.6.1. Analysis of variance

Different general linear models (GLM) of analysis of variance (ANOVA) were used depending on the design and objectives of the observational and interventional studies and the experimental design on a laboratory scale:

- For the study of the relationships between cheese yield and the technological conditions used during cheesemaking in small rural dairies, the effect of individual dairy (l = 8), season (l = 2) and year (l = 2) on the technical and analytical variables was investigated. Equation IV.6 shows the GLM applied in this case:

$$Y_{ijkl} = \mu + D_i + S_j + Y_k(S_j) + D^*S_{ij} + \varepsilon_{ijkl} \qquad \text{(IV.6)}$$

  where Y_{ijkl} = technical or analytical variable; μ = intercept; D_i = individual dairy fixed effect; S_j = season fixed effect; Y_k = year fixed effect; and ε_{ijkl} = residual random effect.

- For the study of the characterization of curd grains after the cutting and stirring processes using two-dimensional image analysis, the effect of individual dairy (l = 8), cheese production (l = 3) and curd syneresis process (l = 2) on the technical and analytical variables was investigated. When assessing cheesemaking physico-chemical and technical parameters, the GLM in Equation IV.7 was used:

$$Y_{ijk} = \mu + D_i + CP_j + \varepsilon_{ijk} \qquad \text{(IV.7)}$$

  where Y_{ijkl} were the technical or analytical variables, and D_i and CP_j were the fixed effects of the individual dairy and cheese production, respectively. The D^*CP_{ij} interaction term was included in the model when curd grains size and shape parameters were evaluated. Additionally, the milk fat-to-protein ratio was included as a covariate when actual cheese yield was studied as a dependent variable. When assessing simultaneously the effect of the individual dairy (D_i) and curd syneresis process (S_j) on curd grain size and shape parameters, the fixed effects GLM used was the one described in Equation IV.8:

$$Y_{ijk} = \mu + D_i + S_j + D^*S_{ij} + \varepsilon_{ijk} \quad \text{(IV.8)}$$

For those parameters with significant interaction term D^*S_{ij}, an F-test for the main effects against the interaction term D^*S_{ij} was used.

- For the experimental study on a laboratory scale, ANOVA was used to study simultaneously the effects of curd grain size (*l* = 2) and cooking temperature (*l* = 2) on the composition, microstructure, and texture properties of curd and cheese samples, and cheese yield parameters measured for the different cheese trials. Equation IV. 9 shows the GLM applied:

$$Y_{ijk} = \mu + GS_i + CGS_j + CT_k + day_l + CGS^*CT_{jk} + \varepsilon_{ijkl} \quad \text{(IV.9)}$$

Y_{ijkl} = analytical variable; μ = intercept; GS_i = gel strength at the cutting point which was used as a covariate; CGS_i = curd grain size fixed effect; CT_k = cooking temperature fixed effect; day_l = cheesemaking day block; and ε_{ijkl} = residual random effect.

In all cases, normality and homoscedasticity of technical and analytical variables were checked. Tukey's honest significance difference test was used for pairwise comparison from mean values with equality of variance, and Games-Howell post-hoc test was applied in case of heteroscedasticity. When covariate was included in the GLM, estimated marginal means were compared using the Fisher's Least Significant Difference test.

2.6.2. Student's t-test

For the interventional study, the Student's t-test was used to separately assess the effect of cutting and cooking conditions (*l* = 2; moderate or high intensity processes), or that of curd syneresis (*l* = 2; after cutting or cooking), on the composition and properties of curd grains, whey and cheese samples. Normality and homoscedasticity of technical and analytical variables were checked.

2.6.3. Bivariate correlations

Pearson's bivariate correlations were calculated for several variables, such as milk and whey composition or textural and microstructural parameters of cheese samples.

2.6.4. Principal component analysis

Principal component analyses (PCA) were performed in order to reduce the number of variables and to look for relationships between technical and analytical variables associated to the PCs extracted by the analysis. PCAs were carried out on self-scaling variables and relationships between composition of milk, curd grain, whey, cheese after pressing and 2 months of ripening, curd grain size and shape parameters, technical settings used for cutting and cooking and cheese yield were investigated. The Kaiser criterion (eigenvalue > 1) was applied to extract the PCs, and the Varimax method was used to rotate the factors and ease interpretation of the results. The spatial distribution of the cheese productions carried out in the small rural dairies was plotted using the two-dimensional coordinate system defined by the first two PCs.

2.6.5. Partial least square regression analysis

For the interventional study, partial least square regression (PLSR) analyses were used to investigate relationships between the milk properties together with the technological settings used during cheesemaking, and the curd, whey and cheese properties, and cheese yield and whey losses obtained in the selected small rural dairies. PLSR analyses were carried out on self-scaling variables and only those variables showing scores for variable importance in the projection higher than 0.8, and loadings higher than 0.5 in the latent dimensions were included.

2.6.6. Linear regression analysis

For the observational study, a simple regression analysis was applied to evaluate the relationship between particle size parameters (area, perimeter and Feret's diameter) and the log of total revolutions used in the cheese productions carried out in the small rural dairies.

2.6.7. Non-parametric analysis

Non-parametric analyses were applied to the median values of particle size and PSD parameters in FCG and SCG samples. Mann-Whitney´s U non-parametric test was used to pairwise compare among dairies the median value of the area of FCG and SCG, separately, and to assess the significance of the differences in mean values of PSD parameters between FCG and SCG sample groups.

Chapter V

Results and Discussion

1. Commercial cheesemaking in small rural Idiazabal dairies

1.1. Observational study: assessment of the cheesemaking technical conditions used in small rural Idiazabal dairies and the effect on cheese yield and whey losses.

Publication I and Manuscript II accurately describe the cheesemaking process carried out in small rural dairies that manufacture Idiazabal cheese and the impact of the technical conditions on curd, whey, cheese composition and yield. In particular, the cutting and cooking conditions were evaluated, and a detailed characterization of the size, shape and size distribution of the curd grains was carried out using a 2-dimensional image analysis (**Publication I**). Additionally, the effect of the seasonal changes in milk composition between late winter and early summer was also observed, together with its influence on cheese yield and milk compound losses in the whey. The results of this study contribute to the improvement of the cheesemaking process in small rural Idiazabal dairies, where the facilities are limited and the role of the cheese-maker is crucial. In this regard, some general recommendations about the control of the technical parameters were provided.

Additionally, another informative article with some preliminary results obtained in this project was published in a journal directed towards the dairy sector in Spain (**Appendix A**). The article explains the environmental problems that the discharge of the whey can cause and the limitations in this regard for the small rural dairies. Some preliminary results regarding the relationship between technological parameters and cheese yield obtained in the small rural dairies were provided. In order to transfer some of these results to the dairy sector, whey composition and cheese yield data was shown and some general recommendations were suggested for a possible improvement of the cheesemaking process.

Publication I

Characterization of curd grain size and shape by 2-dimensional image analysis during the cheesemaking process in artisanal sheep dairies

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Characterization of curd grain size and shape by two-dimensional image analysis during the cheesemaking process in artisanal sheep dairies

Ane Aldalur*, María Ángeles Bustamante*, Luis Javier R. Barron*,[1]

*Lactiker Research Group, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain.

CHANGES IN CURD GRAIN PROPERTIES DURING CHEESEMAKING

IS. Changes in curd grain properties during artisanal cheesemaking – Aldalur. The technical settings employed for cutting and stirring steps during cheesemaking causes differences in the size and shape of curd grains. Artisanal cheese-makers use visual observation of curd grains to decide the end of the cutting and stirring processes. This study addresses changes in curd grain size and shape found in eight artisanal sheep dairies during Idiazabal cheese production. Image analysis of curd grains gives useful information for determining characteristics related to cheese yield and quality, and may contribute to improving and controlling the cheesemaking process in small artisanal dairies.

[1] Corresponding author: luisjavier.rbarron@ehu.eus

ABSTRACT

The size and shape of curd grains are the most important parameters used by cheese-makers to decide the end of the cutting or stirring processes during cheesemaking. Thus, two-dimensional image analysis was used to measure the characteristics of curd grains in commercial cheese productions carried out by artisanal sheep dairies. Dairies used different technical settings for cutting and stirring steps causing differences in the size and shape of curd grains. A linear relationship between total revolutions used for cutting and stirring and curd particle size was established. However, particle size distributions after curd cutting and stirring were highly heterogeneous. Actual cheese yield was correlated to particle size and cutting revolutions, while curd grain shape and fat loss were associated to stirring conditions by a multivariate approach. Image analysis of the size and shape of curd grains gives useful information for determining characteristics related to cheese yield and quality, and may contribute to improving and controlling the cheesemaking process in small artisanal dairies.

Key words: image analysis, cheese yield, artisanal sheep dairy, commercial cheese production

INTRODUCTION

The measurement of the size and shape of curd particles as well as the particle size distribution (PSD) is an effective tool for controlling the cutting and stirring steps during the cheesemaking process. Previous studies have investigated the effect of cutting and stirring on curd syneresis, cheese yield, and protein and fat losses in the whey using the sieving method for curd PSD measurement (Kosikowski, 1962; Johnston *et al*., 1991; Akkerman, 1992; Johnston *et al*., 1998; Everard *et al*., 2008; Mateo *et al*., 2009). This method based on passing the curd grains through a battery of different size mesh sieves showed strong deviations (around 20 times) and limitations (maximum 5 sieves) to separate the grains with different size (Johnston *et al*., 1991; Igathinathane *et al*., 2009). Furthermore, the sieving method shows other difficulties due to stickiness of the curd grains, lump formations, and breakage of curd particles during the mechanical handling (Jablonka and Munro, 1985; Johnston *et al*., 1991). This makes it difficult to compare PSD data amongst different studies or to calculate parameters to describe PSD curves accurately. Recently, other authors have used a computer vision based on a two-dimensional image analysis method to study the curd grains during the cheesemaking of some Italian cheeses (Iezzi *et al*., 2012). Image analysis enables a more detailed measurement of curd PSD and the management of accurate data of different particle size and shape features. This method allows ampler information and looking for new variables for PSD and particle characterization (Du and Sun, 2004; Igathinathane *et al*., 2009).

Most previous studies have focused on the particle size to characterize curd grains during the cheesemaking process but particle shape can be a parameter as important as size because it describes the particle more thoroughly, and it can help to develop a better understanding of curd grain syneresis during cutting and stirring. Kammerlehner (2009) described the shape of the curd grains after stirring as similar to that of seeds and fruits but, to the best of our knowledge, very few quantitative data is nowadays available on the shape description of curd particles (Rüegg and Moor, 1987).

Small artisanal dairies generally use manual or semi-automatic vats for cutting and stirring the curd. Therefore, cheese-makers are guided by their own experience to choose the cutting and stirring parameters, but it is crucial for each dairy to determine the optimal processing conditions for its facilities and for the cheese type that is being manufactured (Kammerlehner, 2009). This study monitored the cheesemaking process carried out in small artisanal dairies belonging to the Idiazabal cheese Protected Denomination of Origin (PDO). This cheese is a semi-hard variety made from raw sheep milk in the Basque Country and Navarre (northern Spain) where most cheese dairies are of small production volume and artisan type (Idiazabal PDO, 2017). The Idiazabal PDO regulation (Ministerio de Agricultura, Pesca y Alimentación, 1993) indicates briefly that the curd grains after the cutting step must achieve a diameter of 5 to 10 mm and afterwards, the grains must be stirred until a maximum temperature of 38ºC. This regulation does not determine the characteristics of the final curd grains after stirring, even though it is a more relevant parameter to cheese quality and yield than the one currently described (Dejmek and Walstra, 2004; Everard *et al*., 2008).

Therefore, the aim of the present study was to investigate the changes in particle size and shape that occurred during the curd grain syneresis after the cutting and stirring steps in small artisanal sheep dairies using two-dimensional image analysis. Specific particle shape parameters were described for the first time in curd grain characterization and the relationships between particle size and shape, cheese yield and technological parameters used for the cutting and stirring were investigated.

MATERIAL AND METHODS

Artisanal dairies and commercial cheese productions

Eight Idiazabal PDO small artisanal dairies participated in the study. Three commercial cheese productions made in different days were monitored in each dairy during the cheese processing season from March to June. The facilities in the artisanal dairies included automatic milking machines, milk cold storage tanks, hydraulic presses, and controlled ripening chambers. All dairies used stainless steel vats (from 300 to 1,500 L) with heating and agitation systems including thermal sensors and potentiometers for the measurement of temperature and rotation speed of knives and stirrers, respectively. Small dairies carried out one cheese production per day. All the dairies, except dairy 1, used open oval-shaped vats, which had one or two axes of rotation where stirrers or cutting frames were placed. The shafts worked in circular motion (in the same or opposite direction when the two axes worked at the same time), but shifted from side to side of the vat while rotation occurred to prevent demixing of the curd. Cutting frames were equipped with 32 to 42 metallic or plastic wires in vertical alone or separately arranged vertical and horizontal positions with a gap of 2 cm among one another. For stirring, helix- or irregular-shaped metal paddles were totally submerged in the curd and whey mixture. On the other hand, dairy 1 used an open double-O shaped vat with two fixed axes, equipped with vertical blade-cutters which were transformed into stirrers when the desired curd grain size was obtained during the cutting. The blades were separated 6 cm from one another and were sharp in the rotation direction when the cutting was performed, and dull in the opposite direction.

The cheeses were manufactured according to the specifications approved by the Idiazabal PDO Regulatory Council (Ministerio de Agricultura, Pesca y Alimentación, 1993) from raw Latxa sheep milk. Depending on the production month and individual dairy, the commercial cheeses were made with bulk milk from two to five milkings, and the raw milk was stored at 3-8ºC for an average time of 34 h before cheesemaking. Briefly, raw sheep milk was heated and a commercial homofermentative starter culture (Choozit, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added when the milk reaches around 25ºC. An adequate amount of commercial rennet and/or artisanal lamb rennet paste (Bustamante *et al*., 2000) was added to coagulate the milk at around 30ºC. Once the milk was coagulated, the curd was cut into grains at a constant temperature of around 30ºC and immediately after the curd grains were stirred until approximately 36ºC was reached. The time and rotation speed used for cutting and stirring depended on the dairy and these parameters were not specified in the Idiazabal PDO regulation (European Commission, 2015). The whey was initially removed by manually pressing the curd grains in the vat and the formed paste was introduced in plastic moulds. Afterwards, cheeses were pressed at around 20ºC for 3-7 h at

1.5-3.5 kg/cm^2, placed in saturated sodium chloride brine at around 10ºC for 10-24 h, and ripened at 8-12ºC and 85% relative humidity for at least 2 mo.

Milk and curd grain sampling

One sample (0.5 L) of bulk raw milk was taken from the vat before cheesemaking commenced and it was transported to the laboratory in a portable cooler. Subsamples of 50 mL were stored in screw-capped plastic containers at -80ºC. Curd grains were sampled following an adapted method from Iezzi *et al*. (2012) using a round steel mesh sieve with a 22 cm diameter and 0.43 mm mesh openings. The sieve was submerged to a depth approximately halfway between the top and bottom of the vat right after the end of cutting (fresh curd grain, FCG) and stirring (stirred curd grain, SCG), and before letting the curd grains settle. The sampling took from 3 to 5 s and curd grains were extracted by draining the excess amount of whey. The granules were scattered on a 210 x 297 mm^2 black plastic sheet, and disjoined carefully using a palette knife. This procedure prevents the formation of lumps that distort the size and shape of the grains and hinder image analysis. Photographs were taken within the first 5 minutes after extracting the curd grains from the vat because, as syneresis continued especially in the fresh curd grains, the image had to be taken as fast as possible to avoid changes in the extracted curd grains. FCG and SCG were sampled for each cheese production.

Physico-chemical and technical determinations

Milk and cheese fat was determined in duplicate by Gerber and Van Gulik method (ISO/IDF, 2008a, b), respectively, using a Lacter centrifuge (Orto Alresa, Aljavir, Madrid). Milk and cheese protein was analysed by Kjeldhal method (ISO/IDF, 2014; 2008c) using a Kjeltec 2100 distillation unit (Foss, Höganäs, Sweden). Cheese dry matter was measured by drying the samples overnight at 102ºC (ISO/IDF, 2004). Curd pH was measured in triplicate *in situ* at 20ºC using a GLP21 pH-Meter (Crison, Alella, Spain). Milk volume (L) used for cheese production was measured in each artisanal dairy using flowmeters with mean precision values of ± 0.15%. Actual cheese yield (kg of cheese/100 L of milk) *per* vat was estimated as the ratio between the total weight of fresh cheeses after pressing and before brining, and the milk volume used in the vat. Commercial weighting scales with mean precision value of ± 1 g available in the dairies were used. Adjusted cheese yield (Y_{ca}) was defined as the moisture-adjusted cheese yield *per* 100 L of cheese milk adjusted to reference levels of fat (6.8%) and protein (5.30%) and was calculated using the equation (1) described by Guinee *et al*. (2006):

$$Y_{ca} = Y_a \times ((F_r+P_r)/(F_c+P_c)) \times ((100-M_a)/(100-M_r)) \quad (1)$$

Where Y_a is the actual cheese yield; F_r and P_r are the percentages of fat and protein in the reference cheese milk; F_c and P_c are the actual fat and protein contents of the cheese milk; and M_a and M_r are the actual and reference (42.5%) moisture contents of cheese, respectively. Adjusted cheese yield was used to estimate the effect of cheesemaking conditions on cheese yield minimizing the intra- and inter-dairy variability in milk composition and cheese moisture during the cheese production season. The amount of fat lost in the whey on milk basis was calculated as described in equation 2 (Guinee *et al*. 2006):

$$\%FL = (W_W \times W_F)/(M_W \times M_F) \times 100 \quad (2)$$

where W_W and M_W were the weight (kg) of the drained whey and milk, respectively, and W_F and M_F were the fat content (wt/wt) of whey and milk, respectively. The amount of whey produced during each processing was estimated as the mass balance between the amount of milk and that of fresh cheese produced *per* vat. The amount of milk *per* vat was calculated from the milk volume and density measured using a thermolactodensimeter (Gerber instruments AG, Effretikon Switzerland). Time and temperature (T) measurements for milk coagulation, and curd cutting and stirring were monitored *in situ* with a Digitaler 2-fach timer digital chronometer (TFA Dostmann, Wertheim, Germany) and a N16B Glas mercury thermometer (Ludwig Schneider, Wertheim, Germany). Stirring T rate was established as the ratio between the difference of final and initial stirring T and time. Rotation speed for cutting and stirring was determined in triplicate from the time measurement that the tools (wire knives and stirrers) take on a full 360º turn. Total revolutions used in each cheese production and artisanal dairy were calculated as the sum of rotation speed x time for cutting and stirring steps (Johnston *et al*., 1991).

Curd grain two-dimensional image analysis

Photographs were taken with a D 80 digital camera (Nikon Corporation, Tokyo, Japan) at a 2896 x 1944 pixels resolution and the curd grain images were analysed with ImageJ software (National Institutes of Health, Bethesda, MD). The best-captured photograph *per* cheese production was used to analyse the curd grains. A 215 x 310 mm^2 graph paper (model 81718, Unipapel Int., Tres Cantos, Spain) was used as a reference to set the image scale. First, the quality of these images was improved by adjusting brightness and contrast to enhance the edge of each curd grain. Then, the image was thresholded, segmenting the picture into features of interest (curd grains) and background. As brightness conditions were not the same in the artisanal dairies, the thresholding step was customized for each photograph taking into account the boundaries of the curd grains. Each granule that was clearly separated from one another or that its boundary was easily detectable for the analyst was selected. Before measuring the particle size and shape, the adjustment of the selection area to the curd grain was individually checked. Those particles with area < 1 mm^2 were not taken into account in the curd grain image analysis.

The following size and shape parameters were measured: area (mm^2); perimeter (mm) which is particularly useful for discriminating between particles with simple and complex shapes (Du and Sun, 2004); Feret's diameter (mm) defined as the longest distance between any two points along the particle boundary; reciprocal aspect ratio (RAR) defined as RAR= B/A, where A and B are the major and minor axes of the best fitting ellipse of the particle, respectively, with a RAR value closer to 0 indicating elongated shape while closer to 1 no flatness; rectangularity (RTY) defined as RTY= AREA/(H×W), where H and W are the height and the width of the smallest rectangle enclosing the particle, respectively, with RTY = 1 indicating a perfect rectangle shape; circularity (CTY) defined as $CTY=4\pi\,[AREA]/[PERIMETER]^2$ with CTY values ranging from 1 to 0 indicating a perfect circle to an increasing elongated shape, respectively. The reduction of the size or shrinkage of the curd particles during stirring was used as an indicator of curd syneresis. Reduction rate mean values for area, perimeter and Feret's diameter were defined as the ratio between FCG and SCG values for each parameter.

Particle size distribution (PSD) analysis of FCG and SCG samples from each artisanal dairy cheese production was based on the area based percentile dimensions from 5 to 95th. According to Igathinathane *et al*. (2009), the following PSD parameters based on percentile dimensions were calculated: uniformity index (I_u) with a percentage value close to 100 indicating homogeneous distribution; size range variation coefficient (S_v) with a percentage value furthest from 0 indicating heterogeneous distribution; relative span (S_l) and coefficient of uniformity (C_u) with a value closer to 1 indicating homogeneous distribution; and graphic skewness (S_g) and kurtosis (K_g) with different numerical values (Folk and Ward, 1957) to indicate the asymmetry and peakiness degree of the distribution, respectively.

Statistical analysis

The IBM-SPSS Statistics software version 24.0 (New York, NY) was used for statistical analysis. The general linear model of ANOVA was used to study the effect of individual dairy (inter-dairy effect; I = 8), cheese production (intra-dairy effect; I = 3) and curd syneresis process (I = 2) on technical and analytical variables monitored during cheesemaking in the artisanal dairies. The fixed effects model $Y_{ijk} = \mu + D_i + CP_j + \varepsilon_{ijk}$ was applied to study simultaneously the effect of individual dairy (D_i) and cheese production (CP_j) on cheesemaking physico-chemical and technical parameters, whereas $(D*CP)_{ij}$ interaction term was included in the model in the case of FCG and SCG particle size and shape parameters. The milk fat/protein ratio was included as a covariate in the general linear model to study the actual cheese yield as dependent variable. The fixed effects model $Y_{ijk} = \mu + D_i + S_j + (D*S)_{ij} + \varepsilon_{ijk}$ was applied to study simultaneously the effect of curd syneresis process (S_j) and D_i on curd grain size and shape parameters. For those parameters with significant interaction term $(D*S)_{ij}$, an F-test of S_j against $(D*S)_{ij}$ was applied. Tukey's honest significant difference test was used to pairwise comparison for mean values from data with equality of variance, and Games-Howell post-hoc test was applied in the case of heteroscedastic data. Mann-Whitney U nonparametric test was applied to assess separately the D_i effect on median values of particle size parameters in FCG and SCG samples, respectively. On the other hand, the nonparametric Kruskal-Wallis test was applied to investigate statistical differences in PSD parameters between FCG and SCG sample groups. Simple regression analysis was used to investigate the relationship between particle size parameters and the log of total revolutions used in the cheese productions, and Pearson's correlation coefficient (r) was calculated for some pairs of variables. In addition, a principal component analysis (PCA) was performed on self-scaling variables corresponding to curd grain size and shape parameters, cutting and stirring conditions, fat loss and actual cheese yield in order to establish relationships among them. Kaiser criterion (eigenvalue > 1) was applied to extract PCs and the distribution plot using the two-dimensional coordinate system defined by the first two PCs was used to study the intra- and inter-dairy variability. Statistical significance was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Physico-chemical and technical parameters used for cheesemaking

Table 1 summarises the most relevant physico-chemical and technical parameters related to the curd syneresis process occurred in artisanal dairies during the Idiazabal PDO cheese processing season from March to June. The main technical parameters monitored in artisanal dairies were related to time, temperature and rotation speed used for curd cutting and stirring steps. As described above, cheese-makers used mechanized vats with different vat volume capacity, and therefore, the amount of milk handled by the cheese-maker ranged from 270 to 1330 L. No significant differences ($P > 0.05$) were found among the artisanal dairies in the fat/protein ratio of milk with grand mean values of 1.3. Accordingly, cheese composition parameters, actual and adjusted cheese yield did not show statistically significant differences ($P > 0.05$) among dairies. However, variations in actual and adjusted cheese yield up to 3.8 and 2.4 kg of cheese/100 L of milk, respectively, were observed between some artisanal dairies (Table 1). These results indicated a large intra- and inter-dairy variability in the actual and adjusted cheese yield during the cheese processing season. On the other hand, significant differences ($P \leq 0.05$) were observed in fat loss between dairy 1 and the others. Dairy 1, which used the most differentiated

Table 1. Physico-chemical and technical parameters (mean ± standard deviation) used in different cheese productions (*I* = 3) by artisanal dairies during Idiazabal PDO cheese processing season from March to June.

	Dairy								
	1	2	3	4	5	6	7	8	Grand mean
Milk volume (L)	1197^{a} ± 181	753^{b} ± 197	499^{bc} ± 70	549^{bc} ± 213	383^{c} ± 115	532^{bc} ± 165	454^{bc} ± 68	498^{bc} ± 122	608 ± 279
Milk fat (g/100 mL)	6.8 ± 0.7	6.5 ± 1.2	6.9 ± 0.9	7.5 ± 1.4	6.7 ± 1.2	6.9 ± 1.1	6.7 ± 0.6	6.1 ± 1.0	6.8 ± 0.9
Milk protein (g/100 mL)	5.29 ± 0.46	5.21 ± 0.45	5.43 ± 0.46	5.56 ± 0.82	5.08 ± 0.20	5.29 ± 0.52	5.28 ± 0.21	5.17 ± 0.33	5.29 ± 0.42
Milk fat/protein ratio	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
Cheese fat (g/100 g)	32.5 ± 0.7	31.8 ± 1.1	33.4 ± 0.5	31.6 ± 0.5	31.0 ± 1.2	31.4 ± 0.3	32.1 ± 1.3	29.8 ± 2.3	31.7 ± 1.3
Cheese protein (g/100 g)	20.8 ± 0.4	22.2 ± 3.0	20.2 ± 0.5	20.9 ± 1.1	19.4 ± 0.8	20.2 ± 1.8	19.5 ± 0.2	21.5 ± 2.2	20.6 ± 1.5
Cheese moisture (g/100 g)	41.6 ± 1.2	42.0 ± 1.1	40.8 ± 1.0	42.8 ± 2.2	44.6 ± 0.6	43.4 ± 0.8	43.0 ± 0.7	42.4 ± 0.6	42.6 ± 1.4
Coagulation temperature (ºC)	30.0 ± 1.4	30.8 ± 1.1	30.0 ± 0.0	30.5 ± 0.7	28.5 ± 0.7	29.0 ± 1.4	30.5 ± 2.1	29.5 ± 0.7	29.8 ± 1.2
Coagulation time (min)	35^{ab} ± 4	39^{ab} ± 4	48^{a} ± 6	36^{ab} ± 5	33^{ab} ± 3	42^{ab} ± 8	31^{b} ± 7	33^{ab} ± 5	37 ± 7
Curd pH	6.60^{ab} ± 0.02	6.53^{b} ± 0.07	6.59^{ab} ± 0.05	6.55^{ab} ± 0.08	6.49^{b} ± 0.03	6.60^{ab} ± 0.08	6.57^{ab} ± 0.07	6.66^{a} ± 0.08	6.58 ± 0.07
Curd grain pH after stirring	6.56 ± 0.03	6.54 ± 0.01	6.60 ± 0.08	6.56 ± 0.06	6.52 ± 0.04	6.69 ± 0.03	6.60 ± 0.06	6.69 ± 0.11	6.59 ± 0.08
Cutting temperature (ºC)	30.0 ± 0.0	32.2 ± 1.9	30.7 ± 0.6	31.7 ± 0.6	29.7 ± 0.6	32.0 ± 1.0	31.7 ± 0.6	30.7 ± 1.5	30.9 ± 1.1
Cutting time (min)	13^{b} ± 2	28^{a} ± 9	4^{bc} ± 1	4^{c} ± 2	6^{bc} ± 1	7^{bc} ± 1	4^{c} ± 1	3^{c} ± 2	9 ± 9
Cutting speed (rev/min)	8.9^{a} ± 0.4	21.2^{bc} ± 0.5	20.9^{bc} ± 3.0	16.2^{b} ± 3.1	22.1^{bc} ± 1.7	23.8^{c} ± 1.8	18.8^{bc} ± 1.3	20.6^{bc} ± 4.9	18.6 ± 5.6
Stirring initial temperature (ºC)	30.3^{a} ± 0.6	30.3^{a} ± 0.6	30.7^{ab} ± 0.6	33.0^{b} ± 1.7	30.5^{ab} ± 0.9	32.7^{ab} ± 0.6	31.7^{ab} ± 0.6	31.3^{ab} ± 0.6	31.3 ± 1.2
Stirring final temperature (ºC)	35.7 ± 0.6	36.7 ± 0.6	36.3 ± 1.2	36.8 ± 0.3	35.3 ± 0.6	36.2 ± 0.3	36.3 ± 0.6	35.0 ± 1.0	36.0 ± 0.8
Stirring temperature rate (ºC/min)	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
Stirring time (min)	19^{ab} ± 2	40^{a} ± 10	33^{ab} ± 10	18^{b} ± 6	33^{ab} ± 6	25^{ab} ± 9	18^{b} ± 8	29^{ab} ± 2	27 ± 10
Stirring speed (rev/min)	10^{d} ± 0	17^{bc} ± 1	14^{cd} ± 0	28^{a} ± 1	22^{b} ± 2	16^{bcd} ± 6	14^{cd} ± 0	14^{cd} ± 0	17 ± 6
Total revolutions (rev)	308^{a} ± 35	1249^{c} ± 111	549^{ab} ± 107	569^{ab} ± 177	838^{b} ± 162	557^{ab} ± 119	328^{a} ±120	449^{a} ± 38	606 ± 311
Whey amount (kg)	992^{a} ± 160	646^{b} ± 179	421^{bc} ± 63	456^{bc} ± 204	317^{c} ± 101	444^{bc} ± 134	364^{bc} ± 49	421^{bc} ± 113	508 ± 237
Actual cheese yield (kg cheese/100 L milk)	20.0 ± 1.3	17.5 ± 3.2	18.7 ± 1.0	21.2 ± 4.3	20.5 ± 1.7	19.4 ± 2.3	21.3 ± 1.6	18.9 ± 2.6	19.8 ± 2.6
Adjusted cheese yield (kg cheese/100 L normalised milk)	20.2 ± 1.0	18.6 ± 2.3	19.3 ± 1.0	19.2 ± 0.1	19.8 ± 0.3	18.5 ± 0.5	20.9 ± 1.4	20.3 ± 0.8	19.5 ± 1.2
Fat loss (g/100 g of milk fat)	5.79^{b} ± 0.98	11.1^{a} ± 0.6	14.2^{a} ± 1.7	12.4^{a} ± 3.1	12.6^{a} ± 1.6	11.2^{a} ± 2.3	11.7^{a} ± 1.1	12.8^{a} ± 0.7	11.5 ± 2.8

[a - d] Means with different superscripts in the same row indicate statistically significant differences among individual artisanal dairies ($P \leq 0.05$).

equipment and settings compared to the others, lost less than 6 g/100 g of fat *per* cheese production while the others released almost double (Table 1). No significant differences in fat loss ($P > 0.05$) were found among dairies 2 to 8, but variations of 3 g/100 g *per* cheese production were observed. These variations in fat loss were not reflected in cheese yield parameters but showed the opportunity for improving cheesemaking efficiency and compound recovery.

Regarding the coagulation step, Table 1 only shows the temperature and time used in each dairy for milk coagulation. The amount of rennet used in each dairy was not detailed because, as described above, most cheese-makers used lamb rennet paste prepared by themselves and so, the amount of rennet was not the reflection of the coagulation capacity. Bustamante *et al*. (2000) characterized the coagulating activity of artisanal lamb rennet pastes prepared according to the traditional method for Idiazabal cheese, and reported that milk coagulating activity varied between 155 and 363 U/g of tissue depending on the preparing conditions. Therefore, due to the difficulty of controlling these wide variations, the cheese-makers make the decision to start the cutting process according to the sensory observation of the curd consistency, and this implied that coagulation time varied from 24 to 55 min. Previous studies using raw milk from commercial flocks showed that curd firmness (curd resistance to compression) at cutting point varied from around 82 to 115 g during Idiazabal cheese processing season (Nájera *et al*., 2009; Abilleira *et al*., 2010). As expected, all cheese-makers used a coagulation temperature close to 30ºC and very few significant ($P \leq 0.05$) differences were found for curd pH among artisanal dairies (mean pH values ranged between 6.49 and 6.65). Both temperature and pH parameters are known to strongly affect milk coagulation process (Nájera *et al*., 2003) but the cheese-makers collaborating in this study managed these two factors in a similar way during the cheesemaking. As well as curd pH values, no significant differences ($P > 0.05$) were found in the pH of stirred curd grains among dairies (Table 1). Furthermore, pH values did not significantly change from curd to stirred curd grains (up to 0.09 units) and, in consequence, curd grain syneresis was not influenced by acidification during stirring process.

Due to the specific conditions established by Idiazabal PDO rules (Ministerio de Agricultura, Pesca y Alimentación, 1993), temperature settings during the cutting and stirring processes were very similar ($P > 0.05$) for all of the artisanal dairies. However, other technical settings were established by the cheese-maker and hence, there were significant ($P \leq 0.05$) variations in time and rotation speed for curd cutting and stirring among dairies (Table 1). Cutting time ranged between 3 and 28 min and stirring time varied widely from 9 to 51 min. The cutting and stirring speeds were also highly variable factors among artisanal dairies (Table 1). The lowest speed used for both cutting and stirring was used in the artisanal dairy with the highest milk volume processed but no significant correlation ($r < 0.20$; $P > 0.05$) was observed between milk volume and cutting or stirring speed for the other dairies (milk volume < 1,000 L). The highest value of total revolutions used during cheesemaking corresponded to the dairy which extended the time for cutting and stirring up to around 70 min (dairy 2), whereas the rest of the artisanal dairies set the time below 40 min and the total revolutions used for cheesemaking were less than 850 (Table 1). As discussed below, this conditioned the curd grain syneresis process and, in consequence, the particle size of FCG and SCG was strongly affected (Table 2). In general, the wide scale of cutting and stirring technical settings employed by the cheese-makers showed the current variability and also the challenges and opportunities to optimize the cheesemaking process in the artisanal dairies.

Curd grain size

Table 2 shows the mean, standard deviation and median area values of the extracted FCG and SCG measured by image analysis from the three cheese productions carried out during Idiazabal cheese processing season in each artisanal dairy. Perimeter and Feret's diameter of curd grains were also measured (data not shown) and high statistical correlation ($r > 0.92$; $P \leq 0.001$) was found between these parameters and particle area. Accordingly, particle surface area was selected as the most representative parameter of curd grain size. As could be expected, cheese production effect and *D*CP* interaction term were statistically significant ($P \leq 0.001$) for a particle area for both FCG and SCG due to the high intra-dairy variability in technical parameters used for cheesemaking, particularly in the cutting and stirring time and rotation speed, or even in the milk volume handled by cheese-makers (Table 1). However, regardless of this intra-dairy variability during the cheese processing season, significant differences (*P*

≤ 0.05) were found in FCG and SCG areas among artisanal dairies depending mainly on cutting and stirring settings used for cheesemaking. Regarding the cutting step, FCG mean area values ranged from 10.01 to 43.11 mm^2, and those of median values from 6.73 to 32.18 mm^2, showing great differences ($P \leq 0.05$) among artisanal dairies. These differences indicated that the particle area was smaller as cutting revolutions increased ($r \geq |0.50|$; $P \leq 0.01$), for example in the case of dairies 2 and 7 for which adjusted cheese yield was also significantly different (Tables 1 and 2). Mean Feret's diameter values of FCG varied from 4.10 to 8.40 mm confirming, in general, the compliance with the curd grain diameter after the cutting process specified in the Idiazabal PDO cheese regulation (between 5 and 10 mm), although the curd grain size variability within the same cheese production was very high as discussed later.

Table 2. Mean, SD and median area values (mm^2) of curd grains after cutting (FCG) and after stirring (SCG) obtained in different cheese productions (l = 3) by artisanal dairies during Idiazabal PDO cheese processing season from March to June.

Dairy	FCG			SCG		
	n	Mean ± SD	Median	n	Mean ± SD	Median
1	331	39.78^d ± 38.02	27.90^e	606	25.79^c ± 31.77	14.24^e
2	374	10.01^a ± 9.22	6.73^a	782	8.59^a ± 7.64	6.10^a
3	166	25.52^{bc} ± 31.65	16.11^{cd}	606	11.09^b ± 11.59	7.53^b
4	209	19.84^b ± 22.26	12.30^b	451	11.70^b ± 12.35	8.05^{bc}
5	250	22.27^{bc} ± 19.88	16.11^{cd}	554	12.67^b ± 11.67	9.00^d
6	353	18.50^b ± 14.57	14.41^{bc}	473	13.17^b ± 14.28	8.41^{cd}
7	175	43.11^d ± 39.12	32.18^e	368	22.76^c ± 20.97	16.50^e
8	135	36.04^{cd} ± 53.97	19.65^d	396	22.98^c ± 30.15	12.58^e
Total	1993			4236		
Grand mean or median		24.98	16.11		15.36	9.18
SEM		0.68			0.30	
Significance of *CP* effect		***			***	
Significance of *D*CP* term		***			***	

n; number of curd grains measured by image analysis; SD; standard deviation; SEM; standard error of the mean; *CP*; cheese production; *D*; individual dairy; *** $P \leq 0.001$.

[a-e] Mean and median values with different superscripts in the same column indicate statistically significant differences among individual artisanal dairies ($P \leq 0.05$).

Regarding the stirring step, SCG mean area values ranged from 8.59 to 25.79 mm^2, and those of median values from 6.10 to 16.50 mm^2, also showing great differences ($P \leq 0.05$) among artisanal dairies (Table 2). In comparison to FCG, overall variability (values range amplitude) for SCG size parameters decreased due to the shrinkage of the particles (syneresis) with stirring and heating up to

36ºC. Figure 1 depicts the reduction rate of curd grain size parameters before and after stirring. Reduction rate mean values for area, perimeter and Feret's diameter of curd grains were 1.86 ± 0.77, 1.33 ± 0.26 and 1.38 ± 0.33, respectively. The shrinkage of particles, and therefore, the whey syneresis of curd grains, caused significant differences ($P \leq 0.01$) in particle size parameters between FCG and SCG independently of stirring settings used in the artisanal dairies (Figure 1). In this sense, dairy 2 showed the smallest reduction rate for curd grain size (1.26 ± 0.40) as expected, since despite the constant temperature (around 30ºC), the lengthening of the cutting time (20 min longer than the grand mean value of the cutting time; Table 1) led to an important part of the curd grain syneresis happening before stirring.

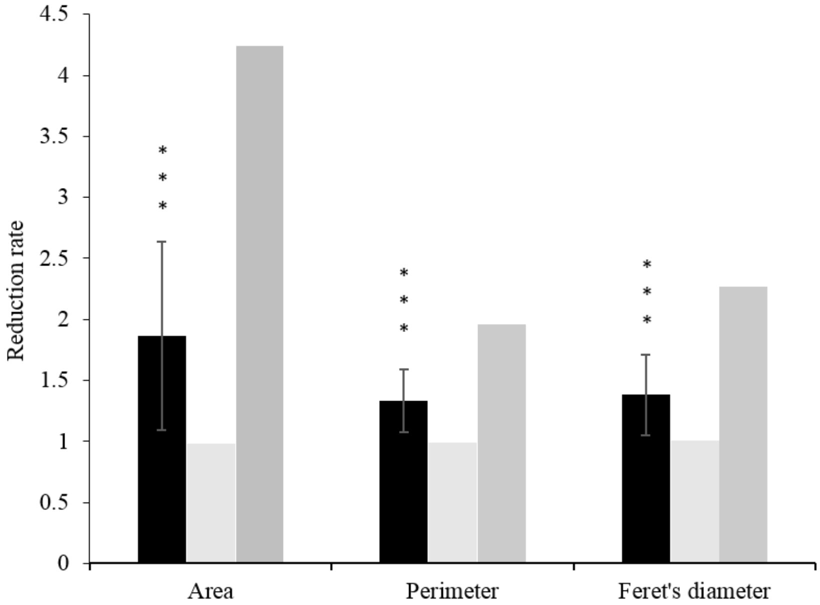

Figure 1. Reduction rate for area, perimeter and Feret´s diameter of curd grains during stirring. Bars depict mean (black), minimum (light gray) and maximum (dark gray) values. Error bars represent the overall standard deviation all dairies (*l* = 8) and cheese productions (*l* = 3). *** Significant difference ($P \leq 0.001$) in particle size parameters before and after stirring.

