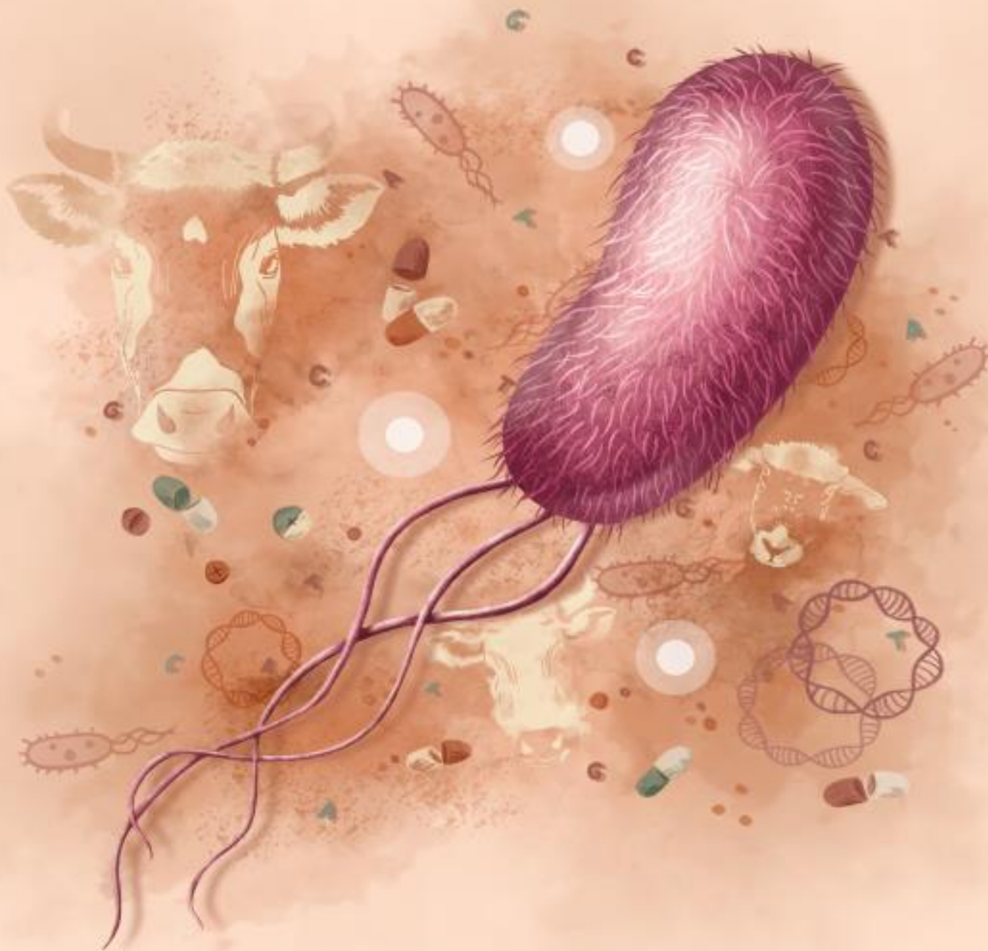
ESBL-, AmpC- and carbapenemase-producing commensal *Escherichia coli* in domestic ruminants:

Phenotypic and whole genome-based characterization to investigate antimicrobial resistance diversity and within-farm transmission dynamics

Universidad del País Vasco
Euskal Herriko Unibertsitatea



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PhD Thesis

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ESBL-, AmpC- and carbapenemase-producing commensal *Escherichia coli* in domestic ruminants: Phenotypic and whole genome-based characterization to investigate antimicrobial resistance diversity and within-farm transmission dynamics

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LIST OF ABBREVIATIONS

aac	Aminoglycoside acetyl transferase gene
aad	Adenylyltransferase gene
acr	TolC-associated efflux pumps gene
adk	Adenylate kinase gene
AIEC	Adherent-invasive *E. coli*
AMEG	Antimicrobial Advice Ad Hoc Expert Group
AMP	Ampicillin
ANOVA	Analysis of variance
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ant	Aminoglycoside nucleotidyltransferase gene
APEC	Avian pathogenic *E. coli*
aph	Adenylyltransferase gene
ARG	Antimicrobial resistance gene
ARG-ANNOT	Antibiotic resistance gene-annotation database
AST	Antibiotic susceptibility testing
BLAST	Basic local alignment search tool
BLEE	From Spanish “betalactamasas de espectro extendido”
bp	Base pairs
BPW	Buffered peptone water
CARD	Comprehensive antimicrobial resistance database
cat	Chloramphenicol acetyltransferase gene
CAV	From Spanish "Comunidad Autónoma Vasca"
CC	Clonal complex
CDS	Coding sequences
cfr	Chloramphenicol-florfenicol resistance gene
CFU	Colony forming units
CGE	Center for Genomic Epidemiology
cgMLST	Core-genome multilocus sequence typing
cgST	Core-genome sequence type
CHL	Chloramphenicol
CI	Confidence interval
CIA	Critically important antimicrobial
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
CLV	Clavulanic acid
cmlA	Chloramphenicol resistance gene
COL	Colistin
CP	Carbapenemase
CTX-M	CTX-M–type β-lactamases
DAEC	Diffusely adherent *E. coli*
DCT	Intramammary dry-cow therapy
DEC	Diarrheagenic *E. coli*
dfr	Dihydrofolate reductase gene
DGR	From Spanish "determinante genético de resistencia"
DNA	Desoxyribonucleic acid
E. coli	*Escherichia coli*
EAE	From Basque language "Euskal Autonomia Erkidegoa"
eae	Intimin gene
EAEC	Enteroaggregative *E. coli*

EARS-Net	Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off values
EFSA	European Food Safety Authority
e.g.	For example
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMA	European Medicines Agency
ent	Siderophore enterobactin gene
EPEC	Enteropathogenic *E. coli*
erm	Macrolide resistance gene
ESBL	Extended Spectrum β-lactamases
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee for Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
EZB	From Basque language "espektro zabaleko betalaktamasak"
FAO	Food and Agriculture Organization
FDA	United States Food and Drug Administration
fep	Ferric enterobactin transport gene
fimH	Type 1 fimbrial adhesine gene
floR	Chloramphenicol resistance gene
fos	Fosfomycin resistance gene
FOT	Cefotaxime
FOT/CLV	Cefotaxime/clavulaic acid
FOX	Cefoxitin
fumC	Fumarate hydratase gene
GDR	Genetic determinant of resistance
GEN	Gentamicin
glpt	Glycerol-3-phosphate transporter gene
gyrA	DNA gyrase A gene
gyrB	DNA gyrase B gene
HAC	High accuracy mode
HGT	Horizontal gene transfer
hlyE	Hemolysin E encoding gene
IBR	Infectious bovine rhinotracheitis
icd	Isocitrate/isopropylmalate dehydrogenase gene
ICE	Integrated conjugative elements
i.e.	That is
IMP	Imipenem
In	Integron
Inc	Incompatibility groups
InPEC	Intestinal pathogenic *E. coli*
IS	Insertion sequences
ISO	International Organization for Standardization
Kb	Kilobase
Km2	Square kilometre

L	Litre
lnu	Lincomycin resistance gene
M	Million
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of fly
Mb	Mega base pairs
mcr	Plasmid-mediated colistin resistance gene
mdf	Multidrug efflux protein encoding gene
mdh	Malate dehydrogenase gene
MDR	Multidrug-resistant
mer	Mercury resistance gene
mg	Milligram
mg/L	Milligrams per liter
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
mL	Millilitre
MLST	Multilocus sequence typing
MPEC	Mammary pathogenic *E. coli*
mph	Macrolide resistance gene
MRSA	Meticillin-resistant *Staphylococcus aureus*
NA	Not applicable
NAL	Nalidixic acid
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding RNA
NMEC	Neonatal meningitis-associated *E. coli*
NPV	Negative predictive value
NS	Not significant
nt	Nucleotide
OIE	World Organisation for Animal Health
ompA	Outer membrane protein A encoding gene
ONT	Oxford nanopore technology
OR	Odds ratio
ORadj	Adjusted odd ratio
***p*-value**	Probability-value
parC	DNA topoisomerase IV subunit encoding gene
parE	DNA topoisomerase IV subunit encoding gene
PBPs	Penicillin-binding proteins
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
PCU	Population correction unit
PFGE	Pulsed field gel electrophoresis
pMLST	PCR-based replicon subtyping
PMQR	Plasmid-mediated quinolone resistance
PPV	Positive predictive value
PRAN	Prom Spanish “Plan nacional de resistencia a antibióticos”
purA	Adenylosuccinate dehydrogenase gene
qnr	Ciprofloxacin resistance gene
qacE	Quaternary ammonium compounds resistance gene
RAM	From Spanish "resistencia a los antimicrobianos"

recA	ATP/GTP binding motif gene
RepA	Plasmid replication protein encoding gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
spp.	Species
SRA	Sequence read archive
ST	MLST sequence type
stb	Plasmid partitioning gene
STEC	Shiga toxin-producing *E. coli*
str	Aminoglycoside resistance gene
sul	Sulfonamide resistance gene
SUS	Susceptible
TAZ	Ceftazidime
TAZ/CLV	Ceftazidime/clavulanic acid
tet	Tetracycline resistance gene
TET	Tetracycline
Tn	Transposon
tra	Conjugative transfer gene
tRNA	Transfer ribonucleic acid
uhpA/T	Fosfomycin uptake system protein encoding genes
uidA	β-glucuronidase structural protein encoding gene
UPEC	Uropathogenic *E. coli*
UPGMA	Unweighted pair group method with arithmetic mean
UTI	Urinary tract infection
VF	Virulence factors
VFDB	Virulence factors database
WGS	Whole genome sequencing
WHO	World Health Organization
ZMW	Zero-mode waveguide
µL	Microlitre
µm	Micrometre
°C	Degree Celsius

LABURPENA

Antimikrobianoen aurkako erresistentzia (AMR, ingelesezko siglen arabera) osasun publikoan arazo larria da, gizakiei, animaliei eta ingurumenari eragiten baitie. Izan ere, elikagaiak ekoizten dituzten animaliak antimikrobianoekiko erresistenteak diren bakterioen gordailu garrantzitsu bat dira, gizakiengana zabal daitezkeenak. Enterobakterioetan, β-laktamikoen aurkako erresistentzia, bereziki hirugarren eta bigarren belaunaldiko zefalosporina eta karbapenemikoena, kezkagarria da. Aipaturiko antimikrobianoen aurkako erresistentziaren mekanismo garrantzitsuenak, Espektro Zabaleko Betalaktamasen (EZB), AmpC zefalosporinen eta karbapenemasen (CP) ekoizpena da. *E. coli* bakterio komentsalak erresistentzia-joerak kontrolatzeko maiz erabiltzen dira. Alde batetik EZB-, AmpC- eta CP-kodifikazio geneen gordailu izan daitezkeelako eta bestetik gene horiek enterobakterio komentsaletara edo patogenoetara sakabanatu ditzaketelako. EZB eta AmpCak ekoizten dituzten *E. coli*-ak gero eta gehiago isolatzen ari dira elikagaiak ekoizten dituzten animalietatik, CPak berriz oso gutxitan agertzen dira. Sentikortasun metodo fenotipikoak dira normalean erabiltzen direnak AMRaren detekzioa eta zaintza egiteko, hala nola mikrodiluzioa-saldan metodoa (erreferentzia metodoa). Metodo hauek erresistentziaren determinatzaile genetikoak (EDR) detektatzeko metodo molekularrekin konbinatzen dira askotan. Azken urteotan, teknologia honetan egindako hobekuntzengatik eta kostuen murrizketengatik, genoma osoen sekuentziazioa (WGS, ingelesezko siglen arabera) ohiko tresna molekularra bihurtu da bereizmen txikiagoko beste metodo batzuk ordezkatuz. Izan ere, EFSAk (Elikagaien Segurtasunerako Europako Agintaritza) AMRaren monitorizazioan, etorkizunean metodo fenotipikoak ordezkatzeko, WGSaren mailaz mailako integrazioa proposatu du. Testuinguru horretan, doktorego-tesi honen helburu orokorra Euskal Autonomia Erkidegoko (EAEko) hausnarkarietan agertzen diren EZBak, AmpCak eta CPak ekoizten dituzten *E. coli* komentsalen prebalentziari eta epidemiologiari buruzko ezagutza lortzea izan zen, WGS teknikak erabiliz isolatuen erresistentzia fenotipiko antimikrobianoa zehaztuz eta EDRen karakterizazioa eginez.

Doktorego-tesi honen lehenengo azterlana (**I. Azterlana**) EAEko esnetarako behi, haragitarako behi eta ardi aziendetan zefotaximarekiko erresistentea den *E. coli*-ren prebalentziari buruzko datu eguneratuak lortzeko egin zen. Horretarako, zeharkako

azterketa sektorial bat diseinatu zen, non 2014ko otsailetik 2016ko ekainera bitartean 300 hausnarkarien aziendetan (104 haragitarako behi-azienda, 82 esnetarako behi-azienda eta 114 ardi-azienda) ondesteko gorozkien laginak jaso ziren. Isolamendu selektiboko hazkuntza medioak erabiliz, EZB/AmpCak ekoizten dituzten *E. coli*-ak esnetarako behi-azienden % 32,9an detektatu ziren, haragitarako behi-azienden % 9,6an eta ardi-azienden % 7,0an. Hala ere, CPak ekoizten dituzten *E. coli*-rik ez zen detektatu. Antimikrobianoen aurkako sentikortasun fenotipikoa zehazteko, salda bidezko mikrodiluzioaren metodoa erabili zen, 135 isolatuentzako gutxieneko inhibizio-kontzentrazioak (MICak, ingelesezko siglen arabera) zehaztu ziren. Zefalosporinekiko erresistenteak ziren isolatu gehienak EZB fenotipoa erakutsi zuten. Bestalde, AmpC fenotipoa noizean behin identifikatu zen eta EZB+AmpC fenotipoa oso gutxitan aurkitu zen. β-laktamikoez gain, isolatu gehienak beste antimikrobiano klase batzuekiko erresistenteak ziren. Hortaz, erresistentzia oso altua izan zen tetraziklina eta sulfametoxazolarentzat, trimetoprima, ziprofloxazino, azido nalidixiko, kloranfenikol eta gentamizinarentzat. Ez hala azitromizinarentzat. Isolatu guztiak bakterio Gram (-) multi-erresistenteak eragindako infekzioak tratatzeko erabiltzen diren azken baliabideko antimikrobianoekiko sentikorrak izan ziren, hala nola imipenem, meropenem, kolistina eta tigeziklina. Isolatuen % 84,4ak (114/135) multi-erresistenteak ziren. EDRak identifikatzeko, hautaturiko 66 isolatuen genoma sekuentziatu zen irakurketa-zati laburreko Illumina WGSa erabiliz. Orokorrean, WGSaren eta erresistentzia fenotipikoaren profilen arteko konparazioak oso akordio ona erakutsi zuen (Kappa>0,90). Salbuespen bakarrak kloranfenikola (Kappa = 0,77) eta zefepimea (adostasun-balio txikiena izan zuena (Kappa = 0,14)) izan ziren. Andui gehienek $bla_{\text{CTX-M}}$ motako geneak zeramatzaten, $bla_{\text{CTX-M-14}}$ (n = 27) genea ohikoena izan zen EZB fenotipoarentzat eta berriz $bla_{\text{CMY-2}}$ (n = 9) genea AmpC fenotipoarentzat. EZB/AmpC gene gehienak IncI1 plasmidoetan aurkitzen ziren, beste AMR gene askorekin batera. Azterlan honen emaitzak EAEko hausnarkariak, batez ere esnetarako behiak, EZBak ekoizten dituzten *E. coli* multi-erresistenteen gordailuak direla plazaratu zuen.

Lehenengo azterlanean ikusi genuenez, esnetarako behi-aziendetan zefalosporinekiko erresistenteak ziren *E. coli* anduien prebalentzia handiagoa izan zen haragitarako behi-aziendetan eta ardi-aziendetan detektatutakoarekin konparatuz. Horren ondorioz, esnetarako behien aziendetan EZBak, AmpCak eta CPak ekoizten dituzten *E. coli*-en epidemiologia sakonago aztertzeko beharra nabarmendu zen. Doktorego-tesi honen bigarren azterlanean (**II. Azterlana**) EAEko abeltzaintza-jarduerak adierazten zituzten 5 esnetarako behi-azienda (F1, F2, F3, F4 eta F5) monitorizatu ziren, EZBak, AmpCak eta CPak ekoizten dituzten *E. coli*-en presentzia eta transmisio-dinamika zehazteko. Hamabi laginketa egin ziren 16 hilabete arteko epean, itxuraz osasuntsuak ziren animalien ondesteko gorozkien laginak jasoz hiru adin-taldeetako animalietan (txahaletan, bigantxetan eta esnealdiko behietan). Lagin fekalak isolamendu selektiboa jasan zuten zefotaxima zuten hazkuntza medioak erabiliz eta MICak zehaztuz, isolaturiko 197 *E. coli*-etan erresistentzia-profil fenotipikoak aztertu ziren. Bost aziendetan EZBak ekoizten zituzten *E. coli*-ak detektatu ziren, baina isolamendu-maiztasuna eta erresistentzia-profilak azienda eta adin-taldeen artean aldatzen zirela antzeman zen, oro har, erresistentzia anitzeko anduien presentzia ohikoa izanik. Beraz, bigantxen aldean, esnealdiko behietan eta txahaletan prebalentzia handiagoa zen. Erresistentzia fenotipoetan aniztasun txikiena (F1) eta altuena (F4) zuten bi aziendetako 41 isolatu genetikoki karakterizatu ziren, ONT (Oxford Nanopore Technology) irakurketa-zati luzeko WGS teknologia erabiliz. Lehenengo azterlanean ikusi genuen bezala, zefalosporinekiko erresistentzia plasmidoetan kodetutako bla_{CTX-M} geneen ondorio izan zen batez ere. Hala ere, azienda bakoitzean EZB geneen transmisio eredu ezberdinak antzeman ziren. F1-ean bi *E. coli* andui ezberdinek luzaroan iraun zuten aziendan (seguruenik hedapen klonikoarengatik), biek EZB-kodifikazio gene bera ($bla_{CTX-M-1}$), IncX1 plasmidoan kokatua zeramaten. Bestalde, F4-an *E. coli* genotipoen aniztasun handia zegoen, ziurrenik iturri anitzeko kutsadura-gertaeren ondorio gisa. Bi azienden artean desberdintasunak antzeman ziren arren, EZBak ekoizten dituzten *E. coli*-en genotipo desberdinetan erresistentzia-gene kopuru bera duten zenbait plasmido-mota berdin hauteman ziren. Honen agerpenak, aziendetan zirkulatzen duten anduien arteko plasmido-transferentzia horizontala ohikoa izan zela adieraz

dezake, erresistentzien barreiadura erraztuz. Azterlan honetan, zaintza genomikoa esnetarako behi-aziendetan gertatzen den multi-erresistentzia hedatzearen azpian dagoen epidemiologia konplexua aztertzeko funtsezkoa dela frogatu zen.