As mentioned above, both cutting and stirring conditions affected curd grain size, and relationships between technical parameters used by the cheese-makers and particle size were investigated. Linear relationships were found between curd grain size parameters and the logarithm of total revolutions used during cheesemaking. Total revolutions included the total time used for cutting and stirring steps but not the stirring temperature rate, and as it has been previously reported for cooked pressed cheeses (Iezzi *et al*., 2012), the heating contribute to casein aggregation and whey syneresis. However, Idiazabal PDO cheese artisanal dairies used similar initial and final stirring temperatures (from around 30 to 36ºC, respectively), and the stirring temperature rate did not significantly change among dairies (Table 1). Therefore, it could be estimated that the potential heating effect on curd grain syneresis was similar during cheesemaking in the artisanal dairies.

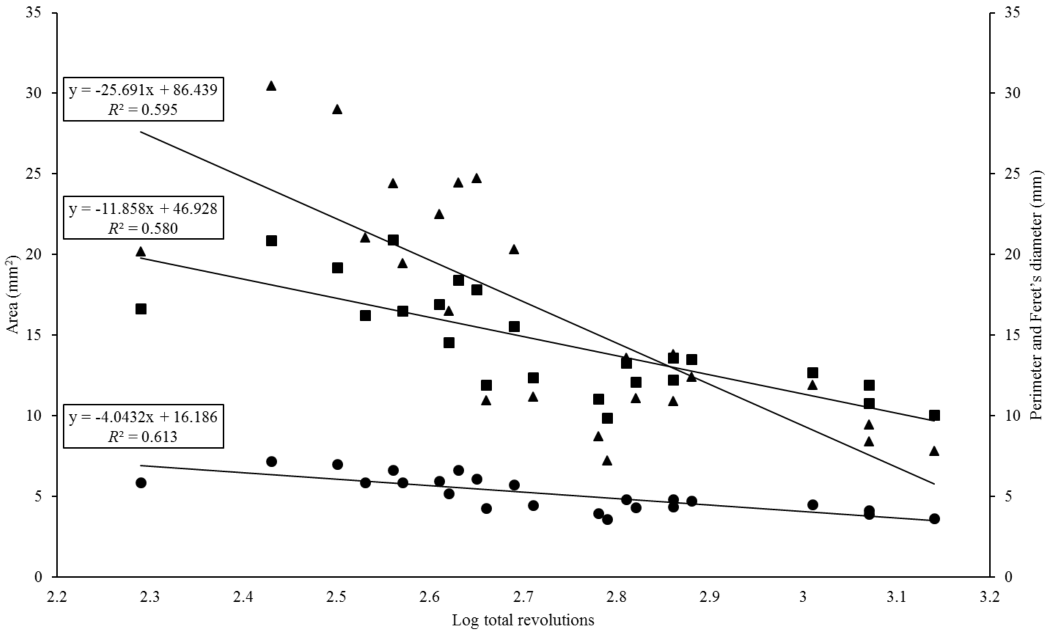

Figure 2. Linear relationship between area (▲), perimeter (■) and Feret's diameter (●) of curd grains and the log of total revolutions used during the cheesemaking (*l* = 3) in artisanal dairies (*l* = 8). R^2, determination coefficient.

The linear regression models showed that the total revolutions had a significant effect ($P \leq 0.001$) on the area, perimeter and Feret´s diameter, and that the percentages of explained variance were around 60% (Figure 2). The relationships pointed in the same direction, as the total revolutions increased, the curd grain size parameters decreased. These results agreed with those reported by Johnston *et al*. (1991) confirming that curd particle size is determined not only by the cutting step but by a combination of cutting and stirring conditions. No direct correlation was observed in the present commercial study between curd grain parameters and cheese composition probably due to the high intra- and inter-dairy variability, but other authors reported lower cheese moisture when curd grain size was smaller (Johnston *et al*., 1991). Therefore, SCG size analysis could be a control point during cheesemaking to achieve uniform curd particle size as well as homogeneous fresh cheese moisture. On the other hand, as Table 2 shows, after the stirring step the artisanal dairies could be classified into three different groups depending on SCG size (area): small (< 10 mm^2), big (> 20 mm^2) and medium (between 10 and 20 mm^2) area. These data could also be useful to differentiate the dairies within Idiazabal cheese PDO depending on SCG size in order to optimize the cheesemaking conditions used by each cheese-maker, as consumers appreciate the artisan process held by small rural dairies, and therefore, the intrinsic sensory properties of the artisanal cheeses and the differences between them. On the other hand, these results can contribute to a deeper knowledge on the relationship between cutting and stirring conditions and curd grain size and the syneresis process.

Curd particle size distribution analysis

PSD analysis was performed for curd grains after the cutting and stirring steps due to the high variability observed in the size of curd particles in the artisanal dairies. As reported, the higher or lower homogeneity of curd grain PSD after the cutting and stirring process could also affect the binding of curd grains during pressing, the moisture distribution and texture of fresh cheese (Akkerman *et al*., 1993; Iezzi *et al*., 2012). Table 3 shows mean values for parameters used to assess the uniformity or unevenness of curd PSD after cutting and stirring in the artisanal dairies. All the PSD parameters confirmed that the curd grains obtained after the cutting and stirring steps were highly heterogeneous. The effect of curd syneresis was only statistically significant ($P \leq 0.05$) for some PSD parameters and the effect was, as expected, dependent on the individual artisanal dairy. None of the dairies showed a clear improvement in all the PSD parameters. Moreover, some values (i.e. size range variation (S_v) and relative span (S_l)) indicated a slight increase of the heterogeneity after the stirring step, whereas the values of the uniformity distribution parameters (I_u and C_u) raised or lowered during curd grain syneresis. In contrast to this study, Iezzi *et al*. (2012) reported an enhanced PSD homogeneity after the cooking process for Italian cheeses by the relative span values, even with a high variability among dairies. The lack of improvement and, in some cases, the impairment of the homogeneity of curd grains after stirring in Idiazabal PDO cheeses is probably due to the existence of a small amount of big SCG despite the general particle size reduction during stirring (Figure 3). This heterogeneity led to a smaller size reduction of the bigger particles and a higher syneresis rate of the smaller particles during stirring. This agrees with other authors (Unger *et al*., 2000; Dejmek and Walstra, 2004) who reported that the grain size was one of the most important factors determining the shrinkage of the curd grain during the initial stage (up to 80 min) due to Darcy´s law.

Table 3. Mean values for particle size distribution parameters of curd grains after cutting (FCG) and after stirring (SCG) obtained in different cheese productions (*l* = 3) by artisanal dairies during Idiazabal PDO cheese processing season from March to June. The number of curd grains measured by image analysis in each dairy is detailed in Table 2.

Dairy	FCG						SCG					
	I_u	S_v	S_l	C_u	S_g	K_g	I_u	S_v	S_l	C_u	S_g	K_g
1	4.14	107.70	3.06	6.23	0.48	1.23	4.17	134.52	3.71	5.61	0.60	1.45
2	6.92	94.74	2.55	4.59	0.39	1.09	8.22	107.52	2.92	4.01	0.52	1.21
3	5.13[a]	91.21	2.78	5.03	0.34	1.50	7.59[b]	102.83	2.85	4.29	0.52	1.27
4	4.42[a]	86.63	2.61	6.37	0.36	1.16	7.97[b]	106.09	2.87	4.06	0.51	1.17
5	9.15	86.19	2.32	3.79	0.47	1.33[a]	7.29	121.97	2.83	4.41	0.50	1.01[b]
6	9.11	84.36[a]	2.24[a]	3.84	0.39	1.03	6.21	122.23[b]	3.18[b]	5.04	0.55	1.13
7	5.65	95.06	2.78	4.57	0.49	1.64[a]	6.38	104.80	2.93	4.56	0.49	1.15[b]
8	6.60	118.91	3.21	4.56	0.39	1.17	4.50	133.93	3.98	5.44	0.61	1.44
Grand mean	6.39	95.60[a]	2.70[a]	4.87	0.41[a]	1.27	6.54	116.74[b]	3.16[b]	4.68	0.54[b]	1.23
SEM	0.52	4.59	0.15	0.27	0.22	0.10	0.40	4.22	0.13	0.18	0.02	0.04

I_u, uniformity index (I_u=D_5/D_{90}*100); S_v, size range variation coefficient (S_v=(D_{84}-D_{16})/2D_{50}*100); S_l, relative span (S_l=(D_{90}-D_{10})/D_{50}); C_u, coefficient of uniformity (C_u=D_{60}/D_{10}); S_g, graphic skewness (S_g=[(D_{84}+D_{16}-2D_{50})/2(D_{84}-D_{16})]+[(D_{95}+D_5-2D_{50})/2(D_{95}-D_5)]); K_g, graphic kurtosis (K_g=[(D_{95}-D_5)/2.44(D_{75}-D_{25})]); where the D_x is the x^{th} percentile dimension of the area distribution in each dairy and cheese production; SEM, standard error of the mean.

[a,b] Mean values with different superscripts in the same row indicate statistically significant differences between FCG and SCG particle size distribution parameters ($P \leq 0.05$).

PSD asymmetry (S_g) and peakiness (K_g) were also measured for FCG and SCG. All artisanal dairies showed S_g values higher than 0.30 and 0.49 for FCG and SCG, respectively (Table 3), classified by Folk and Ward (1957) as very positive-skewed values. This meant that FCG distribution was characterized by a big amount of small curd particles whose number increased ($P \leq 0.05$) during stirring. Concerning K_g values, PSD could be classified from mesokurtic to very leptokurtic (Folk and Ward, 1957) confirming again the presence of a high number of small particles in both FCG and SCG.

Despite the fact that each artisanal dairy had a different behaviour in curd PSD parameters, the general trend suggested that PSD was mainly determined by cutting, whereas stirring could cause slight changes in PSD most likely due to a higher size reduction and increased amount of small particles comparing to the bigger curd grains. These results agree with those found by other authors who reported that technical settings used during cutting and stirring determines the curd grain size after stirring, while curd PSD is essentially derived from the cutting action (Johnston *et al*., 1991; Iezzi *et al*., 2012).

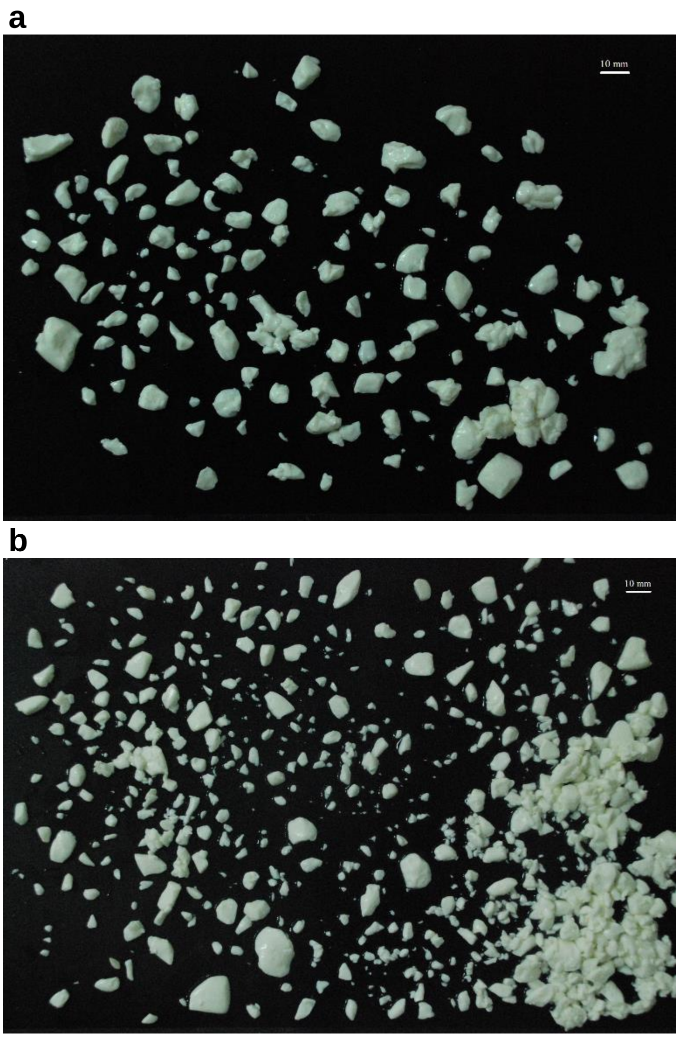

Figure 3. Two-dimensional digital image of curd grains after cutting (a) and stirring (b) from the same cheese production carried out in an artisanal cheese dairy. The scale bars are 10 mm in length.

Curd grain shape

Curd grain shape has not been a widely studied feature mostly due to the sampling methods used until now, especially the sieving method (Johnston *et al*., 1991). In general, the shape of curd grains is usually described as irregular, as particles do not fit regular geometric shapes such as circles, ellipses, rectangles, squares or triangles, and this makes it difficult to apply some known image analysis methods to identify and classify them (Igathinathane *et al*., 2008). Kamerlehner (2009) described curd grain shapes obtained in several cheese types as comparable to walnuts, hazelnuts, corn, barley and others, while Akkerman (1992) characterized them as angular, rounded or disc shaped. More recently, Iezzi *et al*. (2012) used the elongation as a shape indicator of curd grains. In the present study, a major effort has been made to characterize in-depth the irregular shape of the curd grains after the cutting and stirring processes during cheesemaking. Table 4 shows the mean values for RAR, RTY and CTY shape parameters to describe, respectively, elongation, rectangularity and circularity degree of the curd grains after cutting (FCG) and stirring (SCG) during cheesemaking in artisanal dairies. In general, curd grains showed irregular shapes very different from one another (Figure 3) with slightly higher values of circularity than elongation or rectangularity (see grand mean values for RAR, RTY and CTY in Table 4). Significant differences ($P \leq 0.05$) were found for cheese production and individual dairy effects, and *D*CP* interaction term, in the most particle shape parameters of both FCG and SCG. As previously mentioned for particle size parameters, these results pointed out the high intra-and inter-dairy variability in technical parameters used for cheesemaking. In addition, and very prominently for particle shape parameters, the differences in the design of cutting and stirring tools among artisanal dairies were most likely responsible for changes in curd grain shape. The surface area and shape of the cutting and stirring devices, and the number and arrangement of the wires could be important design factors affecting curd grain breakdown. In this regard, it has been suggested that rotating cutting tools lead to several shapes distinct to cube-shape curd grain (Kammerlehner, 2009). However, contrary to what was observed for curd grain size parameters, no clear pattern related to technical parameters

Table 4. Mean ± standard deviation values for particle shape parameters of curd grains after cutting (FCG) and after stirring (SCG) obtained in different cheese productions (*l* = 3) by artisanal dairies during Idiazabal PDO cheese production season from March to June. The number of curd grains measured by image analysis in each dairy is detailed in Table 2.

Dairy	FCG			SCG		
	RAR	RTY	CTY	RAR	RTY	CTY
1	$0.688^{a} \pm 0.144$	$0.682^{a} \pm 0.069$	$0.738^{a} \pm 0.080$	$0.668^{a} \pm 0.141$	$0.680^{a} \pm 0.072$	$0.747^{b} \pm 0.084$
2	$0.706^{ab} \pm 0.135$	$0.689^{ab} \pm 0.067$	$0.774^{bc} \pm 0.076$	$0.701^{bc} \pm 0.128$	$0.692^{bc} \pm 0.063$	$0.777^{c} \pm 0.077$
3	$0.746^{c} \pm 0.123$	$0.712^{c} \pm 0.052$	$0.787^{bc} \pm 0.083$	$0.705^{bc} \pm 0.140$	$0.694^{bc} \pm 0.064$	$0.784^{cd} \pm 0.074$
4	$0.737^{bc} \pm 0.127$	$0.699^{bc} \pm 0.060$	$0.775^{bc} \pm 0.075$	$0.707^{bc} \pm 0.131$	$0.701^{c} \pm 0.063$	$0.795^{d} \pm 0.075$
5	$0.737^{bc} \pm 0.117$	$0.700^{bc} \pm 0.057$	$0.789^{c} \pm 0.060$	$0.719^{c} \pm 0.132$	$0.694^{bc} \pm 0.064$	$0.781^{cd} \pm 0.073$
6	$0.713^{abc} \pm 0.127$	$0.696^{ab} \pm 0.060$	$0.784^{bc} \pm 0.070$	$0.700^{bc} \pm 0.138$	$0.687^{ab} \pm 0.066$	$0.776^{c} \pm 0.076$
7	$0.739^{bc} \pm 0.129$	$0.706^{bc} \pm 0.065$	$0.769^{bc} \pm 0.069$	$0.693^{ab} \pm 0.138$	$0.687^{ab} \pm 0.065$	$0.726^{a} \pm 0.099$
8	$0.708^{ab} \pm 0.131$	$0.704^{bc} \pm 0.061$	$0.754^{ab} \pm 0.120$	$0.705^{bc} \pm 0.130$	$0.698^{bc} \pm 0.059$	$0.776^{c} \pm 0.074$
Grand mean	0.718	0.696	0.771	0.699	0.691	0.771
SEM	0.003	0.001	0.002	0.002	0.001	0.001
Significance of *CP* effect	ns	ns	***	*	***	***
Significance of *D*CP* term	ns	*	***	**	**	***

RAR, reciprocal aspect ratio; RTY, rectangularity; CTY, circularity; SEM; standard error of the mean; *CP*; cheese production; *D*; individual dairy; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns; not significant.

[a-c] Mean values with different superscripts in the same column indicate statistically significant differences among individual artisanal dairies ($P \leq 0.05$).

during cheesemaking was found to explain differences in particle shape parameters of both FCG and SCG among artisanal dairies (Tables 1 and 4).

On the other hand, in spite of the high variability observed, significant differences ($P \leq 0.001$) were found in RAR and RTY between FCG and SCG, which meant that changes in curd grain shape occurred during stirring. Curd grain RAR showed a decreasing trend during stirring in all dairies, meaning that particles were slightly more elongated after stirring, whereas RTY pointed at different directions depending on the dairy (Table 4) being indicative of a strong *D*S* interaction ($P \leq 0.05$). Iezzi *et al*. (2012) also measured the elongation of the curd particles in Italian cooked pressed cheeses but no changes on this parameter were reported before and after the cooking, although it should be pointed out that particles showed high elongation values in FCG and SCG. In summary, cutting and stirring processes caused the breaking and shrinkage of particles and especially the stirring step could affect the final curd grain shape and consequently the binding of curd grains during pressing, and the formation and texture of fresh cheese (Akkerman *et al*., 1993; Iezzi *et al*., 2012).

Relationship between cheese yield and technological parameters

As multiple parameters were involved, a PCA was performed to investigate the relationships between the actual cheese yield, fat loss, curd grain size and shape parameters, and technological conditions used in the artisanal dairies during cheese processing. Two PCs were extracted explaining the 67.82% of the total variance (Figure 4). PC1 was highly correlated (factor loadings > |0.7|) with FCG and SCG size parameters and cutting revolutions, and moderately (factor loading ~ |0.5|) with actual cheese yield and stirring revolutions. Therefore, actual cheese yield and FCG and SCG size parameters were inversely correlated to the cutting and stirring revolutions used during cheesemaking. PC2 was highly correlated (factor loading > |0.7|) with SCG and FCG elongation and fat loss, and to a lesser extent with stirring revolutions and SCG size parameters (factor loading ~ 0.6). Curd grain elongation was positively correlated to stirring revolutions meaning that the stirring process could be the main responsible for the final shape of the curd grains, as discussed above. Additionally, fat loss was interestingly correlated to these parameters, indicating that when higher stirring revolutions were used during cheesemaking fat release was enhanced. This agrees with other authors that demonstrated that an insufficient cutting process leads to the shattering of curd particles during stirring, and consequently, increased fat losses (Johnston *et al*. 1991). As biplot graph shows (Figure 4), high intra- and inter-dairy variability was confirmed during the cheese processing season, but whereas some artisanal dairies showed a major spread between their own cheese productions (dairies 2, 4 or 8), others presented quite homogeneous results (dairies 1, 5 or 6).

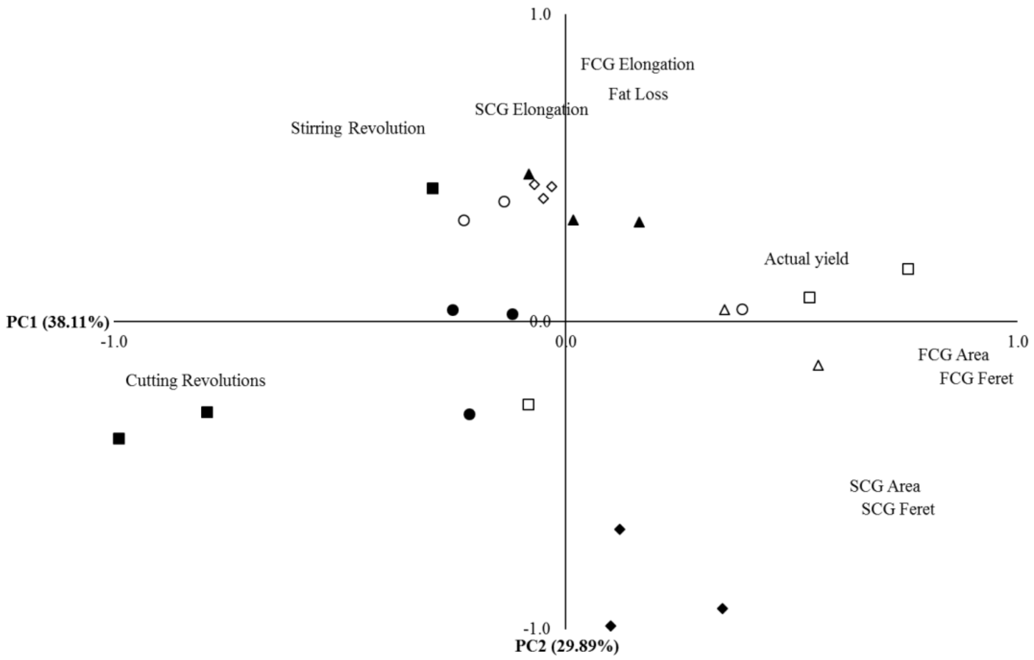

Figure 4. Principal component (PC) analysis biplot depicting variable loadings and cheese production distribution in the two-dimensional coordinate system defined by PC1 and PC2. ♦ dairy 1; ■ dairy 2; ▲ dairy 3; ○ dairy 4; ◊ dairy 5; ● dairy 6; △ dairy 7; □ dairy 8.

CONCLUSIONS

Two-dimensional image analysis appears to be a useful tool to characterize and monitor the curd grain syneresis during cheesemaking. The wide scale of technical settings used in commercial cheese manufacturing caused differences in curd grain size and shape among artisanal dairies. Regardless of the high variability observed during the cheese production season, cutting and stirring conditions were correlated to curd grain size and shape parameters, particle size distribution, fat loss and cheese yield. Higher values of total revolutions used by cheese-makers during the cutting and stirring steps significantly reduced the curd grain size. However, the stirring process was the main responsible for the fat loss during cheesemaking, probably due to the curd shattering effect of overdone revolutions. However, no clear pattern related to technical settings was found to explain differences in particle shape parameters during curd grain syneresis, even though stirring showed to be the main factor responsible for the final shape of curd grains. In general, curd particle size distributions were highly heterogeneous with an increasing number of small particles during stirring and a variable amount of big grains initially formed by curd cutting. Even though each artisanal dairy showed a different behaviour in particle size distribution the general trend suggested that curd syneresis during the stirring step had a very small effect on curd particle size distribution. The data reported in this study may contribute to improving and controlling cheesemaking process in small artisanal dairies where the role of the cheese-maker is crucial. Furthermore, using two-dimensional image analysis approach in controlled experimental designs could help in the interpretation of the effect of interactions between milk composition and cheesemaking technical settings, on curd grain parameters, cheese composition and yield.

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Manuscript II

Impact of technological settings on yield, curd, whey and cheese composition during the cheesemaking process from raw sheep milk in small rural dairies: emphasis on cutting and cooking conditions.

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Impact of technological settings on yield, curd, whey and cheese composition during the cheesemaking process from raw sheep milk in small rural dairies: emphasis on cutting and cooking conditions

Ane Aldalur*, María Ángeles Bustamante*, Luis Javier R. Barron*[,1]

*Lactiker Research Group, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain.

CHEESE YIELD AFFECTED BY TECHNOLOGICAL CONDITIONS

IS. Cheese yield affected by cheesemaking technological conditions – Aldalur. Cheese yield and compound losses in the whey are important parameters that are closely related to the potential economic success and environmental improvement of the small rural dairies. This study addresses the technological conditions, especially those used during cutting and cooking processes that affect cheese yield, composition and losses in the whey in eight small rural sheep dairies during Idiazabal cheese production. The results contribute to the improvement of the cheesemaking process where the facilities are limited and the role of the cheese-maker is crucial.

[1] Corresponding author: luisjavier.rbarron@ehu.eus

Abstract

Cheesemaking technological conditions affect cheese yield and compound losses in the whey, especially the ones regarding cutting and cooking processes. Although significant compositional and functional differences have been reported among animal species, there is a lack of studies of the influence of the cheesemaking technology in cheese yield and losses for sheep milk. Thus, the effect of the cheesemaking settings used in eight small rural dairies working with raw sheep milk on cheese yield and compound losses in whey along the cheese production season was studied. Actual cheese yield varied in 2-3 kg of cheese/100 kg of milk among dairies due to the cheesemaking conditions used, being particularly important the duration of cutting and cooking, and the final cooking temperature. The combination of the conditions used during cutting and cooking specially determined the fat losses in the whey. Fat losses were enhanced with high speed and short cutting time settings together with high stirring speed and long duration of cooking. Additionally, cheese-makers should adapt the cutting and cooking conditions to the seasonal variations of milk composition, especially during early summer when fat losses in the whey are higher. In this regard, the results suggest that it could be useful employing around 10-15 min of cutting time, and using moderate cooking speed and duration. The data reported in this study may assist the improvement of the cheesemaking process in small rural dairies using sheep milk where the facilities are limited and the role of the cheese-maker is crucial.

Key words: curd cut size, cutting and cooking speed, cooking temperature, cheese yield, fat loss.

Introduction

Cheese yield is an essential indicator of the efficiency of the cheesemaking process and the potential economic success of a dairy, particularly of those small dairies subjected to strong seasonal variation in milk production and composition (Barron *et al*., 2001; Walstra *et al*., 2006; Banks, 2007). Factors affecting cheese yield have been extensively reviewed and they could be classified in two main groups: those related to the composition of milk and the technological conditions used during cheesemaking (Lucey and Kelly, 1994; Everard *et al*., 2008). Fat and protein contents of milk are largely responsible for the cheese yield rate, and they vary depending on several factors such as, animal species, breed, genetic variants of proteins, lactation stage, feeding system, animal management or environmental conditions (Lucey and Kelly, 1994; Abilleira *et al*., 2010; Abd El-Gawad and Ahmed, 2011). Sheep milk is largely produced in some European countries such as Greece, Spain, Italy and France (EUROSTAT, 2017), and it has higher concentrations of protein, fat and total solids than the milk of cows and goats. It is characterized by enhanced coagulation properties, such as faster curd formation rate or higher rennet sensitivity, making it very suitable for cheesemaking (Park *et al*., 2007, Park, 2007). However, sheep flocks are usually managed in seasonal calving systems, so milk composition changes widely from the start to the end of the milking period due to the concurrent influence of the above mentioned factors, particularly lactation stage and diet (Barron *et al*., 2001; Nájera *et al*., 2009).

The technological conditions that affect cheese composition, yield and compound losses in the whey encompass a wide variety of processes such as, cold storage of milk, heat treatment, standardization, coagulation, curd cutting and cooking, draining, pressing, salting or ripening (Kammerlehner, 2009). Especially curd cutting and cooking processes are critical steps since syneresis is enhanced, curd moisture varied, and fat and protein losses in the whey inevitably occur, greatly affecting cheese yield (Lucey and Kelly, 1994). Some authors reported that when the cutting process was insufficient a consequent shattering effect occurred during cooking, generating higher fat and protein losses in the whey (Johnston *et al*., 1991; Everard *et al*., 2008). On the contrary, a too intensive cutting process can also damage the curd favoring the production of mini gel particles and incrementing losses in the whey (Kammerlehner, 2009). Fat losses can also be especially enhanced when the stirring speed during cooking is increased (Johnston *et al*., 1998; Aldalur *et al*., 2019). Additionally, different moisture contents of curd can also be achieved varying the technological settings used during cutting and stirring (Whitehead & Harkness, 1954; Everard *et al*., 2008), but other processing parameters could be changed in order to diminish potential yield losses (Gilles, 1976). Most studies assessing the effect of cheesemaking technological conditions on cheese yield and compound losses in the whey have been carried out using bovine milk on a laboratory scale (10 L) or large scale (20,000 – 30,000 L) (Johnston *et al*., 1991; 1998; Everard et a., 2008). Investigations on this topic are scarce in milks other than cow. Some authors evaluated differences in milk composition as well as the influence of cold storage on coagulation properties of milk from different species (Calvo and Balcones, 2000; Raynal and Remeuf, 2000; Park, 2007; Park *et al*., 2007).

Idiazabal PDO cheese is a traditional semi-hard cheese from the Basque Country region of northern Spain. This cheese is manufactured using raw milk from Latxa sheep breed, and it has to be ripened for a minimum of 2 months before consumption. Most of Idiazabal dairies (85%) are small family farms that manage the whole production chain, from the flock management to the commercialization of the cheeses in local markets. Therefore, their technological facilities are limited and the role of the cheese-makers during the cheese manufacture is crucial.

The objective of the current study was to investigate the effect of some cheesemaking conditions of Idiazabal PDO cheese on cheese yield and compound losses in whey along the production season in eight small rural dairies. Cutting and cooking parameters were thoroughly monitored during cheesemaking trials, and multifactorial relationships among technological settings, compositional parameters, curd grain characteristics and cheese yield were investigated.

Material and methods

Manufacture of Idiazabal PDO cheeses and samplings

Idiazabal PDO cheeses were manufactured in 8 small rural dairies, during a 7-month period, from January to July. The commercial production of each dairy was monitored four times, twice during late winter (mid lactation) and 2 more times during early summer (late lactation) in consecutive years. Dairies participating in this study used similar flock management and feeding systems. During winter indoor feeding was mainly used, whereas from spring onwards, sheep were grazed in a part-time system. At the end of the lactation period, extensive grazing management was used. A detailed description of the facilities used by the dairies is provided in Aldalur *et al*. 2019. Briefly, all the artisanal dairies used stainless steel vats (600 to 1,500 L) including automatic heating and agitation systems. Dairy 1 used an open double-O shaped vat with two fixed axes and equipped with vertical blade-cutters separated 6 cm from one another. The blades were transformed into stirrers when the rotation direction was inverted. The rest of the dairies used open oval-shaped vats with one or two axes of rotation to place the cutting frames or stirrers. Cutting frames were armed with wires separated by 2 cm among one another in vertical or separately arranged vertical and horizontal disposition. Helix- or irregular-shaped paddles were used for stirring the whey and curd grain mixture. Horizontal hydraulic presses were used for pressing the cheeses. Ripening was carried out in rooms under controlled temperature and relative humidity.

The commercial cheeses were manufactured using raw sheep milk and following the specifications of the Idiazabal PDO Regulatory Council (Ministerio de Agricultura, Pesca y Alimentación, 1993). Bulk milk from two to five milkings was stored between 3 and 8 ºC for an average time of 34 h before cheesemaking. Raw sheep milk was warmed up to 25 ºC and a commercial homofermentative starter culture (~0.02 g/L, Choozit, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added. Temperature was then raised to around 30 ºC and commercial rennet and/or artisanal lamb rennet paste was added. The minimum coagulation activity of commercial rennets was 1300 IMCU/g of rennet and an amount of around 0.03 g/L of milk was added. Milk clotting activity of artisanal rennet pastes prepared by Idiazabal PDO cheese-makers ranged between 155 and 363 U/g of tissue (Bustamante *et al*., 2000), and rennet concentration varied from 0.10 to 0.15 g/L of milk. Previous studies showed that curd firmness at cutting point for raw sheep milk used for Idiazabal PDO cheese varied from around 82 to 115 g during the processing season (Nájera *et al*., 2009; Abilleira *et al*., 2010). The cheese-maker started the cutting process guided by his/her know-how and the sensory observation of the curd consistency. Cutting was performed generally without raising the temperature at around 30 ºC for a variable amount of time and rotation speed. Cutting frames were then switched with stirrers and temperature was increased to around 36 ºC while stirring. The cheese-maker chose the cooking time and speed conditions that he/she believed to be suitable, as these parameters are not defined in the specifications (OJEU, 2015). Stirrers were exchanged for metal panels with holes when cooking finished and these were manually pressed against the curd grains inside the vat creating a big mass of curd. The whey was then drained from the vat and the mass of curd was cut into blocks and manually introduced in plastic moulds. Finally, cheeses were pressed at around 20 ºC for 3-7 h at 1.5-3.5 kg/cm^2, placed in saturated sodium chloride brine at around 10 ºC for 10-24 h, and ripened at 8-12 ºC and 85% relative humidity for at least 2 mo. The most relevant technical parameters used by the small rural dairies for the cheesemaking trials (n = 4) are presented in Table 1.

Samples of milk, whey, curd grains after cutting (fresh curd grains, FCG), curd grains after cooking (stirred curd grains, SCG) and cheese after pressing were taken during the cheesemaking trials in each dairy. A sample (0.5 L) of bulk raw milk was taken from the vat before cheesemaking commenced. Two samples (0.5 L each) of the whey that the cheese-makers decided to cast aside were taken during the whey draining. FCG and SCG samples were obtained submerging a round steel mesh sieve to a depth approximately halfway between the top and bottom of the vat right after the end of cutting (FCG) and cooking (SCG). For compositional analyses, FCG and SCG samples were directly placed in 0.5 L containers, and for image analysis, the sampling methodology suggested by Aldalur *et al*. (2019) was followed. A whole cheese (~1.5 kg) was taken after pressing for each trial. Two cheese samples 2-month aged were sampled for each trial. All the samples were carried to the laboratory in a portable cooler.

Subsamples of milk, whey, FCG and SCG were stored in 50 mL screw-capped plastic containers at -80 ºC, whereas each cheese was cut into six similar wedges, vacuum packed and frozen to -80 ºC until analysis.

Physico-chemical and technical determinations

Milk and whey fat was determined in duplicate by Gerber method (ISO/IDF, 2008a) and Van Gulik method (ISO/IDF, 2008b) was used for the fat content of curd grain and cheese samples. Milk, whey, curd grain and cheese protein content was analyzed by Kjeldahl method (ISO/IDF, 2008c, 2014). Total solids was measured by drying the samples overnight at 102 ºC for milk and whey samples (ISO/IDF, 2010) and at 105 ºC for curd grain and cheese samples (ISO/IDF, 2004). For determination of the content of calcium, magnesium and phosphorous, milk, whey, FCG, SCG and cheese samples were digested using a Speedwave Four microwave oven (Berghof, Harretstraße, Germany) as described by De la Fuente *et al*. (1997). Calcium and magnesium contents were measured by atomic absorption spectroscopy (A Analyst 200, Perkin Elmer, Mathews, NC, USA) (De la Fuente *et al*., 1997) and the phosphorous content by photometric method (AOAC, 2005).

During cheesemaking trials, pH was measured in triplicate at 20 ºC in milk, curd, FCG, SCG and whey using a GLP21 pH-Meter (Crison, Alella, Spain). Likewise, time and temperature for milk coagulation, and curd cutting and cooking were measured *in situ* in the small rural dairies. Cutting and stirring rotation speed (rpm) was determined in triplicate from the time that wire knives and stirrers needed for a full 360º turn. Knife density was calculated for each dairy as the ratio between the number of wires or knives in the cutting frame *per* the area of the cutting frame. Cutting tip speed (CTS, m/min) was defined as in Equation (1).

$$CTS = 2\pi r(NCF)(CS) \tag{1}$$

where r was the radius (m) of the cutting frame, NCF was the number of cutting frames used in the vat, and CS was the cutting speed (rpm). Cutting density was calculated multiplying the CTS and knife density used in each production. Cutting, stirring or total revolutions used during the cheese production were calculated multiplying the rotation speed and time of cutting, stirring or as the sum of cutting and stirring revolutions, respectively (Johnston *et al*., 1991). Cooking temperature rate was established as the ratio between the difference of final and initial cooking temperature and time. The amount of whey produced during each cheesemaking trial was estimated as the mass balance between the amount of milk and that of fresh cheese produced *per* vat. Other technical parameters related to pressing, brining, airing and ripening of cheeses were also collected during each cheese production.

Actual cheese yield (kg of cheese/100 kg of milk) was determined as the ratio between the total weight of the cheeses after pressing, and the amount of milk used in the vat. Composition-adjusted cheese yield (Y_{CA}) was also calculated to remove the effect of milk composition changes and cheese moisture and allowing the assessment of the isolated effect of technological parameters on cheese yield. This was calculated as described by Guinee *et al*. (2006), using reference values of milk fat (6.7%), milk protein (5.2%) and cheese moisture (42.7%). Cheese yield efficiency was calculated comparing the actual yield to the theoretical cheese yield estimated for each production (Barbano and Sherbon, 1984). The Van Slyke formula was used to determine the theoretical yield (Emmons and Modler, 2010). Fat, protein, calcium, magnesium and phosphorous loss in the whey on milk basis were estimated as in Equation 2 (Guinee *et al*. 2006).

$$\% \text{ Loss} = (W_W \times W_C)/(M_W \times M_C) \times 100 \qquad (2)$$

where W_W and M_W were the weight (kg) of the drained whey and bulk milk, respectively, and W_C and M_C were the amount of fat, protein, calcium, magnesium or phosphorous in whey and milk, respectively, depending on the compound being calculated. The total mineral loss used in the principal component analysis was calculated adding the amount of each mineral (calcium, magnesium and phosphorous) in whey and milk to W_C and M_C, respectively.

Curd grain two-dimensional image analysis

Curd grain image analysis for FCG and SCG samples was carried out as previously reported by Aldalur *et al*. (2019). Briefly, photographs of the curd grains scattered on a black plastic sheet were captured and analyzed. The brightness and contrast were adjusted to enhance the edge of each curd grain, and then, pictures were thresholded and measured. Particles with an area < 1 mm^2 were excluded from image analysis. Size and shape parameters such as, area, elongation, rectangularity and circularity were obtained for each curd grain and were defined in Aldalur *et al*. (2019).

Statistical analysis

Statistical analysis was performed using the statistical package SPSS (Version 25.0, IBM, New York, NY, USA). The General Linear Model of Analysis of Variance (ANOVA) was used to investigate the effect of individual dairy (*I* = 8), season (*I* = 2) and year (*I* = 2) on technical and analytical variables monitored during cheesemaking trials in the small rural dairies. Equation (3) shows the statistical model applied.

$$Y_{ijkl} = \mu + D_i + S_j + Y_k(S_j) + D{*}S_{ij} + \varepsilon_{ijkl} \quad (3)$$

where Y_{ijkl} = technical or analytical variable; μ = intercept; D_i = individual dairy fixed effect; S_j = season fixed effect; Y_k = year fixed effect; and ε_{ijkl} = residual random effects. Technical and analytical variables were checked for normality and homoscedasticity, and Tukey's honest significance difference test was used to pairwise comparison from mean values of individual dairies. Pearson bivariate correlations were calculated for the compositional variables of milk and whey obtained from the cheese productions. Principal composition analyses (PCA) were separately performed on self-scaling variables in order to study the relationships between the composition of milk, curd grain, whey, cheese after pressing and 2 months of ripening, technical settings used for cutting and cooking, and cheese yield. The Kaiser criterion (eigenvalue > 1) was applied to extract the principal components. Significance was declared at $P \leq 0.05$.

Results and discussion

Milk composition and cheesemaking technological parameters

Table 1 shows the mean values of milk composition and processing conditions used by the small rural dairies and the significance of the impact of the individual dairy, season and year effects on these variables. Milk composition varied significantly ($P \leq 0.05$) depending on the season and year. Milk collected in early summer had a major content of fat (6.1 *vs* 7.2 g/100 mL), protein (4.84 *vs* 5.50 g/100 mL), calcium (127.8 *vs* 150.3 mg/100 mL), magnesium (13.5 *vs* 17.0 mg/100 mL), phosphorous (133.7 *vs* 144.8 mg/100 mL), and consequently total solids (16.40 *vs* 17.85 g/100 mL) in comparison to the milk collected in late winter. The fat-to-protein ratio, a parameter highly related to the efficiency of the cheesemaking process, did not vary with season and year (Addis *et al*., 2018). Several authors have widely reported seasonal and year-to-year variations in milk composition due to the stage of lactation, diet, physiological state of the animals or climatic conditions among others (Barrón *et al*., 2001; Nájera *et al*., 2009; Abd El-Gawad and Ahmed, 2011). As explained above, dairies participating in this study used traditional flock management and feeding systems; indoor feeding during winter, part-time grazing from spring onwards and, extensive grazing at the end of the lactation period. Therefore, milk composition did not show significant differences ($P > 0.05$) among dairies (Table 1).

The main cheesemaking parameters, however, differed significantly ($P \leq 0.05$) among dairies, but in general, data did not show seasonal or year-to-year variation (Table 1). This suggests that cheese-makers do not significantly change their cheesemaking process depending on milk composition or controlled environmental factors, but that they follow their own cheesemaking recipe within Idiazabal PDO regulation, which varies according to uncontrolled factors. It is worth noting that the technical parameters related to cutting and cooking, such as time and speed, especially differed among dairies (Table 1), and that there is no specific recommendations for these settings within Idiazabal PDO Regulation. For the cutting process, mean cutting time and speed ranged broadly from 3 to 27 min and 8.5 to 23.8 rpm, respectively, affecting the revolutions used for cutting the curd (from 56 to 569 rev). Similarly, cooking time and speed greatly varied

Table 1. Milk composition and technical parameters (mean values, n = 4) used by small rural dairies during Idiazabal cheese manufacture made in different seasons (late winter and early summer) in two consecutive years.

	Dairy								SEM	Significance			
	1	2	3	4	5	6	7	8		*D*	*S*	*Y*	*D*S*
Milk fat (g/100 mL)	6.7	6.2	6.9	7.2	6.7	6.9	6.7	6.1	0.2	ns	***	**	ns
Milk protein (g/100 mL)	5.14	5.06	5.37	5.31	4.98	5.29	5.25	5.17	0.08	ns	***	**	**
Milk fat/protein ratio	1.3	1.2	1.3	1.3	1.3	1.3	1.3	1.2	0.02	ns	ns	ns	ns
Milk total solids (g/100 mL)	17.23	16.92	17.28	17.63	17.10	17.28	17.18	16.66	0.19	ns	***	*	ns
Milk Calcium (mg/100 mL)	140.7	135.8	137.6	147.7	137.1	127.0	141.1	143.0	3.4	ns	***	***	ns
Milk Magnesium (mg/100 mL)	15.6	14.7	15.5	15.7	15.5	13.9	15.7	15.2	0.4	ns	***	***	ns
Milk Phosphorous (mg/100 mL)	137.1^{abc}	137.2^{abc}	142.9^{bc}	144.7^{bc}	148.0^{c}	127.2^{a}	136.3^{abc}	134.9^{ab}	3.0	*	***	***	*
Milk pH	6.68	6.62	6.61	6.60	6.58	6.64	6.66	6.68	0.01	ns	ns	ns	ns
Milk amount (L)	1266^{c}	809^{b}	512^{a}	614^{ab}	388^{a}	532^{a}	466^{a}	498^{a}	56	***	***	ns	ns
Whey amount (kg)	992^{c}	646^{b}	421^{a}	456^{ab}	317^{a}	444^{a}	364^{a}	421^{a}	48	***	***	ns	ns
Coagulation temperature (ºC)	30.3^{ab}	30.8^{b}	30.0^{ab}	30.0^{ab}	28.7^{a}	29.0^{ab}	30.3^{ab}	29.5^{ab}	0.2	*	ns	ns	*
Coagulation time (min)	36^{a}	38^{ab}	52^{b}	35^{a}	33^{a}	42^{ab}	33^{a}	33^{a}	1	**	ns	ns	ns
Coagulation pH	6.58^{bc}	6.54^{ab}	6.58^{abc}	6.55^{ab}	6.48^{a}	6.60^{bc}	6.57^{abc}	6.66^{c}	0.13	**	*	**	ns
Cutting temperature (ºC)	30.3	31.6	30.5	31.0	29.8	32.0	31.3	30.7	0.2	ns	ns	*	ns
Cutting time (min)	13^{b}	27^{c}	4^{ab}	4^{ab}	6^{ab}	7^{ab}	4^{ab}	3^{a}	2	***	ns	ns	ns
Cutting speed (rpm)	8.5^{a}	21.4^{bc}	21.8^{bc}	17.1^{b}	22.2^{bc}	23.8^{c}	18.7^{bc}	20.6^{bc}	0.9	***	ns	ns	ns
Cutting tip speed (m/min)	72.5^{a}	97.5^{ab}	107.0^{ab}	169.5^{c}	110.0^{b}	118.1^{b}	102.8^{ab}	113.1^{b}	5.5	***	ns	ns	ns
Cutting revolutions (rev)	109^{a}	569^{b}	94^{a}	60^{a}	132^{a}	170^{a}	70^{a}	56^{a}	32	***	ns	ns	ns
Cooking time (min)	20^{ab}	45^{b}	30^{ab}	24^{ab}	32^{ab}	25^{ab}	19^{a}	29^{ab}	2	*	ns	ns	ns
Cooking speed (rpm)	9.7^{a}	16.6^{bc}	13.9^{ab}	28.1^{d}	21.1^{c}	15.9^{bc}	13.8^{ab}	13.5^{ab}	1.1	***	ns	ns	ns
Cooking revolutions (rev)	192^{a}	747^{b}	410^{ab}	684^{b}	671^{ab}	387^{ab}	264^{ab}	393^{ab}	47	*	ns	ns	ns
Cooking initial temperature (ºC)	30.5^{ab}	30.3^{a}	30.5^{ab}	32.3^{ab}	30.4^{a}	32.7^{b}	31.3^{ab}	31.3^{ab}	0.2	*	ns	ns	ns
Cooking final temperature (ºC)	35.8^{abc}	36.8^{c}	36.0^{abc}	36.6^{bc}	35.5^{ab}	36.2^{abc}	36.0^{abc}	35.0^{a}	0.2	**	**	ns	**
Cooking temperature rate (ºC/min)	0.27	0.15	0.20	0.19	0.17	0.15	0.29	0.13	0.02	ns	ns	ns	ns
Total revolutions (rev)	300^{a}	1316^{d}	504^{abc}	744^{bc}	803^{c}	557^{abc}	334^{ab}	449^{abc}	65	***	ns	ns	ns
Pressing time (min)	308^{bc}	203^{a}	308^{bc}	315^{bc}	315^{bc}	330^{c}	360^{c}	250^{ab}	11	***	ns	**	ns
Pressing pressure (bar)	2.5^{b}	3.3^{cd}	3.6^{d}	2.4^{ab}	2.9^{bc}	2.8^{bc}	1.8^{a}	2.5^{b}	0.1	***	ns	ns	ns
Brining time (h)	18^{b}	19^{b}	23^{c}	12^{a}	14^{a}	13^{a}	12^{a}	14^{a}	1	***	ns	ns	ns
Brine concentration (ºBè)	18^{b}	19^{b}	14^{a}	18^{b}	12^{a}	19^{b}	17^{b}	19^{b}	1	***	ns	ns	ns
Airing time (d)	5^{a}	11^{a}	19^{ab}	31^{b}	1^{a}	np	12^{a}	2^{a}	2	***	ns	ns	ns
Ripening temperature (ºC)	10.8^{abc}	9.0^{a}	11.0^{bc}	9.8^{ab}	11.8^{c}	10.3^{abc}	11.3^{bc}	11.0^{bc}	0.2	**	*	ns	ns

$^{a-d}$ Means with different superscripts in the same row indicate statistically significant differences among individual dairies ($P \leq 0.05$).*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant $P > 0.05$; np, no airing process. *D*: dairy; *S*: season; *Y*: year; SEM, standard error of the mean.

from 19 to 45 min and 9.7 to 28.1 rpm, respectively, resulting in 192 to 747 rev during the cooking process. Additionally, the temperature at the end of the cooking process was significantly different ($P \leq 0.05$) among dairies and season, although the variation range was small (from 35.0 to 36.8 ºC) since the temperature at the end of the cooking step is fixed by the Idiazabal PDO Regulation. The energy required to increase the temperature during late winter was higher than in early summer and, as cheesemaking settings generally did not change between seasons, lower temperatures at the end of cooking were recorded during the coldest season (35.8 *vs* 36.2 ºC).

These results open up a range of possibilities to further investigate the multifactorial relationships between technical and compositional parameters, cheese yield and compound losses in the whey in the small rural dairies.

Cutting and cooking processes

Table 2 shows the mean values of composition, size and shape parameters of curd grains after cutting and cooking processes obtained from the cheesemaking trials in the small rural dairies. Fat, protein and mineral content of FCG and SCG did not differ significantly ($P > 0.05$) among dairies and generally, did not show remarkable seasonal variations, even though milk composition greatly varied from late winter to early summer (Table 1). Conversely, moisture and area parameters of both FCG and SCG especially differed ($P \leq 0.05$) among dairies, being dairy 2 the one that had the lowest values for both parameters (Table 2). The differences on the mean curd grain size after cutting was up to 5 times among some dairies (between 11.66 to 56.82 mm^2) while after cooking the size varied from 8.20 to 27.23 mm^2. Two clear groups could be discerned when assessing the mean size of the SCG, where dairies 1, 7 and 8 had areas bigger than 20 mm^2, and dairies 2, 3, 4, 5, and 6 had areas smaller than 13 mm^2. Regarding the shape of the curd grains, FCG showed statistically significant ($P \leq 0.05$) differences among dairies for elongation and rectangularity parameters. However, during the cooking process the shape of the curd particles tend to homogenise, and as a result, no statistical differences ($P > 0.05$) were found among dairies for elongation, rectangularity and circularity parameters (Table 2). These results agree with those reported by other authors that concluded that curd

Table 2. Composition, size and shape parameters (mean values, n = 4) of curd grains after cutting (FCG) and curd grains after cooking (SCG) used by small rural dairies during Idiazabal cheese manufacture made in different seasons (late winter and early summer) in two consecutive years.

	Dairy								SEM	Significance			
	1	2	3	4	5	6	7	8		*D*	*S*	*Y*	*D*S*
Curd grains after cutting													
Fat (g/100 g)	17.5	20.6	18.2	16.6	18.3	19.2	18.4	15.3	0.4	ns	ns	*	ns
Protein (g/100 g)	11.72	14.46	13.28	12.0	11.83	12.64	11.86	12.90	0.29	ns	ns	**	ns
Moisture (g/100 g)	63.42^{ab}	57.08^{a}	61.83^{ab}	63.47^{ab}	61.66^{ab}	60.94^{ab}	61.75^{ab}	64.76^{b}	0.63	*	ns	*	ns
Calcium (mg/100 g)	382.4	417.3	391.7	388.5	380.5	406.8	380.8	311.3	9.4	ns	ns	***	ns
Magnesium (mg/100 g)	26.7	27.4	27.8	27.2	27.3	27.4	25.9	22.4	0.5	ns	*	ns	ns
Phosphorous (mg/100 g)	289.2	319.5	300.9	292.3	293.2	299.9	272.0	257.3	6.0	ns	ns	***	ns
FCG pH	6.59^{ab}	6.54^{a}	6.58^{a}	6.57^{a}	6.54^{a}	6.62^{ab}	6.58^{a}	6.70^{b}	0.01	*	ns	ns	ns
FCG area (mm^2)	41.61^{ab}	11.66^{a}	27.37^{ab}	23.27^{ab}	20.89^{ab}	18.26^{ab}	43.34^{ab}	56.82^{b}	3.36	*	ns	ns	ns
FCG elongation	0.687^{a}	0.711^{ab}	0.736^{b}	0.734^{ab}	0.735^{ab}	0.712^{ab}	0.735^{ab}	0.717^{ab}	0.004	*	ns	ns	ns
FCG rectangularity	0.681^{a}	0.694^{ab}	0.709^{b}	0.698^{ab}	0.703^{ab}	0.695^{ab}	0.703^{ab}	0.694^{ab}	0.002	*	ns	ns	ns
FCG circularity	0.735	0.783	0.758	0.761	0.790	0.783	0.771	0.702	0.010	ns	ns	*	ns
Curd grains after cooking													
Fat (g/100 g)	21.2	23.0	22.5	21.4	22.6	21.6	22.6	19.8	0.4	ns	ns	*	ns
Protein (g/100 g)	13.50	15.63	15.16	14.84	14.55	13.47	14.81	15.09	0.24	ns	ns	ns	ns
Moisture (g/100 g)	60.21^{a}	55.03^{b}	55.84^{b}	58.15^{ab}	57.20^{ab}	58.65^{ab}	56.38^{b}	60.19^{a}	0.70	*	**	**	*
Calcium (mg/100 g)	426.9	456.7	462.1	456.7	433.2	414.3	423.5	450.4	7.7	ns	ns	***	ns
Magnesium (mg/100 g)	29.3	30.1	32.4	30.9	30.3	28.9	29.7	28.9	0.4	ns	*	ns	ns
Phosphorous (mg/100 g)	353.5	373.5	390.4	367.3	351.6	315.0	342.2	333.0	8.7	ns	***	***	ns
SCG pH	6.58^{ab}	6.53^{a}	6.61^{ab}	6.58^{ab}	6.51^{a}	6.69^{b}	6.60^{ab}	6.70^{b}	0.02	*	ns	ns	ns
SCG area (mm^2)	27.23^{b}	8.20^{a}	10.66^{a}	10.72^{a}	12.43^{a}	12.96^{a}	22.84^{b}	22.53^{b}	1.36	***	ns	ns	ns
SCG elongation	0.672	0.701	0.705	0.697	0.718	0.700	0.692	0.702	0.003	ns	ns	ns	ns
SCG rectangularity	0.681	0.694	0.697	0.694	0.695	0.687	0.691	0.699	0.002	ns	*	ns	ns
SCG circularity	0.754	0.788	0.792	0.787	0.784	0.779	0.738	0.783	0.007	ns	**	**	ns

$^{a-b}$ Means with different superscripts in the same row indicate statistically significant differences among individual dairies ($P \leq 0.05$).*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant $P > 0.05$. *D*: dairy; *S*: season; *Y*: year; SEM, standard error of the mean.

shape is clearly determined by the design, number and arrangement of wires and movement of the cutting knives. In this regard, the technical settings used during cooking were the main responsible for the final shape of the curd grains obtained during the cheesemaking trials in the small rural dairies (Kammerlehner, 2009; Aldalur *et al*., 2019). Additionally, some significant differences ($P \leq 0.05$) were observed in curd grain shape parameters due to the season and year, as the cheese-makers did not change the technological parameters when the composition of milk varied from late winter to early summer (Table 1).

In order to study the relationships between technical parameters and the composition and properties of curd grains during the cutting process, a PCA was carried out including FCG composition, FCG size and shape parameters and technological settings. Two PCs were extracted explaining the 61.71% of the total variance (Figure 1A). FCG moisture content and area were negatively correlated (factor loadings > |0.8|) with PC1, whereas fat, protein and mineral content and cutting time showed high positive correlation (factor loadings > 0.6). A higher duration of cutting, as well as cutting revolutions, made curd grains smaller, enhancing syneresis and reducing the moisture content of curd grains. These results agree with other experimental studies on a laboratory scale that reported that the curd grain size was the main responsible for a higher syneresis rate, especially during the first 10 min after cutting at a constant temperature of 33 ºC (Unger Grundelius *et al*., 2000). In the present study, temperature during cutting did not significantly differ ($P > 0.05$) among dairies (Table 1), but certain negative correlation between temperature (factor loading ~ 0.4) and curd grain moisture was observed within PC1. On the other hand, PC2 encompassed variables related to curd grain shape and technical settings, particularly elongation and cutting speed and knives arrangement (factor loadings > |0.7|). At higher speed and number of wires *per* cutting surface (cutting density), the FCG generated were less elongated and more circular (Figure 1A). Additionally, Figure 1A plots the scores corresponding to the cheesemaking trials of each dairy showing a high inter-dairy and, in some cases, intra-dairy variability. For instance, dairies 1 and 6 showed homogeneous cheesemaking trials closer to each other, while others (e.g. dairies 3 and 8) showed larger distances among cheese productions. At this stage, dairies could be segmented into 3 different groups depending on the duration of the cutting process (Figure 1A): short cutting time (shorter than 8 min), moderate cutting time (between 10 and 15 min) and long cutting time (longer than 20 min).

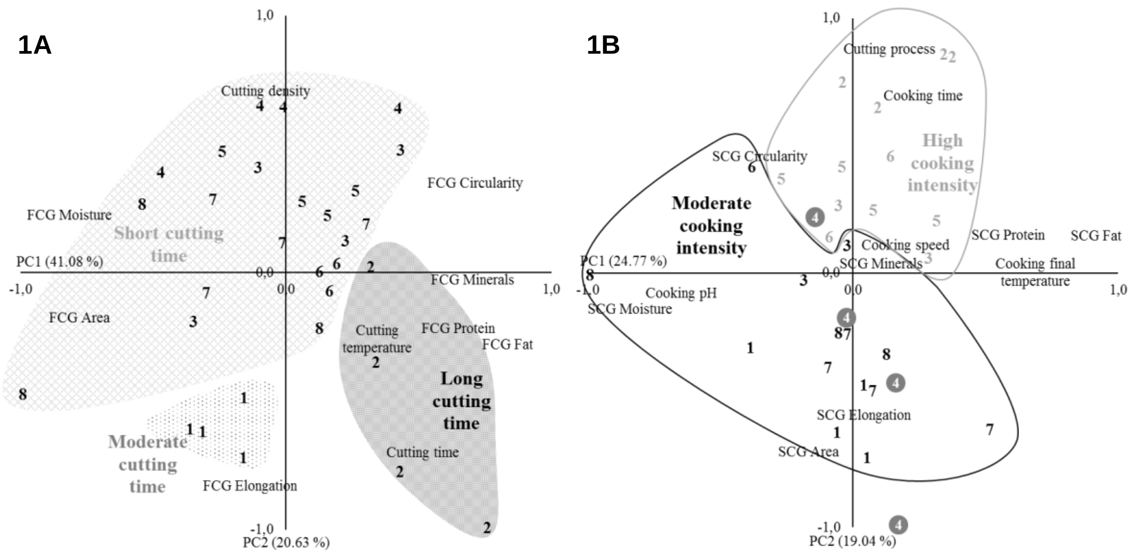

Figure 1. Principal component analysis (PCA) biplot showing variable loadings and cheese production scores distribution in the two-dimensional coordinate system defined by PC1 and PC2 for the cutting (1A) and cooking (1B) processes. Each number indicates the location of the cheesemaking productions for idem dairy. For 1A biplot, cheesemaking productions grouped with different patterns are defined as: cutting time less than 8 min (short cutting time, checked pattern), cutting time between 10 and 15 min (moderate cutting time, dotted pattern) and cutting time higher than 20 min (long cutting time, dark pattern). For the 1B biplot different colors indicate cheese productions using a moderate cooking intensity process (172-413 cooking revolutions; in black) or a high cooking intensity process (440-1210 cooking revolutions; in grey). In 1B, dairy 4 is highlighted (circled) to indicate that the scores are located in a different plane to the rest of the productions. FCG, curd grains after cutting; SCG, curd grains after cooking; Cutting density: cutting tip speed x knife density; Cutting process; cutting tip speed x cutting time x knife density.

As for the cutting step, a second PCA was applied to technical parameters, composition, size and shape of curd grains after the cooking step. Four PC were extracted accounting 74.78% of the total variance. In this case, not only were the cooking technological parameters included in the analysis but also those of the cutting process, since SCG are the combined result of both steps (Figure 1B). The PCA results showed that a higher temperature at the end of the cooking (mean values between 35.0 and 36.8ºC, Table 1) produced curd grains with a reduced amount of moisture and consequently, a higher fat and protein concentration (Figure 1B, PC1). During the cooking step, temperature was a much more relevant factor affecting syneresis than the curd grain size, in agreement with other authors (Dejmek & Walstra, 2004). SCG size was highly and negatively correlated (factor loading > |0.7|) to PC2 whereas the opposite occurred to the previous cutting conditions employed and to the duration of the cooking process (factor loadings > 0.7). A longer and more intense cutting process together with a longer cooking time generated curd grains that were smaller, less elongated and more circular. The other two PCs encompassed a positive correlation between the protein content and mineral content of SCG (PC3), and a negative correlation between the cooking speed and the SCG area (PC4). At the end of the cooking step, cheese productions could be differentiated depending on the cooking revolutions used. In Figure 1B, which depicts PC1 and PC2, cheese productions using between 172 and 413 rev during cooking (moderate cooking intensity) and were differentiated from productions using between 440 and 1210 rev (high cooking intensity). However, dairy 4 trials were an exception and were located along PC4 dimension due to the high correlation of cooking speed to PC4, and the significantly ($P \leq 0.05$) higher cooking speed employed in dairy 4 (mean value 28.1 rpm, Table 1). The separation into moderate and high cooking intensity groups was related to the final size of the curd grains, as discussed above. These results indicated that artisanal cheese-makers looked for different curd grain sizes after cooking even thought they were producing the same type of PDO cheese.

Whey and cheese composition

Table 3 presents data on the composition of whey, cheese after pressing and cheese after 2 months of maturation. Season and year factors were statistically significant ($P \leq 0.05$) for whey components, having a direct positive correlation with the composition of milk ($r > 0.7$, $P \leq 0.05$). During early summer, the amount of fat and protein in whey was 37 and 23% higher, respectively, comparing to late winter. Fat concentration in whey and, accordingly total solids, clearly differed ($P \leq 0.05$) among dairies, being dairy 1 the one with the lowest amount of fat and total solids in whey (Table 3). For the cheese after pressing, the different technological settings employed from cutting to pressing by each dairy significantly affected ($P \leq 0.05$) the content of moisture. However, these differences were more easily observable in the cheeses after 2 mo of ripening, where dairy, season and year factors significantly influenced ($P \leq 0.05$) the compositional variables. As expected, the differences in cheese composition

Table 3. Composition of whey, cheese after pressing and cheese after 2 months of ripening (mean values, n = 4) obtained by small rural dairies during Idiazabal cheese manufacture made in different seasons (late winter and early summer) in two consecutive years.

	Dairy								SEM	Significance			
	1	2	3	4	5	6	7	8		*D*	*S*	*Y*	*D*S*
Whey													
Fat (g/100 mL)	0.4^{a}	0.8^{ab}	1.1^{b}	1.0^{b}	1.0^{b}	1.0^{b}	0.9^{b}	1.0^{b}	0.1	***	***	***	ns
Protein (g/100 mL)	1.43	1.35	1.52	1.51	1.52	1.57	1.54	1.50	0.04	ns	***	**	ns
Total solids (g/100 mL)	7.55^{a}	7.80^{ab}	8.49^{b}	8.25^{b}	8.23^{b}	8.30^{b}	8.17^{bc}	8.40^{b}	0.10	***	***	***	*
Calcium (mg/100 mL)	46.1	43.5	42.7	44.5	44.0	42.4	45.9	43.4	1.0	ns	ns	***	ns
Magnesium (mg/100 mL)	11.6	10.5	10.9	11.3	11.1	11.0	11.3	11.0	0.2	ns	***	***	ns
Phosphorous (mg/100 mL)	50.9	53.3	57.8	53.5	62.4	50.9	50.7	50.2	1.6	ns	ns	***	ns
Whey pH at draining	6.51	6.50	6.52	6.51	6.47	6.49	6.53	6.56	0.01	ns	*	*	ns
Cheese after pressing													
Fat (g/100 g)	32.0	32.1	32.2	30.8	30.9	31.5	31.7	30.3	0.2	ns	ns	ns	ns
Protein (g/100 g)	20.64	21.17	21.09	21.63	19.91	20.49	19.89	21.62	0.23	ns	ns	ns	ns
Moisture (g/100 g)	42.50^{ab}	41.94^{a}	41.57^{a}	43.07^{ab}	44.17^{b}	43.25^{ab}	43.13^{ab}	42.33^{ab}	0.22	**	ns	*	ns
Calcium (mg/100 g)	547.0^{abc}	564.0^{bc}	565.1^{bc}	586.7^{c}	516.3^{a}	517.1^{a}	517.0^{a}	537.7^{ab}	9.8	***	**	***	*
Magnesium (mg/100 g)	37.0	36.2	38.4	38.0	36.0	34.1	35.1	36.7	0.4	ns	**	*	ns
Phosphorous (mg/100 g)	487.1^{bcd}	501.0^{cd}	506.3^{d}	507.1^{d}	453.1^{ab}	455.0^{abc}	440.7^{a}	479.3^{abcd}	8.4	**	***	***	ns
Cheese 2 months													
Fat (g/100 g)	36.9^{b}	35.7^{ab}	35.9^{ab}	35.3^{ab}	36.2^{ab}	34.5^{ab}	33.3^{a}	33.1^{a}	0.5	**	***	**	ns
Protein (g/100 g)	23.32	24.03	24.02	24.25	22.75	22.27	22.77	24.67	0.25	ns	ns	*	ns
Moisture (g/100 g)	34.05^{a}	34.88^{ab}	34.81^{ab}	35.35^{ab}	35.92^{ab}	37.79^{b}	37.24^{ab}	36.42^{ab}	0.46	*	***	ns	*
Calcium (mg/100 g)	579.5^{abc}	613.6^{bcd}	621.0^{cd}	642.1^{d}	557.5^{ab}	530.2^{a}	554.6^{ab}	582.6^{abcd}	9.8	***	ns	***	*
Magnesium (mg/100 g)	41.2^{abc}	42.3^{bc}	43.0^{c}	44.1^{c}	41.3^{abc}	37.7^{a}	39.0^{ab}	40.7^{abc}	0.7	***	***	**	*
Phosphorous (mg/100 g)	484.3^{ab}	503.4^{ab}	503.4^{ab}	535.0^{b}	460.0^{ab}	430.5^{a}	446.6^{a}	497.3^{ab}	9.1	**	ns	*	*

$^{a-b}$ Means with different superscripts in the same row indicate statistically significant differences among individual dairies ($P \le 0.05$).*, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$; ns, not significant $P > 0.05$. *D*: dairy; *S*: season; *Y*: year; SEM, standard error of the mean.

among dairies were due to the cheesemaking technological and ripening conditions employed in each dairy (Table 1). Seasonal and year-to-year variation in the content of cheese fat, moisture and magnesium changed according to the composition of milk. In this regard, cheeses manufactured in early summer had a higher content of fat (33.6 *vs* 36.8%) and consequently lower amount of moisture (37.21 *vs* 34.23%). Protein, calcium and phosphorous content, however, did not statistically ($P > 0.05$) vary between late winter and early summer (Table 3).

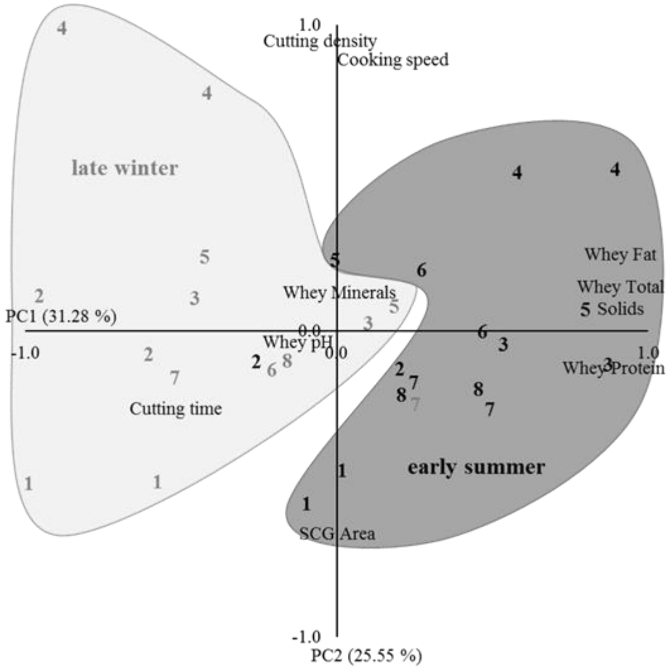

Figure 2. Principal component analysis (PCA) biplot showing variable loadings and cheese production scores distribution in the two-dimensional coordinate system defined by PC1 and PC2 for whey composition, and cutting and cooking technical parameters. Each number indicates the location of the cheesemaking productions for idem dairy. Different colors indicate cheese productions made in early summer (dark grey) and late winter (light grey). SCG, curd grains after cooking; Cutting density: cutting tip speed x knife density.

The multifactorial relationships associated with the cheesemaking conditions and composition of the whey were studied by a new PCA, and three components were extracted accounting for the 77.09% of the total variance. From the variables correlated to PC1, it was observed that employing a shorter time during cutting resulted in a higher concentration of fat, protein and therefore, total solids in the whey (Figure 2). Other authors also reported higher fat and protein losses when cutting was insufficient due to a shattering effect during the stirring step (Johnston *et al*., 1991, 1998; Everard *et al*., 2008; Aldalur *et al*., 2019). However, the correlation observed in PC1 did not explain the lower fat losses obtained in dairy 1 (cutting time mean value 13 min) compared to dairy 2 (27 min). This specific situation could have happened due to an excessive time and speed used during cutting and cooking processes by dairy 2 (Table 1), generating fat losses comparable to the ones obtained for dairies using short cutting time. In PC2, cutting speed, knife density and the total revolutions applied during cooking were mainly related to the final size of the curd grain, as previously mentioned (Figure 2). Additionally, the third component accounting for the 18.99% of the total variance showed high negative correlation (factor loadings > |0.8|) with whey pH at draining and the total mineral loss. It has been previously reported that lower pH at draining affects curd demineralization and increases mineral content in the whey (Lucey and Fox, 1993). The seasonal effect on cheese productions was clearly visible in the biplot of Figure 2 showing that cheese productions carried out in early summer were associated to higher losses of fat and protein in the whey, as also indicated in Table 3.

Relationship between cheese yield and technological parameters

Component losses in the whey in a milk weight basis together with actual cheese yield, composition-adjusted cheese yield and cheese yield efficiency after pressing and after 2 mo of ripening are shown in Table 4. Fat loss statistically differed ($P \leq 0.05$) among dairies, dairy 1 showing mean fat loss around 5% in a milk basis, while at least twice this value was measured for the rest of the dairies. Despite these differences, protein and mineral losses did not show significant differences ($P > 0.05$) among small rural dairies. Seasonal and year-to-year effects, however, were once again significant ($P \leq 0.05$) with higher fat (9.1 *vs* 11.9%) and protein (21.72 *vs* 23.99%) losses in early summer than in late winter. The higher amount of total protein losses during early summer was probably due to an increased content of non-casein proteins in whey (Abilleira *et al*., 2010). On the other hand, the total mineral losses were proportionally lower during early summer, as casein content in milk increased during this season. A higher amount of calcium and phosphorous was retained forming the protein matrix of cheese, and therefore, a similar content of calcium and phosphorous was measured in the whey (Table 3).

Actual cheese yield after pressing and after 2 mo of ripening showed significant differences ($P \leq 0.05$) between seasons and years, according to changes observed in milk and whey composition (Table 1 and 3). Mean actual cheese yield after pressing was 17.2 kg/100 kg in late winter, while in early summer was

Table 4. Component losses in the whey in milk basis and cheese yield parameters (mean values, n = 4) by small rural dairies during Idiazabal cheese manufacture made in different seasons (late winter and early summer) in two consecutive years.

	Dairy								SEM	Significance			
	1	2	3	4	5	6	7	8		*D*	*S*	*Y*	*D*S*
Fat loss (%)	5.2^{a}	10.2^{b}	12.8^{b}	11.0^{b}	11.9^{b}	11.1^{b}	10.4^{b}	12.7^{b}	0.6	***	***	**	ns
Protein loss (%)	22.25	21.86	22.89	22.23	24.21	23.96	22.96	23.48	0.32	ns	***	**	ns
Calcium loss (%)	26.5	26.6	25.3	24.6	25.8	25.1	25.5	24.7	0.5	ns	***	**	*
Magnesium loss (%)	60.1	59.7	57.2	57.8	57.8	60.8	56.9	59.4	0.8	ns	***	***	ns
Phosphorous loss (%)	29.8	32.1	32.7	29.6	33.9	30.1	29.3	30.3	0.7	ns	**	*	ns
Actual pressed cheese yield (kg/100 kg)	19.2	16.7	18.1	19.6	19.4	18.9	19.8	18.3	0.4	ns	***	*	ns
Actual 2 month cheese yield (kg/100 kg)	16.2	14.7	15.5	16.6	14.8	15.2	16.9	15.1	0.3	ns	***	**	ns
Adjusted pressed cheese yield (kg/100 kg)	19.4	18.0	17.9	18.5	19.2	18.2	19.2	19.4	0.2	ns	ns	ns	ns
Adjusted 2 month cheese yield (kg/100 kg)	16.6	15.7	15.2	15.8	14.9	14.2	16.3	15.5	0.2	ns	ns	ns	ns
Cheese yield efficiency (%)	101.3	95.1	94.1	95.6	97.5	92.2	100.7	101.7	1.1	ns	ns	ns	ns

$^{a-b}$ Means with different superscripts in the same row indicate statistically significant differences among individual dairies ($P \le 0.05$).*, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$; ns, not significant $P > 0.05$. *D*: dairy; *S*: season; *Y*: year; SEM, standard error of the mean.

20.0 kg/100 kg. After 2 mo of ripening these values decreased due to the loss of moisture, but the differences remained high for yield values between late winter and early summer (14.6 *vs* 16.5 kg/100 kg, respectively). Although actual cheese yield values among dairies were not statistically different ($P > 0.05$) due to the intra-dairy variability observed, the actual pressed cheese yield varied almost in 3 kg/100 kg, while this difference was reduced to around 2 kg/100 kg for the actual 2 mo cheese yield. However, variations of 2-3 kg/100 kg between cheese productions could have a major impact on the profitability of the small rural dairies, as usually the price *per* kg of cheese hardly varies among Idiazabal PDO cheese small rural dairies during the cheese production season.

In order to compare the differences in yield due to the cheesemaking technology, the effect of milk composition and cheese moisture was removed using the composition-adjusted cheese yield (Guinee *et al*., 2006). The effect of the season and year on the composition-adjusted cheese yield for both fresh and 2 mo ripened cheeses was not statistically significant ($P > 0.05$) (Table 4). The differences among small rural dairies remained large but were not significant ($P > 0.05$) due to the high intra-dairy variability, as observed previously for the actual cheese yield. The same result was obtained for cheese yield efficiency but this parameter was useful for comparative purposes. For instance, dairies 1, 9 and 10 showed mean cheese yield efficiency values around 100% whereas the rest of the dairies were less effective during their cheese production.

For a better understanding of the relationships between technological parameters, milk composition, compound losses in the whey and actual cheese yield, a new PCA was carried out. Four PC were extracted accounting for 73.06% of the total variance. As observed in Figure 3A, which plots PC1 and PC2, a higher protein and fat content of milk together with a lower total mineral loss in the whey were highly correlated to an increased actual cheese yield in PC1 (factor loadings > |0.8|). As observed before, a clear seasonal effect could be discerned (Figure 3A) in the cheese productions, and a higher actual cheese yield was obtained in early summer due to the compositional changes in milk. SCG size was negatively correlated to the cutting speed, knife density and cooking speed in PC2. Likewise, SCG size showed negative correlation with the time employed during cutting and cooking together with the final cooking temperature in PC3.

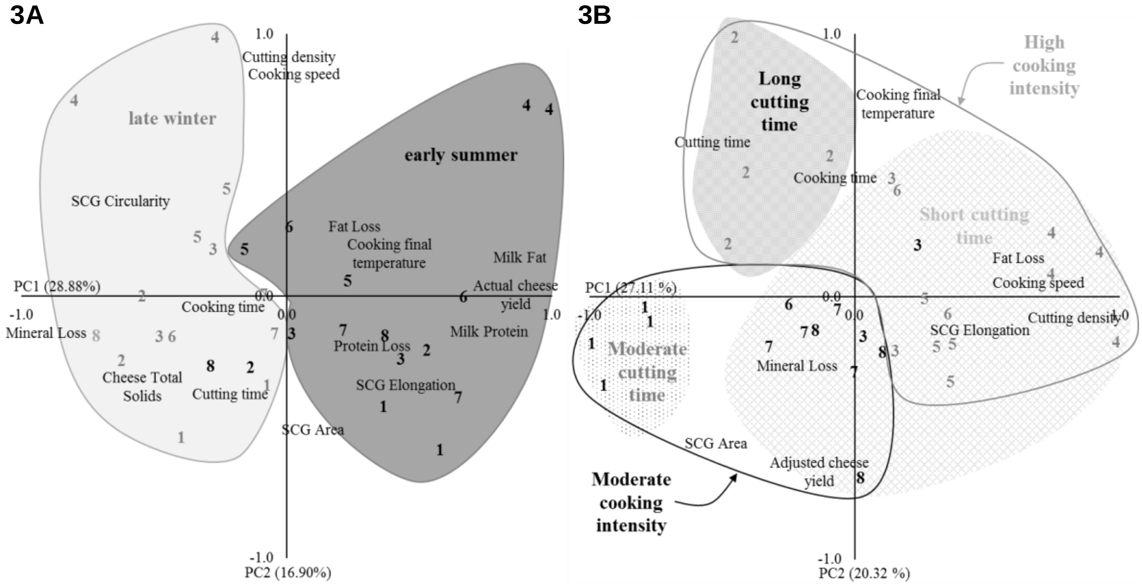

Figure 3. Principal component analysis (PCA) biplot showing variable loadings and cheese production scores distribution in the two-dimensional coordinate system defined by PC1 and PC2 related to technical settings, cheese yield and composition. Each number indicates the location of the cheesemaking productions for idem dairy. The 3A biplot shows the relationships between the actual cheese yield and cheesemaking conditions and depicts the seasonal factor with different colors for early summer (dark grey) and late winter (light grey). The 3B biplot shows the relationships between the composition-adjusted cheese yield and cheesemaking conditions and illustrates the combined effect of cutting and cooking steps. Cheesemaking productions grouped with different patterns are defined as: cutting time less than 8 min (short cutting time, checked pattern), cutting time between 10 and 15 min (moderate cutting time, dotted pattern) and cutting time higher than 20 min (long cutting time, dark pattern). Different colors indicate cheese productions using a moderate cooking intensity process (172-413 cooking revolutions; in black) or a high cooking intensity process (440-1210 cooking revolutions; in grey). SCG, curd grains after cooking.

Additionally, fat and protein losses and SCG elongation were correlated to PC4 showing that in cheesemaking processes where less elongated SCG were generated, usually associated to smaller particles, higher fat and protein losses were obtained. To remove the seasonal effect on milk composition and looking for direct relationships between cheese yield and cheesemaking technological conditions, another PCA was carried out using the composition-adjusted cheese yield. Three PCs were extracted explaining the 64.87% of the total variance. Using higher cutting speed, knife density and cooking speed (factor loadings > |0.6|) resulted in a smaller curd grain size after cooking and enhanced fat losses in the whey along the PC1 (Figure 3B). However, in PC2 the composition-adjusted cheese yield increased when the area of SCG was bigger, temperature at the end of cooking was lower, and cutting and cooking durations were shorter (factor loadings > |0.5|). The cheese production scores are shown in Figure 3B and the seasonal effect plotted in Figure 3A was no longer visible, being now distinguishable the segmentations for the combined effect of cutting duration and cooking revolutions described previously in Figures 1A and 1B, respectively. When combining moderate cutting time and moderate cooking intensity lower fat losses were obtained. Conversely, short cutting time combined with a high intensity cooking process was specially related to higher fat losses. Long cutting time and high cooking intensities were associated to lower composition-adjusted cheese yield values. Therefore, this suggests that Idiazabal small rural dairies could improve the cheesemaking process by changing some of the technological settings currently used by the cheese-makers, decreasing fat losses in the whey and avoiding potential cheese yield reductions.