EDRak detektatzeaz gain, WGSak bakterioak identifikatzeko eta genomikoki karakterizatzeko duen erabilgarritasuna frogatu zen I. eta II. azterlanetan egindako laginketetatik eratorritako bi isolatuen profil genomikoen karakterizazio sakonaren bidez. Horrela, **III. Azterlanean**, II. Azterlanean detektaturiko eta karbapenemekiko erresistentea zen isolatu baten genoma (*E. coli* EC1110) berreraiki zen Illumina – ONT sekuentziazio eta muntaketa hibrido bat erabiliz. Andui honek IncC motatako plasmido bat zeraman (pEC1110_NDM-1), zeinak $bla_{\text{NDM-1}}$ genea eta beste AMR gene batzuk kodifikatzen zituen. $bla_{\text{NDM-1}}$ genea ARI-A erresistentzia anitzeko eskualdean kokatzen zen. Alboetan IS*Aba*125 txertatze-sekuentzia eta bleomicina-erresistentzia genea aurkitzen ziren (ble_{MBL}), azkenengo honen ondoren etendako $\Delta bla_{\text{DHA-1}}$ gene bat topatzen zelarik. ARI-A eskualdeak AMR gene gehiago ere bazituen: sulfonamidaren erresistentzia-genearen bi kopia (*sul1*), trimetoprimaren erresistentziari lotutako *dfrA*12 genea, eta amikazinari (*aph(3')-VI*) eta estreptomizinari (*aadA2*) erresistentzia ematen dieten geneak. Sulfonamidaren erresistentzia *sul2* genea beste erresistentzia anitzeko eskualde batean kokatzen zen, ARI-B. pEC1110_NDM-1 plasmidoaren egiturak antzekotasun handia zuen $bla_{\text{NDM-1}}$ geneak kodifikatzen zituzten beste IncC plasmidoekin, baita elikagaiak ekoizten zituzten animalietatik eratorritako eta CP-generik ez zuten beste enterobakterio batzuetan aurkitzen ziren IncC plasmidoekin ere. Dakigunez, hau da behietan NDM-1 ekoizten zuen *E. coli* baten lehen deskribapena, IncC plasmido batean $bla_{\text{NDM-1}}$ genea daramana. CPak ekoizten zituzten beste *E. coli* batzuk isolatzeko egin ziren hurrengo saiakerak ez zuten arrakastarik izan. Honek iradokitzen du animalien arteko zirkulazioa ez zela ohikoa eta noizbehinkako aurkikuntza zela.

IV. Azterlanean, I. Azterlanaren esparruan haragitarako behi-azienda batetik berreskuratutako isolatu baten (E690) identifikazio zehatza eta karakterizazio genomikoa egiteko irakurketa-zati laburrerako Illumina WGS erabili zen. Hasiera batean isolatu hori *E. coli* gisa identifikatu zen isolamendu selektiboko metodoetan, morfologia

bateragarrian, profil biokimikoan eta PCRan oinarritutako *uidA* genearen detekzioan oinarrituta. Hala ere, genoma Illumina WGS erabiliz sekuentziatu zenean *E. coli* espeziearekin antzekotasun maila baxua zuela antzeman zen. E690 genoma genetikoki pareka konparatu zenean maila genomikoan pareko anduiekin, *E. marmotae*-ren genoma hurbileneko kointzidentzia zela identifikatu zen. Gainera, bi genoma horien arteko konparazio intergenomikoak (DNA-DNA hibridazio digitalaren eta G+C edukiaren bidez egin zena) E690 anduia *E. marmotae* espeziekoa zela baieztatu zuen. *E. marmotae* espeziea 2015ean deskribatu zen lehen aldiz *Marmota himalayana*-ren gorozkien laginetan isolatu eta gero, baina ez zen deskribatu Himalayako marmota ez zen beste animalia ezta gizakietan ere doktorego-tesi hau proposatzerakoan. Ikerketa honetan, WGSak fenotipikoki bereizezinak ziren bi hurbil-hurbileko *Escherichia* espezieak bereizteko duen gaitasuna frogatu zuen.

Laburbilduz, doktorego-tesi honen emaitzek, EAEko hausnarkariak eta, bereziki, esnetarako behiak EZBak ekoizten dituzten *E. coli* multi-erresistenteen gordailuak direla baieztatzen dute. Era berean, aziendetan AMRaren hedatzearen atzean epidemiologia konplexu bat dagoela ikusi da. Azkenik, EDRen detekzioan eta zaintzan, eta baita AMR bakterioaren identifikazioan eta karakterizazio genomikoan ere WGSaren erabilgarritasuna frogatu zen.

RESUMEN

La resistencia a los antimicrobianos (RAM) es un importante problema para la salud pública que afecta a los seres humanos, los animales y al medio ambiente. Los animales de abasto son un importante reservorio de bacterias resistentes a los antimicrobianos y fuente de infección para los humanos. En la familia de las enterobacterias, la resistencia a los β-lactámicos, en particular la resistencia a las cefalosporinas de tercera y cuarta generación y los carbapenemes, es motivo de preocupación. Entre los mecanismos de resistencia a los β-lactámicos, la producción de β-lactamasas de espectro extendido (BLEE), cefalosporinasas AmpC y carbapenemasas (CP) son los más importantes. Los aislados de *E. coli* comensales, por su capacidad de adquirir genes de resistencia y por su potencial de transferirlos a otras enterobacterias (comensales o patógenas), son considerados indicadores y se utilizan con frecuencia para monitorizar la evolución de las resistencias. En los animales de abasto se aíslan de manera frecuente cepas de *E. coli* productoras de BLEE y AmpC, mientras que las cepas productoras de CP solo se detectan de manera ocasional. Para la detección y vigilancia de la RAM se emplean de manera habitual pruebas de susceptibilidad fenotípica (el método de referencia es la microdilución en caldo) que se combinan de manera ocasional con métodos moleculares para la detección de determinantes genéticos de resistencia (DGR). Durante estos últimos años, la secuenciación de genomas completos (WGS, por sus siglas en inglés) se ha convertido en una herramienta molecular cada vez más utilizada, llegando a reemplazar otros métodos de menor resolución. La EFSA (Autoridad Europea de Seguridad Alimentaria) ha propuesto una integración gradual de WGS en la monitorización de la RAM con objeto de reemplazar a los métodos fenotípicos en el futuro. En este contexto, el objetivo general de esta tesis doctoral es estudiar la epidemiología de *E. coli* comensal productor de BLEE, AmpC y CP en rumiantes domésticos de la CAV (Comunidad Autónoma Vasca) a través de la determinación de su resistencia fenotípica y su caracterización molecular utilizando diferentes técnicas de WGS.

El primer estudio de la presente tesis doctoral (**Estudio I**) se planteó con objeto de estimar la prevalencia a nivel de rebaño de *E. coli* comensal productor de BLEE, AmpC y CP en ganado bovino de leche, bovino de carne y ovino de la CAV. Para ello se

diseñó un estudio transversal en el que se tomaron muestras de heces rectales en 300 rebaños (104 de bovino de carne, 82 de bovino de leche y 114 de ovino) entre febrero de 2014 y junio de 2016. Mediante aislamiento en medio selectivo con cefotaxima se aisló *E. coli* productor de BLEE/AmpC en el 32,9 % de los rebaños de bovino lechero, el 9,6 % de los rebaños de bovino de carne y en el 7,0 % de los rebaños de ovino, mientras que en ninguno de esos rebaños se aisló *E. coli* productor de CP. Para la determinación de los perfiles fenotípicos de resistencia se utilizó el método de microdilución en caldo, y los valores de concentración mínima inhibitoria (CMI) obtenidos para un total de 135 aislados se interpretaron utilizando los valores de corte epidemiológicos (ECOFF) establecidos por EUCAST (European Committee on Antimicrobial Susceptibility Testing). La mayoría de los aislados mostraron un fenotipo BLEE, mientras que el fenotipo AmpC se identificó solo ocasionalmente y el fenotipo BLEE+AmpC de manera muy puntual. Una gran proporción de los aislados fueron co-resistentes a otras clases de antimicrobianos además de a los β-lactámicos. Así, la resistencia fue muy alta a tetraciclina y sulfametoxazol, alta a trimetoprima, ciprofloxacino, ácido nalidíxico, cloranfenicol y gentamicina y baja a azitromicina. En total, el 84,4% de los aislados (114/135) fueron multi-resistentes. Sin embargo, todos los aislados fueron susceptibles a antimicrobianos que son utilizados como último recurso para el tratamiento de infecciones causadas por bacterias Gram (-) multi-resistentes tales como imipenem, meropenem, colistina y tigeciclina. Con el fin de identificar los DGR, se secuenció el genoma completo de una selección de 66 aislados utilizando la tecnología Illumina. La comparación entre los DGR detectados y los perfiles de resistencia fenotípica mostró, de manera general, una concordancia muy buena (Kappa>0,90), excepto para cloranfenicol (Kappa=0,77) y cefepima, que presentó el valor de concordancia más bajo (Kappa=0,14). La mayoría de las cepas caracterizadas genéticamente portaban genes de tipo $bla_{CTX\text{-}M}$, siendo el gen $bla_{CTX\text{-}M\text{-}14}$ (n=27) el más común para el fenotipo BLEE, mientras que el gen $bla_{CMY\text{-}2}$ (n=9) fue el DGR predominante para el fenotipo AmpC. La mayoría de los genes BLEE/AmpC estaban ubicados en plásmidos IncI1, en los que también se localizaban una gran variedad genes de resistencia a otros antimicrobianos. Los resultados de este estudio mostraron que los rumiantes en la CAV, y en particular

el ganado bovino lechero, son importantes reservorios de cepas de *E. coli* productoras de BLEE y multi-resistentes.

En vista de la mayor prevalencia de cepas de *E. coli* resistentes a la cefotaxima en el bovino de leche en comparación con el bovino de carne y ovino observada en el Estudio I, se planteó la necesidad de explorar más a fondo la epidemiología de la infección en los rebaños de bovino lechero. Por consiguiente, en el **Estudio II**, se seleccionaron cinco granjas de bovino lechero (F1, F2, F3, F4 y F5) representativas de las prácticas de manejo de la CAV que se monitorizaron a lo largo de 12 muestreos durante un período de hasta 16 meses para determinar la presencia y la dinámica de transmisión de *E. coli* productor de BLEE, AmpC y CP. En cada muestreo se tomaron muestras de heces rectales de animales aparentemente sanos pertenecientes a tres grupos de edad (terneras, novillas y vacas en lactación) que se sometieron a aislamiento selectivo de *E. coli* en medio con cefotaxima y se determinaron los valores de CMI de 197 aislados. De esta manera, se aislaron *E. coli* resistentes a cefotaxima en las cinco granjas, aunque la frecuencia de aislamiento y los perfiles de resistencia fenotípica variaron entre granjas y grupos de edad. En general, la prevalencia fue mayor en vacas en lactación y terneras que en novillas y en las granjas F1 y F2 en comparación con las otras tres. Una selección de 41 aislados procedentes de las granjas con la diversidad de perfiles de resistencia fenotípicos más baja (F1) y más alta (F4) se caracterizaron genéticamente utilizando la tecnología de WGS de lectura de fragmentos largos ONT (Oxford Nanopore Technology). Tal y como observamos en el Estudio I, la resistencia a las cefalosporinas de tercera y cuarta generación se asoció principalmente a la presencia de genes *bla*$_{CTX\text{-}M}$ que estaban codificados en plásmidos. No obstante, se observaron diferentes patrones de diseminación de *E. coli* productores de BLEE en cada granja. En F1 predominaron dos cepas distintas, presentes a lo largo de múltiples muestreos y en todos los grupos de edad, y ambas portadoras del mismo gen BLEE (*bla*$_{CTX\text{-}M\text{-}1}$) ubicado en plásmidos de tipo IncX1, posiblemente como resultado de la persistencia y expansión clonal de unas pocas cepas. Por el contrario, en F4 se observó una gran diversidad de genotipos con una amplia variedad de genes BLEE en diferentes tipos de plásmidos, probablemente como resultado de varios eventos de

contaminación con distintos orígenes. A pesar de las diferencias observadas entre ambas granjas, la presencia de ciertos tipos de plásmidos con el mismo repertorio de genes de resistencia en diferentes genotipos de *E. coli* BLEE indica que la transferencia horizontal de plásmidos entre las cepas que circulan dentro de las granjas podría haber sido frecuente, favoreciendo así la diseminación de genes de resistencia. En general, este estudio demostró que las herramientas de secuenciación masiva aplicadas a la vigilancia genómica son muy útiles para estudiar la compleja epidemiología subyacente a la diseminación de la resistencia a múltiples antimicrobianos en las granjas de ganado bovino lechero.

Más allá de su utilidad en la detección de los DGR, la secuenciación de genomas completos puede ser también fundamental para la identificación y caracterización bacteriana como se demostró en los **Estudios III y IV**. En el **Estudio III**, se combinaron las tecnologías de secuenciación Illumina-ONT para conseguir un ensamblaje híbrido y reconstruir el genoma de un *E. coli* resistente a los carbapenemes (EC1110) aislado de heces de terneras en una de las granjas monitorizadas en el Estudio II. Esta cepa portaba un plásmido IncC (pEC1110_NDM-1) que contenía el gen *bla*$_{NDM-1}$ junto con otros genes de resistencia a varios antimicrobianos. El gen *bla*$_{NDM-1}$ se localizaba en la región de multi-resistencia ARI-A, flanqueada aguas arriba por secuencia de inserción *ISAba*125 y aguas abajo por el gen de resistencia a bleomicina *ble*$_{MBL}$, seguido del gen truncado *Δbla*$_{DHA-1}$. La región ARI-A también contenía otros genes de resistencia: dos copias del gen de resistencia a las sulfonamidas *sul1*, el gen *dfrA*12 asociado con la resistencia a la trimetoprima y genes que confieren resistencia a la amikacina (*aph(3')-VI*) y la estreptomicina (*aadA2*). Además, en una segunda isla de resistencia antimicrobiana (ARI-B) se detectó el gen de resistencia a las sulfonamidas *sul2*. El plásmido pEC1110_NDM-1 compartía un alto grado de similitud estructural con otros plásmidos IncC portadores de genes *bla*$_{NDM}$ además de con plásmidos de enterobacterias aislados de animales de abasto que no contenían genes de resistencia a los carbapenemes. Esta es la primera descripción en ganado bovino de una cepa de *E. coli* portadora del gen *bla*$_{NDM-1}$. Los sucesivos intentos de aislar otras cepas de *E. coli*

productoras de CP en el rebaño no tuvieron éxito, lo que sugiere que se trató de un hallazgo puntual y esta cepa no estaba circulando en la granja.

En el **Estudio IV**, se empleó la tecnología de WGS Illumina para la identificación y la caracterización del perfil genómico de un aislado (E690) procedente de un rebaño de bovino de carne incluido en el Estudio I. Este aislado se identificó originalmente como *E. coli* en base a los métodos de aislamiento selectivo, su morfología compatible, el perfil bioquímico y la detección del gen *uidA* por PCR. Sin embargo, se observó que el genoma compartía un nivel de similitud relativamente bajo con la especie *E. coli*. El análisis filogenético del genoma E690 y los genomas de las cepas tipo de especies genéticamente relacionadas en el servidor TYGS (Type Strain Genome Server) permitió identificar al genoma de *Escherichia marmotae* como el más cercano. Además, la comparación intergenómica de ambas cepas (hibridación digital DNA-DNA y contenido en G+C) confirmó que la cepa E690 pertenecía a la especie *E. marmotae*. Esta nueva especie del género *Escherichia* se describió por primera vez en 2015 a partir de las heces de *Marmota himalayana*, pero en el momento en el que se propuso esta tesis doctoral no se había descrito ni en humanos ni en otros animales distintos a la marmota del Himalaya. En este estudio se demostró el poder discriminatorio de la técnica de WGS para diferenciar estas dos especies pertenecientes al género *Escherichia* que están estrechamente relacionadas y que son fenotípicamente indistinguibles.

En conclusión, los resultados de esta tesis doctoral confirman que los rumiantes de la CAV, y en particular el bovino lechero, son importantes reservorios de *E. coli* productor de BLEE y multi-resistente. Asimismo, se han aportado datos para dilucidar la compleja epidemiología que subyace a la diseminación de la RAM en las granjas de rumiantes. Por último, la técnica de WGS ha demostrado ser útil para la detección y vigilancia de las resistencias, así como para identificación y caracterización genómica de bacterias resistentes a los antimicrobianos.

SUMMARY

Antimicrobial resistance (AMR) is an urgent global health threat that affects humans, animals, and the environment. In fact, food-producing animals are a relevant reservoir of AMR bacteria that can be disseminated to humans. In Enterobacteriaceae, resistance to β-lactams, particularly resistance to third- and higher-generation cephalosporins and carbapenems, is a matter of concern, being the production of β-lactamases (extended spectrum β-lactamases (ESBLs), AmpC-type β-lactamases and carbapenemases (CP)) the most important resistance mechanism. Commensal *E. coli* is frequently used as an indicator for monitoring resistance trends as it can act as reservoir of ESBL-, AmpC- and CP-encoding genes that can be transferred to other commensal or pathogenic Enterobacteriaceae. ESBL- and AmpC-producing *E. coli* are isolated with increasing frequency from food-producing animals, whereas CPs are only occasionally detected. For AMR detection and surveillance, phenotypic susceptibility testing methods such as broth microdilution (gold standard method) are commonly used, occasionally combined with targeted molecular methods for the detection of genetic determinants of resistance (GDRs). Due to the continuous improvement and decrease in costs, whole genome sequencing (WGS) has become a common molecular tool, replacing other lower-resolution methods. In fact, the EFSA (European Food Safety Authority) has proposed to follow a gradual integration of WGS in AMR monitoring to replace phenotypic methods. In this context, the general objective of this PhD Thesis was to generate knowledge on the prevalence and epidemiology of ESBL-, AmpC- and CP-producing commensal *E. coli* in domestic ruminants in the Basque Country, determining their antimicrobial phenotypic resistance and characterizing the GDRs using WGS techniques.

The first study of the present PhD Thesis (**Study I**) was carried out to specifically obtain updated data on herd-prevalence of cefotaxime-resistant commensal *E. coli* in the dairy cattle, beef cattle and sheep population in the Basque Country. For this purpose, a cross sectional study was designed and rectal faecal samples were collected in 300 ruminant herds (104 beef cattle, 82 dairy cattle, and 114 dairy sheep) between February 2014 and June 2016. Using selective isolation media, ESBL-/AmpC-producing *E. coli* were recovered from 32.9% of dairy cattle herds, 9.6% of beef cattle herds and

7.0% of sheep flocks. On the contrary, no CP-producing *E. coli* were detected. Phenotypic antimicrobial susceptibility was determined by broth microdilution, obtaining minimum inhibitory concentrations (MIC) from a total of 135 isolates. MIC values were interpreted using epidemiological cut-off values (ECOFF) as developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Most of the cefotaxime-resistant *E. coli* isolates recovered from ruminants showed an ESBL phenotype, the AmpC phenotype was occasionally identified and the ESBL+AmpC phenotype was nearly absent. A large proportion of the isolates exhibited co-resistance to other antimicrobial classes besides β-lactams. Thus, resistance was very high to tetracycline and sulfamethoxazole, high to trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol and gentamicin, and low to azithromycin. Multidrug resistance was found in 84.4% of the isolates (114/135). However, all isolates were susceptible to last-resort antimicrobials currently used to treat infections caused by multi-drug resistant Gram (-) bacteria such as imipenem, meropenem, colistin, and tigecycline. The genome of a selection of 66 isolates was sequenced using Illumina short-read WGS and screened for GDRs. Comparison of WGS and phenotypic resistance profiles showed an overall very good agreement (Kappa>0.90), the only exceptions being chloramphenicol (Kappa=0.77) and cefepime, which presented the lowest agreement value (Kappa=0.14). Most of the isolates carried $bla_{\text{CTX-M}}$ type genes, being $bla_{\text{CTX-M-14}}$ (n=27) the most common gene responsible for the ESBL phenotype, whereas $bla_{\text{CMY-2}}$ (n=9) was the prevailing resistance determinant of the AmpC phenotype. Most ESBL/AmpC genes were located in IncI1 plasmids, which also carried a great variety of other AMR genes. These results showed that ruminants in the Basque Country, in particular dairy cattle, are reservoirs for MDR ESBL-producing commensal *E. coli*.