Conclusions

The cheesemaking technological settings used in Idiazabal PDO dairies were largely different, especially those regarding the cutting and cooking technology. Although milk composition changed with season, the small rural dairies did not substantially change the cheesemaking conditions. Cheese yield was influenced by season, increasing during early summer when the amount of milk fat and protein were higher. The main technological settings that impaired cheese yield were high cooking temperatures and long cutting and cooking processes, due to an enhanced moisture loss in the cheese. However, higher fat losses in the whey were related to very intense and short cutting time causing tearing of the curd, big curd grains and a subsequent shattering effect during cooking. Additionally, using a higher stirring speed during cooking specially enhanced fat losses in the whey. Some technological parameters, such as cutting time, inversely affected cheese yield and compound losses in whey. Therefore, it is essential to achieve a balance of these conditions to improve the cheesemaking process since both yield and losses are important economic and environmental issues for the small rural dairies. Furthermore, cheese-makers should adapt cutting and cooking conditions to the seasonal variations of milk composition, especially during early summer, considering the possibilities of their own equipment and facilities.

Acknowledgments

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1.2. Analysis of the microstructure of the dairy matrices during the cheesemaking process in small rural Idiazabal dairies

The microstructure of milk, curd (coagulated milk), curd grains and cheese samples was monitored throughout the cheesemaking process in Idiazabal cheese dairies. The size of the fat globules of Latxa sheep milk was measured by a 3-dimensional image analysis and the volume-weighted mean diameter (D[4,3]) was 5.50 ± 0.27 µm in early summer. A 3D representation of the milk fat globules and the size frequency and the volume contributed by each size category are plotted in Figure V.1. Milk fat globules with a diameter of 2 to 4 µm were the most numerous but droplets between 5 to 7 µm were the ones contributing the highest amount of fat volume.

During coagulation, the casein network was developed, which entrapped fat globules and serum pockets in its structure (Figure V.2). The protein matrix of the coagulated milk had a denser structure than other milk gels reported for bovine milk due to the higher casein and fat content of ovine milk (Lopez *et al*., 2007; Ong *et al*., 2010). At this stage, fat globules behaved as inner fillers, that is, they did not interact with the protein matrix (Lopez *et al*., 2007). Some of the fat globules formed aggregates, but in general, no coalescence was observed during this process (Figure V.2). During cutting, the whey was released and the protein

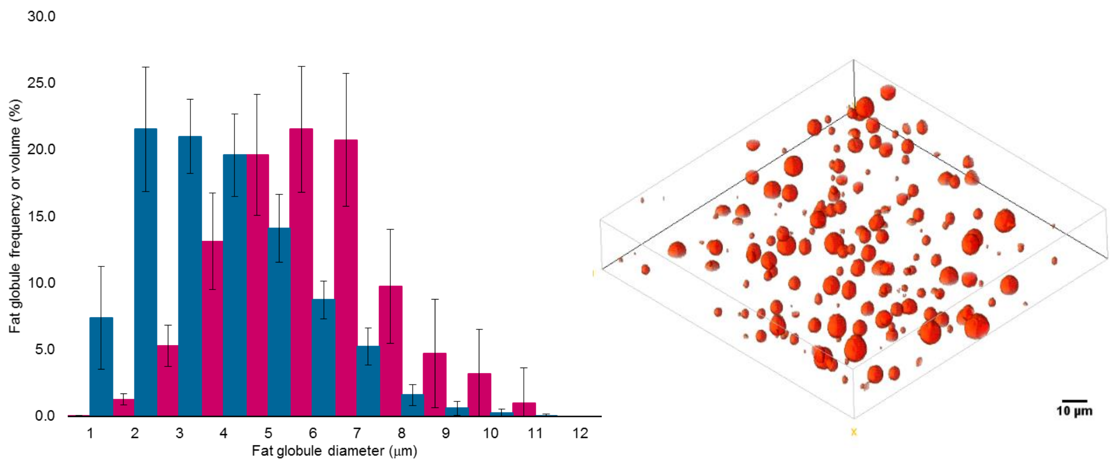

Figure V.1. Fat globule size distribution graph (left) and a 3-D reconstruction of the milk fat globules measured by confocal laser scanning microscopy (right). The graph represents the frequency (blue) and the volume (magenta) contributed by each fat globule size category of Latxa sheep milk used in Idiazabal cheese production during early summer. The scale bar is 10 µm in length.

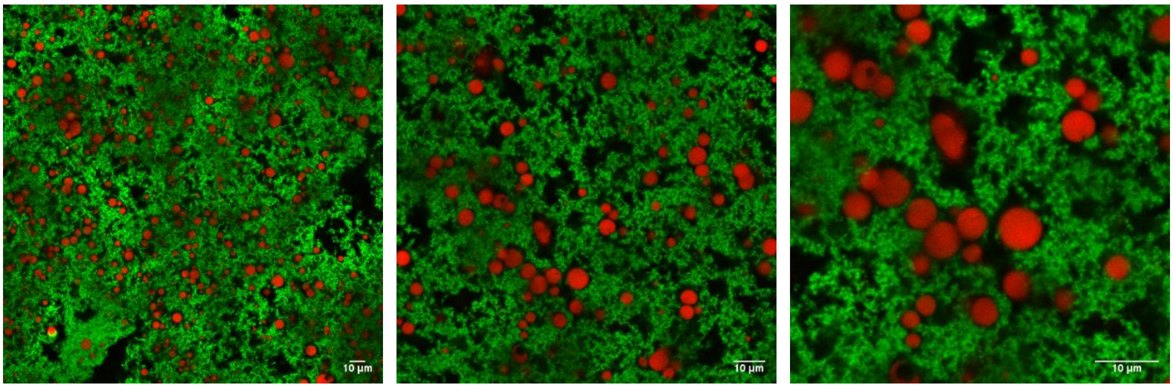

Figure V.2. Confocal laser scanning micrographs of coagulated milk during Idiazabal cheese manufacture. Fat appears red and protein appears green in these images. Images were captured at 1x (left), 2x (middle) and 4x (right) magnifications with a 63x objective lens. The scale bars are 10 µm in length.

network shrunk creating a more compact structure. However, it could be observed that the edges of the curd grains after cutting and some pores of the protein matrix next to the border appeared devoid of fat (Figure V.3, A and B). Since fat droplets are entrapped in the protein matrix but they do not generally interact, cutting the curd released the fat globules. Therefore, the amount of fat released to the whey in the high cutting process was higher, as measured, due to the increased surface area to volume ratio (Banks, 2007).

Curd grain cooking caused a significant ($P \leq 0.05$) reduction of the protein network porosity, especially when higher temperatures, speed and time were employed. Depending on the technical conditions used, the volume of non-globular and globular fat changed in the curd grains after cooking. The temperature is one of the main technical cooking settings that affects syneresis and, consequently, the curd grain microstructure due to the development of more compact protein strands and a higher fat melting degree of the droplets (Fagan *et al*., 2017; Lopez *et al*., 2006). The edges of the curd grains after cooking, these are shown in Figure V.3 (C and D). Once more, a lower amount of fat globules near the edge of the curd grains is observed and, in some cases, these droplets were captured escaping from the protein matrix (Figure V.3, D).

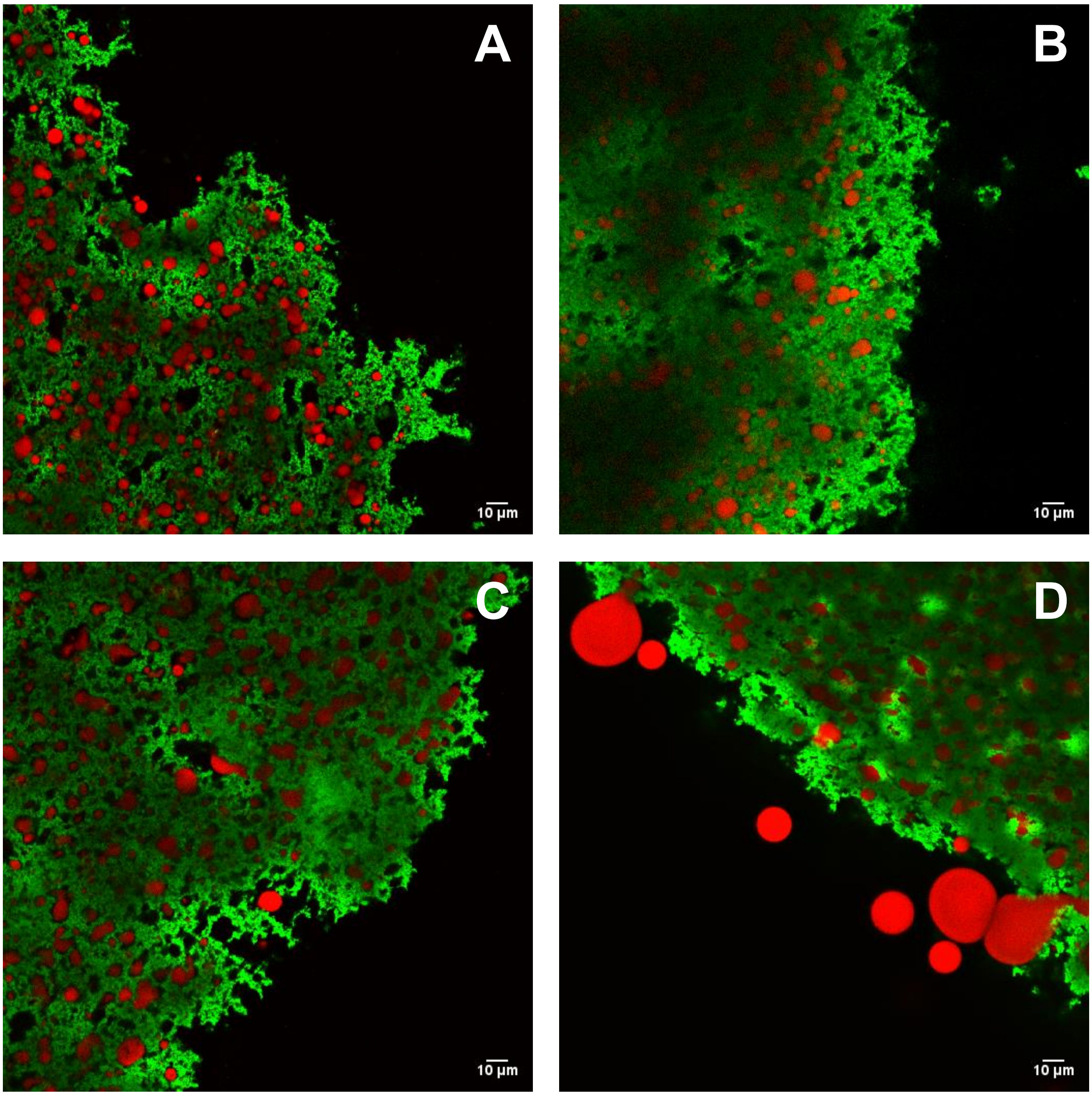

Figure V.3. Confocal laser scanning micrographs of the edges of the curd grains after cutting (A, B) and after cooking (C, D). Fat appears red and protein appears green in these images. Images were captured at 1x magnification with a 63x objective lens. The scale bars are 10 µm in length.

Pressing significantly ($P \leq 0.05$) changed the microstructure of the dairy matrix, increasing the density of the casein network and disrupting the conformation of some fat globules (Figure V.4, A-C). CLSM micrographs showed that fat droplets adopted different forms after pressing: native fat globules, aggregated fat globules, coalesced fat droplets, and free fat. Some authors reported that the disruption of the fat globules during pressing depends on the size of the fat globules, aggregation level, composition of fat, the properties of the casein network and the conditions used during pressing (Lammichhane *et al*., 2018; Lopez *et al*., 2007).

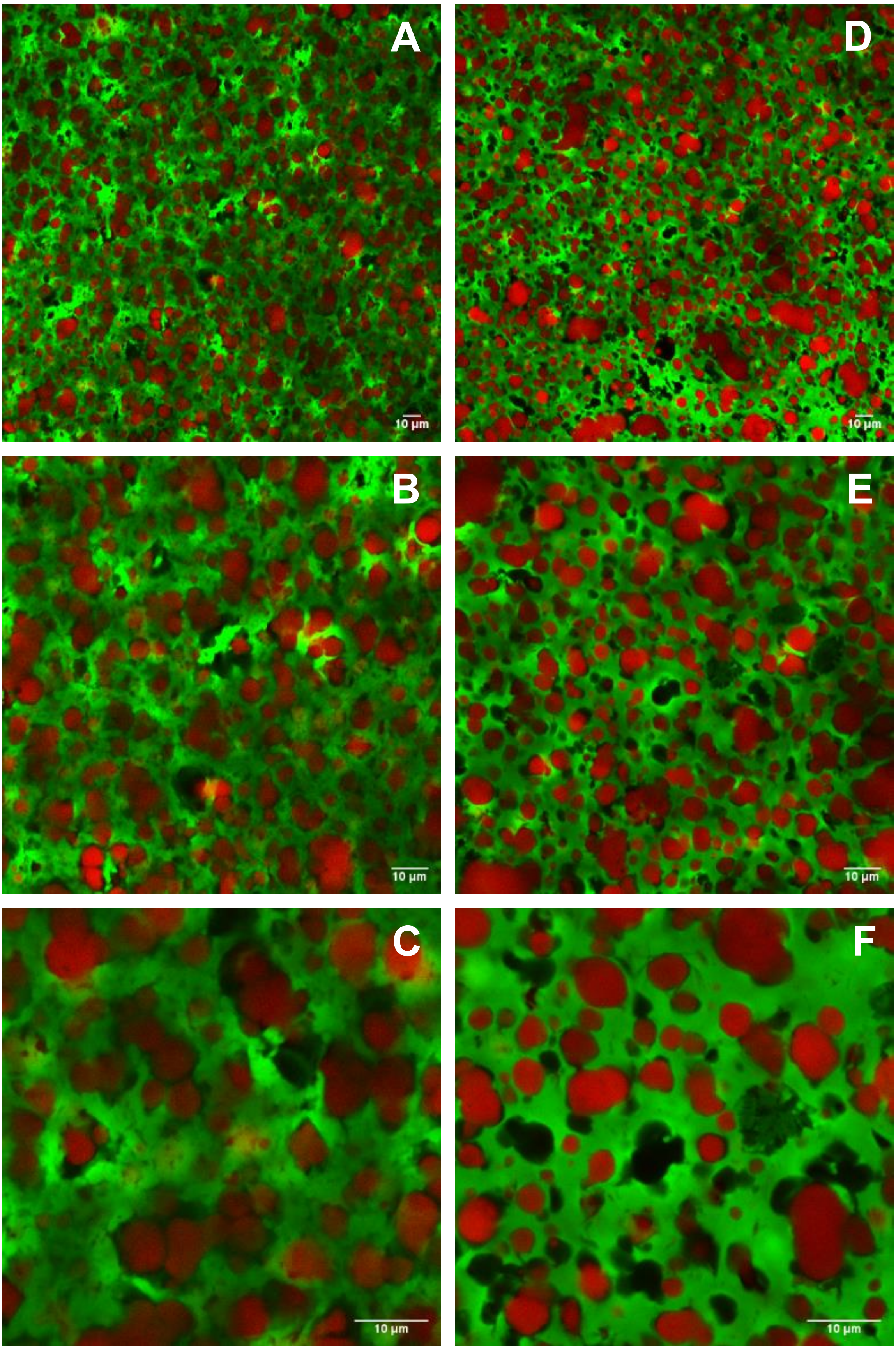

Figure V.4. Confocal laser scanning micrographs of pressed (A-B) and 1-month ripened (D-F) cheese made in small Idiazabal dairies. Fat appears red and protein appears green in these images. Images were captured at 1x, 2x and 4x magnification with a 63x objective lens. The scale bars are 10 µm in length.

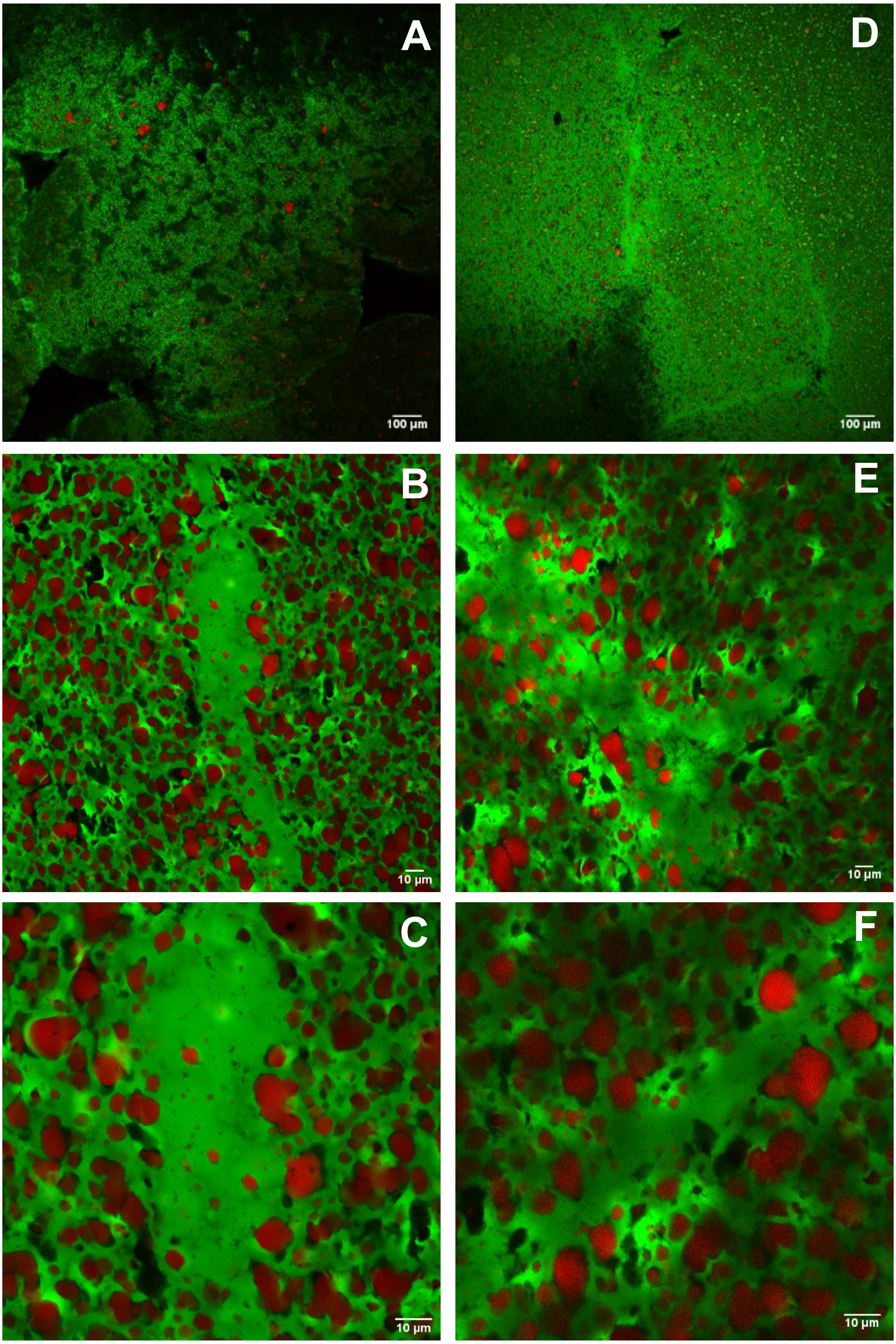

Figure V.5. Confocal laser scanning micrographs of curd junctions in pressed Idiazabal cheese made in small dairies. Fat appears red and protein appears green in these images. Images were captured at 1x with a 10x objective lens (A,D) and 1x (B,E) and 2x (C,F) magnification with a 63x objective lens. The scale bars are 100 µm in length for pictures captured with a 10x objective lens and 10 µm for micrographs captured with a 63x objective lens.

During ripening the microstructure of Idiazabal cheese changed as shown in Figure V.4 (D-F). Generally, an increased amount of coalesced and non-globular fat was observed in the 1-month ripened cheeses. This suggests that during ripening the conformation of fat droplets changes most likely due to hydrolysis of the fat globule membrane (Lopez *et al*., 2007). The protein network considerably changed during ripening, from fibrous chains of casein aggregates to a smoother and compact structure (Figure V.4, C and F). Some authors have suggested that these changes could happen due to the proteolysis of α_{s1}-casein and κ-casein caused by rennet enzymes and the solubilisation of calcium, consequently developing cheese texture to the desired characteristics (Everett & Auty, 2008; Hickey *et al*., 2015; Lamichhane *et al*., 2018).

In cheeses after pressing and after 1 month of ripening the curd grain junctions were clearly visible (Figure V.5 and V.6). These junctions formed due to the fusion of curd grains, which, as shown before, were devoid of fat globules in the edges, creating protein rich areas. At lower magnifications the whole junction surrounding a single curd grain was visible (Figure V.5, A, D; Figure V.6, D), and due to the small size of the particles, this could be the evidence of the existence of casein fines in the cheese matrix. Figure V.5 (A) also showed the fusion of several curd grains, but in this particular case the macroscopic air pockets were also observed between the particles. This could be due to a low deformability degree of the curd grains, or to an incorrect pressing step, leading to a reduced contact area among curd grains, impairing their fusion (Akkerman *et al*., 1993; Fagan *et al*., 2017). Only 1-month ripened cheeses showed crystalline inclusions, most likely calcium phosphate, and generally located in the curd junctions (Figure V.6). Some authors have suggested that this could happen due to the incomplete aggregation of curd grains and the presence of a supersaturated concentration of solutes in the whey between the curd particles. These solutes could act as nuclei and lead to the formation of spherulite crystals in the protein network (D'Incceco *et al*., 2016; Shtukenberg *et al*., 2011), as shown at high magnification in Figure V.7. This structural arrangement has been previously reported in several types of cheeses such as, Cheddar, Grana Padano or Parmigiano-Reggiano (Brooker *et al*., 1975; D'Incceco *et al*., 2016; Ong *et al*., 2015).

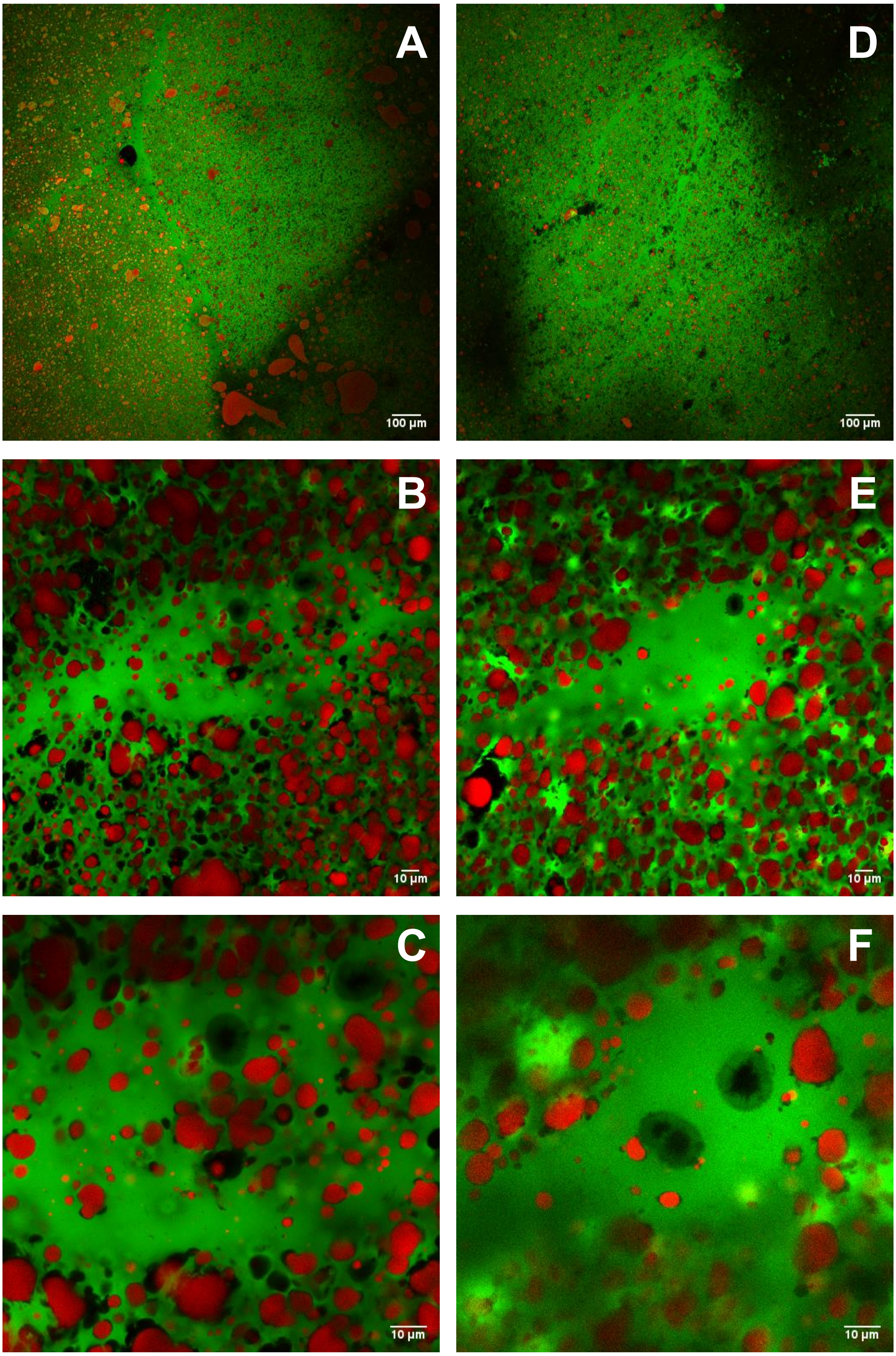

Figure V.6. Confocal laser scanning micrographs of curd junctions in 1-month ripened Idiazabal cheese made in small dairies. Fat appears red and protein appears green in these images. Images were captured at 1x with a 10x objective lens (A,D) and 1x (B,E) and 2x (C,F) magnification with a 63x objective lens. The scale bars are 100 µm in length for pictures captured with a 10x objective lens and 10 µm for micrographs captured with a 63x objective lens.

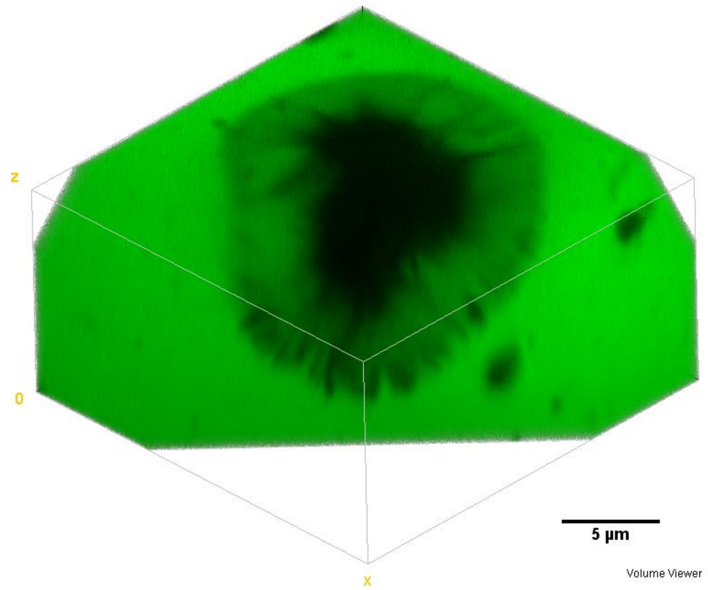

Figure V.7. 3-D reconstruction of a crystalline inclusion in the protein matrix of 1-month ripened Idiazabal cheese observed by confocal laser scanning microscopy. Protein appears in green in this image. Images were captured at 10x magnification with a 63x objective. The scale bar is 5 µm in length.

To the best of the author's knowledge, this is the first time the characterization of the development of the microstructure in Idiazabal PDO cheese production has been carried out. This description is the first step for the future application of this technique in Idiazabal cheeses, where it could be particularly useful for specific studies related to textural properties.

1.3. Interventional study: effect of cutting and cooking intensity on milk compound losses in the whey, yield, microstructure and texture of Idiazabal cheese made in small rural dairies.

Manuscript III studied the effect of moderate and high intensity cheesemaking processes currently carried out in small Idiazabal dairies on the microstructure, curd grain properties, whey losses, cheese yield, composition and texture. This study shows the importance of a correct cheese manufacturing process and the consequences in the composition and properties of the whey and cheese generated. The assessment of technical parameters for cutting and cooking that are easily controllable for cheese-makers is especially interesting in order to improve the process in small rural dairies.

Manuscript III

Effect of high and moderate cutting and cooking intensities on curd, whey and cheese properties and yield during commercial sheep cheese manufacturing.

Manuscript sent to LWT – Food Science and Technology

The supplementary material of this article is in **Appendix B.**

Manuscript submission date: 22nd May, 2019.

Effects of high and moderate cutting and cooking intensities on curd, whey and cheese properties and yield during commercial sheep cheese manufacturing

Ane Aldalur[a], María Ángeles Bustamante[a], Jesús Salmerón[b], Luis Javier R. Barron[a,*]

[a]Lactiker Research Group, [b]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.

[*] Corresponding author: luisjavier.rbarron@ehu.eus

Abstract

The combination of cutting and cooking settings during cheese manufacture affect whey losses and cheese composition, texture and yield. However, the settings combination is endless and the information regarding the effect on texture and microstructure of cheese is scarce. The effect of moderate and high intensity procedures on whey losses, curd grain characteristics, microstructure and cheese properties and yield were analysed. Three trials were monitored in two different small dairies during the cheesemaking of Idiazabal PDO cheese from sheep milk. Higher fat and casein losses were generated due to a combination of excessively hardened gel at cutting point together with high cutting and cooking intensity processes. The moderate intensity process was positively associated with a higher cheese yield. However, no remarkable differences were observed in cheese composition, although cheese after pressing was less porous and texture was more brittle and adhesive when the high intensity process was applied. This could be associated with the composition, characteristics and size distribution of curd grains due to differences in the compaction degree during pressing. These results could help to modify specific parameters used in cheesemaking and especially improving the process in those small dairies where the role of the cheese-maker is crucial.

Keywords: Cheese yield, fat and casein losses, curd grain size, microstructure, cheese texture.

1. Introduction

Cutting and cooking settings used during cheesemaking have a direct effect on whey losses, curd grain properties, cheese texture and yield. Some authors have reported that particularly fat and casein losses are enhanced with the combined effect of an insufficient cutting process followed by higher stirring speed settings (Johnston *et al*., 1998; Everard *et al*., 2008). Other authors have also suggested that an excessive cutting causes high fat and casein losses in the whey (Kammerlehner, 2009; Aldalur *et al*., in press, b). A recent study carried out in commercial small dairies assessed the interactions between several cheesemaking technological settings, whey losses and cheese yield and composition (Aldalur *et al*., in press,a). The results confirmed that fat losses were enhanced by both, insufficient and excessive cutting processes and high stirring speeds, although cheese yield was impaired with longer cutting and cooking times and higher cooking temperatures mainly due to moisture loss. Additionally, cutting and cooking settings inevitably affect the properties of curd grains, which could be a very interesting parameter to control the cheesemaking process. The properties of the particles could affect their deformability during pressing, which enables the fusion of the curd grains and therefore, a correct development of the cheese texture. The size distribution of curd particles has been reported to be very heterogeneous in artisanal dairies (Iezzi *et al*., 2012; Aldalur *et al*., 2019), which could cause moisture distribution problems and defects in cheese texture.

Microstructure greatly changes during the cheesemaking process developing with the course of the process. Confocal laser scanning microscopy (CLSM) allows for observing and quantifying in 3-dimensions the changes in casein compaction and fat globule conformation, which could have further implication in the texture and flavour of cheese (Everett and Auty, 2017). Recently, it has been suggested that curd grain size could be related to a higher non-globular fat amount due to a higher level of compaction during pressing (Aldalur *et al*., in press, b). Additionally, cheese texture is determined by its microstructure and physico-chemical composition (Lamichhane *et al*., 2018), but the effect of in-vat cheesemaking settings on microstructure and texture has not been widely reported.

The aim of this work was to study the effect of the cutting and cooking intensities currently employed during cheesemaking on compound losses in whey and cheese yield, microstructure and texture. Additionally, curd grain characteristics, microstructure properties and milk component losses in the whey was monitored during the cheesemaking process in small dairies.

2. Materials and methods

2.1. Commercial cheese productions and sampling

Two small dairies that manufacture Idiazabal PDO cheese were selected for the study. One of the dairies used moderate cutting and cooking conditions while the other employed intense cutting and cooking settings during the cheese manufacturing. Table 1 shows the technological settings used for cheese productions by the small dairies. Both dairies used milk from their own flocks for the cheesemaking and shepherds managed their flocks in a comparable way, resulting in a quite similar milk composition (Supplementary Table S1). The dairy with moderate intensity cheesemaking process used an open double-O shaped vat equipped with two vertical cutting frames with a separation of the knives of 6 cm from one another. These were turned into stirrers when the direction of the rotation was inverted. The high intensity processing dairy used an open oval-shaped vat with two vertical cutting frames with a wire separation of 2 cm from one another. Two irregular shaped stirrers were placed for stirring the whey and curd grains mixture during the cooking process. Three cheese productions were monitored for each dairy in three consecutive weeks from late May to early June. During the monitoring of the cheese processing several physico-chemical parameters such as temperatures, times, cutting and stirring speed, pressing force, relative humidity of chambers, milk, curd and whey pH, or cheese weight were measured in situ as described in Aldalur *et al*. (in press, a).

The commercial cheeses were made with bulk raw sheep milk and following the Idiazabal PDO bid specification (Ministerio de Agricultura, Pesca y Alimentación, 1993). Briefly, a commercial homofermentative starter culture (Choozit, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added when milk was at 25 ºC and temperature was increased to ~ 30 ºC while stirring. Artisanal lamb rennet paste

Table 1. Technical parameters (mean ± standard deviation) used during cheese manufacturing (n = 3) for moderate and high intensity cutting and cooking processes in Idiazabal PDO cheese production in small dairies.

	Moderate intensity	High intensity
Amount of milk (kg)	1192 ± 63[a]	678 ± 52[b]
Storage temperature (ºC)	5.0[2]	6.0[2]
Storage time (h)	60[2]	15[2]
Coagulation temperature (ºC)	29.3 ± 0.6[b]	31.0 ± 0.0[a]
Coagulation pH	6.50 ± 0.04	6.51 ± 0.02
Coagulation time (min)	50 ± 5[a]	34 ± 1[b]
Rennet concentration (g/100L milk)	0.15[2]	0.12[2]
Milk-clotting activity (RU/g rennet)	63.07[3]	149.79[3]
Gel strength (g·s)	844 ± 19	923 ± 94
Cutting temperature (ºC)	30.3 ± 0.6	31.0 ± 0.0
Cutting pH	6.50 ± 0.07	6.49 ± 0.01
Cutting time (min)	12 ± 1[b]	28 ± 7[a]
Cutting speed (rpm)	8.4 ± 1[b]	22.1 ± 0.3[a]
Cutting tip speed (m/min)	71.5 ± 8.8[b]	100.7 ± 1.2[a]
Cutting revolutions (rev)	101 ± 16[b]	611 ± 144[a]
Cutting process (m·number wires/m^2)[1]	23768 ± 3812[b]	189321 ± 44691[a]
Cooking initial temperature (ºC)	30.0 ± 0.0	30.3 ± 0.6
Cooking final temperature (ºC)	35 ± 1[b]	38 ± 0[a]
Cooking temperature rate (ºC/min)	0.23 ± 0.03[a]	0.11 ± 0.00[b]
Cooking pH	6.49 ± 0.01[a]	6.45 ± 0.01[b]
Cooking time (min)	22 ± 2[b]	68 ± 3[a]
Cooking speed (rpm)	9.2 ± 0.3[b]	15.7 ± 0.3[a]
Cooking revolutions	200 ± 20[b]	1063 ± 32[a]
Total revolutions	301 ± 18[b]	1674 ± 175[a]
Whey draining pH	6.50 ± 0.01[a]	6.45 ± 0.00[b]
Pressing time (min)	298 ± 29[a]	163 ± 23[b]
Pressing temperature (ºC)	20[2]	20[2]
Pressing force (bar)	2.5[2]	2.8[2]
Airing temperature (ºC)	10[2]	11[2]
Airing relative humidity (%)	80[2]	75[2]
Airing time (d)	4[2]	9[2]
Ripening temperature (ºC)	11[2]	10[2]
Ripening relative humidity (%)	85[2]	88[2]

[a-b] Means with different superscripts in the same row indicate statistically significant differences between both cheesemaking technological processes.
[1] Cutting process was calculated as: cutting tip speed x cutting time x knife density. Knife density refers to the ratio between the number of cutting knives in the cutting frame *per* the area of idem.
[2] Average data provided by the cheese-makers for the three cheese productions.
[3] Data corresponding to the milk clotting activity of the rennet used for the three cheese productions.

was added and the mixture was blended. The cheese-maker began the cutting process depending on the sensory observation of the coagulated milk. Cutting was carried out at constant temperature but with variable time and speed, and then cutting frames were switched for stirrers. During stirring, temperature was raised to 36-38 ºC. Curd grains after cooking were pressed in the vat using holey metal panels enhancing whey and curd separation. The continuous curd mass was then cut into cubes, introduced in plastic moulds with a linen cloth and pressed in hydraulic horizontal presses until cheese pH dropped to ~5.5. After that, cheeses were immersed in a saturated sodium chloride brine solution and ripened for 1 month in temperature and humidity controlled chambers.

Samples of bulk milk, rennet paste, curd grains after cutting (fresh curd grains, FCG) and cooking (stirred curd grains, SCG), whey generated after cutting (WFCG), whey generated after cooking (WSCG), whey after draining, cheese after pressing, and 1-month ripened cheese were collected. Milk samples (1 L) were taken from the vat before cheesemaking started and two samples (0.5 L each) of whey were collected after the draining process. FCG and SCG samples together with the whey generated during cutting and cooking processes were collected submerging a 0.5 L plastic container to a depth approximately halfway between the top and bottom of the vat while stirring, and curd grains and whey were separated and weighed in the laboratory for further analysis. For the image analysis of curd grain features, FCG and SCG samples were obtained submerging a mesh sieve halfway between the top and bottom of the vat right after the end of cutting and cooking, respectively (Aldalur *et al*., 2019). Artisanal rennet paste (~50 g) was collected from each dairy, stored in plastic containers and kept in refrigeration (4 ºC) until analysis. Two whole cheeses after pressing and two more after 1-month of ripening were collected, and each cheese was cut into 6 similar wedges. For CLSM analysis, FCG and SCG samples were immersed in a formaldehyde solution at 0.5% (w/v) to prevent structure changes until analysis, while milk, pressed cheese and 1-month ripened cheese were kept in refrigeration (4 ºC). For the simulation of curd texture analysis, milk was kept in refrigeration for no longer than 5 h until analysis. For cheese texture analysis, one wedge of each 1-month ripened cheese was kept in refrigeration (4 ºC) for 3 days until analysis. For other analysis, samples were frozen at -80 ºC until analysis.

2.2. Physico-chemical analysis

Milk, drained whey, WFCG, WSCG, pressed cheese and 1-month ripened cheese were analysed using a near infrared spectrometer (NIR) (SpetrAlyzer 2.0, ZEUTEC GmbH, Rendsburg, Germany). This method was used to analyse fat, protein, dry matter, calcium, magnesium and phosphorous in all the samples, and additionally lactose content was measured in milk samples. Milk casein was determined separating the nitrogen fractions by acidification and analysing by Kjeldahl (ISO/IDF, 2004a). FCG and SCG were analysed for fat, protein and dry matter using Van Gulik (ISO/IDF, 2008a), Kjeldahl (ISO/IDF, 2008b) and oven drying (ISO/IDF, 2004b) methods, respectively. Mineral content of milk, drained whey and pressed cheese were also determined by microwave digestion followed by flame atomic absorption spectroscopy for calcium and magnesium (de la Fuente *et al*., 1997) and photometric method for phosphorous (AOAC, 2005b).

2.3. Rennet milk-clotting activity and gel strength at cutting point

The milk-clotting activity of rennet was determined as previously described by Bustamante (2002). The method is based on the visual determination of the appearance of the first floccules produced by the addition of a known amount of clotting enzyme in a known amount of standardized milk at constant conditions. Briefly, reconstituted skimmed milk at 12% (Chr Hansen, Hoersholm, Denmark) was poured into test tube and an adequate dilution of the rennet extract was added. Test tubes were placed in a rotating device at an angle of 30º and at constant temperature at 30 ºC. The time from the addition of rennet to the appearance of the first floccules was measured and results were expressed as rennet units (RU) *per* g of rennet paste.

For the measurement of gel strength at cutting point, milk coagulation conditions (pH, temperature, rennet concentration and time until curd cutting point) employed during the cheese manufacturing were simulated in the laboratory using the raw milk and rennet paste collected in the small dairies at the same day. A compression-extrusion test was carried out using a texture analyser (TA-

XT2i; Stable Micro Systems, Godalming, UK) armed with a 5 kg load cell and a 25 mm diameter cylindrical probe (Nájera *et al*., 2009). Milk and rennet were poured into a plastic container (28 mm diameter) and after clotting simulating the real conditions, the test was carried out using a test speed of 2 mm/s and a penetration length of 10 mm. Gel strength was defined as the area of the curve (g·s) measured during penetration. Eight samples were analysed for each simulated production conditions and the mean value was used.

2.4. Curd grain properties

Curd grain size, shape and particle size distribution (PSD) properties were measured by 2-dimensional image analysis as previously described by Aldalur *et al*. (2019). The values for area, perimeter, maximum Feret diameter, elongation, rectangularity and circularity were measured for FCG and SCG. Likewise, PSD parameters which described the degree of homogeneity of the curd particle sizes such as uniformity index and coefficient, size range variation, relative span, and graphic skewness and kurtosis were measured for curd grains after cutting and cooking.

2.5. Cheese yield, milk compounds recoveries and texture

The actual cheese yield (Y_A) (kg of cheese/100 kg of milk) was calculated as the ratio between the weight of fresh cheese after pressing, or after 1-month of ripening, and the amount of raw milk used in the processing. Composition-adjusted cheese yield (Y_{CA}) was measured to assess the effect of the technological conditions on cheese yield (Equation 1).

$$Y_{CA} = Y_A \times ((F_R + P_R)/(F_C + P_C)) \times ((100 - M_A)/(100 - M_R)) \quad (1)$$

where F_R and P_R were the reference values for fat (7.2%) and protein (5.0%) in milk, F_C and P_C were the actual fat and protein contents of milk, and M_A and M_R were the actual and reference (42% for cheese after pressing and 38.7% for 1-month ripened cheese) moisture contents of cheese, respectively. The cheese yield efficiency was calculated as the percentage of the ratio between Y_{CA} and the Van Slyke's theoretical cheese yield (Barbano & Sherbon, 1984). The percentage of fat, protein, calcium, magnesium and phosphorous recoveries to cheese were calculated as the total amount of the compound recovered to the cheese in a milk basis (Guinee *et al*., 2006).

Cheese texture analysis was carried out in 1-month ripened cheese samples using a single uniaxial compression test at 50%. A texture analyser (TA-XT2i; Stable Micro Systems, Godalming, UK) equipped with a 25 kg cell and 25 mm diameter cylindrical probe at a constant speed of 1 mm/s was employed for the analysis. Cheese samples were tempered (~25 ºC) for 1 h and cut into cubes of 1.2 cm in each side casting aside ~1 cm from the rind of the cheese. Ten cheese cubes were analysed *per* cheese wedge and two different cheeses were tested for each cheese manufacturing. The textural parameters measured are explained in Figure 1.

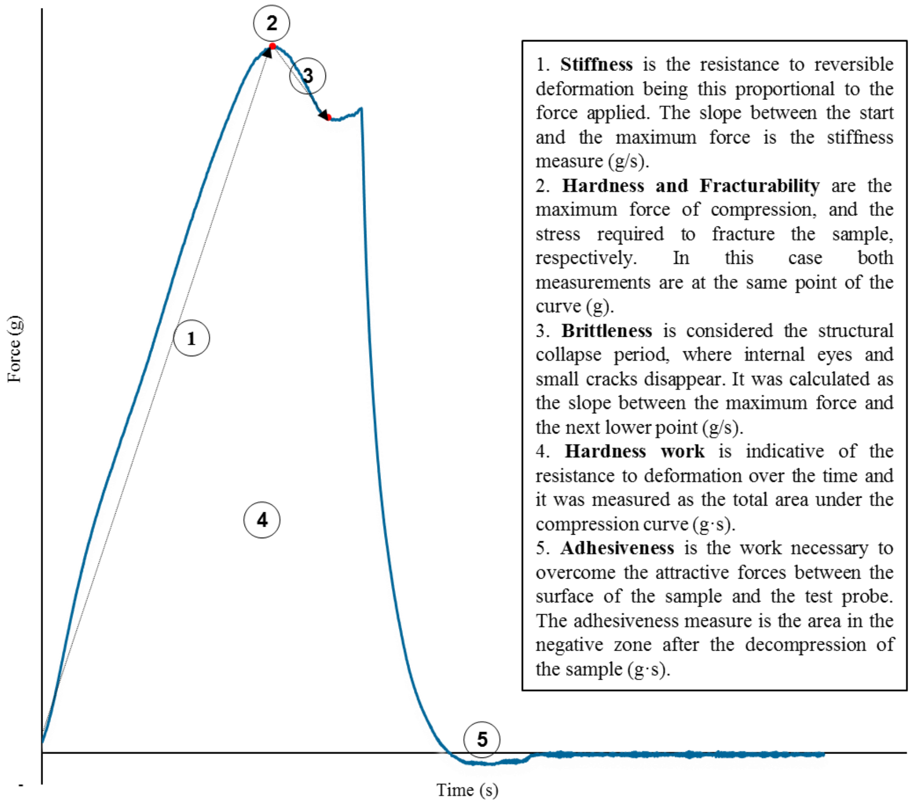

Figure 1. Standard curve obtained in the single uniaxial compression test. Numbers indicate the parameters defined above.

2.6. Whey losses and chemical oxygen demand

The fat, protein, casein, calcium, magnesium and phosphorous loss in the whey after cutting, cooking and draining in a milk basis were measured as the percentage of the ratio between the total amount of the compound in whey and the initial total amount in milk (Guinee *et al*., 2006). The total amount of whey in the intermediate samples was estimated as the total milk weight multiplied by the whey yield, calculated as the ratio between the weight of whey and the weight of curd and whey after cutting and cooking (Everard *et al*., 2008).

The content of casein fines in WFCG, WSCG and drained whey samples was measured using the method described by Everard *et al*. (2008). Briefly, the whey sample was centrifuged for 15 min at 1,500 g. Fat was then removed and supernatant liquid was poured without disturbing the pellet. Distilled water was added to the tube and the centrifugation and cleaning process was repeated. Finally, distilled water at 40 ºC was added and the whole content of the tube was filtered onto a previously oven-dried filter paper with a Buckner funnel under vacuum conditions. The filter paper was then dried in an oven at 102 ºC for 1 h and weighed. Results were expressed as mg of casein fines *per* kg of whey.

The chemical oxygen demand (COD) of whey samples was determined by the dichromate method in small-scale sealed-tubes (ISO, 2002). An adequate dilution of the whey samples was introduced in the sealed-tubes (HI94754C-25, Hannah Instruments Inc., Woonsocket, USA), mixed and digested at 150ºC for 2 h. Absorbance was measured using an spectrophotometer (Spectronic 20D, Milton Roy, France) and results were compared against a standard solution of potassium hydrogen phthalate. Samples were measured in duplicate and results were expressed as mg of oxygen *per* litre of whey.

2.7. Confocal laser scanning microscopy and image analysis

CLSM was performed using an inverted confocal microscopy (SP2; Leica Microsystems, Heidelberg, Germany) to analyse the microstructure of milk, FCG, SCG, cheese after pressing and cheese after 1-month ripening using as previously reported (Ong *et al*., 2011). Briefly, FCG and SCG samples were immersed in a phosphate buffered saline solution (pH 7.4) for at least 1 h to rinse the formaldehyde solution. Solid samples were cut into cubes of approximately 5 x 5 x 2 mm and stained at ~4 ºC using fast green FCF (0.1 mg/mL) and Nile Red (0.1 mg/mL) (both from Sigma-Aldrich, Steinheim, Germany). For milk samples, fast green FCF (0.02 mg/mL), Nile Red (0.02 mg/mL), agarose (0.5%) (Sigma-

Aldrich) and milk were mixed and placed in the imaging dish (0.17 mm thick). Excitation wavelengths of 638 and 488 nm were used for protein and fat, respectively.

Three-dimensional (3D) image analysis was performed for the CLSM micrographs using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as previously reported by Aldalur *et al*. (in press, b). Briefly, a minimum of 20 adjacent micrographs were used for the reconstruction of 3D images and with an observation depth of minimum 10 µm. The quality of the 3D images was improved, then thresholded and quantified by 3D Object Counter analysis (ImageJ software). Fat volume, fat globule (FG) diameter, sphericity and protein network porosity were determined. As previously described by Aldalur *et al*. (in press, b), two fat globule populations were defined: globular fat droplets (FG diameter smaller than 6 µm) and non-globular or coalesced fat droplets (droplet diameter bigger than 10 µm). The percentage of fat volume contributed by globular or non-globular fat was quantified.

2.8. Statistical analysis

The IBM-SPSS version 25.0 (New York, USA) and XLSTAT (Paris, France) softwares were used for statistical analysis. The Student's t-test was used to separately assess the effect of cutting and cooking conditions (I = 2; moderate or high intensity) and curd syneresis (I = 2; after cutting or cooking) on the composition and properties of curd grains, whey and cheese. Partial Least Square Regression (PLSR) analysis was used to investigate relationships between the raw milk properties together with the technological settings used during cheesemaking, and the curd, whey and cheese properties, and cheese yield and whey losses obtained in the small dairies. PLSR analyses were carried out on self-scaling variables and only those variables showing scores for variable importance in the projection higher than 0.8, and loadings higher than 0.5 in the latent dimensions were included. Statistical significance was declared at $P \leq 0.05$.

3. Results and discussion

3.1. Coagulation and cutting process

Figure 2 shows the relationships obtained by PLSR analysis between technical, compositional and microstructural variables involved before and after the cutting process during the cheesemaking trials in the small dairies. In this context, higher cutting speed and time generated higher ($P \leq 0.05$) fat loss in the whey after the cutting process (Table 2). The loss of casein fines also showed an increasing trend when cutting intensity increased, although not statistically significant differences were observed due to the high intra-variability of the high intensity trials in the small dairy. This probably happened due to the changes in the gel strength values at cutting point, which appeared positively correlated to casein fines loss in the PLSR graph (Figure 2). Therefore, these results suggested that the combined effect of gel strength and cutting settings greatly affected the loss of casein fines after cutting. This agrees with other studies that reported that the gel should not harden excessively in order to avoid casein and fat losses when manufacturing semi-hard cheeses (Kammerlehner, 2009; Lucey, 2011). Losses of mineral compounds in the whey after cutting were specially correlated to the

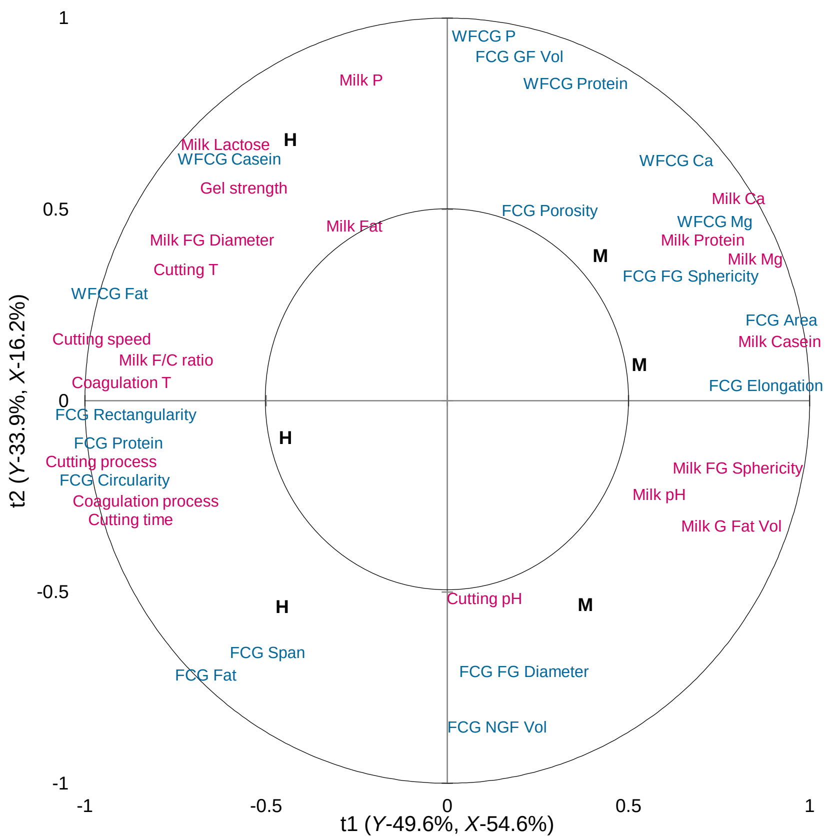

Figure 2. Partial least square regression plot showing the relationships between technological settings of coagulation and cutting together with the composition and microstructure of milk (*X*-variables, in magenta), and the composition, microstructure, size, shape and distribution parameters of curd grains after the cutting process (FCG) (*Y*-variables, in blue). Each cheese manufacturing is bold faced and labelled as M (moderate intensity processing) and H (high intensity processing). The inner circle indicates the correlation value 0.5. WFCG, whey after the cutting process; FG, fat globule; GF, globular fat; NGF, non-globular fat; Vol, volume; F/C, fat-to-casein; Span, relative span; T, temperature. Cutting process was calculated as cutting speed x cutting time x knife density; coagulation process was calculated as coagulation time x rennet activity x rennet concentration.

initial amount of each mineral in milk, since calcium, magnesium and phosphorous salts are highly water-soluble compounds and they are not retained in the protein matrix (Figure 2).

Regarding curd grain size and shape, the higher intensity process generated significantly ($P \leq 0.05$) smaller, more circular and less elongated particles at the end of the cutting process (Figure 2, Table 2). Additionally, the size distribution of FCG was highly heterogeneous regardless of the cutting intensity employed, as shown by the relative span value (Table 2) and other PSD parameters (Supplementary Table S2). Other studies have also reported high PSD heterogeneity for curd grains, and suggested that the cutting process was the main cheesemaking process influencing particle distribution (Iezzi *et al*., 2012; Aldalur *et al*., 2019).

Microstructure parameters of FCG did not show remarkable differences between moderate and high intensity cutting processes (Table 2; Figure 3, A and E). However, FCG microstructure parameters were correlated in the latent dimension t2 (Y-axis) with some milk compounds, whey compound losses and cutting pH, which might confirm the importance of milk composition and acidity on the formation of curd structure and the losses of water-soluble compounds in the whey (Ong *et al*., 2012; Ong *et al*., 2013).

3.2. Cooking process

During cooking, curd grains released fat, protein, casein, calcium, magnesium and phosphorous to the whey, increasing the mean values for all the compound losses in milk basis (Table 2). However, significant increases ($P \leq 0.05$) were only measured for protein and calcium losses when both moderate and high intensity cooking procedures were applied, and for fat, magnesium and phosphorous losses when the high intensity cooking was employed. Curd grain composition also changed during cooking mainly due to the reduction in moisture content, particularly when high intensities were used, since syneresis was especially enhanced (Table 2). Additionally, curd grain size shrunk ($P \leq 0.05$) despite the cooking intensity used, with a higher overall size reduction for the moderate intensity cooking (3.3 *vs* 1.5 times) due to the bigger initial FCG size and the

Table 2. Values (mean ± standard deviation) of composition, microstructure, size, shape and distribution parameters of curd grains, and whey losses, after cutting and cooking for the moderate and high intensity processes during Idiazabal PDO cheese productions (n = 3) in small dairies. Significance values of the curd syneresis s factor for both intensities are also provided.

		After cutting		After cooking		Syneresis factor Significance	
		Moderate Intensity	High Intensity	Moderate Intensity	High Intensity	Moderate Intensity	High Intensity
Curd grains	Fat (g/100g)	13.33 ± 0.80	14.33 ± 0.76	15.92 ± 0.76^{b}	17.50 ± 0.66^{a}	*	**
	Protein (g/100g)	7.75 ± 0.26^{b}	9.22 ± 0.49^{a}	8.13 ± 0.43^{b}	12.56 ± 0.50^{a}	ns	***
	Moisture (g/100g)	71.25 ± 0.38	71.63 ± 0.66	68.88 ± 1.09^{a}	66.77 ± 0.58^{b}	*	***
	Area (mm^2)	64.69 ± 18.05^{a}	10.47 ± 1.10^{b}	19.87 ± 1.39^{a}	7.12 ± 0.90^{b}	**	**
	Elongation	0.672 ± 0.004^{b}	0.736 ± 0.006^{a}	0.678 ± 0.008^{b}	0.705 ± 0.009^{a}	ns	**
	Rectangularity	0.679 ± 0.003^{b}	0.709 ± 0.002^{a}	0.685 ± 0.002^{b}	0.692 ± 0.003^{a}	ns	***
	Circularity	0.736 ± 0.014^{b}	0.798 ± 0.007^{a}	0.765 ± 0.012	0.778 ± 0.004	ns	**
	Relative span[1]	2.40 ± 0.13	2.54 ± 0.20	3.18 ± 0.17^{a}	2.63 ± 0.30^{b}	**	ns
	Fat volume (%)	14 ± 3	15 ± 3	15 ± 3^{b}	22 ± 2^{a}	ns	*
	Porosity (%)	45 ± 4	43 ± 5	42 ± 3^{a}	34 ± 3^{b}	ns	*
	FG sphericity	0.56 ± 0.01^{a}	0.53 ± 0.01^{b}	0.52 ± 0.02	0.50 ± 0.01	ns	**
	FG diameter (µm)	10.1 ± 2.1	9.3 ± 0.9	9.6 ± 1.8	12.6 ± 1.3	ns	*
	Volume of globular fat (%)	19 ± 5	17 ± 6	18 ± 9	7 ± 1	ns	*
	Volume of non-globular fat (%)	46 ± 19	40 ± 16	45 ± 18	68 ± 8	ns	*
Whey	Fat loss (%)	6.9 ± 0.6^{b}	11.0 ± 0.6^{a}	7.5± 0.8^{b}	12.4 ± 0.6^{a}	ns	*
	Protein loss (%)	23.98 ± 1.39	23.07 ± 2.14	28.49 ± 1.88	29.01 ± 1.57	*	*
	Casein fines loss (%)	0.83 ± 0.40	3.15 ± 2.40	1.11 ± 0.25^{b}	5.77 ± 0.24^{a}	ns	ns
	Calcium loss (%)	18.16 ± 0.54	15.63 ± 2.76	21.34 ± 1.68	21.04 ± 2.10	*	*
	Magnesium loss (%)	49.56 ± 2.23^{a}	40.58 ± 4.42^{b}	58.13 ± 6.16	51.90 ± 3.96	ns	*
	Phosphorous loss (%)	24.33 ± 2.43	23.95 ± 4.54	28.23 ± 3.74	33.60 ± 4.14	ns	*

$^{a\text{-}b}$ Means with different superscripts in the same row and process step (cutting or cooking) indicate statistically significant differences ($P \leq 0.05$) between moderate and high intensity processes.

[1] Relative span = $(D_{90}-D_{10})/D_{50}$, where D_x is the x^{th} percentile dimension of the curd grains area distribution for each cheese production. Values closer to 1 indicate more homogeneous particle size distribution.

FG, fat globule.

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant $P > 0.05$.

physical difficulty of the smaller FCG to further reduce in size (Iezzi *et al*., 2012). Regarding the shape of the curd grains, these particularly changed ($P \leq 0.05$) when high intensity cooking was employed, presumably due to the increased probability of collision between particles, or particles and equipment (van den Bijgaart, 1988). Contrarily, PSD did not significantly ($P > 0.05$) change when high intensity cooking was employed, but the relative span value increased ($P \leq 0.05$) for the moderate intensity cooking, indicating that the PSD heterogeneity increased (Table 2). This probably occurred due to the presence of a small amount of large SCG together with the general reduction of curd grain size, which affected PSD parameters (Supplementary Table S2), as previously reported (Aldalur *et al*., 2019).

The microstructure of the curd grains significantly ($P \leq 0.05$) changed during cooking but only when high cooking intensity was employed. The porosity of the protein network significantly ($P \leq 0.05$) decreased (Table 2; Figure 3, E and F) due to the increased syneresis (reduced SCG moisture) induced by the long time, high speed, temperature raise (38 ºC) and consequent pH drop during cooking (Figure 4), as observed by other authors (Ong *et al*., 2011; Aldalur *et al*., in press, b). The size, shape and distribution of the fat globules also changed ($P \leq 0.05$) during cooking, decreasing the sphericity and volume of globular fat, and increasing the non-globular or coalesced fat volume (Table 2). The observation of microstructural changes in curd grains for the high intensity process and the lack of differences in the moderate cooking process could also be related to the size of the curd grains. Small curd grains shrink more and faster than the large ones due to Darcy's law (Fagan *et al*., 2017), and this difference was probably observed in the SCG micrographs when moderate and high intensity processes were compared (Figure 3, B and F).

3.3. Combined effect of cutting and cooking processes

The composition and properties of the curd grains and whey generated after cooking is the result of the combination of cutting and cooking processes. Therefore, Figure 4 plots the relationships obtained by PLSR analysis between the compositional, microstructural, physical and technical parameters for both processes. After the cooking process, statistically higher ($P \leq 0.05$) fat and casein

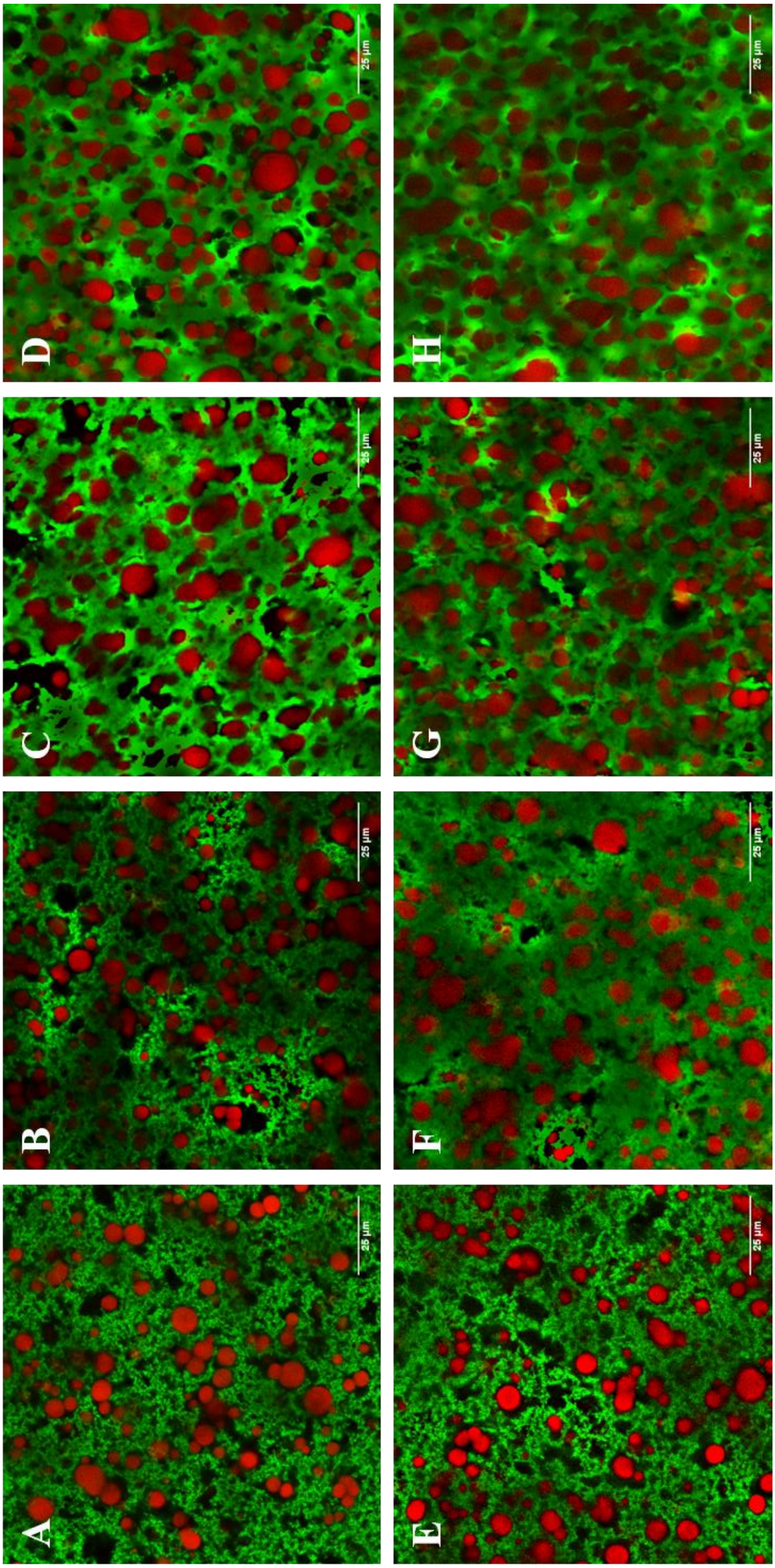

Figure 3. Confocal laser scanning microscopy images of curd grains after cutting (A, E), curd grains after cooking (B, F), cheese after pressing (C, G) and cheese after 1 month of ripening (D, H) for moderate (A-D) and high (E-H) intensity cutting and cooking processes. Images were obtained using a 63x objective lens and digitally magnified 2x. The scale bars are 25 µm in length. Fat appears in red and protein appears in green.

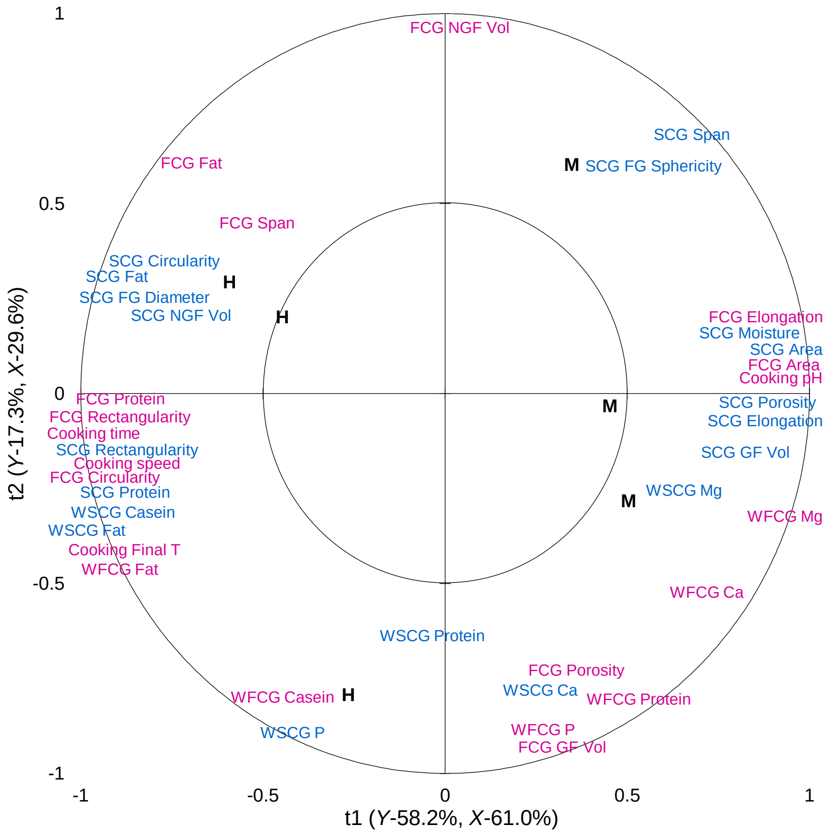

Figure 4. Partial least square regression plot showing the relationships between the technological settings of cooking together with the composition, microstructure and size, shape and distribution parameters of curd grains after cutting (FCG) (*X*-variables, in magenta), and the composition, microstructure, size, shape and distribution parameters of curd grains after the cooking process (SCG) (*Y*-variables, in blue). Each cheese manufacturing is bold faced and labelled as M (moderate intensity processing) and H (high intensity processing). The inner circle indicates the correlation value 0.5. WFCG, whey after the cutting process; WSCG, whey after cooking process; FG, fat globule; GF, globular fat; NGF, non-globular fat; Vol, volume; Span, relative span, T, temperature.

losses in whey were measured for the high intense process (Table 2), regardless of the size of the curd grain at the start of the cooking (FCG). In this case, bigger FCG cooked with a moderate intensity process resulted in lower fat and casein losses than smaller FCG cooked with a high intensity procedure. This suggested that the shattering of curd grains, and consequent fat and casein losses in whey during cooking, was especially enhanced by the cooking intensity employed and, to a lesser extent, by the FCG size at the start of the cooking process (Figure 4). Thus, reducing the stirring intensity during cooking is recommendable to especially decrease fat losses in whey, as previously suggested (Johnston *et al*., 1998; Aldalur *et al*., 2019).

At the end of the cooking process, SCG were smaller, more rectangular, less elongated, and showed more homogeneous PSD when high intensity cutting and cooking processes were used. The difference in the curd grain physical and compositional properties could affect the deformation capacity of curd grains and therefore, the subsequent compaction of the curd at pressing (Fagan *et al*., 2017). Regarding SCG microstructure, high and moderate cutting and cooking processes led to microstructurally different ($P \leq 0.05$) curds (Figure 3, B and F), particularly remarkable for porosity and total fat volume (Table 2). Additionally, Figure 4 showed high correlations between the technical settings used during cooking and the microstructural properties of SCG, unlike for the cutting process (Figure 2). The high intensity cooking process increased the non-globular fat volume and mean diameter of the droplets, while decreased the amount of globular fat, porosity of the protein network and the sphericity of the fat droplets. Therefore, the microstructure of curd grains was mostly affected by the cooking process, although the result of the technical settings used during cutting (i.e. the curd grain size) could remarkably affect the SCG microstructure at the end of cooking, as mentioned before.

3.4. Cheese properties, yield and whey losses

Figure 5 shows the results of the PLSR analysis on the relationships between milk properties and cheesemaking technical settings, and the properties of the cheeses after pressing and ripened for 1-month, and the composition of drained whey. Generally, all variables correlated to the cheesemaking technical settings were located along the latent dimension t1 (X-axis). High cutting and cooking intensities were negatively correlated to the actual and composition-adjusted

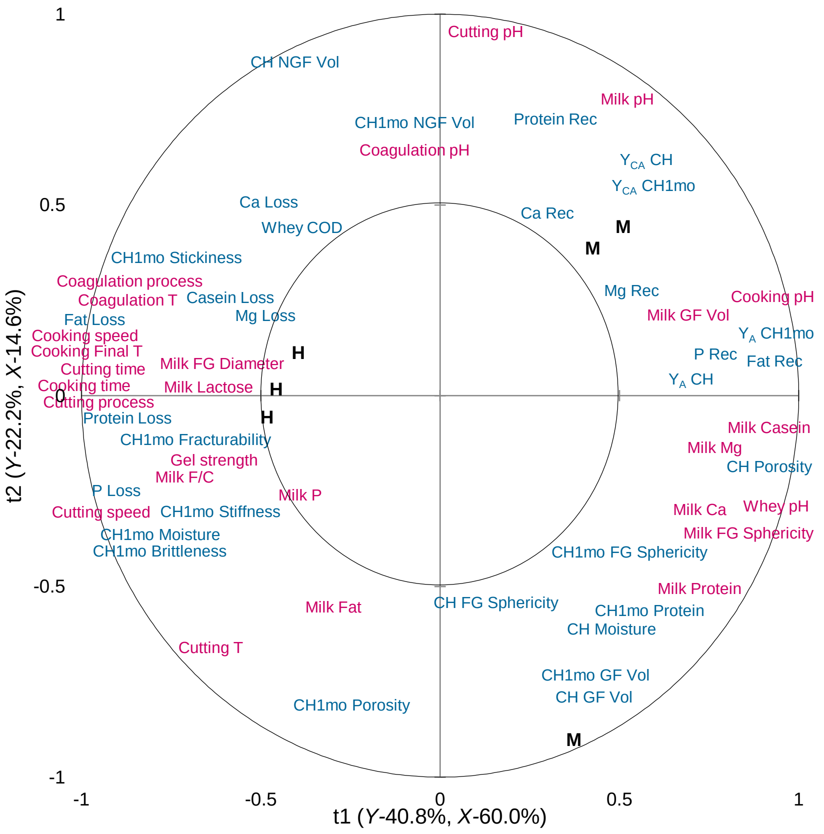

Figure 5. Partial least square regression plot showing the relationships between the technological settings using during cheesemaking together with the composition and microstructure of milk (*X*-variables, in magenta), and the composition, microstructure and texture of pressed cheese (CH) and 1-month ripened cheese (CH1mo) samples, and losses and chemical oxygen demand (COD) in whey after draining (*Y*-variables, in blue). Each cheese manufacturing is bold faced and labelled as M (moderate intensity processing) and H (high intensity processing). The inner circle indicates the correlation value 0.5. FG, fat globule; GF, globular fat; NGF, non-globular fat; Vol, volume; F/C, fat-to-casein; Span, relative span; Y_A, actual yield; Y_{CA}, composition-adjusted cheese yield; Rec, Recovery; Cutting process was calculated as cutting speed x cutting time x knife density; coagulation process was calculated as coagulation time x rennet activity x rennet concentration.

cheese yield, although only significant ($P \leq 0.05$) differences were observed for the actual yield for 1-month ripened cheeses (Table 3). Both actual and composition-adjusted yields were positively correlated to cheese moisture and compound recoveries in the multivariate approach, particularly protein and calcium recoveries to both Y_{CA}, and fat and phosphorous to Y_A (Figure 5) On the contrary, higher cutting and cooking intensities led to increased milk compound losses and COD in the whey. Whey COD did not significantly differ ($P > 0.05$) between intensity processes due to the high intra-process variability in the cheesemaking trials, but mean values were rather different (77.2 *vs* 96.2 g/L for moderate and high intensities, respectively). However, final casein fines losses did not differ ($P > 0.05$) between different intensity processes (Table 3), since after the high intensity process a filter was used to recover casein fines, causing a significant reduction when compared to the whey generated after cooking (Table 2). Therefore, casein fines were recovered in the curd mass but this could cause an uneven moisture distribution in cheese, closing the pores and impairing further expulsion of whey (Kammerlehner, 2009; Fagan *et al*., 2017).

Table 3. Values (mean ± standard deviation) of cheese yield (CY), milk compound losses in whey and milk compound recoveries in cheese for the moderate and high intensity cutting and cooking processes during Idiazabal PDO cheese productions (n = 3) in small dairies.

	Moderate Intensity	High Intensity
Milk compound loss in whey (%)		
Fat	8.4 ± 0.7^{b}	13.1 ± 0.8^{a}
Protein	30.28 ± 0.48^{b}	31.80 ± 0.56^{a}
Casein fines	0.68 ± 0.43	1.60 ± 0.88
Calcium	25.25 ± 0.97	26.11 ± 1.36
Magnesium	57.38 ± 1.04	58.50 ± 1.64
Phosphorous	26.09 ± 2.11^{b}	31.67 ± 1.67^{a}
Milk compound recovery in cheese (%)		
Fat	93.89 ± 0.94^{a}	87.15 ± 2.60^{b}
Protein	80.39 ± 3.32	79.04 ± 3.13
Calcium	82.26 ± 2.61	81.11 ± 3.68
Magnesium	49.35 ± 1.73	47.46 ± 2.63
Phosphorous	71.69 ± 0.80^{a}	67.07 ± 2.76^{b}
Actual cheese yield (kg/100 kg)		
Pressed cheese	20.40 ± 0.56	19.16 ± 1.12
1-Month ripened cheese	17.79 ± 0.33^{a}	16.43 ± 0.40^{b}
Adjusted cheese yield (kg/100 kg)		
Pressed cheese	20.13 ± 0.70	19.39 ± 0.82
1-Month ripened cheese	16.62 ± 0.92	15.74 ± 0.46

$^{a-b}$ Means with different superscripts in the same row indicate statistically significant differences ($P \leq 0.05$) between moderate and high intensity processes.

Table 4. Values (mean ± standard deviation) of microstructure, size, shape and distribution parameters of cheeses after pressing and 1 month of ripening for the moderate and high intensity processes during Idiazabal PDO cheese productions (n = 3) in small dairies.

	Cheese after pressing		1-Month ripened cheese	
	Moderate Intensity	High Intensity	Moderate Intensity	High Intensity
Fat Volume (%)	31 ± 2	35 ± 1	37 ± 3	38 ± 1
Porosity	25 ± 2[a]	22 ± 1[b]	14 ± 4	15 ± 2
FG Sphericity	0.52 ± 0.02	0.52 ± 0.01	0.55 ± 0.02	0.54 ± 0.01
FG diameter D[4.3]	26.1 ± 6.7	30.4 ± 1.4	33.1 ± 2.0	37.0 ± 4.9
Volume of globular fat (%)	4 ± 2	3 ± 0	3 ± 1	2 ± 0
Volume of non-globular fat (%)	88 ± 7	92 ± 1	93 ± 3	93 ± 2
Fracturability (g)	-[1]	-[1]	2037 ± 121	2384 ± 239
Stiffness (g/s)	-[1]	-[1]	385.7 ± 32.2	440.1 ± 54.0
Brittleness (g/s)	-[1]	-[1]	124.0 ± 50.1[b]	223.4 ± 41.0[a]
Hardness work (g·s)	-[1]	-[1]	10778 ± 450	11668 ± 1076
Adhesiveness (g·s)	-[1]	-[1]	27.1 ± 6.5[b]	51.5 ± 13.9[a]

[a-b]Means with different superscripts in the same row and type of cheese sample indicate statistically significant differences ($P \leq 0.05$) between moderate and high intensity processes.
[1] No measurements were carried out in these samples.

The composition of the cheeses after pressing and after 1-month of ripening did not significantly ($P > 0.05$) vary between high and moderate intensity settings (Supplementary Table S3), presumably due to differences in the ulterior pressing, brining and ripening processes carried out in the small dairies (Table 1). Therefore, although the cheesemaking process considerably differed among dairies, the resulting cheese composition remained similar, probably because the composition of milk has a bigger influence on the final cheese composition (Aldalur *et al*., in press, a). However, some cheese texture parameters were different depending on the process intensity employed (Table 4) and regardless of cheese composition. A more intense cutting and cooking process produced cheeses that were significantly ($P \leq 0.05$) more brittle and adhesive and with tendency to be stiffer and with a higher fracturability (Figure 5). This could be associated with the curd grain compaction degree in cheeses during pressing due to the different characteristics of the curd grains after cooking. Regarding microstructure, cheeses after pressing made with moderate cutting and cooking intensity were significantly ($P \leq 0.05$) more porous, and the volume of non-globular fat was also slightly higher (Table 4; Figure 3, C and G). During ripening, the differences between high and moderate intensity processes dissipated since casein network compacts and smoothens (Figure 3, D and H), and fat droplets change their structural arrangement due to enzymatic reactions making more difficult to quantify changes in the cheese microstructure (Lopez *et al*., 2007). However, microstructural features were located along the latent dimension t2 (Y-axis) related to the acidity during cheesemaking, protein and calcium recoveries, and composition-adjusted cheese yield. This could suggest that when higher milk protein and calcium recoveries in cheese were obtained, the cheese microstructure was less porous and the volume of non-globular fat increased, obtaining a higher composition-adjusted cheese yield. In this sense, the interactions between cheese microstructure and texture, curd grain characteristics, cheese yield and cheesemaking settings have not been widely reported and further research should be required.