The higher prevalence of cefotaxime-resistant *E. coli* in dairy cattle in comparison to beef cattle and sheep in the cross-sectional Study I highlighted the need to further investigate the epidemiology of transmission of such *E. coli* within dairy cattle herds. Thus, in **Study II**, five dairy cattle farms (F1, F2, F3, F4 and F5) that represented the style of farming in the Basque Country were monitored to determine the occurrence and transmission dynamics of ESBL-, AmpC- and CP-producing *E. coli*.

Twelve samplings were performed over a period of up to 16 months, collecting rectal faeces from apparently healthy animals in three age groups (calves, heifers, and lactating cows). Faecal samples were subjected to selective isolation in cefotaxime-containing media and MICs were determined for 197 isolates. Cefotaxime-resistant *E. coli* were detected in the five farms but isolation frequency and resistance profiles varied among age groups and farms. Overall, prevalence was higher in lactating cows and calves compared to heifers, and lower in F1 and F2 compared with the other three farms. A selection of 41 isolates from the two farms that showed the lowest (F1) and largest (F4) AMR profile diversity were further characterized using long-read Oxford Nanopore Technology (ONT) WGS. Again, resistance to cephalosporins was mainly due to plasmid-encoded $bla_{\text{CTX-M}}$ genes but different patterns of transmission of ESBL genes were observed in each farm. In F1, two distinct strains, both carrying the same ESBL-encoding gene ($bla_{\text{CTX-M-1}}$) located in IncX1 plasmids predominated in the farm probably due to clonal expansion of a few strains that persisted in the farm over a long time. On the other hand, in F4, a large diversity of genotypes was observed, probably as the result of multiple source contamination events. In both farms, the presence of certain plasmid types with the same repertoire of ARGs in different *E. coli* MLST types strongly suggested the occurrence of horizontal transfer of such plasmids among strains circulating within the farms. This study demonstrated the power of genomic surveillance in deciphering the complex epidemiology underlying multidrug resistance dissemination within dairy cattle farms.

Besides the detection of GDRs, the usefulness of WGS for bacterial identification and characterization was further proven by the in-depth genomic profiling of two particular isolates of interest derived from the samplings carried out in Studies I and II. Thus, in **Study III**, the genome of a CP-resistant isolate (*E. coli* EC1110) that was detected in one of the farms monitored in Study II was reconstructed using a hybrid Illumina–ONT sequencing and assembly approach. The strain carried an IncC plasmid (pEC1110_NDM-1) that harboured the $bla_{\text{NDM-1}}$ gene along with several other ARGs. The $bla_{\text{NDM-1}}$ gene was located in the ARI-A multi-resistance region flanked upstream by *ISAba*125 and downstream by the bleomycin resistance gene ble_{MBL},

followed by a truncated *Δbla*$_{\text{DHA-1}}$ gene. The ARI-A region also contained further AMR genes: two copies of the sulfonamide resistance gene *sul1*, the *dfrA*12 gene associated with resistance to trimethoprim, and genes that confer resistance to amikacin (*aph(3')-VI*) and streptomycin (*aadA2*). The sulfonamide resistance gene *sul2* was present in a second antimicrobial resistance island, ARI-B. The plasmid pEC1110_NDM-1 shared a high degree of backbone similarity with other *bla*$_{\text{NDM}}$-harbouring IncC plasmids but also with plasmids from Enterobacteriaceace isolated from food-producing animals that did not contain CP-producing genes. To the best of our knowledge, this was the first report of an NDM-1-producing *E. coli* isolated from cattle that carried the *bla*$_{\text{NDM-1}}$ gene in a IncC plasmid. Further attempts to isolate other CP-producing *E. coli* in the herd were unsuccessful, suggesting that the circulation within the herd was improbable and that this was a sporadic finding.

In **Study IV**, short-read Illumina WGS was employed for the accurate identification and the in-depth genomic profiling of an isolate (E690) recovered from beef cattle in the frame of Study I. This isolate was originally identified as *E. coli* based on the selective isolation methods, compatible morphology, biochemical profile, and PCR-based detection of the *uidA* gene. However, the genome which was sequenced using short-read Illumina WGS, shared a relatively unusual low level of similarity with the *E. coli* type species. Genome-scale phylogenetic analysis of the E690 genome and closely related strain genomes performed at the Type (Strain) Genome Server (TYGS), identified an *E. marmotae* genome as the closest match and intergenomic comparison of these two genomes (by digital DNA-DNA hybridization and G+C content) confirmed that strain E690 belonged to the species *E. marmotae*. *E. marmotae* was first described in 2015 from the faeces *of Marmota himalayana* but had not been described in humans or animals other than the Himalayan marmot at the time when this PhD Thesis was proposed. In this study, WGS demonstrated its usefulness to distinguish between these two closely related *Escherichia* species that were phenotypically indistinguishable.

All in all, the results of this PhD Thesis confirm that in the Basque Country, ruminants and in particular dairy cattle, are reservoirs of MDR ESBL-producing

commensal *E. coli* and data have been provided to elucidate the complex epidemiology underlying AMR spread within ruminant farms. The usefulness of WGS in GDR detection and surveillance, as well as in the identification and genomic characterization of AMR bacteria was demonstrated.

1. GENERAL INTRODUCTION

1.1. Antimicrobial use and spread of resistance: a global public health threat

1.1.1. Antimicrobial resistance (AMR)

The term antimicrobial refers to an agent or substance derived from any source (microorganisms, plants, animals, synthetic or semisynthetic) that acts against any type of microorganism: bacteria (antibacterial), mycobacteria (antimycobacterial), fungi (antifungal), parasite (antiparasitic) or virus (antiviral). On the other hand, antibiotics are a type of antimicrobial product produced from microorganisms that can act against another living microorganism (WHO, 2019). Under the action of antimicrobials, susceptible microorganisms are either killed or their growth inhibited.

Alongside vaccines and public health measures to control transmission of communicable diseases, antimicrobials have helped to dramatically reduce mortality from infectious diseases during the 20th century, becoming an extremely valuable resource across the spectrum of modern medicine. In the last decades, the “golden age” of antibiotics and their efficacy is threatened by the global increase in antimicrobial resistance (AMR) and the dwindling supply of new antimicrobials.

AMR is a natural phenomenon that occurs when microorganisms such as bacteria, viruses, fungi and parasites, by modifications in their genetic material become resistant to antimicrobials and can multiply and persist in their presence. The main drivers of AMR are overuse and misuse (inappropriate choices, inadequate dosing and/or poor adherence to treatment guidelines) of antimicrobials both in human and veterinary medicine. Nowadays many bacteria are resistant to several (multi-resistant) and sometimes all (pan-resistant) antimicrobials available for clinical therapy making infections harder to treat and increasing the risk of disease spread, severe illness and death.

Historically, the development of resistance to antimicrobial drugs appeared soon after their introduction. In fact, resistance to penicillin, the first β-lactam antibiotic discovered by Alexander Fleming, was recorded shortly after its onset in clinical use in the 1940s (Abraham and Chain, 1940). Over the years, in order to deal with AMR, new classes of antimicrobials were developed either by the isolation of novel antimicrobials

from nature within a particular class or by the development of synthetic analogs of an existing class. However, nowadays, despite the need of new antimicrobial medicines to treat AMR infections, there has been a drought in research and development implemented for that purpose, especially among big pharmaceutical companies. Therefore, the speed of discovery of new antimicrobials is far slower than the pace of emergence of AMR thus limiting treatment options for some serious and life-threatening diseases.

AMR is a global public health crisis in which humans, animals and the environment are involved. The problem is so great that the World Health Organization (WHO) has declared that AMR is one of the top 10 global public health threats facing humanity (https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019). The impact of antibiotic resistance in terms of mortality and public health cost is quite difficult to estimate, and there are few studies addressing this issue. A review on AMR commissioned by the UK Government argued that AMR could kill 10 million people per year by 2050 (O'Neill, 2014) if actions are not taken to tackle the problem. A more recent systematic analysis based on data from around the world using predictive statistical modelling estimated that in 2019 AMR was directly responsible for 1.27 million deaths globally (*i.e.*, deaths that would not have occurred had the infections been drug-susceptible), but also associated with 4.95 million deaths (*i.e.*, where a drug-resistant infection had been implicated in the death, but resistance itself may or may not have been the direct cause) (Murray et al., 2022).

1.1.2. Antimicrobial use (AMU) in veterinary medicine

The majority of antimicrobial drugs used in veterinary medicine belong to a small number of major classes, and in the past 30 years, few new classes of antimicrobials have been introduced for use in food-producing animals (Prescott, 2018). Only some are limited to veterinary use (*e.g*. flavophospholipols, ionophores) mainly due to its toxicity to humans. Antimicrobials such as colistin (polymyxin), that was predominantly used in food producing animals because of its nephrotoxicity and

neurotoxicity activity in humans, was reintroduced in human medicine as a last resort to treat pan-resistant bacteria.

Like in humans, in food-producing animals, antimicrobials are mainly used for therapeutic purposes. However, in veterinary medicine treatment can be applied to individual animals or a group of animals (metaphylaxis). Thus, when a disease is present within a herd/flock, antimicrobials can sometimes be administered in feed or water to the entire group (*e.g.* pens of pigs, flocks of broilers) (Baptiste and Kyvsgaard, 2017). However, in compliance with the new Regulation (EU) 2019/6 which came into effect on 28 January 2022, metaphylaxis should only be used when the risk of spread of an infection or an infectious agent in the group of animals is high and no other appropriate alternatives are available (Article 107(4)). Similarly, antimicrobials shall not be used for prophylaxis other than in exceptional cases and applying it to a specific animal when the risk of an infection is very high and the consequences are likely to be severe (Article 107(3)).

On the other hand, antimicrobials were widely used for many years in food-producing animals as growth promoters, *i.e.*, substances other than the nutrients added in the ration that increase the rate of growth and improve the conversion rate of healthy and correctly fed animals. This long-term, sub-therapeutic dosage of antimicrobials for the purpose of growth promotion favoured the selection and spread of AMR bacteria. This situation led to changes in the legislation regarding the use of antibiotics as growth promoters during the years and, in 2006, the European Union (EU) completely banned its use. However, policy largely differs in different regions of the world and 42 countries still reported the use of antimicrobial agents for growth promotion (OIE, 2021).

In Europe, the European Medicines Agency (EMA) annually collects data on antimicrobial use (AMU) in animals from European countries and elaborates a report describing levels and trends in consumption of veterinary antimicrobials along the years. According to the last ESVAC (European Surveillance of Veterinary Antimicrobial Consumption) report published in 2021 (EMA, 2021), which includes data from 2010 to 2020, Spain was the second country in the EU behind Cyprus with the highest levels of

AMU in livestock from 2010 to 2015, reaching in 2014 a peak of 418.8 kg/PCU (Population Correction Unit, technical unit of measurement used for estimated weight at treatment of livestock and of slaughter animals; 1 PCU = 1 kg). A large decrease in AMU was reported from 2015 onwards (EMA, 2021) after the implementation in June 2014 of a national plan to combat resistance (Plan Nacional frente a la Resistencia a los Antibióticos, PRAN). The sales of colistin, which is almost exclusively used in food-producing animals (ECDC et al., 2021), declined by nearly 70% between 2011 and 2018 in food-producing animals in the EU and Spain (EMA, 2021). The decrease in the use of colistin in Spain was mainly attributed to a nearly 100% reduction in use in the pig sector (https://www.resistenciaantibioticos.es/es/programa-reduce-porcino). According to the antimicrobials sales data for Spain included in the last ESVAC report, tetracyclines (279.9 tonnes of active substance) and penicillins (425.1 tonnes of active substance) were the highest selling classes of antimicrobials in 2020 for food-producing animals. Other antimicrobials such as fluoroquinolones and third- and fourth-generation cephalosporins were less used in food-producing animals (the sales in mg/PCU accounted for 2.5% and 0.2% of the total sales respectively). For fluoroquinolones a decrease in their sales was observed (from 10.2 mg/PCU in 2012 to 3.7 mg/PCU in 2020) whereas the sales of third and fourth-generation cephalosporins remained at low and stable levels during the years (around 0.3 mg/PCU since 2011 to 2020) (ECDC et al., 2021; EMA, 2021). The overall mean sales of antimicrobials for food-producing animals in the EU in 2020 was 89.0 mg/PCU, compared with 154.3 mg/PCU in Spain. This indicates that it is still necessary to continue implementing measures to promote the prudent use of antimicrobials in veterinary medicine in Spain.

AMR has limited the therapeutic choices available and this has in turn led to an increased pressure on human and veterinary medicine to reduce and prudently use antimicrobial agents. Action plans against AMR propose other preventive and therapeutic options while restricting the use of antimicrobials to those situations where they are indispensable. Antimicrobials should not be used to compensate for poor hygiene or inadequate animal husbandry (FAO, 2016; OIE, 2016). When needed,

antimicrobials must be prudently used, and drug classifications determining priority of use are an important and practical tool.

Institutions such as the WHO, OIE (World Organisation for Animal Health), EMA or FDA (U.S. Food and Drug Administration) have created different lists ranking antimicrobials for use in humans and animals. Those lists might vary depending on the region of application (among other factors), but they are all based on the WHO List of Critically Important Antimicrobials for Human Medicine (WHO CIA List) (WHO, 2019) that classify antimicrobial classes into three categories (critically important, highly important or important) according to two criteria:

- Criterion 1: The antimicrobial class is the sole or one of limited available therapies to treat serious bacterial infections in humans
- Criterion 2: The antimicrobial class is used to treat infections in humans caused by either: (i) bacteria that may be transmitted to humans from non-human sources, or (ii) bacteria that may acquire resistance genes from non-human sources

Critically important antimicrobial (CIA) classes meet the first and second criteria (*e.g.* carbapenems, third- and fourth-generation cephalosporins, some penicillins, macrolides, polymyxins and quinolones, between others); highly important antimicrobials meet one of the two criteria (*e.g.* first and second generation cephalosporins, narrow spectrum penicillins, sulfonamides and tetracyclines, between others); and, important antimicrobials meet neither of the two criteria.

The OIE has also produced a list of antimicrobials of veterinary importance (OIE, 2019). In line with the WHO list, the OIE list is divided in veterinary CIAs (aminoglycosides, all generation cephalosporins, macrolides, penicillins, phenicols, quinolones, sulfonamides (alone or in combination with trimethoprim), and tetracyclines), veterinary highly important antimicrobials (ansamycin/rifamycins, fosfomycin, ionophores, lincosamides, pleuromutilins, and polypeptides), and veterinary important antimicrobials (bicyclomycin, fusidic acid, novobiocin, orthosomycins, quinoxalines, and streptogramins).

In the EU, veterinarians are encouraged to check and consider EMA's updated scientific advice on the categorisation of antimicrobials when prescribing these medicines for animals in their care (EMA/AEMEG, 2019). The EMA categorisation of antimicrobials for use in food-producing animals comprises four categories:

- Category A ("Avoid") includes antimicrobials that are currently not authorised in veterinary medicine in the EU. These medicines may not be used in food-producing animals and may be given to individual companion animals only under exceptional circumstances. It includes carbapenems, glycylcyclines, and monobactams, between others.
- Category B ("Restrict") refers to critically important antimicrobials in human medicine whose use in animals should be restricted to mitigate the risk to public health. It includes third- and fourth-generation cephalosporins, polymyxins and fluoroquinolones.
- Category C ("Caution") comprises antimicrobials for which alternatives in human medicine generally exist in the EU, but only few alternatives are available in certain veterinary indications. These antimicrobials should only be used when those in Category D would not be clinically effective. In this category are first and second generation cephalosporins, aminopenicillins with β-lactamase inhibitors, aminoglycosides and macrolides, among others.
- Category D ("Prudence") includes antibiotics that should be used as first line treatments whenever possible. These antibiotics should be used in a prudent manner, *i.e.*, unnecessary use and long treatment periods should be avoided, and group treatment restricted to situations where individual treatment is not feasible. It includes tetracyclines, sulfonamides, narrow-spectrum penicillins and aminopenicillins without β-lactamase inhibitors, among others.

1.1.3. AMR in food-producing animals: surveillance and monitoring programs

Research studies addressing AMR in food-producing animals were first published in the second half of the 20th century. In 1968, a study described infections

caused by multidrug-resistant bacteria in the UK in calves in the United Kingdom (Anderson, 1968). This study was perhaps the first one drawing attention into the human health implications of the careless use of antimicrobials in animal farming. In the following years, the first AMR monitoring programs commenced in various European countries (Martel and Coudert, 1993; Wray et al., 1993). In 1995 the Danish Integrated Antimicrobial Resistance Monitoring Program (DANMAP) was established. This national program forms a successful blueprint that has been replicated by other countries, as it monitors both the resistance in selected food-animal indicator organisms and pathogens, and the use of antimicrobials in human and animal medicine.

Nowadays, most livestock-associated AMR surveillance programs focus on three main sources (cattle, pigs and poultry) and on three categories of bacteria (human and animal pathogens, zoonotic foodborne bacteria and commensal indicator bacteria). In the EU, the Directive 2003/99/EC on the surveillance of zoonoses and zoonotic agents and AMR was reinforced with the publication of Commission Decision 2013/652/EU, which established rules on monitoring AMR in zoonotic and commensal bacteria in food-producing animals and food. After December 31, 2020, it was repealed by Implementing Decision 2020/1729/EU, which establishes new standards for harmonized surveillance and notification of AMR in food-producing animals from 2021 to 2027. Thus, monitoring and reporting of AMR in food-producing animals and food shall cover the following zoonotic and commensal bacteria, which represent the major risks of AMR spread to humans via food, water and environmental contamination:

- *Salmonella* spp., focused on multidrug-resistant *Salmonella*
- *Campylobacter coli* and *Campylobacter jejuni*
- Indicator commensal *E. coli*
- *Salmonella* spp. and *E. coli* producing the following enzymes:
    - Extended Spectrum β-Lactamases (ESBL)
    - AmpC β-lactamases (AmpC)
    - Carbapenemases (CP)

On voluntary basis, the monitoring and reporting of AMR may also cover indicator commensal *Enterococcus faecalis* and *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* (MRSA).

1.1.4. Tackling Antimicrobial resistance from a One Health perspective

One Health is defined as an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. It recognizes that health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and inter-dependent (One Health Commission, 2021). It is essential to address AMR using a One Health approach. In fact, AMR is an ecological problem characterized by complex interactions involving diverse microbial populations. Humans, animals and their associated environments like hospitals, farms and aquaculture environments can act as AMR reservoirs. The cycling of AMR bacteria and their genes can also occur in water, soil, wildlife, and many other ecological niches, due to pollution by sewage, pharmaceutical industry waste, and manure runoff from farms (Fig. 1).

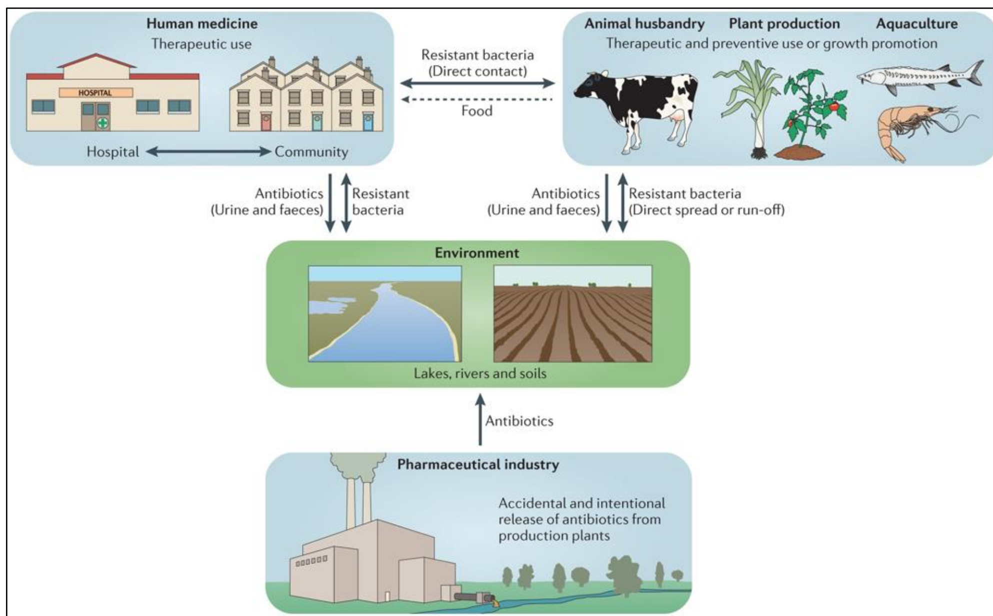

Figure 1. An overview of the ecology of antimicrobials showing how these drugs are cycled between different environments, such as the medical environment, agricultural settings, the aquacultural environment, the pharmaceutical industry and the wider environment. Image reprinted from (Andersson and Hughes, 2014).

AMR control mobilizes multiple sectors, disciplines and communities. As mentioned before, WHO and other international agencies such as FAO, OIE or EFSA have developed actions plans to tackle AMR, all of them using a "One Health" approach (FAO, 2016; OIE, 2016; WHO, 2017; EFSA and ECDC, 2022).

1.2. Escherichia coli

1.2.1. Taxonomic classification and general characteristics

In the family Enterobacteriaceae, classified in the phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales, more than 50 genera and hundreds of species and subspecies have been described (Murray et al., 2020). Enterobacteriaceae are ubiquitous bacteria found worldwide in soil, water, and vegetation and are part of the commensal intestinal flora of most warm-blooded vertebrates (Tenaillon et al., 2010). Within the Enterobacteriaceae family, the genus *Escherichia* includes four species, *Escherichia coli* (Castellani and Chalmers, 1919), *Escherichia fergusonii* (Farmer et al., 1985), *Escherichia albertii* (Huys et al., 2003), and the recently described *Escherichia marmotae* (Liu et al., 2015), along with several cryptic clades (Clermont et al., 2011). *E. marmotae*, formerly ascribed to "*Escherichia* cryptic clade V", was isolated in Qinghai-Tibet plateau in 2012 from the faeces of Himalayan marmot (*Marmota himalayana*) and described as a novel species in 2015 (Liu et al., 2015). *In vitro* studies for virulence testing determined its potential as a human pathogen (Liu et al., 2019). When this PhD Thesis was proposed, *E. marmotae* had not been described in humans or animals other than the Himalayan marmot.

E. coli, a Gram (-), lactose-fermenting, non-sporulating facultative anaerobe rod, is the most common and important member of the genus. *E. coli* cells are typically 1.1–1.5 µm wide, 2–6 µm long and can be either motile or non-motile, producing lateral flagella (rather than polar) when motile. In addition to flagella, many strains produce other appendages such as fimbriae or pili, which play a role in the attachment to other cells or host tissues (Kaper et al., 2004). *E. coli* carry strain-specific antigens such as the O lipopolysaccharide antigen, the H flagellar antigen, whose combination is used for

serotyping (Fratamico et al., 2016) and the K capsular polysaccharide antigen. *E. coli* is one of the best characterized model organisms, key in the advancement of genetics, molecular biology, physiology and biochemistry (Tenaillon et al., 2010). Moreover, *E. coli* has long be an indicator of anthropogenic impact on the environment serving as water quality indicator (Anjum et al., 2021).

1.2.2. Pathogenicity and clinical impact (in humans and animals)

Although most *E. coli* strains play a beneficial or harmless role for their hosts, there are also pathogenic members within this species classified into two main groups depending on the pathology: extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC). While InPEC are accurately distinguished from the commensal strains based on certain virulence factors (VF), this is not as simple with ExPEC since they behave as opportunistic pathogens that can colonize the intestinal environment without causing harm to the host. Within the ExPEC group, uropathogenic *E. coli* (UPEC) is the main cause of urinary tract infections (UTIs), avian pathogenic *E. coli* (APEC) causes colibacillosis in poultry and neonatal meningitis-causing *E. coli* (NMEC) strains are associated with meningitis and lung or wound infections in newborns (Bélanger et al., 2011; Riley, 2014; Poirel et al., 2018; Denamur et al., 2021). Mammary pathogenic *E. coli* (MPEC) is responsible for causing mastitis in dairy animals, including cattle (Shpigel et al., 2008; Richards et al., 2015).

On the other hand, within InPEC, seven main subgroups are recognized: (1) Shiga toxin-producing *E. coli* (STEC), which includes the subgroup of enterohaemorrhagic *E. coli* (EHEC), (2) enteropathogenic *E. coli* (EPEC), (3) enterotoxigenic *E. coli* (ETEC), (4) enteroinvasive *E. coli* (EIEC), (5) enteroaggregative *E. coli* (EAEC), (6) diffusely adherent *E. coli* (DAEC) and (7) disease-associated adherent-invasive *E. coli* (AIEC) (Nataro and Kaper, 1998; Kaper et al., 2004; Denamur et al., 2021). ETEC stains are associated with enteric pathologies in animals, causing diarrhoea in calves (Kolenda et al., 2015), whereas in humans EPEC and STEC (including EHEC subgroup that cause from self-limited diarrhoea to more severe illness such as haemorrhagic colitis or haemolytic uremic syndrome) are considered as food-borne

pathogens for which cattle can constitute a reservoir (Kolenda et al., 2015; Mughini-Gras et al., 2018; Oporto et al., 2019). Combinations of virulence factors among the classic *E. coli* pathotypes have been described. Some examples of these hybrid pathovars are ExPEC/STEC strains that cause diarrhoea and extraintestinal infections simultaneously or those have been involved in important outbreaks such as the highly virulent EAEC/STEC (Santos et al., 2020).

1.2.3. *E. coli* as an indicator of AMR

E. coli has several characteristics which make it particularly suitable as AMR indicator. *E. coli* has a widespread distribution as it is a very commonly detected commensal enteric bacteria in both humans and animals and can also be found in nature and food (Dorado-García et al., 2018; EFSA and ECDC, 2022). *E. coli* acts as a potential reservoir of transferrable AMR genes; it can acquire AMR genes from other bacteria and transfer them to other commensal and pathogenic Enterobacteriaceae (Blake et al., 2003; Poirel et al., 2018). Furthermore, *E. coli* is a versatile, easily cultured and one of the most studied and well characterized microorganisms. In consequence, this bacterium is frequently used as an indicator of resistance used in surveillance studies to monitor resistance trends in food-producing animals (Aarestrup et al., 1998; EFSA, 2012; ECDC et al., 2021; EFSA and ECDC, 2022).

1.2.4. Genomics and Phylogeny

E. coli presents great plasticity, genetic diversity and its population structure and evolution have been studied in depth over time (Tenaillon et al., 2010). The *E. coli* chromosome shows a G+C content of 50.4% - 50.8%, includes 3,900-5,800 genes and it can range in size from 4.2 to 6.0 Mb (average of 5 Mb) (Denamur et al., 2021). Variations in the chromosome size are predominantly derived from recombination events in which large (> 10 kb) DNA fragments (also known as genomic islands) are acquired (Lukjancenko et al., 2010). All the strains of the species share a group of approximately 2,000 genes, known as the core genome, while the pan-genome (the total number of

genes found in the different strains of this species) is expanding as more strains are sequenced (Denamur et al., 2021). A recent pan-genome study included a total of 55,039 genes (Horesh et al., 2021).

The phylogenetic structure of *E. coli* has been intensely studied. Historically, four phylogroups have been recognized, A, B1, B2, and D (Clermont et al., 2000; Tenaillon et al., 2010) and three more were added later, phylogroups C (closest relative to B1), F (similar to phylogroup B2), and E to which many D members were reassigned (Clermont et al., 2013). Later, phylotype G was characterized as an intermediate phylogroup between B2 and F (Clermont et al., 2019). In a recent analysis using whole genome sequencing (WGS), 12 *E. coli* phylogroups (G, B2-1, B2-2, F, D1, D2, D3, E2(O157), E1, A, C, and B1) were differentiated (Abram et al., 2021). Strains from different phylogroups vary in metabolic characteristics and the presence of virulence genes. Overall, B2, D, G, and F, usually associated with ExPEC strains, carry a higher number of virulence genes compared to phylogroups A, B1, C and E that include commensal strains and those that cause digestive pathology (Denamur et al., 2021).

Currently, *E. coli* population genetic structure studies are predominantly based on multilocus sequencing typing (MLST). The Achtman *E. coli* scheme is the most widely used for this species (Clermont et al., 2015) in which partial sequences of seven house-keeping genes (*adk*, *fumC*, *gyrA*, *icd*, *mdh*, *purA* and *recA*) are sequenced (Wirth et al., 2006). Different sequences of each gene fragment are assigned a distinct allele number and the combination of alleles for the seven loci defines the allelic profile or sequence type (ST). MLST profiles for the *Escherichia* genus and other Enterobacteria are stored and curated at the Enterobase database (Zhou et al., 2020), which currently includes more than 13,400 STs (last access: 13/07/2022). On the other hand, based on the same principle, core genome multilocus sequence typing (cgMLST) uses *in silico* detection of 2,513 core genes (last access: 13/07/2022) from *E. coli* WGS data to assign an allelic number to each gene sequence and a core genome sequence type (cgST) to the combined profile (Zhou et al., 2020).

1.3. Antimicrobials and AMR mechanisms in *E. coli*

1.3.1. AMR mechanisms and types of resistance

Resistance in bacteria can be intrinsic or acquired. Bacteria are intrinsically resistant to a certain antimicrobial when they lack its target of action (*e.g.* the lack of a cell wall in *Mycoplasma* makes it intrinsically resistant to β-lactams and all antimicrobials that target the cell wall) or as a consequence of other functional or structural characteristics inherent to the species (IFT, 2006). *E. coli* is intrinsically susceptible to almost all clinically relevant antimicrobial agents but has a great capacity to accumulate resistance genes and acquire resistance. Resistance acquisition occurs by vertical transmission (chromosomal mutations such as single point mutations or regulatory mutations that usually affect gene expression mechanisms) or through horizontal transmission (horizontal gene transfer, HGT) mechanisms (Poirel et al., 2018). HGT occurs when genes are transferred from one cell into another through three main mechanisms: (a) transformation, when parts of free DNA are taken up by the bacteria from the external environment; (b) transduction, when bacteria-specific viruses (bacteriophages) transfer DNA between two closely related bacteria; and (c) conjugation, when DNA is mobilized from a donor bacterium to a recipient bacterium, requiring physical contact and conjugative machinery. Conjugation is thought to have the greatest influence on the dissemination of antimicrobial resistance genes (ARGs) in a broader host bacteria range, while transformation and transduction are deemed less important, although recent discoveries suggest their role may be larger than previously thought (von Wintersdorff et al., 2016; Liu et al., 2022). Passage of resistance can occur through the transfer of a single ARG or combinations of ARGs inserted in mobile structures also known as mobile genetic elements (MGE).

To inhibit bacterial growth, antimicrobials should pass through the cell wall without being metabolized in order to reach their target sites at a sufficient concentration to exert their action. The major antibiotic targets and mechanisms of resistance that bacteria have evolve to overcome their action are indicated in Figure 2.

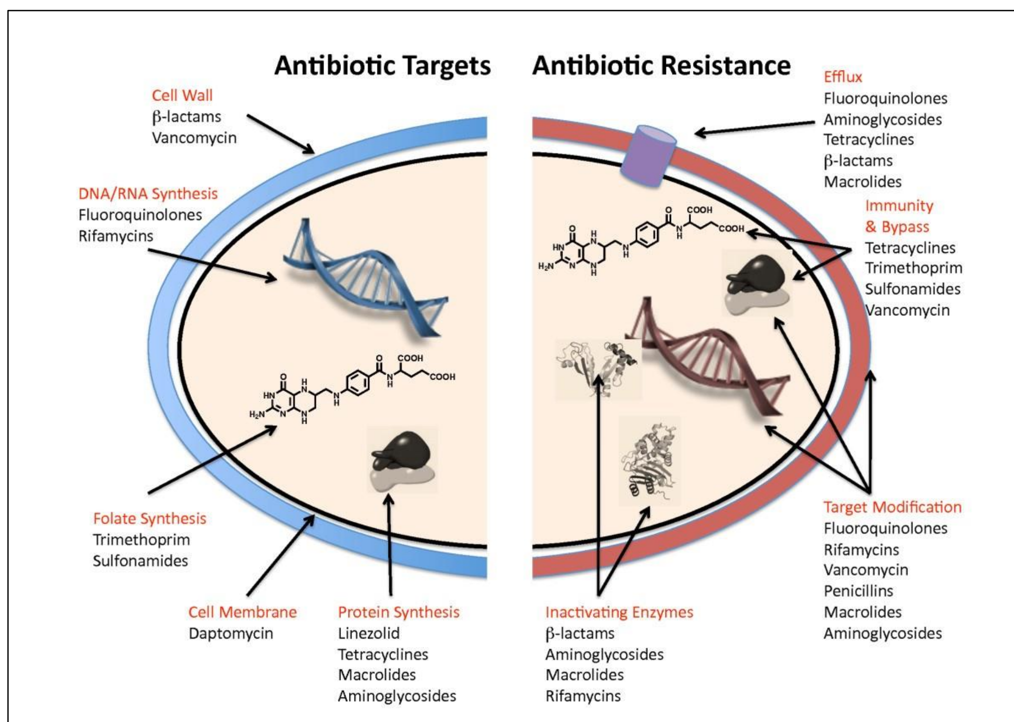

Figure 2. Antibiotic targets and mechanisms of resistance. Image reprinted from (Wright, 2010).

1.3.2. Resistance to β-lactam antibiotics

1.3.2.1. β-lactam antibiotics: classification and activity

β-lactam antibiotics are bactericidal agents that interrupt bacterial cell-wall formation as a result of their covalent binding to essential penicillin-binding proteins (PBPs), enzymes that are involved in the terminal steps of peptidoglycan cross-linking in both Gram (-) and Gram (+) bacteria (Bush and Bradford, 2016). This group of antibiotics share a common basic structure called β-lactam ring; a heterocyclic ring formed by cycling of an amide group. According to their β-lactam core ring and chemical structure, β-lactam antibiotics are classified into five important groups with a particular spectrum of activity: penicillins, cephalosporins, monobactams, carbapenems and β-lactams associated with β-lactamase inhibitors.

Even before penicillin was commercialized in the early 1940s, penicillin-resistant β-lactamase expressing strains of *E. coli* were already identified (Abraham and

Chain, 1940). The identification of β-lactam resistance led to the development of extended-spectrum antibiotics such as ceftazidime and cefotaxime (third-generation cephalosporins), carbapenems and β-lactamase inhibitors such clavulanic acid. However, nowadays, no single β-lactam is free from resistance (King et al., 2014).

There are three mechanisms of resistance to β-lactams: (1) enzymatic degradation of the β-lactam ring by β-lactamases; (2) target modification of the PBPs resulting in a lack of β-lactam binding; (3) regulation of β-lactam entry and efflux with porin modification mediated resistance and bacterial efflux pumps (Harder et al., 1981; Lou et al., 2011; King et al., 2014). Of these, the production of β-lactamases is the most common mechanism, especially in Gram (-) bacteria.

1.3.2.2. ESBL, AmpC and carbapenemases (CP) producing Enterobacteriaceae and associated AMR genes

β-lactamases existed even in the absence of the pressure of therapeutic antimicrobials (Hall and Barlow, 2004), but the overuse of β-lactam antibiotics has created an environment in which new β-lactamases readily emerge (Bush, 2018). The first enzyme with β-lactamase activity capable of destroying penicillin (now assumed to be the class C, AmpC chromosomal cephalosporinase from *E. coli*) was reported in 1940 in *Bacillus coli* (now *E. coli*) (Abraham and Chain, 1940). During the late 1970s and early 1980s, a great number of surveillance studies commenced to assess β-lactamase production in both Gram (-) and Gram (+) bacteria and nowadays, more than 2,770 unique, naturally occurring β-lactamases have been documented (Bush, 2018).

With this variety of β-lactamases it is primordial to have reliable nomenclature to refer to these enzymes. Two classification schemes for β-lactamases are currently in use (Table 1). On the one hand, the molecular classification is based on the amino acid sequence and divides β-lactamases into class A, C, and D enzymes which utilise serine for β-lactam hydrolysis, and class B metallo enzymes which require divalent zinc ions for substrate hydrolysis (Ambler, 1980). On the other hand, the Bush–Jacoby–Medeiros functional classification scheme, first proposed in 1995 (Bush et al., 1995) and updated

in 2010 (Bush and Jacoby, 2010), takes into account substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their phenotype.

Penicillinases and extended-spectrum β-lactamases (ESBLs) are part of the Ambler class A β-lactamases which comprise the largest number of enzymes with a very wide spectrum of activity. Two penicillinases commonly found in Enterobacteriaceae, TEM-1 and SHV-1, belong to this group. In fact, carriage of the $bla_{\mathrm{TEM-1}}$ gene was the primary cause of ampicillin resistance in *E. coli* before the emergence in the late 1980s of ESBLs (Bush and Bradford, 2019). ESBLs are capable of hydrolysing penicillins, third-generation cephalosporins and monobactams (*e.g.* aztreonam). They are not active against cephamycins (*e.g.* cefoxitin), carbapenems and β-lactamase inhibitors like clavulanic acid. The first ESBLs to emerge were derivatives of TEM (TEM-10) and SHV (SHV-2 and SHV-5). However, around the year 2000, CTX-M-type ESBL emerged and become dominant, with *E. coli* and *Klebsiella pneumonieae* being their major hosts within the Enterobacteriaceae (Livermore et al., 2007; Bush and Bradford, 2019), not only in humans, but also in food-producing animals (Naseer and Sundsfjord, 2011). In Spain, in the early 2000s, *E. coli* isolates with the ESBL coding genes $bla_{\mathrm{CTX-M-9}}$ and $bla_{\mathrm{CTX-M-14}}$ were encountered widely in the community and hospitalised patients (Bou et al., 2002; Romero et al., 2005) and also in food-producing animals (Briñas et al., 2005). Currently, CTX-M ESBLs enzymes are predominant around the world, in commensal and human pathogens (D'Andrea et al., 2013; Bush and Bradford, 2020), food-producing animals (Briñas et al., 2005; Geser et al., 2012; Seiffert et al., 2013; Dorado-García et al., 2018) and wildlife (Cristóvão et al., 2017; Palmeira et al., 2021a).