4. Conclusions

Cutting and cooking settings used during cheesemaking clearly affected the course of the cheese manufacture process in small dairies. The slightly increased cheese yield obtained in the moderate intensity process was probably connected to higher milk compound recoveries in cheese. Contrarily, higher fat and casein losses in whey were generated when high intensity process was employed. This occurred due to the combined effect of a hardened gel at cutting point and excessive cutting process, together with a too high intensity cooking stage. Therefore, it is highly recommendable to cut the gel at the precise moment and to reduce the intensity during stirring to avoid the shattering effect occurred regardless of the size of the curd grains after cutting. The heterogeneity of the curd grain size distribution, together with the differences found in the size, shape, microstructure and composition of curd grains could have an important effect on their deformation degree and compaction during pressing. Ultimately, this could be the main responsible for the differences observed in the some microstructure and texture features of the final cheese regardless of its composition. The results of this commercial study showed the importance of carrying out a correct cheese manufacturing process, and the consequences of the technical settings employed on the whey losses and on the properties of the final cheese. The modification of cheesemaking technical parameters that are feasible and easily in situ controllable for cheese-makers is especially interesting for small producers in order to improve cheese yield and diminish milk compound losses in the whey.

5. Acknowledgements

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2. Sheep cheese manufacture on a laboratory scale

2.1. Experimental study: evaluation of cheesemaking processing parameters on the microstructure, texture, composition and yield of cheese made from raw sheep milk

Manuscript IV assesses the effect of the cheesemaking technical parameters, curd grain size and cooking temperature on the microstructure, whey losses, cheese texture and yield. The development of the microstructure in the course of the cheesemaking process was analysed and some correlations between microstructure and cheese texture were established. This study contributes with new data on the technical cheesemaking parameters on the microstructure, milk compound losses in the whey and physico-chemical properties of cheese made from sheep milk, which is particularly scarce. Additionally, these results were useful to complement and support the results obtained in the observational and interventional studies carried out in small rural Idiazabal dairies.

Manuscript IV

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Impact of processing conditions on microstructure, texture and chemical properties of model cheese from sheep milk

Ane Aldalur[acd], Lydia Ong[bcd], María Ángeles Bustamante[a], Sally L. Gras[bcd**], Luis Javier R. Barron[a,*]

[a] Lactiker Research Group, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006-Vitoria-Gasteiz, Spain.

[b] Department of Chemical Engineering, The University of Melbourne, Parkville, Vic 3010, Australia.

[c] The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Vic 3010, Australia

[d] The ARC Dairy Innovation Hub, Department of Chemical Engineering, The University of Melbourne, Parkville, Vic 3010, Australia

[*] Corresponding author: luisjavier.rbarron@ehu.eus

[**] Co-corresponding author: sgras@unimelb.edu.au

Abstract

Cutting and cooking settings have a strong effect on curd particle features, whey syneresis and cheese properties. In the present study, the impact of curd grain size and cooking temperature on the microstructure, texture and composition of cheese, whey losses and cheese yield was studied with specific focus on sheep milk. Cooking temperature especially affected cheese microstructure, texture and composition, while cutting process was largely responsible for fat losses in the whey. Additionally, cheese yield increased with a bigger curd grain size and lower cooking temperatures. Higher cooking temperatures reduced the moisture content of the curd grains and cheese and lead to cheeses with reduced porosity and more free fat in their structure, resulting in harder and chewier cheeses. Interactions between the microstructural arrangement of fat and textural parameters were also observed. These results contribute with new data on the relationships between curd grain size and cooking conditions on the microstructure and physico-chemical properties of cheese. In addition, reducing the compound losses in whey would have a direct effect on the improvement of processing, cheese quality and yield, and the ulterior byproduct management.

Keywords: Confocal microscopy, cryo-scanning electron microscopy, fat loss, curd grain size, cooking temperature, cheesemaking settings.

Highlights

- Cutting and cooking settings affect cheese microstructure and texture properties
- A higher cooking temperature raised free fat formation and reduced cheese porosity
- The hardness and chewiness of cheese increased with higher cooking temperature
- A bigger curd grain size increased the globular fat volume in cheese microstructure
- A smaller curd grain size enhanced the fat loss and decreased cheese yield

1. Introduction

The technological conditions used during cheese processing, particularly curd cutting and cooking process, have a direct effect on cheese composition, yield and compound losses in the whey. During cutting and cooking, syneresis of curd occurs and this is affected by several factors well documented in the scientific literature such as: milk composition, firmness of the gel at cutting, gel acidity, temperature, time of cutting, speed of rotation of the cutting and stirring tools and the surface area of the curd (Banks 2007; Dejmek and Walstra 2004; Everard *et al*. 2008; Johnston *et al*. 1991; Johnston *et al*. 1998; Walstra *et al*. 2006).

While some authors (Johnston *et al*. 1991; Whitehead and Harkness 1954) have suggested that the size of the curd grain plays an important role in the moisture of the final cheese product, others have found that different curd cutting intensities have little effect on the extent of syneresis, although enhanced losses of fat and casein fines were reported (Everard *et al*. 2008). The relationship between fat and protein losses from the curd and a reduction in cheese yield with curd grain size has been shown to be influenced not only by the cutting revolutions but by a combination of the total revolutions applied during cutting and cooking processes (Everard *et al*. 2008; Johnston *et al*. 1991; Johnston *et al*. 1998). Even though cheesemaking processing conditions affect chemical properties of cheese, the studies about the interactions of cutting and cooking settings with microstructure and texture are scarce. Additionally, the comparison of cutting and stirring conditions in these prior studies is made difficult due to technical differences in the cheesemaking processes applied. In this study, the curd grain size was assessed by image analysis to provide information about the curd grain size obtained at the end of the cutting or cooking processes (Aldalur *et al*. 2019). This method may reduce some of the comparative issues described by other authors, such as the use of different vat designs and sizes, cutting knives, stirrers and cutting and stirring conditions (Everard *et al*. 2008; Johnston *et al*. 1998).

Temperature is another factor that enhances moisture expulsion from curd grains, as the permeability of the curd matrix increases at higher temperatures (Fagan *et al*. 2007). A rapid increase in temperature, however, may lead to a shrunken outer layer of the curd grain, impairing permeability and slowing syneresis (Dejmek and Walstra 2004). Moreover, temperature greatly affects the properties of fat globules and the spatial arrangement of other components in cheese, which ultimately determine the structure of the cheese and its physico-chemical and sensory properties (Lamichhane *et al*. 2018; Lopez *et al*. 2006). As a result, the microstructural observation of cheese and samples taken during the cheesemaking process can be very useful to predict and control the properties of the final cheese product (El-Bakry and Sheehan 2014). Confocal laser scanning microscopy (CLSM) is a powerful tool, which allows two-dimensional (2D) thin optical sections of a sample to be digitally captured and reassembled to obtain three-dimensional (3D) information (Gunasekaran and Ding 1998). This imaging allows both fat and protein to be visualised and quantified, providing numerical information about the size, shape and distribution of key components of the curd and cheese matrix. In addition, cryo-scanning electron microscopy (cryo-SEM) provides a detailed image of the surface features of hydrated samples without chemical staining and conventional sample drying (Ong *et al*. 2011).

Cheese produced from raw sheep milk is particularly important in European countries such as France, Italy, Greece, Spain and Romania (EUROSTAT 2016). This type of milk is suitable for cheesemaking due to the higher concentration of milk components and improved coagulation properties compared to cow milk (Kammerlehner 2009; Park *et al*. 2007). While considerable differences have been observed, the effects of the conditions used during the cheesemaking process using sheep milk in the cheese product, compound losses and yield have been scarcely reported.

The objective of the current study was to investigate the effect of the processing parameters curd grain size, assessed by image analysis, and cooking temperature on the microstructure, texture, composition and yield of cheese from sheep milk.

2. Materials and methods

2.1. Experimental design and sampling

A two-level full factorial experimental design was employed, which looked at the effects of curd grain size (*CGS*) and cooking temperature (*CT*) on the final cheese product (Table 1). Two levels of *CGS* were selected: big and small, and two levels of *CT*: 36 ºC and 45 ºC, all other cheesemaking conditions remained fixed. The four experimental cheeses were made in two consecutive days using the same bulk raw ewe milk collected from a local farm (Meredith Dairy, Truganina, Victoria, Australia) one day prior to the first day of cheesemaking. Simultaneous treatments were carried out in two identical vats. On the first day the big *CGS* factor level was selected and the *CT* factor was varied in the two identical vats. The following day, the same procedure was followed except the *CGS* factor level selected was small. The experiments were then replicated the following week using a second collection of bulk milk. Online Resource 1 summarises the most relevant physico-chemical and rheological parameters of the milk used for the experimental cheesemaking.

Cheese production was carried out in a small cheesemaking facility located at The Bio21 Molecular Science and Biotechnology Institute (The University of Melbourne, Melbourne, Australia). The cheesemaking process carried out was a typical manufacture of semi-hard sheep cheeses which are usually ripened for a minimum of 2 months. For that purpose, 20 L cubic-shaped stainless-steel double jacketed open vats were used. Each experimental cheese was made using 10 kg of raw sheep milk, which was stored at 5 ºC for 1 or 2 d before cheese production. First, the cheese milk was tempered to 25 ºC in the vat with constant stirring at 30 rpm for ~35 min before adding 0.02 g/kg of freeze-dried mesophilic starter culture, *Lactococcus lactis*, subsp. *lactis* and *Lactococcus lactis* subsp. *Cremoris* (Danisco, Copenhagen, Denmark). The temperature was increased from 25 °C to 30 ºC over 20 min and 0.03 g/kg of rennet (IMCU ~1400, Hansen Naturem, Chr. Hansen, Hørsholm, Denmark) added. Stirring was stopped 2 min after rennet addition to ensure the correct blending of the mixture. The cutting time was set to 40 min after rennet addition and the gel strength of each batch was determined at this time point. Cutting was performed using two manual cheese cutting frames with nylon wires (Grunt, Victoria, Australia) stretching in the horizontal (29 wires) or vertical direction (23 wires). The spacing between the wires in both directions was 1 cm. First, both cutting frames were inserted in the left side of the vat and moved simultaneously from left to right once and removed. The two frames were then inserted again at the back of the vat and were pushed through the curds to the front of the vat. This whole movement was repeated either twice to obtain big curd grains or six times to obtain small curd grains. The assessment of the curd grain size (big and small) was performed after cutting and stirring (Table 1) by image analysis, as previously reported (Aldalur *et al*. 2019) to ensure that two well defined and significantly different curd grain sizes were produced during cheesemaking. After cutting, the curd grain and whey mixture was stirred at 40 rpm for 50 min while the temperature increased from 30 ºC to either 36 °C or 45 ºC, depending on the *CT* selected. The cooking temperature rate was different for both *CT,* with mean values of 0.12 and 0.30 ºC/min when the temperature was raised to 36 °C and 45 ºC, respectively. Whey was then drained from the vat and weighed. The curd was placed manually into a metallic cheese mould with a wet cloth and pressed at 4.2 kg/cm^2 in a horizontal hydraulic press for ~4-5.5 h at room temperature (~ 20 ºC) until the cheese pH dropped to 5.50 ± 0.07. The pressed cheese was removed from the mould and weighed (± 0.5 g). The final cheese had a cylindrical shape 15 cm in diameter and ~12 cm in height, with an approximate weight of 2 kg. Within this study, the brining and ripening typically following cheesemaking were not carried out. The pH values at rennet addition (i.e. coagulation pH), cutting, stirring, whey draining and after pressing are shown in Table 1. The cheese was subsequently cut into four quarters or subsamples, vacuum packed and stored at 5 ºC until physico-chemical and microstructure analyses.

During the cheesemaking process, intermediate samples of curd, curd grains after cutting (fresh curd grains, FCG), curd grains after cooking (stirred curd grains, SCG) and whey were collected. For image analysis of the curd grains, FCG and SCG samples were collected using a round steel mesh sieve 13 cm in diameter with 0.7 mm mesh openings. The sieve was submerged to a depth approximately halfway between the top and bottom of the vat right after the end of either cutting, to collect FCG or after stirring or to collect SCG, the grains were extracted by draining the excess whey. For CLSM analysis, intermediate samples were stored at 5 ºC for no more than 3 h. For compositional analysis, curd grain samples were frozen at -30 ºC until analysis. CLSM, analysis to determine fat globule size and rheological analysis of milk samples was carried out on the day of milk collection. Samples were then kept frozen (-30 ºC) for compositional analysis.

2.2. Physico-chemical analysis

The fat content was determined by the Babcock method (AOAC 2005). Protein content was obtained by analysing the nitrogen content using the combustion method (ISO/IDF 2002) with a nitrogen determinator (TruMac CNS, LECO Corporation, Saint Joseph, MI, USA); this value was then multiplied by a factor of 6.38 (ISO/IDF 2014). Dry matter (DM) was measured by drying the samples overnight (ISO/IDF 2004; 2010). The pH during the cheesemaking process and in the final product was assessed using a pH meter (S220 SevenCompact, Mettler Toledo, Port Melbourne, Australia).

The total calcium, magnesium and phosphorous content of samples was analysed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (720ES; Varian Inc., Palo Alto, CA, USA) (Chen *et al*. 2016). Liquid samples were prepared for the ICP-OES analysis by diluting the sample 100 times in high purity water (resistivity of 18.2 mΩ·cm; Millipore, Billerica, MA, USA) followed by filtering through a 0.22 µm pore size filter (Millipore). Solid samples were dried overnight at 100 °C before ashing at 600 °C for 4 h using a chamber furnace equipped with a temperature controller (FP93, Shimaden, Tokyo, Japan) to remove the organic matter. The ashes where then dissolved in nitric acid (68%) and hydrochloric acid (32%) (1:1; vol/vol; both from Univar, Ingleburn, NSW, Australia) before diluting 1000 times in high purity water and filtering through a 0.22 µm pore size filter for ICP-OES analysis. All physico-chemical analyses were performed in duplicate for each sample.

The particle size distribution of the milk fat globules was measured by Light Scattering (LS) using a particle size analyser (Mastersizer 3000; Malvern Instruments, Malvern, UK), as described previously by Ong *et al*. (2010). Briefly, sheep milk samples were diluted (1:1; vol/vol) in ethylenediaminetetraacetic acid (EDTA, 50 mM, pH 7) 2 hours before analysis. Samples were then added to a circulating cell containing a mixture of water and sodium dodecyl sulphate (SDS, 0.05%). The refractive indices of the milk fat and water were set at 1.460 and 1.334, respectively. The volume weighted mean diameter D[4,3] was calculated by the particle analyser software.

2.3. Coagulation properties and texture

The coagulation properties of the milk at renneting were analysed using a Twin Drive Rheometer (MCR702, Anton Paar, North Ryde, Australia), equipped with a CC27 cup and bob accessory. A sample of milk containing rennet was loaded into the cup while the temperature was maintained at 30 °C. The analysis was carried out at an angular frequency of 0.8 Hz and 0.1% shear strain. Changes in storage modulus (G’), loss modulus (G’’) and loss factor tan (δ) were measured every 10 s for 60 min. The onset of gelation (s) was defined as the time at which the G’ first reached 0.05 Pa, the curd firming rate (Pa/s) was obtained from the maximum slope determined over 12 consecutive points and the final curd firmness defined as the G’ value at 60 min after rennet addition (Logan *et al*. 2014). All samples were analysed in duplicate.

The gel strength at the cutting point was analysed using a texture analyser (TA.HD plus; Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell and a cylindrical probe (10 mm diameter). Immediately after rennet addition and blending, two 50 mL samples of milk were taken directly from the cheese vat, poured into a plastic container 42 mm in diameter and 55 mm in height and incubated for 40 min at 30 °C. A test speed of 1 mm/s was used and the penetration was set to 20 mm with a trigger force of 1g. The maximum force obtained during the penetration was used as the measure of gel strength. The measurement was performed in duplicate on the two samples collected for each cheese vat and the mean value of gel strength was used for each vat.

Texture profile analysis was carried out using the texture analyser following a published method with some modifications (Ong *et al*. 2012). Briefly, cheese samples were taken from the centre of the cheese subsample using a knife and a cylindrical cheese corer to obtain three samples 2.5 cm in diameter and 2.5 cm in height. These were equilibrated at room temperature (~20 °C) for 1 h in a closed container prior to analysis to prevent moisture loss. A 35 mm cylindrical flat probe was used and a 50% compression test was applied to the cheese using two compression cycles at a constant crosshead speed of 2 mm/s. Three analyses were performed for each cheese treatment.

2.4. Determination of cheese yield

The actual cheese yield (kg of cheese/100 kg of milk) *per* vat was estimated as the ratio of the total weight of fresh cheese after pressing to the weight of milk used in the vat. Moisture-adjusted cheese yield (Y_{MA}) was calculated using the equation (1) described by Guinee *et al*. (2006).

$$Y_{MA} = Y_A \times ((100 - M_A)/(100 - M_R)) \quad (1)$$

where Y_A was the actual cheese yield and M_A and M_R were the actual and reference (45.0%) moisture contents of the cheese, respectively. The theoretical cheese yield (Y_T) was calculated using equation (2) described by Van Slyke (Emmons and Modler 2010):

$$Y_T = (0.93 \times F + (C - 0.1)) \times 1.09 \times 100/(100 - (M)) \quad (2)$$

where F and C were the fat and casein content of the milk respectively and M was the desired cheese moisture content, which was 45% in this instance. Casein content of milk was estimated as the 80 % of the total protein (Park *et al*. 2007). The yield efficiency (Y_E) was calculated as the percentage of the ratio between Y_{MA} and Y_T (Barbano and Sherbon 1984). The amount of fat, protein and minerals lost in the whey were calculated on the basis of milk weight following methods described by Guinee *et al*. (2006). Fat loss percentage (FL) was calculated as described in equation 3:

$$\%\,FL = (W_W \times W_F)/(M_W \times M_F) \times 100 \quad 3)$$

where W_W and M_W were the weight (kg) of the drained whey and milk, respectively and W_F and M_F were the fat content (wt/wt) of whey and milk, respectively. The protein and mineral losses were calculated using similar formulas described in the same reference.

2.5. Microstructure analysis

Milk, gel, curd grain and cheese samples were prepared for microstructural examination using CLSM by applying a method reported previously (Ong *et al*. 2011). Briefly, solid samples were cut into approximately 5 x 5 x 2 mm in size using a surgical blade and stained at ~ 4 ºC using Nile Red (0.1 mg/mL) (Sigma-Aldrich, Castle Hill, Australia) and fast green FCF (0.1 mg/mL) (Sigma-Aldrich). For milk samples, Nile Red (0.02 mg/mL), fast green FCF (0.02 mg/mL), agarose (0.5%) (Sigma-Aldrich) and milk were mixed and the stained sample was then placed on a cavity slide and covered with a coverslip (0.17 mm thick) secured with nail polish (Maybelline, LLC, NY, USA). Microstructural analysis was performed using an inverted confocal microscopy (SP8; Leica Microsystems, Heidelberg, Germany) with excitation wavelengths of 488 nm for fat and 638 nm for protein. Leica confocal software was used to acquire two-dimensional images 512 x 512 pixels in size.

Cheese samples were also analysed by cryo-SEM, as described by Ong *et al*. (2011). Briefly, cheese samples 5 x 5 x 2 mm were placed on the sample holder and immersed into a liquid nitrogen slush (-210 ºC) for 10 s. The frozen samples were then transferred into the cryo preparation chamber, which was maintained at -140 ºC under high vacuum conditions ($< 10^{-4}$ Pa). The sample was fractured horizontally using a cooled scalpel blade and etched at -95 ºC for 30 min. The temperature was reduced to -140 ºC and the cheese sample was coated with 10 mA of sputtered gold/palladium alloy (60/40) using a cold magnetron sputter coater (300V) for 120 s generating a layer ~ 6 nm thick. The sample was then placed onto a nitrogen gas cooled module under vacuum, maintained at -140 ºC and observed using a field emission SEM (Quanta; Fei Company, Hillsboro, OR, USA) with a spot size of 2 and acceleration voltage of 15 kV. The detector used for observation was a solid-state backscattered electron detector.

2.6. Image analysis

Three-dimensional (3D) image analysis was carried out for the CLSM micrographs of milk, curd, curd grains and cheese samples. For each sample, a minimum of 30 adjacent planes were acquired with a separation of 0.5 µm and reconstructed for an observation depth of 15 µm. This 3D reconstruction was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, USA). First, the 2D image layers were stacked together to reconstruct the 3D image. Stacks were equalized using Stack Normalizer plug-in and the channels were split to separate the data for fat and protein. For both stacks, the background was subtracted to enhance image quality. For the fat micrograph stack, a 3D Median filter was also used to smooth the image and improve analysis. The stacks were then thresholded using the IsoData thresholding method (Ridler and Calvard 1978). A Watershed function was applied to separate fat globules that appeared coalesced in the samples. To quantify image features, a 3D Object Counter was used to obtain volume and area surface measurements. This procedure allowed the fat globule diameter and sphericity to be determined together with the network porosity. In addition, two distinct populations of fat globules were identified in samples that were quantified by image analysis. These were the population of globular fat droplets and the population of non-globular or coalesced fat droplets. Globular fat droplets were defined as those droplets with a diameter smaller than the mean diameter of the fat globule in milk. The diameter of these fat droplets ranged from 0.57 to 5.93 µm, which were clearly not disrupted by the handling encountered during the cheesemaking process. Non-globular fat droplets were defined as those droplets with a diameter higher than 10 µm, as these larger areas of fat are most likely pools of free fat (Everett and Auty 2008). Fat droplets with a diameter between 6-10 µm did not fit these descriptions and were excluded from this analysis. In each case, the percentage of fat volume corresponding to globular or non-globular fat was quantified.

2.7. Statistical analysis

The IBM-SPSS Statistics software version 24.0 (New York, NY, USA) was used for statistical analysis. The general linear model of Analysis of Variance (ANOVA) was used to study the effects of the two factors, *CGS* (l = 2) and *CT* (l = 2) on the composition, microstructure, texture and yield variables measured in this study. The fixed effects model $Y_{ijk} = \mu + GS_i + CGS_j + CT_k + day_l + (CGS*CT)_{jk} + \varepsilon_{ijkl}$ was applied to study simultaneously the effect of both factors and the gel strength (*GS*) was used as a covariable. The relationship between texture and microstructure parameters of cheese samples were studied using Pearson bivariate correlations. Statistical significance was defined as $P \leq 0.05$.

3. Results and discussion

3.1. Curd grain size and cooking temperature

Cheesemaking experiments were performed where the curd grain size (*CGS*) and cooking temperature (*CT*) were varied to assess the impact of these parameters. The area of the large curd grains assessed by image analysis was significantly ($P \leq 0.05$) greater than the area of the small curd grains (Table 1). As expected, this difference was significant for grains assessed both after cutting (FCG) and after cooking (SCG), indicating the effectiveness of the manual wire mesh technique and number of cuts used to alter curd grain size. Direct comparisons between the curd grain size produced here and in previous studies is made difficult by the use of different measures of curd sizes, including previous indirect measures of knife spacing and curd weight after sieving. Nevertheless the method used here provides a good span of sizes (Aldalur *et al*. 2019; Johnston *et al*. 1991; Whitehead and Harkness 1954).

Table 1. Technical parameters controlled during the experimental cheesemaking design assessing the effect of curd grain size (*CGS*) and cooking temperature (*CT*). Two replicates (n = 2) were carried out for each of the four treatments and the data presented is the mean ± standard deviation.

Factors		FCG area (mm^2)	SCG area (mm^2)	Milk pH	Coagulation pH	Cutting pH	Cooking pH	Whey draining pH	Cheese pH after pressing
CT	*CGS*								
36ºC	Big	52.53^{a} ± 5.30	7.48^{a} ± 0.66	6.54 ± 0.01	6.53 ± 0.01	6.51 ± 0.01	6.49 ± 0.01	6.41 ± 0.04	5.46 ± 0.01
45ºC	Big	45.06^{a} ± 8.35	6.46^{a} ± 1.03	6.53 ± 0.04	6.52 ± 0.01	6.50 ± 0.01	6.48 ± 0.18	6.35 ± 0.04	5.56 ± 0.11
36ºC	Small	14.42^{b} ± 3.21	4.54^{b} ± 1.41	6.56 ± 0.04	6.52 ± 0.00	6.51 ± 0.04	6.38 ± 0.04	6.41 ± 0.04	5.41 ± 0.01
45ºC	Small	12.69^{b} ± 4.44	3.63^{b} ± 1.04	6.54 ± 0.04	6.52 ± 0.01	6.50 ± 0.03	6.40 ± 0.02	6.33 ± 0.03	5.48 ± 0.01

[a,b] Different superscripts in the same column indicate statistically significant differences ($P \leq 0.05$). FCG, curd grain after cutting; SCG, curd grain after cooking.

3.2. Physico-chemical composition and cheese yield

Table 2 shows the physico-chemical parameters measured for the curd grains during cooking (SCG) and cheese from the trials described in Table 1. The significant ($P \leq 0.05$) differences observed among treatments are mainly due to an increase in dry matter content of the curd grains and cheese samples when subjected to higher cooking temperatures, as temperature greatly increases the rate of curd syneresis (Dejmek and Walstra 2004). Regarding the effect of curd grain size, measured with varied indices, previous authors have reported a higher moisture content in cheese made from bigger curd grains (Johnston *et al*. 1991; Whitehead and Harkness 1954). In other studies, however, the effect of curd grain size was reported have no impact on the dry matter content of the cheese (Everard *et al*. 2008). In this study, no significant difference ($P > 0.05$) was observed for the dry matter of the cheese samples using both big and small curd grain sizes, consistent with the results obtained by Everard *et al*. (2008).

The fat in dry matter content in the cheese samples was significantly ($P \leq 0.05$) affected by the curd grain size, indicating that the bigger particles retained more fat (Table 2). Similarly, the fat loss was also higher when the curd grain was smaller (Table 3). Reducing the curd grain size increases the surface area to volume ratio and the loss of fat globules to the whey is consequently enhanced (Banks 2007). This process could be visualised using confocal microscopy, as shown in Online Resource 2, where the fat globules near the edge of the curd grain are observed readily entering the surrounding solution after the protein matrix of the curd is cut and this process is likely responsible for the differences in fat loss observed with different sized curd. No visual differences were observed, however, between the edges of the curd with difference grain sizes.

The cooking temperature was the main factor affecting the mineral composition of the cheese and the curd grain size had no significant effect (Table 2). The calcium, phosphorous and magnesium contents were significantly ($P \leq 0.05$) higher in cheeses cooked at the higher temperature of 45 °C. Consequently, the calcium in the whey was also significantly ($P \leq 0.05$) higher when the cooking temperature was lower at 36 °C (Table 3). Interestingly, these differences occurred despite the similar pH at whey draining (Table 1); another factor known to influence calcium loss during cheesemaking (Johnson and Lucey 2006).

Table 2. General composition (mean ± standard deviation) of curd grains after cooking and cheese samples from the experimental cheesemaking design combining the factors curd grain size (*CGS*) and cooking temperature (*CT*).

	Big *CGS*		Small *CGS*		Significance		
	36 ºC	45 ºC	36 ºC	45 ºC	*CGS*	*CT*	*CGS*CT*
Curd grains after cooking							
Fat, g/100 g	17.1 ± 1.6	24.0 ± 0.7	17.5 ± 1.1	22.5 ± 0.4	ns	**	ns
Fat in dry matter, g/100 g	42.0 ± 1.7	48.0 ± 2.7	44.9 ± 1.5	47.4 ± 1.3	ns	ns	ns
Protein, g/100 g	16.10 ± 1.97	20.50 ± 1.85	14.24 ± 0.24	20.41 ± 0.18	ns	**	ns
Protein in dry matter, g/100 g	39.45 ± 2.72	40.92 ± 2.61	36.55 ± 1.61	43.02 ± 0.89	ns	ns	ns
Dry Matter, g/100 g	40.74 ± 2.17	50.07 ± 1.31	38.97 ± 1.07	47.44 ± 0.57	*	***	ns
Calcium, mg/100 g	661 ± 50	842 ± 11	623 ± 55	874 ± 35	ns	**	ns
Phosphorous, mg/100 g	426 ± 27	527 ± 53	399 ± 32	545 ± 23	ns	**	ns
Magnesium, mg/100 g	42 ± 3	52 ± 1	40 ± 4	53 ± 2	ns	**	ns
Cheese							
Fat, g/100 g	28.5 ± 0.0	30.0 ± 0.7	27.9 ± 0.2	29.0 ± 0.0	ns	*	ns
Fat in dry matter, g/100 g	52.8 ± 0.4	52.6 ± 0.8	51.4 ± 0.6	50.1 ± 0.3	*	ns	ns
Protein, g/100 g	21.90 ± 0.07	24.00 ± 0.26	23.12 ± 0.33	24.27 ± 0.16	*	**	*
Protein in dry matter, g/100 g	40.52 ± 0.44	42.10 ± 0.82	42.66 ± 1.36	41.91 ± 0.54	ns	ns	ns
Dry Matter, g/100 g	54.04 ± 0.40	57.02 ± 0.49	54.22 ± 0.96	57.91 ± 0.36	ns	**	ns
Calcium, mg/100 g	986 ± 28	1062 ± 34	990 ± 1	1104 ± 21	ns	**	ns
Phosphorous, mg/100 g	596 ± 4	648 ± 21	610 ± 2	675 ± 8	ns	**	ns
Magnesium, mg/100 g	56 ± 1	61 ± 2	59 ± 1	65 ± 6	*	**	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant where $P > 0.05$.

Table 3. Actual yield, moisture-adjusted yield, yield efficiency and component losses (mean ± standard deviation) obtained from the experiments assessing the effect of curd grain size (*CGS*) and cooking temperature (*CT*).

	Big *CGS*		Small *CGS*		Significance		
	36 ºC	45 ºC	36 ºC	45 ºC	*CGS*	*CT*	*CGS*CT*
Fat loss (%)	8.60 ± 0.78	9.11 ± 1.46	10.81 ± 0.23	11.35 ± 0.06	*	ns	ns
Protein loss (%)	22.39 ± 0.66	23.82 ± 4.44	22.91 ± 1.01	22.76 ± 0.50	ns	ns	ns
Calcium loss (%)	23.67 ± 4.87	19.83 ± 1.63	22.17 ± 3.37	20.05 ± 0.86	ns	*	ns
Phosphorous loss (%)	36.98 ± 1.71	36.71 ± 2.07	37.23 ± 2.02	36.87 ± 3.38	ns	ns	ns
Magnesium loss (%)	60.20 ± 0.48	60.90 ± 1.17	61.14 ± 1.52	62.50 ± 0.59	ns	ns	ns
Actual yield (kg/100 kg)	19.21 ± 0.74	18.43 ± 0.37	19.16 ± 0.24	17.96 ± 0.48	**	***	**
Moisture-adjusted yield (kg/100 kg)	18.87 ± 0.58	19.10 ± 0.22	18.89 ± 0.09	18.91 ± 0.38	ns	ns	ns
Yield efficiency (%)	97.09 ± 0.63	98.29 ± 2.57	97.23 ± 4.13	97.28 ± 1.67	ns	ns	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant where $P > 0.05$.

The actual yield (Y_A) was statistically affected by both, the curd grain size and cooking temperature ($P \leq 0.05$; Table 3). The yield increased when the curd grain size was bigger and the cooking temperature was lower. The interaction term *CGS*CT* was also statistically significant ($P \leq 0.05$), indicating that both factors had an impact on the cheese yield. These differences were mainly associated with the dry matter content of the cheese samples, however, as once corrected for moisture the moisture adjusted yield (Y_{MA}) was not significantly different for these treatments ($P > 0.05$; Table 3). The relationship between cheese yield and dry matter has been well documented by other authors (Lucey and Kelly 1994; Whitehead and Harkness 1954). The moisture-adjusted yield was expected to be different due to an increased amount of fat loss in the whey observed for small curd grains (Table 3), however, this loss appears to be insignificant compared to the weight of the cheese samples. Consequently, we consider the amount of fat loss a better indicator of cheese yield when observing small differences between temperatures and curd grain sizes in cheesemaking. Other studies have similarly observed fat loss to be a more sensitive measure when operating a small pilot scale (Ong *et al*. 2015). The high fat losses in this study were also reflected in the low cheese yield efficiency (Table 3), as the average values for Y_{MA} were below the calculated theoretical cheese yield (19.44 ± 0.55 kg/100 kg). This again may be due to several factors including the composition of milk, the method of cutting, number of total revolutions and pilot scale of the study.

3.3. *Microstructure of curd and cheese*

Changes in the microstructure of dairy matrices were followed by confocal microscopy during the cheesemaking process to visualise the effects of *CGS* and *CT*. The rennet-induced gel had a compact structure for all cheesemaking treatments, with a mean porosity of 0.36 ± 0.08. This low porosity was expected, due to the high fat and protein content of raw sheep milk (Online Resource 4; Online Resource 1). In comparison, Ong *et al*. (2010) reported 0.49 ± 0.02 porosity for gels formed from raw cow's milk where the fat and protein content were lower with a concentration of 4.45 g/100 g and 3.53 g/100 g, respectively.

Fat droplets were numerous in rennet-induced gel samples. The mean diameter determined by image analysis was 7.25 ± 0.74 µm, which was not significantly ($P > 0.05$) different to the mean diameter of milk samples determined using the same method (5.93 ± 0.48 µm). In agreement with other studies, the fat droplets appear to act as inner fillers, as the casein network was formed around the fat globules with no visible interaction between the protein and fat components (Lopez *et al*. 2007; Michalski *et al*. 2002). Some fat globules formed aggregates or coalesced droplets but generally the globular structure of fat was retained throughout this stage of the cheesemaking process (Online Resource 4).

The protein matrix contracted during the cheesemaking process (Figure 1), as the dry matter content significantly ($P \leq 0.05$) increased due to the syneresis (Table 2). The size of the fat globules was similar to within the gel after cutting (7.58 ± 0.86 µm) but these droplets began to change in shape following cooking and pressing (Figure 1, e to l). Some fat globules fused together during cooking generating coalesced fat globules, whilst some non-globular or free fat could also be observed (Figure 1, f and j). After pressing, the fat volume significantly increased ($P \leq 0.05$) and the structure of nearly all fat globules was disrupted (Figure 1, i to l).

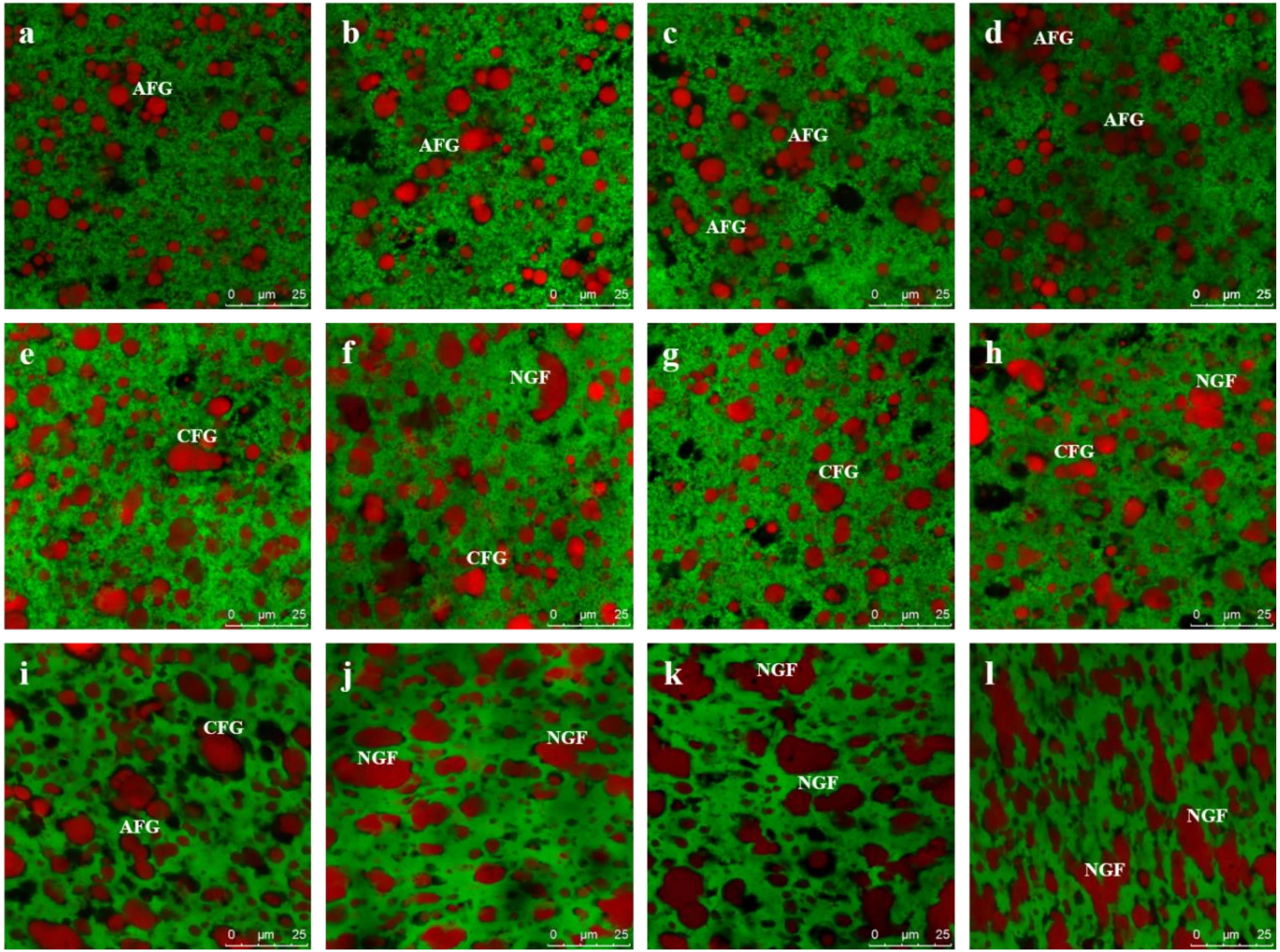

Figure 1. Confocal laser scanning microscopy images of (a to d) curd grains after cutting, (e to h) curd grains after cooking and (i to l) cheese, of the combination of factors curd grain size (*CGS*) and cooking temperature (*CT*) during the cheesemaking process. Cheesemaking treatments were coded as: big *CGS* and 36 ºC *CT* (a, e, i); big *CGS* and 45 ºC *CT* (b, f, j); small *CGS* and 36 ºC *CT* (c, g, k); small *CGS* and 45 ºC *CT* (d, h, l). Images were obtained using a 63x objective lens and digitally magnified 2x. Fat appears red and protein appears green in these images. The scale bars are 25 µm in length. AFG = aggregates of fat globule, CFG = coalesced fat globule, NGF = non-globular fat.

The cooking temperature was found to significantly affect the physical properties of the cheese measured by 3D image analysis of the CLSM micrographs. When the cooking temperature increased to 45 ºC, the volume of non-globular fat increased significantly ($P \leq 0.05$) in cheese samples (Table 4). This was observed by both CLSM (Figure 1; j and l) and cryo-SEM imaging (Figure 2; b, d, f and h). Most of the fat droplets in these micrographs appeared to have lost their globular structure and the fat appeared as coalesced non-globular fat. Conversely, the volume contributed by globular fat significantly decreased ($P \leq 0.05$) when the stirring temperature was 45 ºC; few globular fat droplets were also observed when the curd grain size was smaller (Table 4), indicating that these smaller fat globules fused to form larger coalesced fat globules and free fat (Lopez *et al*. 2006). Additionally, a smaller curd grain size can lead to a higher compaction of the curd during pressing (Walstra *et al*. 2006); this could cause further disruption of the fat droplets, resulting in more non-globular fat as detected by image analysis here.

The effect of temperature on the cheese matrix structure could be also observed by analysing the porosity (Table 4), as the curd grains heated at 36 ºC or 45 ºC had porosities of 0.17 ± 0.03 and 0.10 ± 0.03, respectively, with the higher temperature reducing network porosity ($P \leq 0.05$). This effect of temperature could also be observed visually by cryo SEM, as gaps could be seen at the interface between the fat and protein network of the cheese cooked at 36 ºC with big curd granules (Figure 2e).