AmpC-type β-lactamases belong to Ambler class C (Bush–Jacoby–Medeiros functional group 1). They confer resistance to most penicillins, cephalosporins including expanded-spectrum cephalosporins (such as ceftazidime, cefotaxime and ceftriaxone) and monobactams. However, unlike ESBLs, they are active against cephamycins and resistant to inhibition by clavulanate. AmpC β-lactamases can be encoded in the chromosome or in MGE such as plasmids.

Table 1. Comparison of two β-lactamase classification schemes Modified from (Bush and Bradford, 2019).

Ambler class: catalytic site (spectrum)	Bush–Jacoby–Medeiros group: catalytic site (spectrum)	Substrates	Inhibited by	Examples
A: serine (variable)	2a: serine (penicillinases)	Penicillins	Clavulanate, avibactam, other newer inhibitors*	Penicillinases from Gram (+) bacteria
	2b: serine (penicillinases)	Penicillins and narrow-spectrum cephalosporins	Clavulanate, avibactam, other newer inhibitors*	TEM-1, TEM-2 and SHV-1
	2be: serine (extended-spectrum β-lactamase, ESBLs)	Penicillins and cephalosporins including extended-spectrum	Clavulanate, avibactam, other newer inhibitors*	SHV-2, TEM-10, CTX-M and GES-1
	2br: serine (inhibitor-resistant)	Penicillins	Avibactam, other newer inhibitors*	TEM-30 and SHV-72
	2c: serine (penicillinases)	Penicillins and carbenicillin	Clavulanate, avibactam, other newer inhibitors*	PSE (CARB)
	2f: serine (carbapenemases)	Penicillins, cephalosporins and carbapenems	Avibactam, other newer inhibitors*	KPC, SME, NMC-A and GES-2
B: metallo (carbapenemase)	3: metallo (carbapenemases)	Most β-lactams, including carbapenems, but not monobactams	Chelating agents (EDTA), ANT431	IMP, VIM and NDM
C: serine (cephalosporinases)	1: serine (cephalosporinases)	Penicillins and cephalosporins	Cloxacillin, avibactam, other newer inhibitors*	Chromosomal AmpC, CMY, ACT-1 and DHA
D: serine (oxacillinases)	2d: serine (oxacillinases)	Penicillins and cloxacillin; some include cephalosporins and/or carbapenems	Sodium chloride; by clavulanate, avibactam and other newer inhibitors*	OXA-1/30, OXA-10, OXA-23 and OXA-48

*The term “newer inhibitors” refers to the diazabicyclooctanone and boronic acid inhibitors.

Chromosomal AmpC β-lactamases are produced by many species of Gram (-) bacteria with a generally low basal expression of chromosomal bla_{ampC} gene that can be induced following exposure to some β-lactams (Jacoby, 2009). In *E. coli,* AmpC is non-inducible in response to β-lactam exposure and the expression levels are usually very low, mainly due to an inefficient promoter and the absence of the *ampR* regulator gene (Honoré et al., 1986; Jacoby, 2009). However, high-level producers of this β-lactamase

have been identified, typically as cefoxitin-resistant *E. coli* isolates with point mutations in the promoter of the bla_{ampC} gene, the most important being those at positions -32 and -42 (Caroff et al., 2000). AmpC β-lactamases can also be expressed from plasmids, which have now become widely disseminated. The main plasmid-encoded AmpC enzymes are CMY-, DHA-, and ACC-type β-lactamases, with a higher prevalence of CMY-type enzymes worldwide (Philippon et al., 2002; Jacoby, 2009).

Both ESBLs and AmpCs have poor hydrolytic activity against carbapenems, except when other resistance mechanisms are added, such as porin mutations that reduce influx (outer membrane porin loss) or enhance efflux (efflux pump activation) (Stapleton et al., 1999; Tängdén et al., 2013). However, the major mechanism for carbapenem resistance in Gram (-) bacteria is the production of carbapenem-hydrolysing β-lactamases or **carbapenemases (CP)**. They confer resistance to a broad spectrum of β-lactams, including carbapenems, although the specific spectrum of affected substrates will depend on the specific enzyme. As previously mentioned, carbapenems are last-resort drugs to treat infections caused by multidrug-resistant Gram (-) bacteria. The use of carbapenems is not authorised for food-producing animals, and, subsequently, a selective pressure associated to its use is unlikely. This might explain that, unlike in humans, reports of CP-producing bacteria in animals are scarce (Madec et al., 2017; Kock et al., 2018; Poirel et al., 2018; EFSA and ECDC, 2022). CPs are arranged in different classes (see Table 1):

- Within the serine CPs (group 2f) KPC enzymes, in particular KPC-2 and KPC-3 variants, have the largest distribution worldwide and are found among *Pseudomonas aeruginosa* and many genera of Enterobacteriaceae (Bush and Bradford, 2020). The first bla_{KPC} gene was identified in 1996 from an isolate of *Klebsiella pneumoniae* in the east coast of the United States (Yigit et al., 2001) and now they have become the most common CPs detected globally in humans (Castanheira et al., 2019). In food-producing animals, KPC producing Enterobacteria have been sporadically detected in poultry (Hamza et al., 2016; Qiao et al., 2017)

- Among the Metallo-β-lactamases (group 3), IMP, VIM and NDM variants are the most widespread worldwide in humans (Kazmierczak et al., 2016). They are able to hydrolyse most β-lactams, including carbapenems, but not monobactams (Bush and Bradford, 2016) and are commonly found in Enterobacteriaceae and *P. aeruginosa* (Bush and Bradford, 2016; Kazmierczak et al., 2016). In food-producing animals, VIM and NDM producing Enterobacteria were more frequently detected than IMP, which have been only punctually reported in wildlife and seafood (Madec et al., 2017; Kock et al., 2018; Taggar et al., 2020). In humans, IMP enzymes are most common in Asia-Pacific (Kazmierczak et al., 2016). VIM-type enzymes, which have become widespread especially among human clinical isolates in the Mediterranean region (Kazmierczak et al., 2016, 2020), have been detected in Germany in pig and poultry farms (Kock et al., 2018). NDM enzymes have been detected in cattle in India, Algeria and China (Purkait et al., 2016; Yaici et al., 2016; He et al., 2017b, 2017a) and in pigs in Italy (Diaconu et al., 2020). In humans, the prevalence of NDM producing isolates is higher in South Asia, the Balkans, North Africa, and the Middle East (Wu et al., 2019)
- The OXA-type enzymes traditionally classified as oxacillin-hydrolysing enzymes (class D; group 2d) include several subgroups including OXA-48-like β-lactamases, which are commonly encountered in the Enterobacterales. In *E coli*, the most frequently detected CP is OXA-48 that is disseminated throughout the Mediterranean area, Turkey and Russia (Castanheira et al., 2019; Kazmierczak et al., 2020). OXA-48 CP has much greater activity against imipenem than over other carbapenems like meropenem and ertapenem (Hirvonen et al., 2021). In food-producing animals, OXA-48 enzymes were detected in dairy cattle farms from Egypt (Braun et al., 2016), in ruminants and in animal derived food products in Algeria (Mairi et al., 2019), in raw milk in Lebanon (Diab et al., 2017) and more recently in a German pig farm (Irrgang et al., 2020).

1.3.2.3. Mobile genetic elements (MGE) associated with the spread of ESBL, AmpC and carbapenemase encoding genes

In Enterobacteria, the capture, accumulation, and dissemination of AMR genes are largely due to the actions of MGE. These can be divided into two major groups: (1) intra-cellular elements that are able to move within a given genome, such as insertion sequences (ISs), transposons (Tn) and integrons (In), or (2) inter-cellular elements, that are able to move between bacterial cells, such as plasmids, integrated conjugative elements (ICE), genomic islands and bacteriophages (Frost et al., 2005; Partridge et al., 2018). Intercellular elements are mobilized from the donor to the recipient cell through HGT (see 1.3.1.).

Among the intra-cellular MGE elements, ISs are small transposable elements in bacteria (Mahillon and Chandler, 1998). IS can move neighbouring resistance genes as part of a composite Tn, a region bounded by two copies of the same or related IS that move as a single unit (Partridge et al., 2018). Both ISs and Tns move themselves (and associated genes) randomly to new locations in the genome, whereas other elements, such as In are integrated at a specific recombination site (Gillings, 2014). The emergence and dissemination β-lactamases is associated with several ISs and Tns. Some examples are, IS*Ecp1*-like insertion sequences in association with β-lactamases encoding genes bla_{CTX}, bla_{CMY} and some bla_{OXA} gene variants, IS*CR1* with bla_{CMY} and bla_{DHA} genes, IS*26* with bla_{SHV}, IS*Aba125* with bla_{NDM} and Tn*1*, Tn*2*, Tn*3* with bla_{TEM} gene variants (Bonnet, 2004; Poirel et al., 2008; Liakopoulos et al., 2016; Partridge et al., 2018).

Regarding the inter-cellular MGE elements, plasmids are small, circular, double-stranded DNA molecules that replicate independently from the chromosome, present in both Gram (+) and Gram (-) bacteria, that may vary in size (from less than a kilobase (kb) to several megabases (Mb)), copy number or host range (Shintani et al., 2015). *E. coli* isolates frequently carry plasmids, typically two to four plasmids per strain (Denamur et al., 2021). Plasmids are built up by the juxtaposition of different housekeeping functional “backbone” modules, such as replication, stability,

conjugation and establishment. In addition to these, accessory niche-adaptive functional modules may appear. In these accessory regions is where AMR genes and other MGE (such as IS, Tn and In) may be encoded (Partridge et al., 2018). Plasmid incompatibility, which is based on the disability of plasmids sharing the same replication control systems to co-reside in the same cell (Novick, 1987), is used to classify plasmids into different families, and the polymerase chain reaction (PCR) based replicon typing (PBRT) scheme is the standard method for plasmid typing of Enterobacteriaceae (Carattoli et al., 2005) in addition to sequencing-based approaches such as PlasmidFinder (Carattoli et al., 2014). Currently, there are 28 known plasmid types in Enterobacteriaceae distinguished by PBRT. Frequently reported plasmids like IncF, IncI, IncC (formerly known as IncA/C), IncL, IncN and IncH are the ones that bear the greatest variety of AMR genes (including ESBLs AmpC and CP genes) in Enterobacteria (Carattoli, 2013; Rozwandowicz et al., 2018). Certain predominant ESBL gene-plasmid combinations are known to spread epidemically in humans and food-producing animals, such as *bla*$_{CTX-M-1}$/IncI1, *bla*$_{CTX-M-1}$/IncN or *bla*$_{CTX-M-15}$/IncF (Madec and Haenni, 2018). Other ESBL gene-plasmid combinations are more frequently found in animal sources, for example *bla*$_{CTX-M-1}$ gene in IncF plasmids or *bla*$_{CTX-M-14}$ gene in IncK plasmids (Rozwandowicz et al., 2018). IncC plasmids have been associated with the spread of the AmpC β-lactamase CMY-2 in food-producing animals (Carattoli, 2013; Guo et al., 2014; Rozwandowicz et al., 2018) and the spread of NDM-1 CP in humans (Carattoli et al., 2012). Only limited studies have identified carbapenem resistance genes on plasmids in livestock. However, two CP gene-plasmid combinations have been reported repeatedly. This is the case of *bla*$_{VIM-1}$ gene, identified over years in an IncHI2 plasmid in Germany in pigs and poultry farms, and *bla*$_{NDM-5}$ gene in IncX3 plasmids, which has been identified in livestock and food products, mostly in China but also in India and Algeria (Madec and Haenni, 2018).

1.3.2.4. Prevalence of ESBL, AmpC and carbapenemase producing Enterobacteriaceae in humans and food producing animals

According to the last surveillance conducted by the ECDC and EFSA (EFSA and ECDC, 2022) that provide data on the prevalence of ESBL-, AmpC- and CP-producing *E. coli* in the EU member states in food-producing animals (broilers, turkeys, pigs and calves), the ESBL phenotype was more frequently detected than the AmpC phenotype for all animal groups monitored and the detection of CP-producing *E. coli* was still low (only 11 *E. coli* with elevated MIC to meropenem were detected in 2019 and 2020). The mean prevalence remained similar to that estimated in the previous years for calves under 1 year (46.4%) and pigs (42.7%) but has gradually decreased in broilers (from around 60% in 2016 to 38.0% in 2020) and fattening turkeys (from around 40% in 2016 to 30.7% in 2020). However, the prevalence of ESBL and/or AmpC *E. coli* producers varies not only by animal species, but also by country. For instance, the prevalence in calves under 1 year of age ranged from 4.1% in Norway to 99.6% in Italy (Fig. 3). In Spain, albeit having one of the highest rates of ESBL- and/or AmpC-producing *E. coli* in the animal populations monitored, the prevalence decreased 15% between 2015 to 2019 and prevalence in calves is currently below the EU mean (38.9% in Spain in comparison to 46.4% in the EU). According to other studies in ruminants (mostly in cattle), variations were also observed on the herd-level prevalence of ESBL/AmpC-producing *E. coli*. In cattle farms in eastern England (UK) a prevalence of 30.0% (3/10) was observed (Ludden et al., 2019) and 41.0% (41/100) in a cross-sectional survey carried in dairy cattle in The Netherlands (Gonggrijp et al., 2016). In Germany, cefotaxime-resistant *E. coli* were isolated in 70% (42/60) and 85% (44/52) of the farms keeping beef and dairy cattle units, respectively (Hille et al., 2017). However, despite the efforts to conduct AMR surveillance in food-producing animals, some species have received less attention. This is the case of sheep for which there was limited prevalence data at the commencement of this PhD Thesis. Among the few studies available, one conducted in Switzerland reported 6.9% of ESBL-producing *E. coli* in 58 sheep samples (Geser et al., 2012).

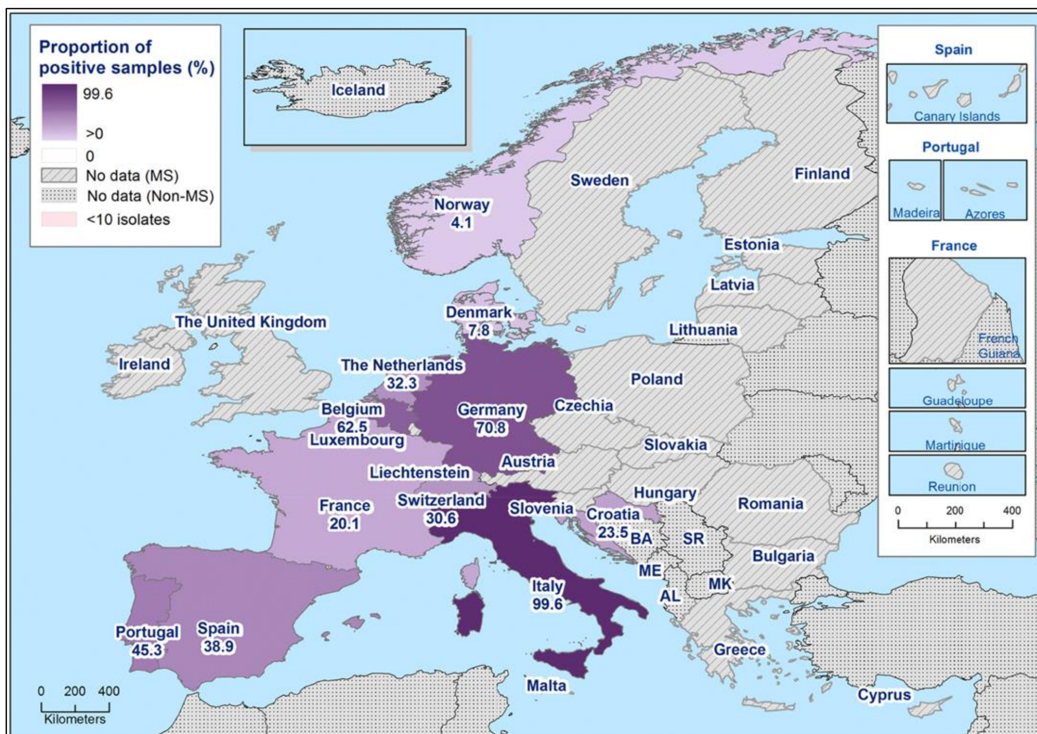

Figure 3. Prevalence of presumptive ESBL and/or AmpC-producing *E. coli* from calves under 1 year of age in 2019, EU Member States and Member States, 2019–2020 (EFSA and ECDC, 2022).

The ECDC annually reports AMR data from invasive (blood and cerebrospinal fluid) human bacteria isolates in the Antimicrobial Resistance Surveillance Network (EARS-Net, https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/report). According to the last EARS-Net report published in 2022 (WHO and ECDC, 2022), a north-to-south and west-to-east gradient was generally observed, with higher AMR percentages in the southern and eastern parts of Europe. Resistance to both third-generation cephalosporins and carbapenems was generally higher in *K. pneumoniae* than *E. coli*. Carbapenem resistance in *K. pneumoniae* remains a major public health challenge, with percentages in the EU being seven-fold higher in 2018 (7.5%) than in 2006 (0.96%), and a large variability between the European countries (0% to 66.3% in 2020) (ECDC et al., 2021; ECDC, 2022). On the other hand, carbapenem resistance in *E. coli* remains low, with a mean percentage of 0.2% in 2020, although there was a significantly increasing trend between 2016 and 2020. In comparison to previous years, a decrease of 0.7% for the resistance to third-generation

cephalosporins was detected in *E. coli*, being the resistant prevalence in 2020 14.9%, notably inferior from that reported in *K. pneumoniae* (33.9%) (WHO and ECDC, 2022).

Considering the parallel occurrence of ESBL/AmpC genes in humans and animals together with their frequent plasmid-mediated spread, the hypothesis of food-producing animals being contributors to the ESBL/AmpC reservoir in humans has been widely studied, giving contradictory conclusions (Leverstein-van Hall et al., 2011; Wu et al., 2013; Huijbers et al., 2014; Day et al., 2016; Dorado-García et al., 2018; Trung et al., 2019; Van Hoek et al., 2020; Giufrè et al., 2021; Miltgen et al., 2022). On the contrary, the situation with CPs is quite different. Considering the low prevalence of these enzymes in food-producing animals they do not seem to be currently circulating in the food chain outside incidental transfers from human sources. However, measures should be taken to prevent a wider dissemination in livestock.

1.3.3. Resistance to other antimicrobials

1.3.3.1. Quinolones and fluoroquinolones

The antibacterial activity of quinolones (*e.g.* nalidixic acid) and fluoroquinolones (*e.g.* ciprofloxacin) is due to inhibition of DNA replication. The primary target in *E. coli* is the DNA-gyrase, which consists of two GyrA and GyrB subunits, and topoisomerase IV (a secondary target) which consists of two ParC and ParE subunits. Resistance mainly occurs due to point mutations within the gyrase and topoisomerase subunits. A single nucleotide point (SNP) mutation in the gene *gyrA* may confer high level resistance to nalidixic acid but further mutations within *gyrA* and/or *parC* genes are needed for high level fluoroquinolone resistance (Hopkins et al., 2005; Poirel et al., 2018). These have been frequently detected in food-producing animals (Poirel et al., 2018). Plasmid-mediated quinolone resistance (PMQR), usually associated with mobilization of transposable elements on plasmids or the chromosome (Jacoby et al., 2014), has also been described to confer low-level resistance. This is the case of Qnr proteins, the aminoglycoside acetyltransferase AAC(6')-Ib-cr, and the efflux pumps

QepA and OqxAB (Hopkins et al., 2005; Munita and Arias, 2016; Raherison et al., 2017; Poirel et al., 2018).