The surface of the curd grains contained little fat content once the curd was pressed and the curd junctions were evident as thick protein strands within the cheese microstructure (Online Resource 5). Several calcium phosphate crystalline inclusions were also observed (white arrows in Online Resource 5) and these generally occurred along the curd junction in all four treatments, as calcium in dry matter basis was not significantly different ($P > 0.05$) in these cheese samples (Online Resource 6). This structural arrangement has also been reported in other cheeses (Brooker *et al*. 1975; D'Incecco *et al*. 2016; Ong *et al*. 2015), with the number of inclusions increasing after calcium addition. D'Incecco *et al*. (2016) attributed the increased number of crystalline inclusions within the

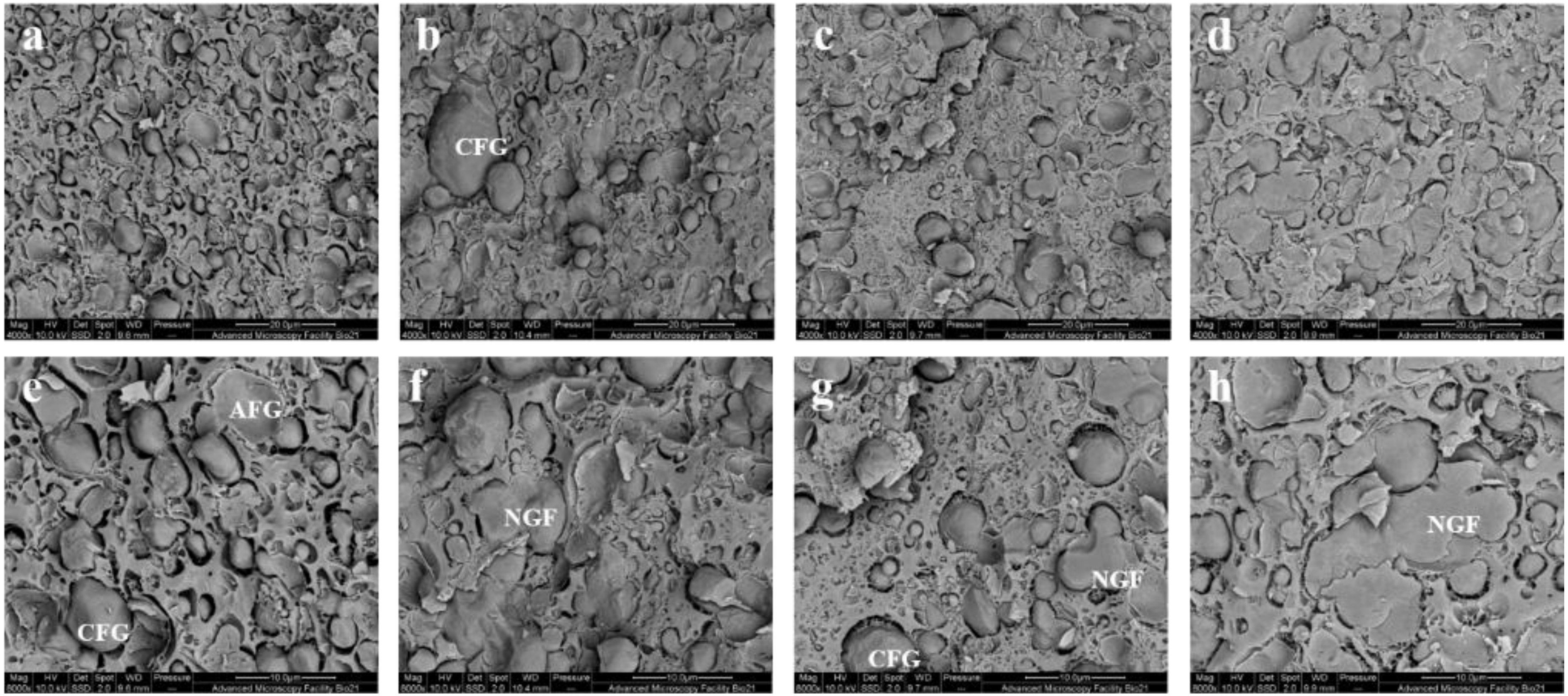

Figure 2. Cryo-scanning electron microscopy images of cheese of the combination of factors curd grain size (*CGS*) and cooking temperature (*CT*) during the cheesemaking. Cheesemaking treatments were coded as: big *CGS* and 36 ºC *CT* (a, e); big *CGS* and 45 ºC *CT* (b, f); small *CGS* and 36 ºC *CT* (c, g); small *CGS* and 45 ºC *CT* (d, h). Representative images were presented at 4000x magnification (a to d) and 8000x magnification (e to h). The scale bars are 20 µm (a-d) and 10 µm (e-h) in length. AFG = aggregates of fat globule, CFG = coalesced fat globule, NGF = non-globular fat.

curd junction to be a result of incomplete aggregation of the curd granules and the higher volume of whey initially present within the curd junctions. The resulting dense spherulite crystals likely arise from non-crystalline branching from a nuclei core. These nuclei could form from the supersaturated concentration of solutes originating in the whey; a condition that is known to lead to spherulite formation in gels (Shtukenberg *et al*. 2011), such as the protein network observed here.

Table 4. Physical properties obtained by 3D image analysis and texture parameters (mean ± standard deviation) of cheese samples from experiments assessing the effect of curd grain size (*CGS*) and cooking temperature (*CT*).

	Big *CGS*		Small *CGS*		Significance		
Physical properties	36ºC	45ºC	36ºC	45ºC	*CGS*	*CT*	*CGS* CT*
Fat sphericity	0.60 ± 0.00	0.58 ± 0.02	0.61 ± 0.04	0.56 ± 0.05	ns	ns	ns
Porosity	0.18 ± 0.05	0.11 ± 0.00	0.17 ± 0.01	0.09 ± 0.05	ns	*	ns
Volume of globular fat (%)	12 ± 1	4 ± 2	7 ± 2	3 ± 2	*	**	ns
Volume of non-globular fat (%)	69 ± 6	92 ± 0	78 ± 11	96 ± 2	ns	*	ns
Cheese texture							
Hardness (g)	5389 ± 518	7796 ± 649	6653 ± 1307	8414 ± 296	ns	*	ns
Chewiness (g)	2989 ± 560	4834 ± 308	3845 ± 1086	5284 ± 403	ns	*	ns
Springiness (s/s)	0.846 ± 0.002	0.858 ± 0.004	0.858 ± 0.007	0.865 ± 0.011	ns	ns	ns
Cohesiveness (g·s/g·s)	0.654 ± 0.056	0.716 ± 0.004	0.669 ± 0.065	0.726 ± 0.022	ns	ns	ns
Resilience (g·s/g·s)	0.338 ± 0.029	0.373 ± 0.000	0.360 ± 0.026	0.391 ± 0.020	ns	ns	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant where $P > 0.05$.

3.4. Cheese texture

Cooking temperature was the main factor found to affect the textural parameters of the cheese (Table 4). A higher cooking temperature (45 ºC) produced cheeses that were significantly ($P \leq 0.05$) harder and chewier than those produced at lower temperatures (36 ºC). Cheese texture is known to be affected by many factors such as the composition, microstructure, fat droplet size and distribution, casein matrix bond strength and interactions between fat globules and the protein matrix (Everett and Auty 2008; Hickey *et al*. 2015). Here, the differences observed with higher temperature were likely due to the higher fat, protein, mineral and dry matter content in the cheeses cooked at 45 ºC (Table 2). Conversely, the curd granule size was not found to significantly impact ($P > 0.05$) on the cheese textural parameters.

In addition, the textural parameters measured were correlated with the microstructural properties of cheese quantified by 3D image analysis. The cheeses in which a higher volume of globular fat was retained (i.e. cheese cooked at 36 °C and or using a larger curd grain size) had lower hardness, chewiness, cohesiveness and resilience (r > |0.7|, $P \leq 0.05$), while those cheeses with a higher volume of non-globular fat (i.e. cheese cooked at 45 ºC) had higher values in textural parameters ($r > 0.7$, $P \leq 0.05$; i.e. were harder, chewier and had greater cohesiveness and resilience). Fat is known to act as an inert filler when the globular structure is maintained, retaining a softer cheese texture (Everett and Auty 2008), as was observed here. Cheese springiness also showed an interesting negative correlation with the porosity of the protein network (r = |0.7|; $P \leq 0.05$), with more porous cheeses demonstrating a reduced ability to spring back on compression; this may be due to the higher number of cavities and network imperfections observed in the most porous cheese structures (e.g. Figure 2e).

4. Conclusions

This study indicates that curd grain size and cooking temperature affect the structural and textural characteristics of cheese made using raw sheep milk, in addition to affecting the yield and composition of the whey generated during the cheesemaking process. The cooking temperature had a major effect on cheese composition, increasing the dry matter content of the cheese due to the enhanced syneresis during cooking. A higher cooking temperature significantly altered the texture of the cheese, increasing hardness and chewiness. Additionally, changes in the microstructure were observed with different cooking temperatures, with high temperatures increasing non-globular fat and reducing the porosity of the cheese protein matrix. These changes of the structural arrangement of fat were correlated with the textural properties of cheese. A small curd grain size was the main factor responsible for increased fat losses in the whey. Consequently, the actual cheese yield was impaired when the cutting process was more intense and cooking temperatures higher. These results contribute with new data on the interactions between curd grain size and cooking conditions on the microstructural, textural and chemical properties of cheese. These insights are useful for the dairy sector in order to improve and control the cheesemaking process and the composition of the whey generated for ulterior processing.

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3. General discussion

This Ph. D. addresses the relationships between cheesemaking technical conditions, especially those regarding the cutting and cooking parameters, cheese properties, yield and losses in the whey at experimental and commercial scale in small rural dairies. Cheese yield and milk compound losses in the whey are essential indicators used in the dairy sector to evaluate the economic and environmental viability of the cheesemaking process. The main objective of this Ph. D. was to contribute to the sustainability of cheese production in small rural dairies, improving cheese yield and reducing the amount and organic load of the whey produced. In that regard, the current cheesemaking practices carried out in small rural dairies that manufacture Idiazabal cheese during the cheese production season were evaluated (Observational study), and the effect of these technical settings on cheese properties, yield and whey losses were studied (Interventional study). Additionally, the interactions between cheese composition and properties, yield and specific technical parameters of the cutting and cooking processes were studied in a model cheese using raw sheep milk (Experimental study), of which information is particularly scarce. The results of this work contribute to the improvement of the cheesemaking process especially where the facilities are limited and where the role of the cheese-maker is crucial.

The first objective was to assess the current cheesemaking practices carried out in small rural Idiazabal dairies and the impact on the composition of whey, cheese properties and yield throughout the cheese production season. In this regard, the relationships between technical conditions, particularly those regarding to cutting and cooking processes, whey and cheese composition and yield were investigated. The present work reported that the technical parameters used for the manufacturing of Idiazabal PDO cheese largely varied among small rural dairies. However, the technical settings used by the cheese-makers did not change with the season, which was considered surprising due to the lack of adjustment in the cheesemaking procedure according to the variations in milk composition during lactation and feeding management. It is well known that the composition of milk can cause changes in the coagulation properties, and that the cutting and cooking procedures should be adapted to the consistency of the curd in order to minimize milk compound losses in the whey (Banks, 2007; Bencini, 2002; Kammerlehner, 2009). The intra-dairy variability, however, was remarkably large among the productions of the same small dairy as seen in the PCA multivariate approach. This suggests that the cheesemaking process of each dairy varied as a result of uncontrolled factors, and not due to the composition of milk or controlled environmental factors, as could be expected. Therefore, these results open a window of opportunity for reducing the variability of the processing conditions used in the productions of the same dairy, and for possibly improving their cheesemaking process depending on their facilities.

Actual cheese yield after pressing ranged from 16.7 to 19.8 kg/100 kg and after 2 months of ripening, which is the moment from which the cheese-makers can start to commercialise the product, it varied from 14.7 to 16.9 kg/100 kg among small Idiazabal dairies. The variations of 2-3 kg/100 kg *per* cheese production observed among dairies could have a great impact on the economic profitability of small rural dairies for their annual cheese production, since the price *per* kg of cheese hardly varies among them. In the observational study (**Manuscript II**), correlations between cheese yield and technical parameters were established by a PCA multivariate approach. Higher cheese yield values were obtained when lower cooking temperatures and shorter cutting and cooking times were employed during the cheesemaking in the small dairies. The cheese yield was also related to the curd grain size after cooking, as it increased with a bigger curd particle. These results were also found in the experimental design on a laboratory scale (**Manuscript IV**), where curd grain size, cooking temperature and the combined effect between them greatly affected actual cheese yield. Other authors have also found significant correlations between stir-out time (time in the cheese vat post-cutting), pressing duration and cheese yield (Everard *et al*., 2011). However, the studies regarding the relationships between cheesemaking technical settings and actual cheese yield are particularly scarce in commercial studies. Additionally, the studies carried out on a laboratory scale generally focus on milk compound losses in the whey and not on the direct cheese yield, and although useful, these parameters do not give the same information, as reported in our study.

The effect of the cutting and cooking technical settings used in small rural dairies on the compound losses in the whey were studied using PCA and PLSR multivariate analyses. The results confirmed that higher cutting and particularly higher cooking speeds remarkably increased fat losses in the whey. Additionally, shorter cutting duration was also correlated to higher fat losses. In the experimental study (**Manuscript IV**), the correlation between enhanced fat losses at high cooking speed was also observed. The results agree with several authors that reported that fat losses increased with higher stirring speeds, especially when the previous cutting process had been insufficient causing the shattering of the curd particles (Johnston *et al*., 1998; Everard *et al*., 2008). However, an excessive cutting procedure with a long cutting time and high speed was also observed to cause an increased amount of fat and casein losses in the whey after cutting (**Manuscript III**). Casein losses were closely related to the consistency of the curd at the cutting point, the losses increasing with firmer curds. Therefore, the gel should not harden excessively when manufacturing semi-hard cheeses like Idiazabal, as also reported by other authors (Kammerlehner, 2009; Lucey, 2011). In all the cases, a larger curd grain surface to volume ratio increased fat losses, because of an excessive or insufficient cutting, and subsequent curd shattering effect. This was confirmed when the curd microstructure was observed by confocal laser scanning microscopy and happened due to the lack of interaction between the protein matrix and fat globules. The higher amount of total losses increased the chemical oxygen demand of the whey, increasing from 77.2 to 96.2 g/L when higher fat, protein and mineral losses were reported.

In the present study, the composition of cheese among small rural dairies varied due to the cutting, cooking, pressing and ripening conditions employed in each dairy. Cheeses after pressing differed significantly among dairies regarding the content of moisture, calcium and phosphorous, while fat and protein content showed large differences that were not statistically significant. Additionally, a high positive correlation between the amount of protein, calcium and phosphorous of the cheese was found, since these minerals are the main ones responsible for the formation of the crosslinks in the casein network of cheese (Johnson & Lucey, 2006). However, no significant correlation between specific technical settings and cheese composition was found. In the experimental study (**Manuscript IV**), the effect of the cooking temperature was the main parameter responsible for the changes in the cheese composition, increasing its fat, protein and mineral content due to the higher moisture loss at higher temperatures.

The relationships between the composition of raw sheep milk, cheese yield and whey composition during the whole cheese production season in small rural dairies were also investigated. The composition of milk did not significantly differ among small dairies most likely because the animal feeding strategy and flock management is generally managed by associations of cheese producers and veterinary inspectors from the regional administration. However, as mentioned above, the composition of milk changed during the cheese production season, increasing the content of fat, protein, calcium, magnesium, phosphorous and total solids from late winter to early summer. This increment in the total solids of milk, which was related to the lactation stage, feeding system, flock management, physiological state or climatic conditions (Barron *et al*., 2001; Nájera *et al*., 2009), remarkably increased cheese yield in early summer (17.2 *vs* 20.0 kg/100 kg for cheeses after pressing, and 14.6 *vs* 16.5 kg/100 kg for 2-months ripened cheeses). Other authors have also reported higher cheese yield values for Idiazabal cheese during early summer, with similar values to the ones presented in this study (Abilleira *et al*., 2009; Barron *et al*., 2001). Additionally, the milk compound losses in the whey also increased in this period of the year, showing a clear seasonal effect as demonstrated by the univariate (ANOVA) and multivariate (PCA) approaches (**Manuscript II**). The seasonal changes of milk composition were reflected on 2-months ripened cheeses, where greater concentration of fat (33.6 *vs* 36.8 g/100g) and reduced amount of moisture (37.21 *vs* 34.23 g/100g) were measured in early summer. Some authors did not find significant differences in the dry matter content of Idiazabal cheeses ripened for 3 and 6 months with the seasonal changes in milk composition (Barron *et al*., 2005; Mendia *et al*., 2000). However, Mendia *et al*. (2000) reported a higher fat concentration and lower protein content in Idiazabal 3-month ripened cheeses manufactured in early summer, as reported in the present work.

The characterization of the size, shape and particle size distribution of curd grains after the cutting and cooking processes, and the relationships with the technical settings were specifically investigated in **Publication I**. In this regard, a 2-dimensional image analysis method was developed and applied for the monitoring of the cheese manufacturing in small Idiazabal dairies. As mentioned above, cutting settings used during cheesemaking widely varied among dairies. These differences in the cutting parameters, together with the arrangement, design and size of the cutting tools, caused large variations in the size, shape and size distribution of the curd grains after cutting. A linear relationship (R^2 = 0.82) between a parameter that encompassed cutting time, tip speed and knife density (cutting process, defined in Figure V.8 and **Manuscript II**), and the surface area of the curd grains after cutting (FCG Area) was established (data not shown in **Publication I**) (Equation V.1; Figure V.8).

$$\log(FCG\ Area) = -0.7599\log(Cutting\ process) + 4.9629 \qquad \text{(V.1)}$$

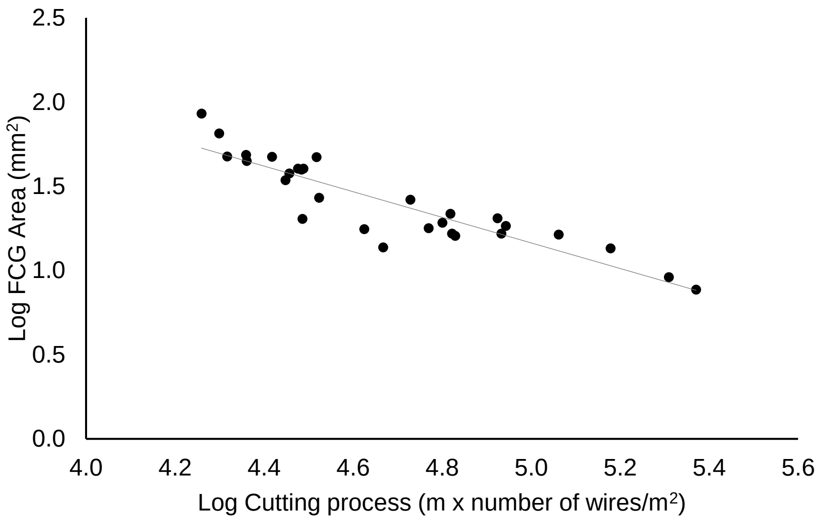

Figure V.8. Linear relationship between the logarithm of the area of the curd grains after cutting and the logarithm of the cutting process (cutting time, tip speed and knife density) used in Idiazabal PDO small rural dairies. Cutting process, cutting time x tip speed x knife density.

As expected, higher cutting time, speed and knife density generated curd particles that were smaller. This shows that the size of the curd grains after cutting could be predicted and easily controlled, adjusting the time and speed during cutting and taking into account the cutting tools available in each dairy. The size of the curd grain at this stage of the cheesemaking has been reported to be the main factor that influences the rate of syneresis at a constant temperature (Panthi *et al*., 2019; Unger Grundelius *et al*., 2000), which has also been observed at the commercial and laboratory scale studies carried out in the present Ph. D. The size distribution indicators of the curd grains showed that the particles generated during cheesemaking in the small dairies were highly heterogeneous regardless of the technical cutting settings used. Other authors using 2-dimensional image analysis for the curd grain analysis in Italian hard cheeses also observed high heterogeneity in the particle size distribution (relative span values up to 5.76) (Iezzi *et al*., 2012). Regarding the shape, three parameters were analysed to assess the elongation, rectangularity and circularity degree of the curd grains. In general, curd grains after cutting were more circular than rectangular and slightly elongated. A relationship between the technical cutting settings and shape parameters was established indicating that a higher cutting speed and number of wires *per* cutting surface (cutting density) generated curd grains that were less elongated and more circular. Other authors have also suggested that the shape of the curd grains is related to the arrangement, design, position and rotation of the cutting frames and wires (Kammerlehner, 2009), but to the best of the author's knowledge no quantitative data has been provided in this regard.

The different cooking technical parameters used in the small rural dairies markedly affected the properties and composition of the curd grains after cooking. It is important to highlight that the result of the cooking process happens due to the combined effect of the cutting and cooking steps. A longer cooking time and higher stirring speed, including a more intense previous cutting process, generated curd particles that were smaller. The longer cooking time causes the shrinkage of the protein network (Fagan *et al*., 2017), while a higher cooking speed increases the average pressure of the turbulent flow and the collisions between the particles or between the particles and the equipment (Patel *et al*., 1972; van den Bijgaart, 1988), further reducing the size of the curd grains. These factors, and especially the final cooking temperature, were closely related to the moisture content of the curd grains, which reduced at higher cooking temperatures. This was also clearly observed in the experimental study (**Manuscript IV**), where the increment to 45 ºC during cooking notably reduced the moisture content of the curd grains. The complexity of the cooking process makes it difficult to control and predict the potential size of the final curd grain. However, a linear relationship between the total revolutions of the cheesemaking process, which comprised cutting and cooking speed and durations, and the area of the curd grains after cooking was established (**Publication I**). The indicators of the particle size distribution did not show a remarkable improvement after cooking, meaning that this process did not improve the homogeneity and that the particle size distribution remained highly heterogeneous. Moreover, some indicators showed a slight impairment of the homogeneity after the cooking process due to the presence of a small amount of big curd grains after cooking together with a general size reduction of the curd particles. Therefore, if the big curd grains do not break during cooking, these particles will reduce their size slower than a small particle due to the longer distance that the whey has to travel to be expelled, as stated by Darcy's law. Iezzi *et al*. (2012) reported improvements on the particle size distribution after the cooking process due to an overall size reduction, but the curd grain areas in their study were in general much smaller (maximum mean area 4.74 *vs* 56.82 mm^2 in the present study). Other authors using the sieving method also found asymmetric distribution of the curd particle sizes (Akkerman, 1992; Johnston *et al*., 1991; Kosikowski, 1963). However, this method consisted of passing the curd grains through a set of sieves with different mesh opening sizes and showed strong deviations and limitations (only up to 5 classification categories) (Igathinathane *et al*., 2009). The shape of the curd grains during cooking changed especially with increased cooking time, generating less elongated and more circular particles due to the collisions between the curd grains and the particles and the equipment. The cutting process, particularly the design and arrangement of the cutting tools, highly determines the shape of the curd grains, but the final shape is the consequence of both cutting and cooking processes. Therefore, considering all the results above mentioned, the utilization of the 2-dimensional image analysis could be a useful tool for supervising the cutting and cooking processes, although its implementation in the dairies as a routine control tool is currently hardly feasible.

The relationships existing between all the technical settings, physico-chemical properties, milk compound losses in the whey, and cheese yield variables involved in the cheesemaking process of Idiazabal PDO cheese in small rural dairies is rather complex. Figure V.9 plots the bivariate Pearson's correlations among technical parameters, composition, pH values, curd grain properties and yield. This figure shows the large amount of significant correlations between the parameters measured in small dairies, making the interpretation of the real situation using univariate analyses extremely complicated. In this sense, the PCA was a very useful tool to simplify this complexity while retaining the information, and additionally, the small rural dairies could be segmented regarding the technical settings used along the cheesemaking process. Dairies were segmented into three different groups regarding cutting time (short, moderate and long) and two groups regarding cooking intensity (moderate and high). In the commercial cheese productions carried out in the observational study, the following four possible combinations of cutting time and cooking intensity were observed: short-moderate, short-high, moderate-moderate and long-high.

For a more detailed study of the relationships between the technical parameters, microstructure, texture and whey losses, two small dairies using moderate-moderate (called moderate) and long-high (called high) intensity cheese processing were selected (interventional study). The changes in the microstructure of the raw sheep milk, curd and cheese samples during the Idiazabal cheese manufactured in the same season (early summer) were analysed. Likewise, the influence of some technical settings in the microstructure and texture of cheese was investigated. The comparison between high and moderate intensity processes showed that the intensity of the cutting did not have marked effects on the microstructure of the curd grains. However, the differences were clearly observed for the cooking process, were the high intensity cooking process increased the total fat volume and the volume of non-globular fat while decreasing the porosity and the amount of globular fat of the curd grains after cooking. After pressing, the porosity of the protein matrix was still different between high and moderate processes, but during ripening the differences dissipated, both processes producing final cheeses with a similar microstructure. Nevertheless, some disparities in the textural properties of brittleness, stiffness and stickiness were observed in the 2-months ripened cheeses. In the experimental study (**Manuscript IV**), the biggest differences observed in the microstructure of pressed cheese were associated with the cooking temperature, which affected the porosity and the structural arrangement of the fat globules. These had an influence on the cheese texture, increasing the hardness and the

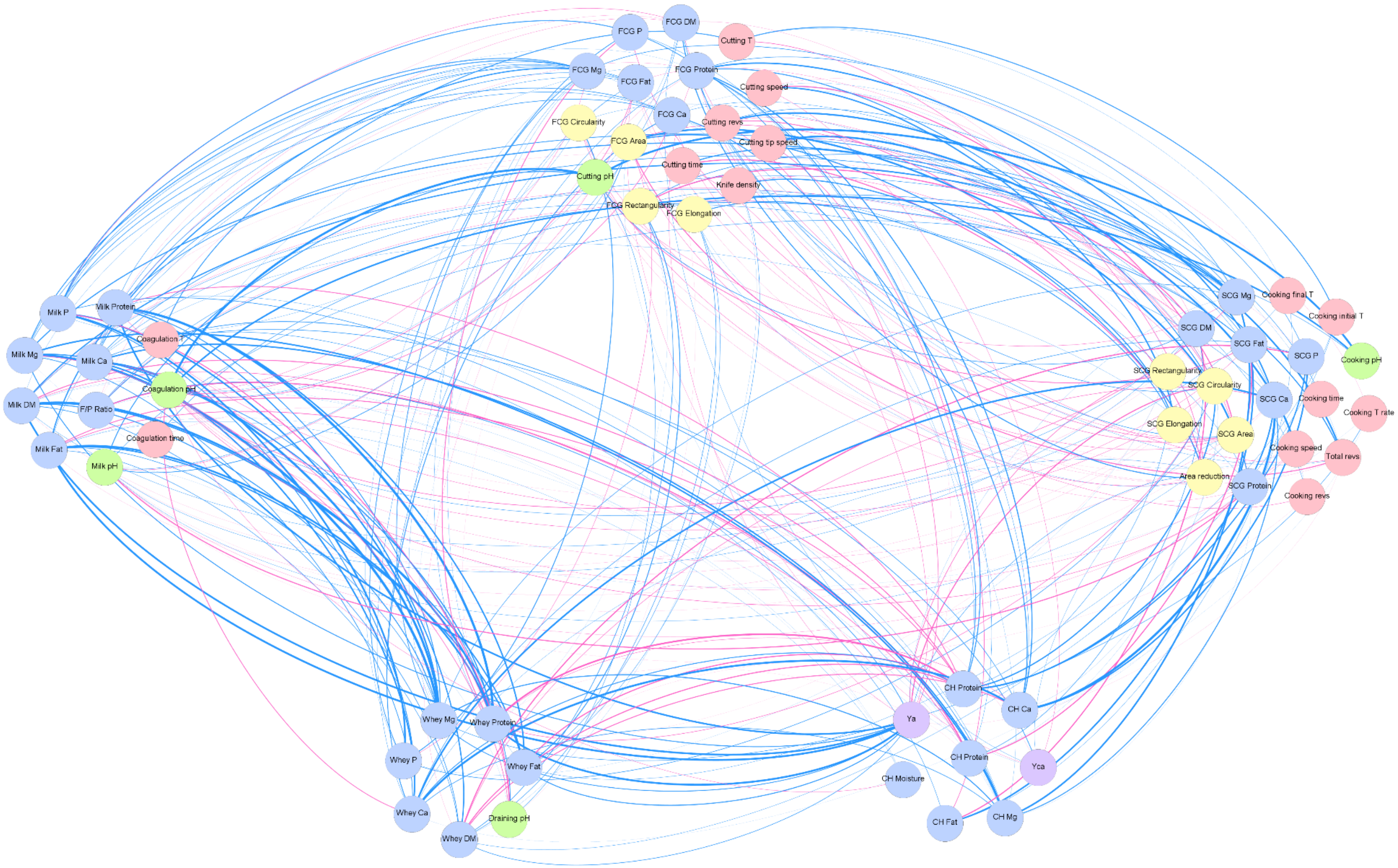

Figure V.9. Representation of significant Pearson's correlations between cheesemaking technical settings, composition, losses in the whey and yield in the manufacture of Idiazabal cheese in small rural dairies. Each circle represents a variable and appear colored depending on their category: composition (blue), technical settings (red), curd grain characteristics (yellow), pH values (green) and yield (purple). Lines in blue show the positive correlations between variables, while magenta lines depict negative correlations. The higher thickness of the lines indicate an increased absolute value of Pearson's correlation. The abbreviations of this figure are explained in the list of abbreviations of this Ph. D.

chewiness when higher temperatures were used, and additionally some correlations between microstructural and textural properties of pressed cheese were observed. Anyhow, further experimental research should be conducted to elucidate the interactions between cheese properties and technical settings.

The results obtained in the present Ph. D. demonstrate that the cheese manufacturing process currently carried out in small rural Idiazabal dairies could be improved by adapting some of the technical parameters studied. The information about the relationships between technical parameters, milk compound losses in the whey, cheese properties and yield provided in this study could be especially useful for cheese-makers in order to adapt the technical settings to their particular facilities during the cheese production season.

Chapter VI

Conclusions

From the results obtained in the present Ph. D. Thesis, the following conclusions are drawn:

1. The data collected in the observational study revealed that the technical parameters used to manufacture Idiazabal PDO cheese varied remarkably among small rural dairies. In general, a large variability of the cheesemaking process within the same dairy during the cheese production season was observed, being particularly high for some dairies. The reduction in the intra-dairy variability by accurately controlling the technical settings used during cheesemaking could contribute to obtain a more homogeneous final product with the desired properties.

2. The seasonal changes in the raw sheep milk composition were more determinant in the variations of cheese yield and milk compound losses in the whey, particularly fat losses, than the cheesemaking technical settings currently employed in the small Idiazabal diaries. However, cheese-makers should adapt the cutting and cooking conditions to the seasonal variations of milk composition, especially during early summer when milk fat content strongly increases, considering the facilities and equipment available in each dairy. This would potentially reduce fat and protein losses in the whey improving the cheesemaking efficiency and yield.

3. The technical parameters used during the cutting and cooking processes clearly affected cheese yield and milk compound losses in the whey. Adjusting the cutting time to 10-15 minutes and using moderate cooking speeds would presumably reduce the fat losses in the whey. Likewise, reducing the cutting time, and the cooking time and temperature may increase cheese yield. Therefore, it is important to achieve a balance of the technical conditions employed, and to adapt them to each dairy.

4. Two-dimensional image analysis is a useful tool for supervising the cutting and cooking processes, although its implementation in small rural dairies as a routine control tool does not currently seem feasible. The size, shape and distribution of the curd grains together with the composition and microstructure, are responsible for the degree of deformation in the

particles. Therefore, this could affect the degree of compaction during pressing and seems to be related to the final properties of the cheese.

5. The cooking temperature appears to be the main technical factor affecting the microstructure of cheese, which changes the structural arrangement of the fat globules and the porosity of the protein network, regardless of its composition. This has important implications for the textural properties of the cheese, but further research should be conducted to study the interactions between the microstructural and textural properties.

6. Overall, the results obtained in this work are remarkably valuable since the information about the effects of the technical settings on curd, cheese, and whey properties, are particularly scarce in commercial dairies. The data reported is especially useful to improve the cheese yield and to reduce the amount and the organic load of the whey, contributing to the economic and environmental sustainability of small rural dairies.

Chapter VII

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Appendix A

Informative article in Alimentaria

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Sostenibilidad de pequeñas queserías rurales: rendimiento quesero y problemática del lactosuero

Ane Aldalur, María Ángeles Bustamante, Luis Javier R. Barron*

Grupo de Investigación Lactiker-Calidad y Seguridad de Alimentos de Origen Animal, Facultad de Farmacia, Universidad del País Vasco/Euskal Herriko Unibertistatea, Paseo de la Universidad 7, 01006-Vitoria-Gasteiz.

*Autor de correspondencia: luisjavier.rbarron@ehu.eus

Introducción

Las pequeñas queserías rurales presentan una problemática ligada a sus limitadas instalaciones, su dispersión en amplias zonas geográficas, en muchos casos zonas montañosas, y su lejanía de infraestructuras industriales o logísticas. Estas explotaciones elaboran queso a partir de volúmenes de leche que pueden oscilar entre 250 y 1400 litros. La sostenibilidad de estas queserías depende de diversos factores, entre ellos, el rendimiento quesero de la explotación y la gestión del lactosuero producido. En general, las infraestructuras disponibles en las queserías rurales, además de la prohibición normativa existente en algunas Denominaciones de Origen, dificultan la realización de tratamientos previos de la leche con objeto de maximizar el rendimiento quesero, como se lleva a cabo habitualmente en las grandes empresas transformadoras. Del mismo modo, la reutilización del lactosuero como materia prima para la obtención de otros productos resulta complicada por limitaciones económicas, de equipamiento y de personal. Así pues, la producción de queso de oveja en este contexto está fuertemente influenciada por factores estacionales y tecnológicos que tienen un importante impacto sobre el rendimiento quesero, la calidad del producto final, la cantidad y composición del lactosuero producido, y, en definitiva, sobre la sostenibilidad tanto económica como medioambiental de la pequeña explotación.

Tanto el control y mejora del rendimiento quesero como la gestión del lactosuero son dos aspectos que, a día de hoy, no están resueltos en gran parte de las queserías rurales. En este artículo se analizan y discuten los principales factores que afectan al rendimiento quesero y a la composición del lactosuero en pequeñas queserías rurales de la Comunidad Autónoma del País Vasco (CAPV) destinadas, fundamentalmente, a la elaboración de queso de oveja de Denominación de Origen (DO) Idiazabal a partir de leche cruda. Como en otras zonas geográficas de España, en la CAPV una parte importante (en torno al 60%) de la leche de oveja que se produce se utiliza en la propia explotación o se vende a pequeñas y medianas queserías para la elaboración de queso. Según datos del Gobierno Vasco (10), en los últimos años, la producción anual de leche de oveja se sitúa en torno a los 8 millones de litros, lo cual supone la fabricación anual de unas 1.300 toneladas de queso Idiazabal. Estos datos parecen indicar que, con la excepción conocida aunque no cuantificada de que una pequeña parte se utiliza para alimentación animal, la mayor parte del lactosuero producido, estimado entre 3,5 y 4,5 toneladas al año, es vertido al medio ambiente.

Problemática del lactosuero

El lactosuero es un efluente altamente contaminante debido a sus altos valores de demanda biológica y química de oxígeno, los cuales pueden suponer el principal problema medioambiental de las pequeñas queserías rurales. El vertido de este subproducto a aguas fluviales supone un crecimiento exponencial de los microorganismos por su alta carga orgánica (70-80 g/L) (18), con la consiguiente disminución de la concentración de oxigeno disponible en las aguas. Además, la descarga no controlada de lactosuero en el campo puede poner en peligro la estructura físico-química de los suelos, provocando una reducción del rendimiento de los cultivos y problemas de contaminación del agua subterránea (15).

En función del tipo de queso y del proceso de fabricación se producen entre 7 y 12 kg de lactosuero por cada kilogramo de queso, lo que representa alrededor del 85-90% del volumen de la leche utilizada en el proceso de elaboración (14). Según datos recogidos por nuestro grupo de investigación, entre el 78 y 90% del volumen de leche de oveja utilizada para elaborar queso Idiazabal en pequeñas queserías se transforma en lactosuero. Este subproducto contiene principalmente lactosa (4,5%), proteína (0,8%), sales minerales (0,6%) y grasa (0,5%) (3), aunque su composición química varía considerablemente dependiendo del tipo de leche, época de elaboración ligada al período de lactación, y del proceso tecnológico de fabricación. En la Tabla 1 se muestran datos de composición media del lactosuero obtenido en distintas épocas de elaboración en queserías rurales pertenecientes a la DO Idiazabal, así como los porcentajes de pérdida de grasa, proteína y sales minerales de la leche en el lactosuero. Se encontraron diferencias estadísticamente significativas ($P \leq 0,05$) en los parámetros de composición entre el lactosuero de verano e invierno, lo que puso de manifiesto la influencia de los cambios estacionales en la composición de la leche cruda de oveja sobre la composición final del lactosuero. Sin embargo, también se observó que en queserías que utilizaron leche con valores similares de la relación grasa/proteína, la cantidad de grasa en el lactosuero variaba desde 0,36 a 0,96% en la misma época de producción, siendo, como posteriormente se discute, los parámetros tecnológicos empleados en cada quesería la principal causa de esta diferencia.

Tabla 2. Composición del lactosuero (media ± desviación estándar) y porcentajes de pérdida de componentes de la leche en el lactosuero generado en fabricaciones comerciales de queso DO Idiazabal en dos épocas distintas de elaboración (invierno/verano) en pequeñas queserías rurales (n=10).

	Invierno		**Verano**	
	Composición	**% Pérdida**	**Composición**	**% Pérdida**
Grasa (%)	0.74 ± 0.20^{a}	12.85 ± 3.14	1.05 ± 0.31^{b}	14.68 ± 3.56
Proteína (%)	1.36 ± 0.14^{a}	27.85 ± 2.12	1.65 ± 0.14^{b}	30.06 ± 1.91
Calcio (mg/L)	387.53 ± 13.51^{a}	32.73 ± 1.41	488.03 ± 24.41^{b}	29.72 ± 2.16
Magnesio (mg/L)	99.94 ± 2.99^{a}	79.02 ± 2.04	126.96 ± 7.69^{b}	70.51 ± 1.71

^{a,b} Letras superíndice distintas indican diferencias estadísticamente significativas ($P \leq 0.05$) en la composición del lactosuero entre invierno y verano.

En las pasadas décadas se ha realizado un notable esfuerzo de investigación y de transferencia tecnológica para la reutilización del lactosuero como materia prima, bien para la obtención de productos de alto valor añadido como proteínas, compuestos bioactivos, enzimas y biocombustibles, o bien como ingrediente de otros productos alimenticios (2, 4). Sin embargo, estas alternativas están fuera del alcance de las pequeñas queserías rurales, en comparación con las grandes empresas transformadoras de leche. En este sentido, estudios recientes (20) han diagnosticado la situación de la gestión del lactosuero producido en el sector lácteo de la CAPV y se han propuesto algunas medidas correctoras. Entre ellas se indicó la posibilidad de recolección de parte del lactosuero generado por algunas pequeñas y medianas queserías para su aprovechamiento en una potencial planta centralizada, la incentivación del uso del lactosuero líquido para alimentación de ganado, o incluso se sugirió la opción de desarrollar algunos productos alimentarios de consumo humano y piensos para animales. Sin embargo, los estudios señalan también serias limitaciones para la aplicación real de las alternativas de aprovechamiento o transformación del lactosuero en las pequeñas queserías rurales debido fundamentalmente, al pequeño volumen de lactosuero generado, y como se ha comentado anteriormente, a la localización dispersa de las instalaciones y a los escasos recursos financieros y de capacitación de personal del que disponen. Por lo tanto, una de las opciones más factibles se centra en la gestión *in situ* del lactosuero en la propia quesería rural, empezando por reducir la cantidad de lactosuero generado así como su carga orgánica, disminuyendo el impacto ambiental, y facilitando los tratamientos posteriores, y/o su vertido controlado al medio ambiente.

Rendimiento quesero

La reducción de la cantidad y carga orgánica de lactosuero producido en la quesería es consecuencia del incremento del rendimiento quesero, que se define como la relación entre la cantidad de queso (kg) obtenido por 100 L (o kg) de leche (1, 17). Se diferencian dos tipos de rendimiento: por un lado, el rendimiento tecnológico (RT), donde se expresa la cantidad de queso fresco (después del prensado) por 100 L de leche, y por otro, el rendimiento comercial o final (RF), que hace referencia a la cantidad de queso obtenido tras el proceso de maduración correspondiente. Es importante la diferenciación de estos dos términos ya que, con el primero, se pueden valorar las pérdidas (o la no retención) de los compuestos de la leche durante la elaboración de queso, mientras que con el segundo, las pérdidas ocasionadas vienen dadas únicamente por la pérdida progresiva de humedad durante la maduración, además de ser el RF real del queso. Estos dos parámetros están altamente correlacionados ya que cuanto mayor es el RT, o la retención de los compuestos en el queso, mayor suele ser también el RF, aunque este último también se ve afectado por las condiciones de maduración.

Los datos obtenidos por nuestro grupo de investigación sitúan el RT de elaboraciones comerciales de queso Idiazabal en pequeñas queserías rurales entre 15 y 25 kg de queso por 100 L de leche, mientras que el RF está entre 12,5 y 19,5 kg de queso por 100 L de leche.

Varios de los factores que afectan tanto al rendimiento quesero como a la composición del lactosuero dependen de la genética y del estado fisiológico de los animales, mientras que otros están directamente relacionados con el proceso tecnológico de fabricación. Entre los primeros, los más significativos son los cambios que se producen en la composición de la leche, en particular en la relación grasa/proteína debido al estado de lactación de los animales, a cambios en el manejo y alimentación del rebaño, y a diferentes variantes genéticas y polimorfismos de las caseínas de la leche (1, 17, 21). Entre los factores tecnológicos más importantes se encuentran la temperatura de almacenamiento y el tratamiento térmico de la leche antes del procesado, las condiciones de coagulación, corte y desuerado de la cuajada, el prensado, y las condiciones de maduración del queso (1, 9, 21).

Resultados en pequeñas queserías rurales

El proceso de elaboración del queso con DO Idiazabal se resume en la Figura 1. Cada una de las etapas puede ocasionar cambios en la composición de la leche, la cuajada, el queso y el lactosuero, y por lo tanto, en el rendimiento quesero. Los elaboradores de queso intervienen de forma notable en el proceso de elaboración de queso mediante la realización de cambios en parámetros como el tiempo, la temperatura, la velocidad o la fuerza aplicada en las operaciones de cuajado, corte, recalentamiento y prensado. Por lo tanto, es muy importante ajustar dichos parámetros en función de la composición de la leche, el tipo de cuajo y cultivo iniciador, así como de las características finales del queso, con objeto de conseguir un rendimiento óptimo. La Tabla 2 muestra los parámetros tecnológicos más relevantes utilizados en queserías rurales que elaboran queso DO Idiazabal.

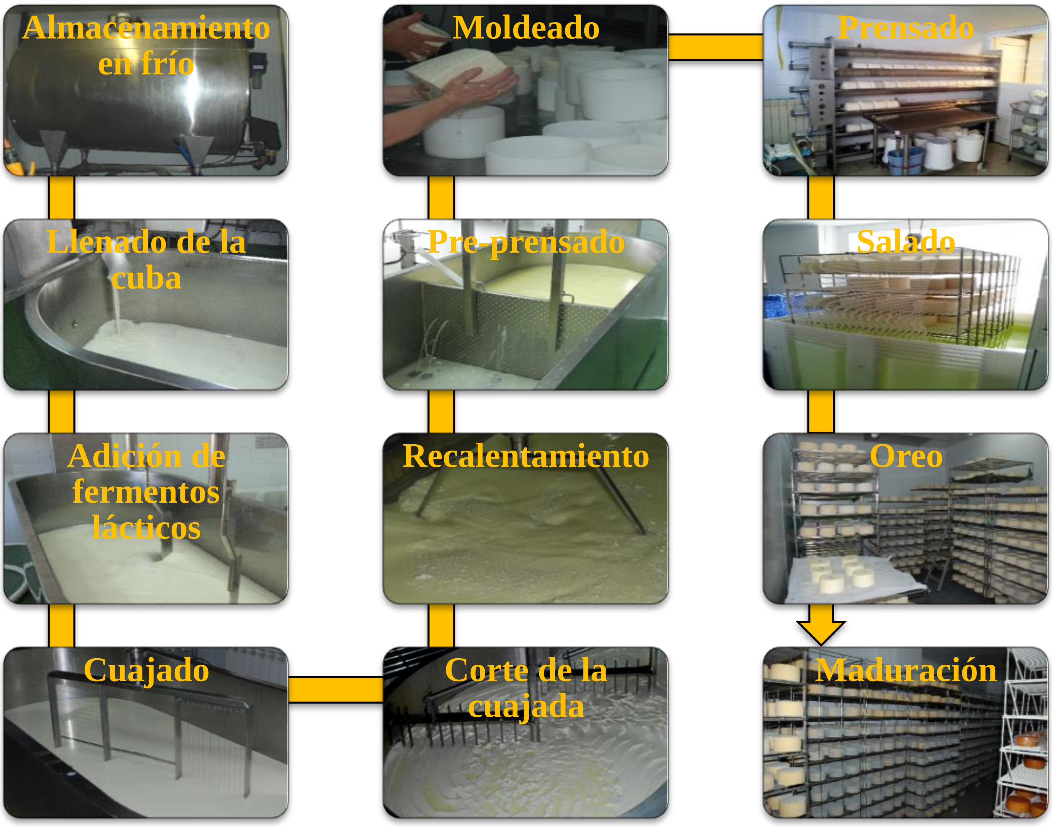

Figura 1. Etapas de elaboración del queso DO Idiazabal en pequeñas queserías rurales.

El almacenamiento en frío de la leche es una práctica habitual en las queserías rurales. Durante la refrigeración (especialmente entre 2 y 6 ºC), las bacterias psicrótrofas presentes en la leche cruda proliferan, produciendo enzimas proteolíticos y lipolíticos extracelulares que pueden degradar las proteínas y los glóbulos grasos (8), ocasionando pérdidas notables de sólidos durante el proceso de elaboración del queso, especialmente cuando los tiempos en refrigeración son prolongados (11). Los datos recogidos por nuestro grupo de investigación, indican que el tiempo de almacenamiento oscila entre 10 y 60 horas a una temperatura de entre 3 y 8 ºC (Tabla 2). El tiempo de refrigeración es ligeramente superior en verano debido a la disminución de la cantidad de leche producida por el rebaño, y a la consiguiente necesidad de reunir un volumen adecuado de leche para su manejo en la cuba (entre 250 y 1400 L).

Debido a los cambios químicos y microbiológicos mencionados, no es conveniente almacenar la leche durante tiempos superiores a 24 horas, ya que esto puede suponer una disminución importante del rendimiento quesero.

Tabla 3. Parámetros tecnológicos utilizados durante el proceso de elaboración de queso DO Idiazabal en pequeñas queserías rurales (n=10).

Parámetros tecnológicos	Media	DE	Mín.	Máx.
Cantidad de leche (L)	584	281	247	1330
Relación Grasa/Proteína	1,27	0,11	1,07	1,51
Tª tanque de almacenamiento (ºC)	4,8	1,1	3,0	8,0
Tiempo tanque de almacenamiento (h)	34	14	10	60
Tª de coagulación (ºC)	29,8	1,2	28,0	32,0
Tiempo de coagulación (min)	37	7	24	55
Tiempo de corte (min)	9	8	2	35
Tª de corte (ºC)	30,9	1,1	29,0	33,0
Velocidad de corte (rpm)	18,7	5,5	5,9	25,5
Tiempo de recalentamiento (min)	27	9	9	51
Tª inicial de recalentamiento (ºC)	31,3	1,2	30,0	35,0
Tª final de recalentamiento (ºC)	36,0	0,8	34,0	37,0
Velocidad de agitación (rpm)	16,7	6,0	8,1	28,5
Incremento Tª de recalentamiento (ºC/min)	0,2	0,1	0,1	0,6
Tiempo de prensado (min)	301	62	180	420
Tª de prensado (ºC)	20,2	1,0	17,0	22,0
Presión de prensado (bar)	2,7	0,5	1,5	3,5
Tiempo de salado (h)	15	4	10	23
Tª de salado (ºC)	10,8	3,3	6,0	20,0
Concentración de la salmuera (ºBè)	17,2	2,4	13,0	20,0
Tª de oreo (ºC)	12,0	3,0	8,0	20,0
HR de oreo (%)	79,5	9,3	66,0	99,0
Tiempo de oreo (días)	8	8	0	27
Tª de maduración (ºC)	10,5	1,2	8,0	12,0
HR de maduración (%)	87,4	3,3	80,0	92,5
Cantidad de lactosuero (kg)	487	239	210	1122
RT (kg/100 L)	19,8	2,5	14,9	24,7
RF (kg/100 L)	16,2	1,9	12,7	19,2

Tª: temperatura; HR: humedad relativa del aire; RT: rendimiento tecnológico; RF: rendimiento final; DE: desviación estándar; Mín.: valor mínimo; Máx.: valor máximo.

Para el cuajado o coagulación de la leche, los productores de DO Idiazabal suelen utilizar pasta de cuajo de cordero preparado de forma artesanal, bien sólo o mezclado con cuajo comercial estandarizado de origen bovino u ovino. El uso de este tipo de cuajo aporta al queso propiedades sensoriales de sabor (picante) y aroma que son características de este tipo de queso (5). Sin embargo, esta práctica supone una dificultad añadida para el control de la coagulación debido a las posibles variaciones en la actividad coagulante de los cuajos artesanales. Por lo tanto, los elaboradores toman la decisión de comenzar el proceso de corte según la observación sensorial de la consistencia de la cuajada, y esto implica que los tiempos de cuajado utilizados en las queserías varíen considerablemente entre 24 y 55 minutos (Tabla 2).

El corte de la cuajada es un punto crítico de control porque afecta directamente al rendimiento quesero. Durante el cortado, la cuajada pierde agua (sinéresis del gel) y los componentes lácteos de interés, principalmente grasa, caseína y sales minerales, se concentran en los gránulos de cuajada. Algunos de los estudios llevados a cabo hasta ahora a escala de laboratorio o planta piloto señalan que si el corte es insuficiente, bien por escasa velocidad o tiempo, los gránulos formados son de gran tamaño y en la agitación posterior tienden a desgarrarse y desmenuzarse, dando lugar a niveles más altos de pequeñas partículas de caseína y grasa en el lactosuero (6, 13). Asimismo, se ha descrito que cuando el tamaño del gránulo de la cuajada es pequeño, menor es la distancia que tiene que recorrer el lactosuero en su interior para ser expulsado del gel, consiguiéndose una distribución más homogénea de la humedad en la pasta prensada del queso freso (12).

El proceso de recalentamiento con agitación de los gránulos de cuajada ayuda a expulsar el lactosuero debido a la presión ejercida por la velocidad de los gradientes en el líquido, y a la colisión entre sí de los gránulos de cuajada y con los mezcladores, que hace que los gránulos se compriman por cortos periodos de tiempo. La temperatura tiene un marcado efecto sobre la sinéresis ya que, a mayor temperatura de recalentamiento la sinéresis se acelera y, por tanto, el contenido de humedad retenida en los gránulos es menor (7). El mismo efecto parece ocurrir al aumentar el tiempo de agitación, mientras que cuando se utilizan mayores velocidades de agitación se incrementa la pérdida de componentes en el lactosuero (6). En todo caso, los efectos combinados de los parámetros de corte y de recalentamiento parecen ser los más determinantes del contenido de humedad en el queso fresco, así como de la pérdida de compuestos en el lactosuero (6, 13).

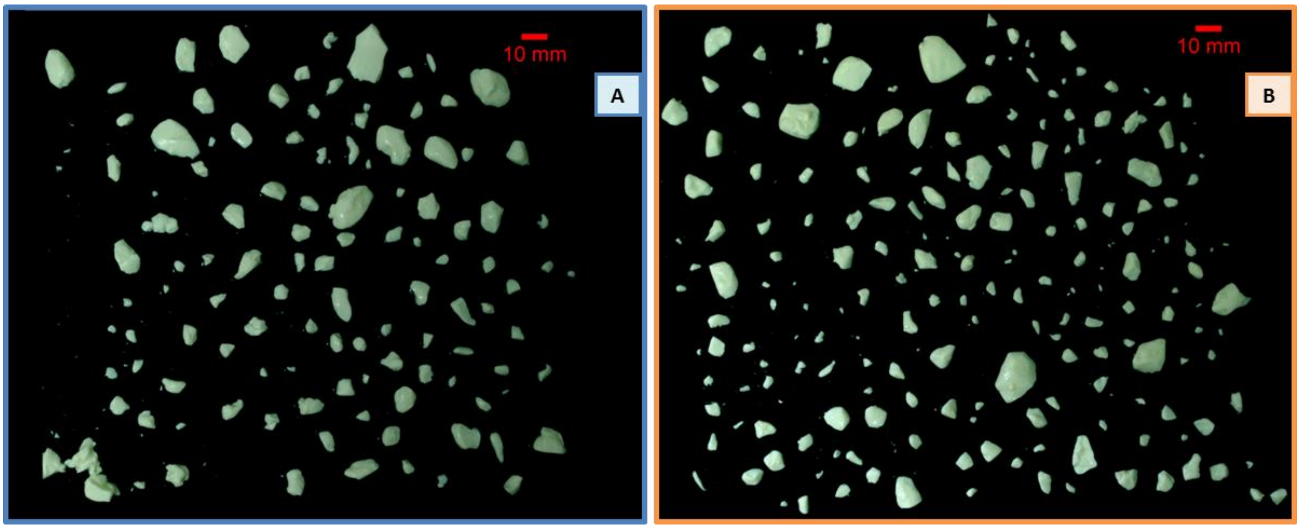

Figura 2. Gránulos de cuajada tras el corte (A) y recalentamiento (B) obtenidos durante la producción de queso DO Idiazabal en una pequeña quesería rural.

En la Tabla 2 se muestran valores de los parámetros tecnológicos de corte y recalentamiento utilizados en queserías rurales de la DO Idiazabal. Tanto en el corte como en el recalentamiento, la temperatura se mantiene constante alrededor de los 30 ºC y 36 ºC, respectivamente, mientras que el tiempo y la velocidad de agitación varían de forma notable en ambos procesos. Teniendo en cuenta la variabilidad observada en los parámetros tecnológicos del corte y recalentamiento de la cuajada, nuestro grupo de investigación está llevando a cabo un estudio de caracterización (forma, tamaño y distribución) de los gránulos de cuajada obtenidos en las fabricaciones comerciales de las pequeñas queserías rurales. El objetivo de la investigación es relacionar los parámetros de corte y agitación con las características físico-químicas de los gránulos de cuajada y del queso fresco obtenido, así como con el rendimiento quesero y con la cantidad y composición del lactosuero producido. La Figura 2 muestra imágenes de gránulos de cuajada obtenidos tras el corte y recalentamiento en una pequeña quesería, y la Figura 3 los correspondientes histogramas de distribución de frecuencia de áreas de los gránulos en cada una de dichas operaciones. Como se puede observar, tras el proceso de corte, las áreas de los gránulos de cuajada varían desde 4 hasta 252 mm^2, mostrando gran heterogeneidad en el tamaño y forma de los mismos. Tras el recalentamiento, los gránulos de cuajada sufren una disminución del tamaño debido a la sinéresis, por lo que aumenta significativamente el número de gránulos de tamaño inferior a 15 mm², aunque sigue manifestándose una alta heterogeneidad en formas y tamaños (Figuras 2 y 3).

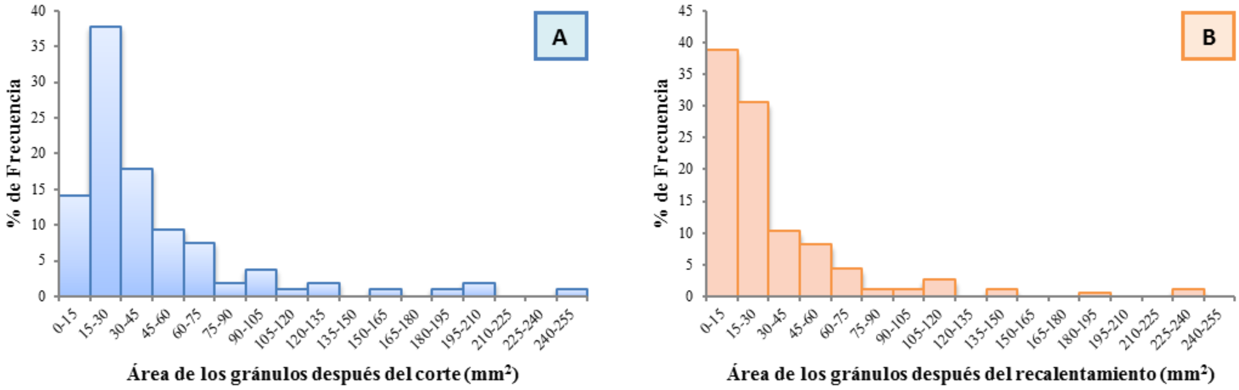

Figura 3. Distribución (expresada como porcentaje de frecuencia) de los tamaños de gránulos de cuajada tras el corte (A) y recalentamiento (B) obtenidos durante la fabricación de queso DO Idiazabal en una pequeña quesería rural.

Como anteriormente se ha comentado, las condiciones de corte y recalentamiento de la cuajada pueden influir de forma muy notable en el desuerado, en la pérdida de componentes de la leche en el lactosuero, y en definitiva en el rendimiento quesero. En la Tabla 3 se muestra la relación entre parámetros de corte y recalentamiento de la cuajada utilizados en queserías rurales de la DO Idiazabal (Tabla 2) sobre las pérdidas de grasa y proteína en el lactosuero, el tamaño de los gránulos de cuajada, y sobre el RT. Esta relación fue estudiada mediante un análisis de componentes principales con datos obtenidos a partir de fabricaciones comerciales en 10 queserías. Los resultados más relevantes mostraron que, en general, la pérdida de grasa y proteína soluble en el lactosuero era mayor cuanto mayor es la relación grasa/proteína de la leche. A mayor velocidad de corte y tiempo de recalentamiento mayor pérdida de grasa en el lactosuero, mientras que la pérdida de proteína en el lactosuero aumentaba con menor tiempo de corte y mayor velocidad de agitación durante el recalentamiento. El RT era negativamente afectado con mayores tiempos de corte y bajas velocidades de agitación durante el recalentamiento. Así mismo, el tamaño de los gránulos se vio reducido por el aumento del tiempo de corte y la velocidad de agitación durante el recalentamiento.

Posteriormente los gránulos se pre-prensan manualmente en la propia cuba (Figura 1) separando la cuajada del lactosuero. El prensado constituye la fase final del proceso de elaboración con interés para el RT del queso, y su finalidad es favorecer la fusión de los gránulos de cuajada en los moldes, expulsar el lactosuero sobrante, darle una forma cilíndrica estandarizada a la pasta de queso, así como favorecer la formación de la corteza (19). Tanto la temperatura, el tiempo y la fuerza de prensado, como la composición (principalmente el contenido en grasa) y pH de los gránulos de cuajada, son factores determinantes en esta etapa (7, 21). En la Tabla 2 se muestran parámetros técnicos de prensado utilizados en queserías rurales de la DO Idiazabal observándose diferencias importantes en los tiempos y fuerzas aplicadas.

Tabla 3. Relación entre parámetros tecnológicos de corte y recalentamiento de la cuajada con variables de composición de leche, pérdidas de componentes en lactosuero, rendimiento tecnológico y tamaño de gránulos de cuajada obtenidos en fabricaciones comerciales de queso DO Idiazabal en pequeñas queserías rurales (n = 10).

	Factores[1]		
Variables	**1**	**2**	**3**
Pérdida de proteína en el lactosuero	0,856		
Rendimiento tecnológico	0,776		
Relación grasa/proteína de la leche	0,727		
Tiempo de corte	-0,602	-0,559	
Tamaño del gránulo de cuajada tras el corte		0,900	
Tamaño del gránulo de cuajada tras el recalentamiento		0,798	-0,527
Velocidad de agitación durante el recalentamiento	0,484	-0,487	
Velocidad de corte			0,888
Pérdida de grasa en el lactosuero	0,500		0,762
Tiempo de recalentamiento			0,589

[1] Valores de correlación (entre -1 y 1) entre variables y factores resultantes del análisis de componentes principales.

Después del prensado, y antes del proceso de maduración, los quesos DO Idiazabal son sometidos a un proceso de salado, generalmente por inmersión en salmuera a temperatura entre 6 y 20 ºC durante tiempos no superiores a 24 horas (Tabla 2). La duración del salado la establece el pequeño elaborador en función del peso del queso, que oscila entre 1 y 3,5 kg, y la concentración de la salmuera, entre 13 y 20 ºBaumé. Este proceso, aunque en menor medida que las operaciones anteriormente descritas, también puede afectar al rendimiento quesero puesto que promueve la sinéresis de la cuajada prensada, reduciendo el contenido de humedad de la misma. La mayor o menor pérdida de agua está también influenciada por otros factores como la composición y compactación de la pasta, su contenido en grasa, calcio y humedad, y la concentración de sal y temperatura de la salmuera (16).

Algunos elaboradores añaden una etapa intermedia entre el salado y la maduración del queso llamada oreo, en la que el queso fresco se mantiene hasta un máximo de 30 días a temperaturas cercanas a 12ºC y humedad relativa del aire en torno al 80%. Durante el oreo y la maduración, el queso va perdiendo humedad progresivamente, lo cual depende del tamaño del mismo y de las condiciones de humedad relativa y temperatura de las cámaras (1). Estas condiciones serán las que determinen el RF del queso madurado (17). En general, las cámaras de maduración de los elaboradores de queso Idiazabal se encuentran a una temperatura entre 8 y 12 ºC, con una humedad relativa variable entre 80 y 90%, lo que conlleva pérdidas de peso de aproximadamente el 18% tras dos meses de maduración.

Conclusiones

El estudio *in situ* de los parámetros tecnológicos utilizados en las pequeñas queserías rurales permite un conocimiento profundo de las operaciones de fabricación y su relación con el rendimiento quesero tecnológico y comercial. Asimismo, las investigaciones en curso están favoreciendo la transferencia de buenas prácticas de elaboración a las pequeñas queserías rurales para, por una parte, maximizar el rendimiento quesero y reducir la cantidad y carga orgánica del lactosuero producido, y por otra, optimizar los parámetros tecnológicos relacionados especialmente con las operaciones de corte y desuerado en función de la época de producción. En este sentido, algunas prácticas recomendables para las pequeñas queserías rurales son aplicar menor velocidad de corte y tiempo de recalentamiento para disminuir la pérdida de grasa, en particular en la época de verano cuando la leche de oveja presenta mayor cantidad de grasa. Por otra parte, los elaboradores deberían tener en cuenta que el rendimiento tecnológico relacionado principalmente con el contenido de humedad del queso fresco puede afectarse negativamente con mayores tiempos de corte y bajas velocidades de agitación durante el recalentamiento. Dadas las grandes dificultades para la reutilización y/o recogida del lactosuero en las pequeñas queserías rurales, una vía de solución consistirá en la generación de un lactosuero de menor carga orgánica, así como la implementación de un proceso de disminución o eliminación de la materia orgánica *in situ* de importantes cantidades de lactosuero. De esta forma, será posible conseguir una adecuada optimización de los recursos, un aumento de la rentabilidad económica y una mejora de la sostenibilidad medioambiental de la explotación.

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Appendix B

Supplementary material Manuscript III

The supplementary material of the **Manuscript III** is provided in this Appendix.

Supplementary Table S1. Composition, pH and microstructure parameters (mean ± standard deviation) of the raw sheep milk used in the moderate and high intensity cheesemaking processes (n = 3).

	Moderate intensity	High intensity
Fat (g/100g)	7.2 ± 0.15	7.2 ± 0.22
Fat in dry matter (g/100g)	0.41 ± 0.01	0.42 ± 0.01
Protein (g/100g)	5.07 ± 0.16	4.87 ± 0.12
Protein in dry matter (g/100g)	0.29 ± 0.01	0.28 ± 0.00
Casein (g/100g)	3.86 ± 0.04	3.71 ± 0.09
Fat-to-casein ratio	1.86 ± 0.02	1.95 ± 0.06
Dry matter (g/100g)	17.47 ± 0.04	17.41 ± 0.45
Lactose (g/100g)	4.74 ± 0.07[b]	4.94 ± 0.09[a]
Calcium (mg/kg)	1494 ± 18[a]	1436 ± 10[b]
Magnesium (mg/kg)	162 ± 2[a]	155 ± 0.3[b]
Phosphorous (mg/kg)	1334 ± 43	1349 ± 17
Milk pH	6.64 ± 0.07	6.58 ± 0.02
FG sphericity	0.64 ± 0.01	0.63 ± 0.01
FG diameter[1] (µm)	5.39 ± 0.15	5.72 ± 0.32
Volume of globular fat (%)	64.35 ± 5.08	57.07 ± 4.80
Volume of non-globular fat (%)	1.06 ± 1.84	0.00 ± 0.00

[1]Volume-weighed mean diameter measured by 3D image analysis of confocal laser scanning micrographs.

[a-b] Means with different superscripts in the same row indicate significant differences ($P \leq 0.05$) between moderate and high intensity processes.

FG, fat globule.

Supplementary Table S2. Size and distribution parameters (mean ± standard deviation) of curd grains after cutting (FCG) and cooking (SCG) for the moderate and high intensity processes during Idiazabal PDO cheese production (n = 3) in small dairies.

	FCG		SCG	
	Moderate intensity	High intensity	Moderate intensity	High intensity
Perimeter	30.59 ± 4.84[a]	11.88 ± 0.76[b]	16.25 ± 0.76[a]	9.98 ± 0.71[b]
Maximum Feret diameter	10.80 ± 1.63[a]	4.13 ± 0.26[b]	5.82 ± 0.24[a]	3.53 ± 0.23[b]
Uniformity Index	7.91 ± 1.03[a]	6.72 ± 0.36[b]	4.96 ± 1.02[b]	9.20 ± 1.01[a]
Size range variation	99.99 ± 22.50	97.94 ± 5.98	115.23 ± 10.41	101.69 ± 12.43
Coefficient of uniformity	3.72 ± 0.23[b]	4.71 ± 0.41[b]	5.37 ± 0.66[a]	3.87 ± 0.51[b]
Graphic skewness	0.48 ± 0.01	0.44 ± 0.08	0.53 ± 0.06	0.50 ± 0.04
Graphic kurtosis	1.34 ± 0.38	1.11 ± 0.16	1.27 ± 0.05[a]	1.07 ± 0.06[b]

[a-b] Means with different superscripts in the same row and type of sample indicate statistically significant differences ($P \leq 0.05$) between moderate and high intensity processes.

Supplementary Table S3. Composition (mean ± standard deviation) of cheeses after pressing and 1 month of ripening for the moderate and high intensity processes during Idiazabal PDO cheese productions (n = 3) in small dairies.

	Cheese after pressing		1-Month ripened cheese	
	Moderate Intensity	High Intensity	Moderate Intensity	High Intensity
Fat (g/100g)	33.16 ± 0.67	32.98 ± 0.84	33.45 ± 0.49	33.24 ± 0.90
Protein (g/100g)	19.97 ± 0.29	20.09 ± 0.29	22.09 ± 0.28	21.75 ± 0.22
Moisture (g/100g)	42.43 ± 0.78	41.71 ± 0.66	38.15 ± 0.58	39.14 ± 0.63
Calcium (mg/kg)	6023 ± 54	6084 ± 68	-[1]	-[1]
Magnesium (mg/kg)	393 ± 2	384 ± 1	-[1]	-[1]
Phosphorous (mg/kg)	4688 ± 28	4728 ± 107	-[1]	-[1]

[a-b] Means with different superscripts in the same row and type of cheese sample indicate statistically significant differences ($P \leq 0.05$) between moderate and high intensity processes.
[1] No measurements were carried out in these samples.

Appendix C

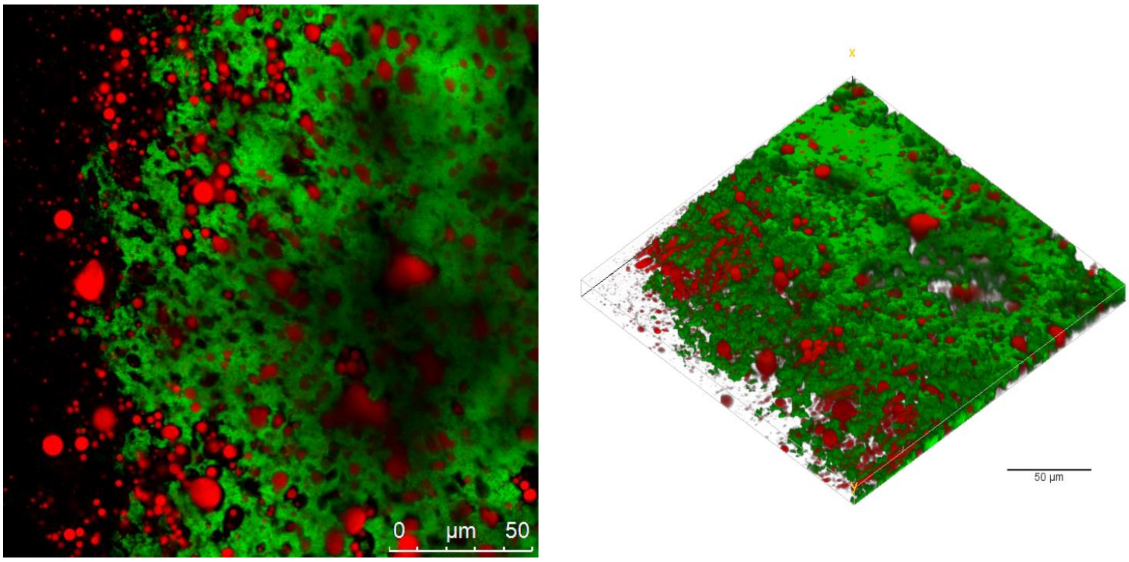
Supplementary material Manuscript IV

The supplementary material of the **Manuscript IV** is provided in this Appendix.

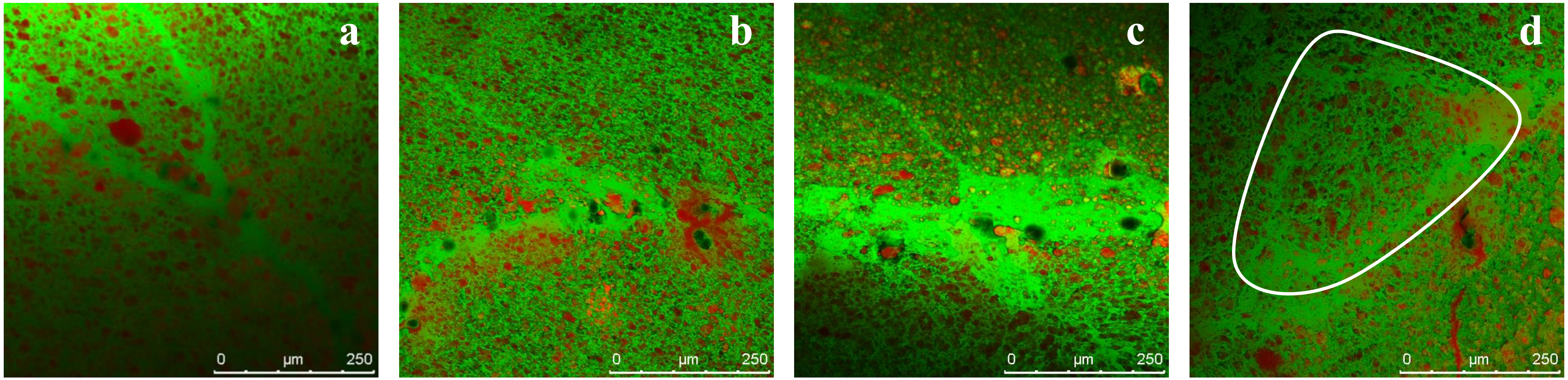
Online Resource 1. Chemical composition, rheological properties and fat globule size (mean ± standard deviation) of the raw ewe's milk used for experimental cheesemaking. Bulk milk was collected from a commercial farm on two consecutive weeks (n = 2).

Fat, g/100 g	6.14 ± 0.17
Protein, g/100 g	5.25 ± 0.14
Dry matter, g/100 g	17.91 ± 0.38
Calcium, mg/100 mL	158 ± 8
Magnesium, mg/100 mL	16 ± 1
Phosphorous, mg/100 mL	134 ± 14
Fat globule size D[4,3], µm[1]	4.29 ± 0.12
Onset gelation, s	882 ± 55
Curd firming rate, Pa/s	0.16 ± 0.03
Final curd firmness, Pa	320 ± 42

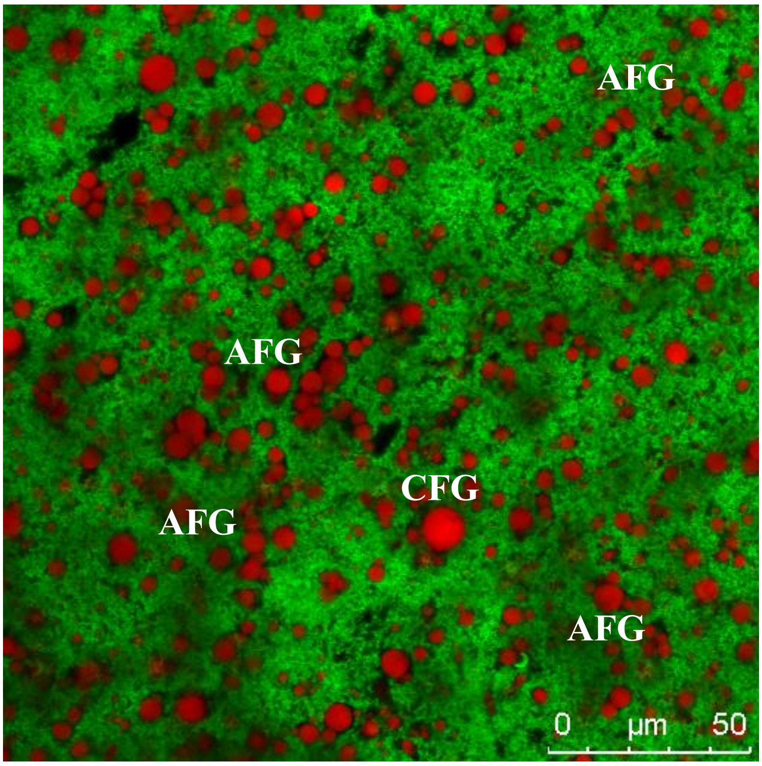
[1]Measured by light scattering.



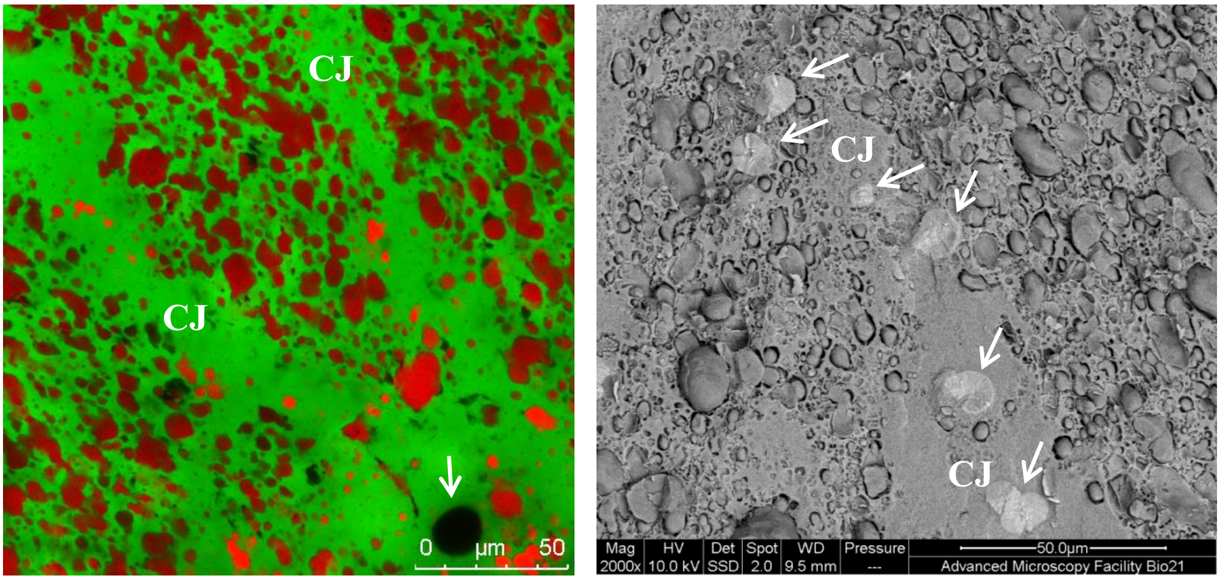
Online Resource 2: Confocal laser scanning microscopy micrograph in 2-D (left) and 3-D reconstruction (right) showing the release of fat globules from the curd grain (small size) after cooking (45 ºC). Images were acquired using a 63x objective lens. Fat appears red and protein appears green in this image. The scale bar is 50 µm in length.



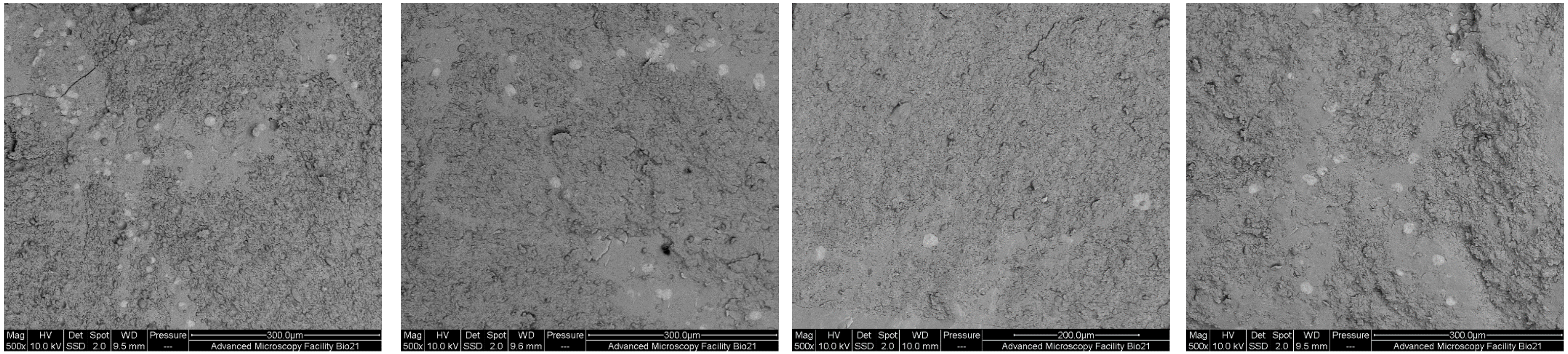
Online Resource 3: Confocal laser scanning microscopy images showing mini curd particles and curd junctions in cheese samples. Cheesemaking treatments were coded as: big *CGS* and 36 ºC *CT* (a); big *CGS* and 45 ºC *CT* (b); small *CGS* and 36 ºC *CT* (c); small *CGS* and 45 ºC *CT* (d). Fat appears red and protein appears green. The scale are 250 µm in length. The white outline indicates a mini curd particle completely surrounded by curd junctions.



Online Resource 4: Confocal laser scanning microscopy image of rennet-induced gel obtained using a 63x objective lens. Fat appears red and protein appears green in this image. The scale bar is 50 µm in length. AFG = aggregates of fat globule, CFG = coalesced fat globule.



Online Resource 5: Confocal laser scanning microscopy (left) and cryo-scanning electron microscopy (right) images showing curd grain junctions in cheese samples. For CLSM image, fat appears red and protein appears green. Scale bars of both images are 50 µm in length. White arrows indicate the presence of crystalline inclusions of calcium phosphate. CJ = curd junction.



Online Resource 6: Cryo-scanning electron microscopy images showing curd grain junctions and crystalline inclusions of calcium phosphate in cheese samples. Cheesemaking treatments were coded as: big *CGS* and 36 ºC *CT* (a); big *CGS* and 45 ºC *CT* (b); small *CGS* and 36 ºC CT (c); small CGS and 45 ºC *CT* (d). Scale bars of images 1, 2 and 4 are 300 µm and of image 3 is 200 µm in length.