1.3.3.2. Aminoglycosides

The most frequently used aminoglycosides in veterinary medicine are neomycin and derivatives of streptomycin as well as gentamicin and kanamycin. The main mechanisms of resistance are (i) the modification of the target site by methylation of residues of the site A of the 16S RNA, (ii) efflux-mediated resistance, and (iii) the enzymatic inactivation conducted by aminoglycoside-modifying enzymes. Currently, three classes of aminoglycoside modifying enzymes are known (acetyltransferases, nucleotidyltransferases, and phosphotransferases), which are further divided into subtypes. The most commonly found subtypes for acetyltransferases in *E. coli* are AAC(3)-II/IV and AAC(6)-Ib (encoded by *aac(3)-II*, *aac(3)-IV* and *aac(6)-Ib* genes, respectively). For aminoglycoside nucleotidyltransferases, ANT(2´´) and ANT(3´´) subtypes (encoded by *aadB* and *aadA* genes, respectively) are commonly found in Gram (-) bacteria and for phosphotransferases, APH(6)-Ia and APH(6)-Id subtypes (encoded by the *strA* and *strB* genes, respectively) are most the commonly observed in *E. coli* (Krause et al., 2016; Poirel et al., 2018). These enzymes have been disseminated globally and have been found in several food-producing animal species (Poirel et al., 2018).

1.3.3.3. Fosfomycin

Fosfomycin inhibits the MurA enzyme involved in peptidoglycan synthesis. This antimicrobial is not authorised as veterinary medicine in Spain; it is only available in a few countries and its use is almost limited to intensive production of broiler chickens and pigs (Pérez et al., 2014). It has recently been reintroduced as an alternative for the treatment of human infections caused by multidrug-resistant Gram (-) pathogens, particularly ESBL-producers and even carbapenem-resistant Enterobacteriaceae (Meletis, 2016). Two major resistance mechanisms have been described in *E. coli* of

animal origin: (i) mutations in the *glpT* and *uhpA/T* genes encoding proteins involved in the fosfomycin uptake system (Tseng et al., 2015; Poirel et al., 2018) and (ii) the acquisition of fosfomycin-modifying enzymes encoded by *fos* genes which are mainly plasmid-borne (Silver, 2017; Poirel et al., 2018).

1.3.3.4. Tetracyclines

Tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Chopra and Roberts, 2001). Nine tetracycline resistance efflux genes (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(J), *tet*(L), and *tet*(Y)), two tetracycline resistance genes encoding ribosome protective proteins (*tet*(M) and *tet*(W)), and one gene coding for an oxidoreductase that inactivates tetracyclines (*tet*(X)) have been identified in *E. coli* (Poirel et al., 2018). These *tet* genes occur at different frequencies in different animal sources and/or geographic regions. In general, the *tet*(A) and *tet*(B) genes are the most prevalent tetracycline resistance genes in *E. coli* of animal origin and they are frequently found on plasmids or other MGE such as transposons (Tn*1721* (*tet*(A)) and Tn*10* (*tet*(B)) together with other AMR genes (Partridge et al., 2018).

1.3.3.5. Phenicols

Phenicols such as chloramphenicol and its derivatives were banned in 1994 in the EU from use in food-producing animals to avoid the risk associated to the presence of residues in carcasses of food-producing animals, whereas others (florfenicol) are employed in food-producing animals (Schwarz et al., 2004). Resistance is mediated by three major mechanisms: (i) enzymatic inactivation by chloramphenicol acetyltransferases encoded by *cat* genes, (ii) active efflux (*cmlA* and *floR* genes), and (iii) target site methylation by an rRNA methylase encoded by *cfr* gene (Schwarz et al., 2004). The genes *catA1*, *cmlA*, and *floR* are often found in plasmids in *E. coli* in animals (White et al., 2000; Bischoff et al., 2005; Travis et al., 2006).

1.3.3.6. Sulfonamides and trimethoprim

Sulfonamides and trimethoprim inhibit different steps in the folic acid synthesis pathway and have been used for decades in animals and humans. Each of these agents acts in a bacteriostatic manner, whereas the combination of both results in synergistic bactericidal action. Acquired resistance mechanisms have been frequently identified in food-producing animals, mainly associated to *sul* genes (*sul1*, *sul2*, or *sul3*), which confer resistance to sulfonamides, or *dfr* genes (*dfrA* and *dfrB*), which confer resistance to trimethoprim (Poirel et al., 2018). These genes are often found together with other AMR genes on gene cassettes, integrons and plasmids (Partridge et al., 2018; Rozwandowicz et al., 2018).

1.3.3.7. Polymyxins

Colistin (polymyxin) targets the lipopolysaccharide in the outer membrane of Gram (-) bacteria (Poirel et al., 2017). This antimicrobial has been widely used in veterinary medicine, but now the use has been limited. Resistance to polymyxins has long been considered to be chromosomally encoded (mutations in *pmrA*, *pmrB*, *mgrB*, *phoP*, and *phoQ* genes). However, in 2015, the first plasmid-mediated polymyxin resistance gene (*mcr-1*) was identified in Enterobacteria on an IncI2 plasmid in China, mainly in livestock (Liu et al., 2016). Currently, additional *mcr* gene variants (from *mcr-2* to *mcr-10*) have been identified (Wang et al., 2020) and the *mcr-1* gene has been found in different types of plasmids in almost all food-producing animal species around the world (Madec and Haenni, 2018), sometimes in combination with cephalosporin resistance genes (Hassen et al., 2019; Dhaouadi et al., 2020).

1.4. Methods to study antimicrobial resistance

There are several methods to identify antimicrobial resistant bacteria (phenotypic resistance) and the associated resistance genes (genotypic resistance). The classical phenotypic antimicrobial susceptibility testing (AST) methods are based on the continuous exposure of a bacterial isolate to a set of antimicrobials followed by a visual

detection of growth. On the other hand, molecular characterization methods attempt to detect the presence of a specific ARG or mutation in the bacterial genome. This method does not determine resistance expression, therefore susceptibility has to be validated with a phenotypic test. The combination of both techniques is now an integral part of many surveillance investigations, whether in humans or animals.

Overall, phenotypic AST methods are divided into diffusion and dilution methods. The most frequently used diffusion method is the agar disk diffusion test (also known as the Kirby-Bauer test) which uses disks of filter paper impregnated with a known concentration of an antimicrobial agent that are placed onto an agar plate uniformly inoculated with a standard concentration of the bacteria to test. After incubation, the inhibition-zone around the disk is measured and the final results are reported categorically as susceptible with standard dosing regimen (S), susceptible with increased exposure (I) or resistant (R) to the antimicrobial agent tested (https://www.eucast.org/newsiandr/). Advantages of disk diffusion are the simplicity and low cost of the test (Gajic et al., 2022) but sometimes the measurement of the diameter of inhibition-zone is relatively imprecise. Gradient diffusion tests, such as E-test, share a similar methodology as agar-disk diffusion test but are more expensive; rather than a single concentration, a predefined exponential gradient of antimicrobial agent is applied to the bottom of a plastic strip which is subsequently placed on an agar medium to generate diffusion of the drug. This strip is then applied to the bacterial culture plate as described above for disk diffusion. A limitation of both diffusion test methods is that only a few antimicrobial agents can be tested per strain in a single agar plate.

On the other hand, broth microdilution is the most widely used dilution method and is considered the gold standard. This method can be standardised and automatised (microdilution plates can be read with an automated reader or a camera), is more accurate, allows testing of several antimicrobial substances in a single assay and results are reported as quantitative data. This method is endorsed by the EUCAST (European Committee on Antimicrobial Susceptibility Testing, https://www.eucast.org/) since 2003 (EUCAST, 2003) performed according to the recommendations from the

International Organization for Standardization, ISO 20776-1:2006 (International Organization for Standardization, 2006) and ISO 20776-1:2019 (International Organization for Standardization, 2019). Briefly, in this technique, a suspension of pure bacterium culture is added at a predetermined concentration to a series of tubes or micro-wells containing increasing concentrations of an antimicrobial agent, usually in two-fold dilution series. Following incubation, the tubes or micro-wells are examined for the presence of visible microbial growth by turbidity or growth of a bacterial pellet. Growth appearance can differ depending on the microorganism and the antimicrobial agent tested. The lowest concentration of the antimicrobial agent that completely inhibits bacterial growth (no turbidity or growth of bacterial pellet) represents the minimum inhibitory concentration (MIC), expressed in mg/L. Broth dilution methods are the most appropriate for the determination of MIC values but they are not the only AST methods that can be used to quantitatively determine resistance. In fact, gradient diffusion methods also provides MIC values (Benkova et al., 2020).

Phenotypic AST results are interpreted according to official guidelines, such as those developed by international susceptibility testing committees like Clinical and Laboratory Standards Institute (CLSI, https://clsi.org/), approved by the U.S. Food and Drug Administration (FDA) and/or the European EUCAST. European countries have gradually adopted the harmonised European guidelines developed by EUCAST that include clinical breakpoints and epidemiological cut-off (ECOFF) values (EUCAST, 2003; Kahlmeter et al., 2003). Taking into account clinical parameters such as pharmacokinetics and pharmacodynamics, clinical breakpoints define the lowest concentration for which a treatment is likely to be successful and are therefore used to guide decision-making on antimicrobial therapy. On the contrary, ECOFF values separate the wild type bacterial population (susceptible isolates, i.e., isolates without acquired resistance mechanisms) from those that possess acquired and/or mutational genetic determinants of resistance (GDRs) (resistant isolates), and are therefore used in surveillance studies. As such, clinical breakpoints and ECOFFs should not be used interchangeably (Schwarz et al., 2010; Kahlmeter and Turnidge, 2022). For AMR surveillance in food-producing animals EFSA adopted EUCAST ECOFFs, according to

Commission Implementing Decision 2013/652/EU (now replaced by the Decision 2020/1729/EU), where the panel of antimicrobial substances and concentration ranges to be tested are detailed along with the ECOFF thresholds for MIC interpretation (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020D1729&from=ES).

Phenotypic AST methods require relatively simple techniques and are cost limited, but often face limitations in terms of testing time or parameters affecting bacterial growth and stability of reagents. Reproducibility also remains a concern since small variations in operators' methodology, laboratory materials (reagents, equipment) and culture conditions may translate into different results and respective interpretations (Wiegand et al., 2008). Furthermore, the agreement between results obtained with different phenotypic methods is not perfect and some tests cannot accurately detect resistance. This is the case of polymyxins, large cationic molecules that diffuse poorly in diffusion-based assays making broth microdilution the only appropriate method (Matuschek et al., 2018; Satlin, 2019) or the poor performance of agar disk-diffusion and broth microdilution for fosfomycin susceptibility testing of Enterobacterales (Mojica et al., 2020).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry is a very versatile method mainly used for early identification of bacteria (Singhal et al., 2015) that has also been evaluated as a rapid AST method. MALDI-TOF is based on the determination of the unique ribosomal protein pattern displayed by each microbial species and its detection through the application of a soft desorption/ionization to bacterial biomass embedded in an organic matrix. Its use as a rapid AST method is based on measuring the β-lactamase activity by visualising the hydrolysis of the β-lactam ring. For example the detection of ESBL and CP-producing Enterobacteriaceae using MALDI-TOF have already been reported (Oviaño et al., 2017; Gato et al., 2022).

Conversely, molecular characterization methods attempt to detect the presence of a specific GDR (AMR gene or mutation). A commonly used method is real-time PCR using specific primers and probes. In addition, due to the continuous improvement and decrease in the cost, whole-genome sequencing (WGS) technologies

are becoming an interesting feasible system for bacterial typing and characterization of GDRs in a single process. This technique enables better standardisation and reproducibility, generating greater inter-laboratory comparability than phenotypic testing. Furthermore, the digital output can be stored, providing the opportunity to be re-analysed in the future when more algorithms are developed (Boostrom et al., 2022) and/or databases updated. Due to all these advantages, WGS is becoming a common tool in molecular epidemiological research and surveillance studies. In fact, the WHO recently suggested to extend the use of WGS for global surveillance of AMR as part of the global action plan (WHO, 2020), and the EFSA proposed to follow a gradual integration of WGS within the harmonised AMR monitoring (EFSA et al., 2019). The EUCAST committee also stated that WGS may replace phenotypic testing for surveillance purposes in the near future (Ellington et al., 2017), but so far it does not support the use of WGS-inferred susceptibility alone as a guide in clinical decision-making. In fact, the practical aspects of applying WGS to investigate the epidemiology of AMR bacteria in food-producing animals are still under development.

1.5. Whole Genome Sequencing: current technologies and bioinformatic tools for bacterial characterization

WGS is used to obtain the complete or nearly complete DNA sequence of an organism, and it can be performed using short-read or long-read sequencing technologies. The most commonly used short-read sequencing technology is Illumina (sequencing platforms include HiSeq, NextSeq, MiSeq, and NovaSeq) that is based on the sequencing by synthesis technology (Goodwin et al., 2016). Illumina DNA sequencing provides two sequencing read options: single-read and paired-end sequencing. Single-read DNA sequencing involves sequencing fragments from one end to the other, whereas with paired-end sequencing the DNA fragment is read from both ends. The paired-end approach is preferred as it produces twice the number of sequencing reads, enabling a more accurate output. Read size for pair-end reads usually ranges from 150 to 300 bp (https://emea.illumina.com/science/technology/next-generation-sequencing/plan-experiments/read-length.html). An alternative technology

for short-read sequencing is semiconductor sequencing, the method used by Ion Torrent devices (Thermo Fisher), where nucleotides in a DNA strand are inferred from pH changes during synthesis (Goodwin et al., 2016). In comparison to Illumina, less bioinformatic tools are available for Ion Torrent data, limiting the subsequent data analysis. The main advantage of short-read sequencing is the low error rate (Reuter et al., 2015; Heather and Chain, 2016). However, complete assembly of the bacterial chromosome and plasmids is very challenging with this methodology, as it cannot resolve repeated elements, resulting in hundreds of sequence fragments per genome (Goodwin et al., 2016). In order to overcome this limitation, long-read sequencing often produce read lengths exceeding the length of repeated sequences in bacterial genomes and enables the reconstruction of complete genomes (Amarasinghe et al., 2020).

The two dominant long-read sequencing technologies include Oxford Nanopore Sequencing (ONT) (MinION, GridION and PromethION) and Pacific Biosciences (PacBio) platforms (Goodwin et al., 2016). Both technologies provide read lengths in the range of 10-30 kb (Amarasinghe et al., 2020), although Nanopore sequencing has achieved the longest peak lengths of more than 2 Mb (Payne et al., 2019). PacBio instrumentation is expensive and non-portable while ONT devices are scalable and portable, with the MinION ONT platform being relatively cheap and highly portable. Both sequencing technologies rely on very distinct principles. PacBio single molecule real-time sequencing is based on the detection of fluorescence-labelled nucleotides added by a polymerase tethered in a nanoscale space called a ZMW (zero mode waveguide), whereas ONT sequencing relies on a nanoscale protein pore (nanopore) that is embedded in an electrically resistant polymer membrane (flow cell) that acts as a biosensor. In ONT sequencing, when a suitable potential is applied across this membrane ions pass through the nanopore. Single-stranded DNA or RNA molecules pass through the nanopore driven by a motor protein which also controls translocation speed, causing changes in the ion current. Each nucleotide provokes specific changes, which are registered as signals and stored as raw data (fast5 files). These data can then be processed by basecalling algorithms to transform the signals into sequence of bases. The most commonly used base-caller is Guppy (provided by ONT within MinKNOW

operating software used to control ONT devices), but newly developed base-callers are also available (Peresini et al., 2021; Wang et al., 2021). Different strategies have been developed to decrease the price of sequencing, such as multiplexing of samples that can be carried out in short-read and long-read sequencing platforms including ONT (Wick et al., 2017a; Taylor et al., 2019; Arredondo-Alonso et al., 2021). Although the error rate is decreasing due to improvements in the chemistry, base calling algorithms and bioinformatic workflows (Taylor et al., 2019), ONT long-read sequencing is still less accurate than short-read sequencing.

Once sequencing reads have been produced, bioinformatics analyses can sometimes become a bottle neck. Researchers with no bioinformatic knowledge might benefit from accessible user-friendly pipelines, such as open-access bioinformatics software available at public genome data centres like the Center for Genomic Epidemiology (CGE) web service (https://www.genomicepidemiology.org/). Scientist with certain bioinformatic expertise can exploit downloadable programs for local installation from GitHub (https://github.com/) or Bitbucket (https://bitbucket.org/) public repositories. Briefly, WGS bioinformatic analysis includes several steps, usually beginning with removal of sequencing adaptors followed by quality assessment and subsequent reads-filtering. These cleaned and high quality sequence reads can be readily used for certain types of WGS analysis of ONT reads like MLST determination using Krocus (Page and Keane, 2018) or single nucleotide polymorphisms (SNPs) calling. The following step is to reconstruct the draft genome from the fragmented raw reads (*i.e.*, genome assembly) to be used in downstream analyses (Segerman, 2020). Genome assembly can be carried out using a reference genome as a guide or be performed *de novo* without a reference genome. Furthermore, depending on the sequencing technology and the type of genome (bacterial, fungal, animal…) different assembly software are used (Segerman, 2020). For instance, for bacteria genomes generated by short-read sequencing assembly is frequently carried out with SPAdes (Bankevich et al., 2012), whereas for long-reads Unicycler (Wick et al., 2017b) or Flye (Kolmogorov et al., 2019) assemblers are preferred. The assembly output consists of a variable number of continuous sequences (contigs) or longer scaffolds (a set of contigs and unresolved gaps

between contigs). A complete genome assembly is accomplished when all gaps are resolved. In order to obtain the most comprehensive results, many studies are based on hybrid assemblies, combining both ONT/PacBio long-reads and Illumina short-reads (Wick et al., 2017a; De Maio et al., 2019; Chen et al., 2021).

Assembled genomes can be screened for the detection of ARGs and the characterization of further genetic features. Widely used bioinformatic tools for the detection of GDR rely on BLAST (Basic Local Alignment Search Tool) searches (Zhang et al., 2000). Generally, each bioinformatic tool integrates its own curated database. This is the case of tools used for the detection of acquired ARGs such as ResFinder (developed by the CGE) (Bortolaia et al., 2020), Comprehensive Antimicrobial Resistance Database (CARD) (Alcock et al., 2020), NCBI AMRFinder (Feldgarden et al., 2019) or Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) (Gupta et al., 2014) or for the detection of point mutations like PointFinder (developed by the CGE) (Zankari et al., 2017). On the other hand, ABRIcate (https://github.com/tseemann/abricate) also uses BLAST but, conversely to other tools, it comes bundled with multiple AMR gene databases developed by others (including NCBI, CARD, ARG-ANNOT, Resfinder and MEGARES databases) and enables users to create custom databases. Tools that provide valuable information for the study of AMR spread include PlasFlow (Krawczyk et al., 2018), that predicts whether GDRs are located on the bacterial chromosome or on plasmids, or the CGE developed tool PlasmidFinder, which enables the identification of plasmid replicons in Enterobacteria (Carattoli et al., 2014). Genome annotation (the description of the function of a predicted gene) is useful to obtain information about the genetic environment of the ARGs. Commonly used bacteria annotation tools include Prokka (Seemann, 2014) or RAST (Brettin et al., 2015). Regarding tools for bacterial typing,MLST and cgMLST can be determined in *E. coli* assembled Illumina genomes in PubMLST (Zhou et al., 2020). *Escherichia* genus strain phylotyping can be carried out using ClermonTyper (Beghain et al., 2018). Furthermore, *E. coli* serotyping can be performed employing the CGE tool SerotypeFinder, which includes a database of O and H-type antigens (Joensen et al., 2015). The identification of *fimH* alleles in *E. coli*, which enable high-resolution subtyping of MLST-based clonal groups, can be

fulfilled the CGE tool FimTyper (Roer et al., 2017) and for the detection of virulence genes the Virulence Factor Database (VFDB) is widely used (Chen et al., 2005). All in all, WGS provides detailed and precise data with great potential for species identification, strain typing and characterization of antimicrobial resistance mechanisms and virulence traits as well as for plasmid characterization.

2. BACKGROUND AND OBJETIVES

Antimicrobial resistance (AMR), mainly driven by overuse and misuse of antimicrobials, is a serious global public health problem that affects humans, animals and the environment. Emergence and dissemination of resistance to β-lactams, particularly to cephalosporins (third- and higher-generation) and carbapenems, are a matter of concern in Enterobacteria, as these are sometimes the unique therapy available to treat multidrug-resistant bacteria in humans. The production of β-lactamases (extended spectrum β-lactamases (ESBLs), AmpC-type β-lactamases and carbapenemases (CP)) is the most common mechanism of resistance to β-lactams in Gram (-) bacteria. These enzymes are often encoded by genes located in mobile genetic elements (MGEs), which is especially worrisome in terms of spread. Commensal *E. coli* are frequently used as indicator of AMR as they can act as reservoir of these and other AMR genes (ARGs) and transfer them to other commensal or pathogenic Enterobacteriaceae.

ESBL/AmpC-producing *E. coli* have emerged in recent years and are now widely distributed in humans and food-producing animals. CP-producing *E. coli*, on the other hand, are far less frequently detected in food-producing animals compared to humans probably because, unlike third- and fourth- generation cephalosporins, the use of carbapenems in food-producing animals is banned in the EU and several other countries (EMA/AEMEG, 2019). Nonetheless, the impact of food-producing animals in the transmission of ESBL-, AmpC- and CP-producing *E. coli* to humans is not easy to measure. In fact, the prevalence of ESBL/AmpC-producing *E. coli* in food-producing animals varies by country and animal species, and data are still very limited for certain animal species like small ruminants (EFSA and ECDC, 2022). This variation on the prevalence rates of ESBL/AmpC-producing *E. coli* in food-producing animals is likely a consequence of the different farm management practices applied, including housing systems (*e.g.* intensive, semi-intensive or extensive farming) and/or antimicrobial treatments used, among other factors. At the commencement of this PhD thesis, there was a lack of data on the prevalence of ESBL-, AmpC- and CP-producing *E. coli* in food-producing animals in the Basque Country. Moreover, long-term surveillance studies of resistant bacteria were limited and little was known about the epidemiology underlying

ESBL-, AmpC- and CP-producing *E. coli* dissemination within farms or the effect of different farming practices and age-related variations.

AMR detection and surveillance have fundamentally focused on reporting phenotypic results using either disk diffusion or broth microdilution (gold standard method), occasionally combined with molecular determination of targeted genetic determinants of resistance (GDRs). In recent years, due to the continuous improvement and decrease in costs, whole genome sequencing (WGS) has provided a practical advantage compared to other commonly used molecular methods, as it allows, in a single assay, the simultaneous detection of GDRs, their association with MGEs and molecular characterization of bacteria. In fact, it has been proposed to gradually integrate the use of WGS within the harmonised AMR monitoring (EFSA et al., 2019; WHO, 2020) with the aim to replace phenotypic testing for surveillance purposes in the near future (Ellington et al., 2017). Nevertheless, at the time this PhD Thesis was proposed, few studies had addressed the practical aspects of applying WGS (*e.g.*, different sequencing methodologies and bioinformatic tools) to investigate the epidemiology of ESBL-, AmpC- and CP-producing *E. coli* in food producing animals.

In this context, the objectives of this PhD Thesis are:

1. To estimate the prevalence of ESBL-, AmpC- and CP-producing commensal *E. coli* in domestic ruminants in the Basque Country, including dairy cattle, beef cattle and sheep, which represent the different farm management systems in the region.
2. To determine the antimicrobial phenotypic resistance profiles of ESBL-, AmpC- and CP-producing commensal *E. coli* isolated from domestic ruminants in the Basque Country by using broth microdilution.
3. To assess the application of different WGS technologies (short-read and long-read) to characterize the genetic determinants of antimicrobial resistance in a selection of ESBL-producing commensal *E. coli* and compare the results with those obtained with the phenotypic method.
4. To assess the within-farm dynamics of ESBL-, AmpC- and CP-producing commensal *E. coli* in dairy cattle farms from the Basque Country and study the

genetic diversity of the circulating strains by performing WGS-based *in-silico* typing (e.g., phylogroup, serogroup, MLST, virulence factors, plasmid replicons).

5. To exploit the usefulness of WGS for bacterial identification and characterization for an in-depth genomic profiling of two particular isolates of interest derived from the field studies, a rare CP-producing *E. coli* and a cryptic *Escherichia* isolate.

3. MATERIALS, METHODS AND RESULTS

3.1. Study I

Prevalence of cefotaxime-resistant *Escherichia coli* isolates from healthy cattle and sheep in northern Spain: phenotypic and genome-based characterization of antimicrobial susceptibility

3.1.1. Background

Antimicrobial resistance (AMR) is considered an emerging problem at a worldwide scale. It has been estimated that unless actions are taken, AMR could cause up to 10 million deaths each year by 2050 (O'Neill, 2014). The WHO list of critically important antimicrobials for human medicine includes third and higher generation cephalosporins as well as carbapenems, as these antimicrobials are either the sole or one of the limited therapies available to treat multidrug resistant (MDR) bacteria in human infections (WHO, 2019). In *Escherichia coli*, the most important mechanism of resistance to those critically important β-lactam antimicrobials is the production of extended spectrum β-lactamases (ESBLs), followed by the production of AmpC cephalosporinases and carbapenemase enzymes. ESBLs are capable of hydrolysing penicillins, third generation cephalosporins, and monobactams (eg. aztreonam). They are not active against cephamycins (eg. cefoxitin) or carbapenems, but are susceptible to β-lactamase inhibitors like clavulanic acid. AmpC-type β-lactamases, unlike ESBLs, are active against cephamycins and resistant to inhibition by clavulanate. Carbapenemases are carbapenem-hydrolysing β-lactamases that confer resistance to a broad spectrum of β-lactams including carbapenems.

ESBL-/AmpC-producing *E. coli* have emerged in recent years, becoming one of the fastest spreading AMR determinants not only in humans, but also in food-producing, companion and wild animals, as well as the environment (Guenther et al., 2011; Dierikx et al., 2012; Gekenidis et al., 2018; EFSA and ECDC, 2019). Despite the wide distribution of ESBL-/AmpC-producing *E. coli* in livestock, their contribution as source of human infection remains controversial (Collis et al., 2019). Although humans seem to be the main source of community-acquired infections of ESBL- and AmpC-producing *E coli*, non-human sources act as important reservoirs that contribute to further spread the infection (Dorado-García et al., 2018; Ludden et al., 2019; Mughini-Gras et al., 2019). Moreover, prevalence of cefotaxime-resistant bacteria in food producing animals varies by country and animal species, and some of them, like small-ruminants, have received less attention than others (EFSA and ECDC, 2019). On the

other hand, carbapenemase (CP)-producing *E. coli* are more prevalent in humans than in animals. The use of carbapenems in livestock is banned and CP producing *E. coli* have only rarely been identified in food-producing animals in Europe (Kock et al., 2018; EFSA and ECDC, 2019).

ESBLs are mostly plasmid-mediated enzymes, with CTX-M-1, CTX-M-14, and CTX-M-15 being the most frequently described in *E. coli* isolated from cattle (Dantas Palmeira and Ferreira, 2020). In fact, the first description of ESBL-producing *E. coli* in cattle in Spain was a CTX-M-1 cephalosporinase-bearing strain isolated from a cattle with mastitis (Briñas et al., 2005). AmpC enzymes in *E. coli* from livestock are mainly encoded by bla_{CMY} genes located in plasmids (Poirel et al., 2018) and also by mutations in the promoter region of the chromosomal *ampC* gene. The latter, normally repressed or only weakly expressed leading to constitutive hyper-expression of the gene resulting in β-lactam resistance (Jørgensen et al., 2010). The most frequently detected carbapenemases in livestock in Europe are OXA-48 and VIM-1, but evidence of the dissemination of NDM, KPC, and IMP carbapenemases has also been reported globally (Kock et al., 2018).

Phenotypic detection of ESBL-, AmpC- and CP-producers among *E. coli* isolates from food-producing animals is important for epidemiological purposes. However, molecular determination of AMR genetic determinants provides an insight into the resistance mechanisms. For this purpose, whole genome sequencing (WGS) has proven to provide a practical advantage compared to other commonly used molecular methods. The aim of this study was to determine the occurrence of ESBL-, AmpC- and CP-producing commensal *E. coli* in dairy cattle, beef cattle, and sheep without clinical signs of disease in farms in the Basque Country (Northern Spain) by using selective isolation methods, and to characterize the AMR profiles of the isolates obtained. Phenotypic antimicrobial susceptibility was tested and a selection of isolates was subjected to WGS in order to assess, not only the carriage of the ESBL-, AmpC- and CP-coding genes, but also, GDRs to other antimicrobials.

3.1.2. Materials and methods

Sampling design. A cross-sectional survey was carried out in ruminant herds in the Basque Country, a 7,234 km^2 region located in northern Spain. Ruminant production is one of the pillars of the rural economy of the region, with *ca.* 260,000 sheep and 135,000 cattle (dairy and beef) according to the 2015 census (https://www.eustat.eus/banku/id_4017/indexLista.html). Dairy cattle are managed under an intensive system, whereas semi-extensive production predominates for sheep and beef cattle; animals graze in farmland pastures in spring and part of the summer, and in communal mountain pastures from the middle of July until the end of November, and are housed in winter. Further details on general husbandry systems for beef cattle, dairy cattle, and sheep in the Basque Country were reported elsewhere (Hurtado et al., 2017; Ocejo et al., 2019).

The census of beef cattle, dairy cattle and sheep farms was obtained from the Department of Agriculture of the Basque Government. Since this survey was part of a larger study designed to also estimate the prevalence of *Salmonella*, *Listeria monocytogenes* (Hurtado et al., 2017), thermophilic campylobacters (Ocejo et al., 2019), and Shiga toxin-producing *Escherichia coli* (STEC) (Oporto et al., 2019), the number of herds to sample was calculated separately for each animal category for an expected herd prevalence of 50%, a 95% confidence level and an accuracy of 10% using Win Episcope 2.0. A sample size of 25 animals per herd was selected after estimating a within-herd prevalence of 10% and a level of confidence of at least 90% in detecting a positive. Thus, a total of 300 herds (104 beef cattle, 82 dairy cattle, and 114 dairy sheep) were sampled once between February 2014 and June 2016. Rectal faecal samples from 25 animals randomly selected per herd were collected with a gloved hand, and analysed in a single 25 g-pool (1 g per animal per herd).

Sample collection was carried out by veterinary practitioners as part of the usual screening scheme performed on farms, strictly following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013).

ESBL-, AmpC- and CP-producing *E. coli* selective isolation. Faeces (25 g of pooled rectal faecal samples) were diluted 1:10 in modified Tryptic Soy Broth (mTSB,

bioMérieux) supplemented with novobiocine (Biolife) and incubated at 41±1°C for 6-7h. For the selective isolation of cefotaxime-resistant *E. coli*, samples were then pre-enriched in MacConkey broth supplemented with cefotaxime 1 mg/L (37±1ºC, 24h) and subcultured onto MacConkey agar with cefotaxime (1 mg/L). For the selective isolation of OXA-48 and other CP-producing *E. coli*, a pre-enrichment with unsupplemented MacConkey broth was carried out (37±1ºC, 24h), followed by subculturing of 50 µL on a bi-plate selective chromogenic media (CHROMID® CARBA SMART, BioMérieux). Both plates were incubated at 37±1°C for 24h. Three colonies per plate were selected based on colony morphology diversity and were further confirmed as *E. coli* by species-specific real-time PCR targeting the *uidA* gene (Frahm and Obst, 2003).

Antimicrobial susceptibility test (AST) determination by broth microdilution. Minimum inhibitory concentrations (MIC) were determined by broth microdilution following recommendations by the Commission Decision 2013/652/EU (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013D0652&from=EN).
Isolates were tested using two Sensititre® MIC Susceptibility Plates (ThermoFisher Scientific, Waltham, MA): one (EUVSEC1) that contains 14 antimicrobial agents (10 classes), and a second panel (EUVSEC2) with 10 antimicrobial substances for the phenotypic characterization of presumptive ESBL-, AmpC- and CP-producers. The second panel includes cefoxitin (FOX) as well as cefotaxime (FOT) and ceftazidime (TAZ) with and without clavulanic acid (CLV) to investigate clavulanate synergy for phenotypic characterization of ESBL and AmpC production, along with imipenem, meropenem, and ertapenem to phenotypically verify the presumptive carbapenemase-producers. MIC results were interpreted using epidemiological cut-off values (ECOFF) as developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org) to define microbiological resistance to the antimicrobial in question, that is, to discriminate those microorganisms with and without acquired resistance mechanisms (non-wild type and wild type, respectively). For azithromycin resistance (no cut-off assigned by EUCAST) a MIC ≤16 mg/L for wild-type isolates was used as reference, as proposed for *Salmonella* spp. (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015). Here, the terms susceptible and

resistant will refer to isolates without (wild-type) and with phenotypically expressed resistance mechanisms (microbiologically resistant), respectively.

Interpretation of resistance profiles for phenotypic detection of ESBL and AmpC production was based on the EUCAST guideline for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance (v 2.0, July 11, 2017; http://www.eucast.org/resistance_mechanisms/). Briefly, an ESBL phenotype was inferred if isolates were resistant to FOT (>1 mg/L) or TAZ (>1 mg/L), but susceptible to FOX (≤8 mg/L) and showed clavulanate synergy with FOT and/or TAZ (≥8-fold reduction in the MIC of the cephalosporin combined with 4 mg/L CLV compared with the MIC of the cephalosporin alone). Isolates were considered to be AmpC phenotype if clavulanate synergy was not shown and they were resistant to FOT (>1 mg/L) or TAZ (>1 mg/L) and FOX (>8 mg/L). An ESBL+AmpC phenotype was inferred if isolates were resistant to FOT (>1 mg/L) or TAZ (>1 mg/L), resistant to FOX (>8 mg/L) and showed clavulanate synergy with FOT and/or TAZ. Meropenem-resistance (>0.12 mg/L) was used to infer a carbapenemase-producing phenotype.

Whole genome sequencing (WGS), genome assembly and analysis. Bacterial genomic DNA was extracted with Wizard genomic DNA purification kit (Promega, Madison, WI, United States). WGS was carried out at a commercial facility using Illumina NovaSeq6000 (150 bp pair-end reads). The quality assessment of the raw reads was performed using FastQC v.0.11.9 quality-control tool (Babraham Bioinformatics, Cambridge, United Kingdom) (Andrews, 2010). Data were analysed by using the automated pipeline TORMES v.1.0 (https://github.com/nmquijada/tormes; Quijada *et al.*, 2019). Briefly, reads were quality filtered using trimmomatic v.0.38 (Bolger et al., 2014) and *de novo* assembled into a draft genome using SPAdes v.3.13.0 (Bankevich et al., 2012) with the default parameters and in careful mode. QUAST v.5.0.2 (Gurevich et al., 2013) was used to evaluate the quality of the assemblies and contigs below 200 bp long were discarded. The draft genomes were screened for acquired AMR genes using BLASTn v.2.7.1+ (Zhang et al., 2000) and ABRicate v.0.8.10 (T. Seemann, https://github.com/tseemann/abricate) against ResFinder (Zankari et al., 2012) (last updated on December 5, 2019). Chromosomal point mutations associated to quinolone

resistance and β-lactams (*ampC* promoter) were investigated using the *E. coli* point mutations database PointFinder (Zankari et al., 2017) (last updated on June 4, 2019). PlasFlow v.1.1 (Krawczyk et al., 2018) was used to predict plasmid- and chromosome-derived contigs. The presence of plasmid replicons was identified using PlasmidFinder v.2.0.1 (last updated on September 4, 2018) (Carattoli and Hasman, 2020). Any hit with coverage below 60% and/or identity below 90% was removed. A dendrogram was generated to illustrate the similarity among isolates based on their AMR pattern. Hierarchical clustering analysis was performed with the unweighted pair-group method with arithmetic mean (UPGMA) based on the Euclidean distance matrix, using the function hclust of the R statistical package v.3.6.3 (R Development Core Team, 2016).

Statistical analysis. Herd-level prevalence was expressed as the percentage of herds/flocks that tested positive in each farm system out of all herds/flocks that were examined in the respective farm system, with 95% confidence intervals adjusted for the population size, using the software EpiInfo2. To assess factors associated with shedding prevalence of cefotaxime-resistant *E. coli*, selected variables were categorized as follows: (i) host species (cattle, sheep), (ii) production system (beef cattle, dairy cattle, and sheep), (iii) sampling season (spring, summer, autumn, winter), (iv) geographical location of the farm (oceanic, continental), (v) presence of other species in the farm such as cattle, sheep, goats, horses (presence, absence), (vi) herd size stratified according to farm system management (beef cattle, <50, 50–100, and >100; dairy cattle, <50, 50–150, and >150; sheep, <150, 150–300, and >300), and (vii) year of sampling (2014, 2015, 2016). First, univariate logistic regressions were conducted to explore the unadjusted association between herd positivity and variables. Only significant factors ($p \leq 0.20$; likelihood-ratio test) were included for further multivariate logistic regression analyses. Test of overall significance (chunk test) was performed to assess any possible effect modifiers that could bias the magnitude of associations, and interactions with a value of $p > 0.05$ were excluded until no significant difference between the full and the reduced models was observed. To identify confounding variables, the measure of association was estimated before and after adjusting for the potential confounder, and variables causing change of ≥ 10% in the estimated measure

were retained. Adjusted odds ratios (OR_{adj}) were used as the measure of association between positivity and the explanatory variable, and were expressed with their confidence interval at 95% (95% CI). To evaluate differences in the distribution of AMR among production systems, simple logistic regressions were performed.

Phenotypic (broth microdilution AST-based) and genotypic (WGS-based) susceptibility results were compared. Resistant WGS genotypes were defined by the presence of one or more resistance genes and/or point mutation for each antimicrobial tested in the AST. The sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values for the genotypic prediction were calculated for each antimicrobial tested for their corresponding phenotypic AST reference. Inter-rater agreement analyses were performed for each antimicrobial using Cohen's kappa (κ) method. Interpretation of Kappa values to assess the strength of agreement between techniques was based on the one proposed by Altman (Altman, 1991), which is as follows: $\kappa \leq 0.20$ = poor, 0.21-0.40 = fair, 0.41-0.60 = moderate, 0.61-0.80 = good, and 0.81-1.00 = very good. Analyses were conducted using statistical software Stata/IC version 13.1 (StataCorp LP, College Station, TX, USA).

3.1.3. Results

ESBL-, AmpC- and CP-producing *E. coli* herd prevalence. *E. coli* was isolated in cefotaxime-containing media in samples collected from 15.0% (45/300) of the herds/flocks, with different prevalence distribution according to production system (Table 2). No *E. coli* isolates were recovered from the chromogenic media used to screen for CPs. Univariate analyses performed to assess factors associated with shedding prevalence of cefotaxime-resistant *E. coli* identified season, presence of other species in the farm, and herd size as potential confounder variables but only season passed to the final model (Table 3). Multivariate logistic regression analysis indicated that bovine were more likely to shed cefotaxime-resistant *E. coli* than sheep (OR_{adj} 3.55 (1.57-8.04), p=0.002). When host was categorized according to production system, herd prevalence of cefotaxime-resistant *E. coli* was significantly higher in dairy cattle compared to beef cattle (OR_{adj} 3.71 (1.60-8.58), p=0.002) and sheep (OR_{adj} 6.11 (2.55-

14.60), *p*<0.001). Shedding of cefotaxime-resistant *E. coli* was higher during any season compared to winter, this difference being larger between autumn and winter (Table 3).

Table 2. Proportion of herds/flocks where *E. coli* was isolated from cefotaxime-containing media and distribution of phenotypically inferred phenotypes.

	Growth in cefotaxime-containing medium			**Inferred phenotype**					
				ESBL		**AmpC**		**ESBL+AmpC**	
	n	**%**	**CI (95%)**	***n***	**%**	***n***	**%**	***n***	**%**
Host									
Beef Cattle (n=104)	10	9.6	4.1-15.2	9	8.7	1	1.0	0	0.0
Dairy Cattle (n=82)	27	32.9	23.8-42.1	20	24.4	5	6.1	2[b]	2.4
Sheep (n=114)	8	7.0	2.8-11.2	7[a]	6.1	1	0.9	1[a]	0.9
TOTAL (n=300)	45	15.0	11.2-18.9	36	12.0	7	2.3	3	1.0

[a] In one sheep flock, two of the three isolates characterized had a ESBL phenotype and another isolate had the ESBL + AmpC phenotype.
[b] Only one was confirmed as ESBL + AmpC by WGS analyses (see text).

Antimicrobial resistance phenotype as determined by broth microdilution (MICs). MICs were determined for a total of 135 isolates (3 per positive herd/flock). Antimicrobials tested, distribution of MICs, and interpretation of results are shown in Table 4. All isolates were susceptible to imipenem, meropenem, tigecycline, and colistin. Since isolates had been obtained by selective isolation in media containing cefotaxime, they were all resistant to cefotaxime and also to ampicillin. Most isolates (97.8%) were also resistant to the fourth-generation cephalosporin cefepime, while resistance to ceftazidime (third-generation cephalosporin) was present in 87.4% of the isolates and to cefoxitin (second-generation cephalosporin) in 20.7% (Fig. 4).

Two isolates were resistant to temocillin (MIC = 32 mg/L). Although no carbapenemase-producing *E. coli* were found, one isolate from beef cattle and three from sheep were resistant to ertapenem (MIC = 0.12 mg/L). In addition to this high resistance to β-lactams, co-resistance to other antimicrobial classes was also observed in most cases (110/135 isolates). Resistance to other antimicrobials included tetracycline (70.4%), sulfamethoxazole (70.4%), trimethoprim (47.4%), ciprofloxacin (41.5%), nalidixic acid (33.3%), chloramphenicol (28.9%), gentamicin (23.7%), and azithromycin (6.7%, MIC >16 mg/L).

Table 3. Univariate and multivariate logistic regression analyses to assess association of different factors with prevalence of FOT-resistant *E. coli.*

Variables	N	Univariate		Multivariate	
		OR (95% CI)	*p* value	OR_{adj} (95% CI)	*p* value
Host Species			0.002		0.002
Ovine (Ref)	114				
Bovine	186	3.29 (1.47-7.35)	0.004	3.55 (1.57-8.04)	0.002
Farm management system			< 0.001		< 0.001
Sheep (Ref)	114				
Beef Cattle	104	1.41 (0.53-3.72)	0.488	1.65 (0.61-4.43)	0.324
Dairy Cattle	82	6.50 (2.77-15.27)	≤ 0.001	6.11 (2.55-14.60)	< 0.001
Sampling season			0.003		0.022
Winter (Ref)	79				
Spring	76	5.22 (1.43-19.16)	0.003	5.19 (1.40-19.18)	0.014
Summer	45	5.48 (1.37-21.86)	0.016	6.20 (1.53-25.14)	0.011
Autumn	100	6.73 (1.93-23.51)	0.003	7.31 (2.07-25.78)	0.002
Geographical location			0.219		
Oceanic (Ref)	204			-	-
Continental	96	1.51 (0.79-2.91)	0.214	-	-
Presence of other species			0.026		
Absence (Ref)	156				
Presence	139	0.47 (0.24-0.93)	0.030	-	-
Herd size			0.098		
Small (Ref)	84				
Medium	109	0.67 (0.28-1.61)	0.375	-	-
Large	96	1.58 (0.72-3.46)	0.254	-	-
Year of sampling			0.709		
2014 (Ref)	113				
2015	136	0.81 (0.40-1.63)	0.548	-	-
2016	51	1.13 (0.47-2.72)	0.784	-	-

OR = Odds ratio, CI = confidence interval, Ref = reference category

Thus, co-resistance to cephalosporins and tetracycline occurred in 70.4% of isolates, co-resistance to cephalosporins, tetracycline and ciprofloxacin in 34.8% and co-resistance to cephalosporins, tetracycline, ciprofloxacin and sulfamethoxazole/trimethoprim was found in 24.4%. Significant differences among hosts in AMR rates were only observed against nalidixic acid, trimethoprim, and sulfamethoxazole, with dairy cattle presenting significantly higher prevalence of resistance (Fig 4). Specifically, proportions of resistant isolates in dairy cattle were higher than in sheep for nalidixic acid (OR=4.81, *p*=0.017) and trimethoprim (OR=4.59, *p*=0.004), and were higher in dairy cattle than in beef cattle for sulfamethoxazole (OR=3.26, *p* = 0.009) and trimethoprim (OR=3.57, *p*=0.006).

When MIC data were used for the phenotypic detection of ESBL and AmpC production, most of the 135 tested isolates presented a characteristic ESBL phenotype (107/135, 79.3%), 21 (15.6%) had an AmpC phenotype, and the remaining 7 (5.2%) had an ESBL+AmpC phenotype. Prevalence of inferred phenotypes within each production system is shown in Table 2.

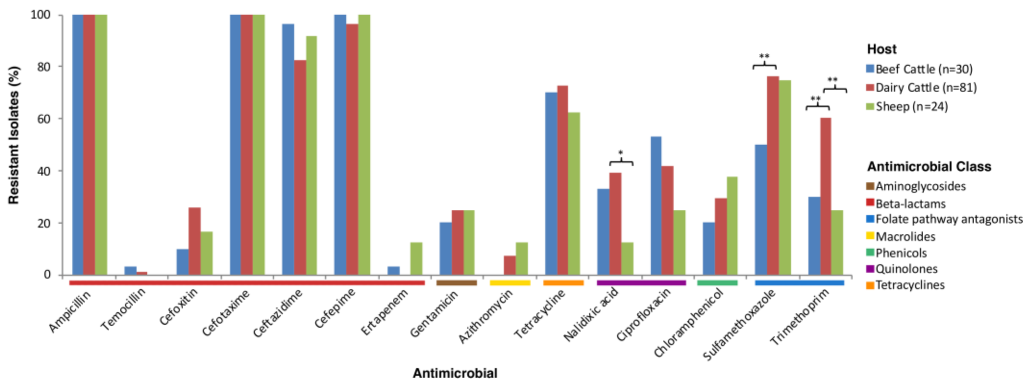

Figure 4. Proportion of isolates microbiologically resistant to different antimicrobials in the different production systems based on phenotypic characterization by broth microdilution. Antimicrobials are grouped according to their corresponding antimicrobial classes, which are colour coded. The asterisks denote significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

Whole genome sequencing (WGS) and antimicrobial resistance genotype. Sixty-six isolates (13 beef cattle, 43 dairy cattle, and 10 sheep) were selected for WGS based on their presumptive phenotypic AMR profiles (52 ESBL, 11 AmpC, and 3 ESBL+AmpC). The sequencing facility provided an average of 8.6M ± 1.6M of reads per sample (range = 5.2-12.1M) corresponding to an average coverage of 258X ± 48X (range = 157-364X) in a 5Mb genome with a mean quality reads of 36.1. The median N50 of assemblies was 154Kb (IQR = 117-185Kb). The median number of contigs recovered per sample were 277 (IQR = 189-337) with an average contig length of 442Kb ± 130Kb (range = 202-736Kb) (Table 5).

Table 4. Distribution of MIC values for the 135 *E. coli* isolates from cefotaxime-containing medium

Antimicrobial Class[a]	Antimicrobial Agent	% Microbiological resistance				No. of isolates at the indicated MIC (mg/L)[b]																
		TOTAL (N=135)	Beef Cattle (N=30)	Dairy Cattle (N=81)	Sheep (N=24)	0.02	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
β-Lactam (Penicillin)	**Ampicillin**	100	100	100	100														135			
	Temocillin[c]	1.5	3.3	1.2	0								2	44	67	20	2					
β-Lactam (Cephalosporin 2nd gen.)	**Cefoxitin**	20.7	10	27.2	16.7								8	60	39	4	8	10	6			
β-Lactam (Cephalosporin 3rd gen.)	**Cefotaxime**	100	100	100	100								3	11	10	15	26	19	51			
	Ceftazidime	87.4	96.7	82.7	91.7					1	16	36	9	13	10	30	20					
β-Lactam (Cephalosporin 4th gen.)	**Cefepime**	97.8	100	96.3	100				3	15	17	3	10	29	25	13	14	6				
β-Lactam (Carbapenem)	**Ertapenem**	3	3.3	0	12.5	86	31	14	4													
	Imipenem	0	0	0	0				53	71	11											
	Meropenem	0	0	0	0		134	1														
Aminoglycoside	**Gentamicin**	23.7	20	24.7	25						52	45	6			5	4	23				
Macrolide	**Azithromycin**[d]	6.7	0	7.4	12.5								1	32	86	7	5	1	3			
Tetracycline	**Tetracycline**	70.4	70	72.8	62.5								29	10	1			29	66			
Glycylcycline	**Tigecycline**	0	0	0	0					116	19											
Quinolone	**Nalidixic acid**	33.3	33.3	39.5	12.5									81	7	2	1		4	40		
(Fluoro)quinolone	**Ciprofloxacin**	41.5	53.3	42	25	35	42	2		11	15				7	23						
Phenicol	**Chloramphenicol**	28.9	20	29.6	37.5										90	6	2	11	17	9		
Polymyxin	**Colistin**	0	0	0	0							134	1									
Folate pathway inhibitor	**Sulfamethoxazole**	70.4	50	76.5	75										4	30	6					95
	Trimethoprim	47.4	30	60.5	25					12	38	18	3					64				

[a]gen, generation

[b]White fields denote range of dilutions tested for each antimicrobial agent. MICs above the range are given as the concentration closest to the range, except for sulfamethoxazole which is indicated as the highest concentration tested. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration tested. Vertical lines indicate European Committee for Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values.

[c]As from March 23rd 2020, the ECOFF for temocillin was fixed at 16 mg/L.

[d]No ECOFF given by EUCAST; reference as indicated by double vertical lines was used for azithromycin (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015).

WGS analyses identified 52 acquired AMR genes along with point mutations in another four genes (Fig. 5) that code for resistance to antimicrobials representing 11 different classes. The presence of ESBL-encoding genes was detected in 55 of the 66 genomes. Most of them carried $bla_{CTX\text{-}M}$ type genes (50/55, 90.9%), the most abundant being $bla_{CTX\text{-}M\text{-}14}$ (n=27), followed by $bla_{CTX\text{-}M\text{-}1}$ (n=9), $bla_{CTX\text{-}M\text{-}15}$ (n=7), $bla_{CTX\text{-}M\text{-}32}$ (n=5), and $bla_{CTX\text{-}M\text{-}14b}$ (n=2). bla_{SHV} genes were only sporadically found ($bla_{SHV\text{-}12}$, n=5). Ten isolates carried the AmpC-encoding genes $bla_{CMY\text{-}2}$ (n=9) and $bla_{CMY\text{-}4}$ (n=1). $bla_{CMY\text{-}2}$ was found in combination with $bla_{TEM\text{-}1B}$ in four isolates and with $bla_{TEM\text{-}1A}$ in one. Additionally, a point mutation in the *ampC* promoter (nt 42 C→T) was found in two isolates, in one of them in combination with $bla_{CTX\text{-}M\text{-}14}$, this being the only isolate co-harbouring more than one ESBL/AmpC genetic determinant. In this isolate, presence of both $bla_{CTX\text{-}M\text{-}14}$ and a mutation in the *ampC* promoter resulted in a much higher MIC value for cefotaxime (MIC > 64 mg/L) compared to the isolate that only carried the *ampC* mutation (MIC = 2 mg/L). However, no difference was observed in ceftazidime and cefoxitin MIC values. The distribution of MIC values and presence of the different ESBL/AmpC coding genes associated to some of the β-lactams tested are shown in Figure 6. Other genes coding only for resistance to narrow-spectrum β-lactamases were also detected, such as TEM type genes (41/60, 68.3%), including $bla_{TEM\text{-}1B}$ (n=30), $bla_{TEM\text{-}1A}$ (n=9), $bla_{TEM\text{-}1D}$ (n=3), $bla_{TEM\text{-}1C}$, and $bla_{TEM\text{-}190}$ (one isolate each), as well as $bla_{OXA\text{-}1}$ (n=2). The majority of ESBL/AmpC genes (61/67) were associated with plasmid-derived contigs; the only chromosomally located ESBL gene was $bla_{CTX\text{-}M\text{-}15}$ in a single isolate. Most of the ESBL/AmpC genes carrying plasmids were identified as IncI1 (Supplementary Table S2). Thus, $bla_{CMY\text{-}2}$, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{SHV\text{-}12}$ were always associated to IncI1 plasmids. On the other hand, $bla_{CTX\text{-}M\text{-}14}$ was not only found in IncI1 (n=3) but also in other *rec* types (IncB/O/K/Z, n=9; IncHI2, n=2), while plasmid-located $bla_{CMY\text{-}4}$ and $bla_{CTX\text{-}M\text{-}15}$ genes were associated with IncQ1 and p0111, respectively.

Table 5. Overview of sequencing raw data and assembly for each sample

Raw Reads Stats			Assembly Stats				
Sample ID	**Total Reads**	**Coverage (X)**	**Number of contigs**	**Genome Length**	**Contig Length**	**N50**	**Mean Q**
E_0670	10449667	316	233	5124332	377229	191127	35.9
E_0684	11288346	341	426	5083178	304329	90141	36.0
E_0685	11594106	350	426	5078030	204263	77542	36.1
E_0688	10172991	307	323	5082618	693943	300597	36.1
E_0696	9003830	272	310	5123383	313965	154413	36.0
E_0699	9422908	285	2190	5730896	385298	154517	36.1
E_0701	5512266	166	219	5002886	531328	184710	36.1
E_0704	10114010	305	1238	5595899	538420	111013	36.1
E_0706	9911660	299	344	5034410	375213	140612	36.1
E_0708	8208039	248	165	5081399	303905	107823	36.1
E_0713	8408546	254	70	4921505	617690	307619	36.1
E_0715	9852072	298	3368	5956319	617690	197414	36.2
E_0718	12075348	365	423	4830241	272238	67637	36.1
E_0721	6704668	202	262	5013913	482556	180338	36.1
E_0722	7537337	228	97	4970888	577591	229561	36.1
E_0724	8511647	257	87	4760759	655571	235006	36.3
E_0727	8576376	259	323	5131117	443643	160931	36.1
E_0730	10255297	310	113	4710397	580758	141547	36.3
E_0731	10392095	314	339	4949673	270088	127956	36.1
E_0736	9425340	285	276	4982195	485749	153062	36.1
E_0738	10739223	324	306	4820705	396548	148051	36.1
E_0740	6058225	183	236	5096373	507731	192883	36.1
E_0741	9450007	285	111	5062833	510463	192873	36.1
E_0744	7767418	235	312	5133630	263722	112961	36.0
E_0745	7547723	228	307	5130514	250954	101135	36.0
E_0746	6795967	205	319	5172150	451627	140357	36.0
E_0747	10466017	316	297	5128206	216206	101135	36.1
E_0748	5771065	174	328	5443211	412575	192367	36.1
E_0750	8771906	265	107	5128618	419721	186340	36.2
E_0751	7626077	230	236	5163695	584633	184313	36.1
E_0753	5694475	172	454	5273242	201498	83199	36.1
E_0754	11306234	341	653	5324785	215631	80823	36.1
E_0756	5210464	157	196	4932972	520968	156744	36.1
E_0757	9543257	288	288	4958350	558770	175344	36.1
E_0760	7766853	235	248	4971802	449956	143812	36.1

E_0773	7039381	213	255	5021684	466502	174061	36.1
E_0775	7622826	230	220	5010855	466502	184846	36.1
E_0776	7099360	214	207	5428724	361337	153395	36.1
E_0780	8518935	257	189	5239524	376463	170363	36.2
E_0781	10143392	306	316	5019607	419338	159869	36.1
E_0786	8427651	255	134	5194995	378190	154413	36.1
E_0787	7786516	235	127	5075828	353718	155777	36.1
E_0789	11655146	352	418	5244828	657124	129801	36.1
E_0790	9170976	277	337	5195112	657124	130992	36.1
E_0792	7643794	231	179	5080889	481791	116603	36.1
E_0795	7826957	236	115	5032537	581058	200385	36.1
E_0796	7038812	213	319	5168309	410150	116911	36.1
E_0798	8087201	244	189	5133473	371154	111618	36.1
E_0802	10290772	311	403	5175436	430083	181418	36.1
E_0803	6638566	200	222	4808631	389149	192918	36.1
E_0806	5990319	181	216	5095474	522750	184343	36.1
E_0811	8353535	252	115	4829516	507071	174720	36.2
E_0812	9455310	286	179	5274771	452705	169624	36.1
E_0817	7330179	221	153	4862709	333424	127725	36.3
E_0818	8048331	243	237	4969464	617690	193574	36.1
E_0820	7978446	241	308	4802893	272238	71218	36.1
E_0821	9186047	277	319	5212464	410150	112991	36.1
E_0822	8387153	253	279	5014700	574565	173293	36.1
E_0825	7820435	236	251	5102287	313965	154413	36.1
E_0826	7944475	240	343	5222936	384714	132476	36.1
E_0828	8698058	263	361	5275849	415492	175277	36.1
E_0832	7919339	239	178	5088265	273665	107823	35.9
E_0833	10201182	308	382	5366702	538420	111889	36.1
E_0834	8458453	255	348	5422047	735840	133443	36.1
E_0836	7339805	222	243	5211472	522852	210039	36.1
E_0837	8979130	271	312	5237591	522852	210039	36.1

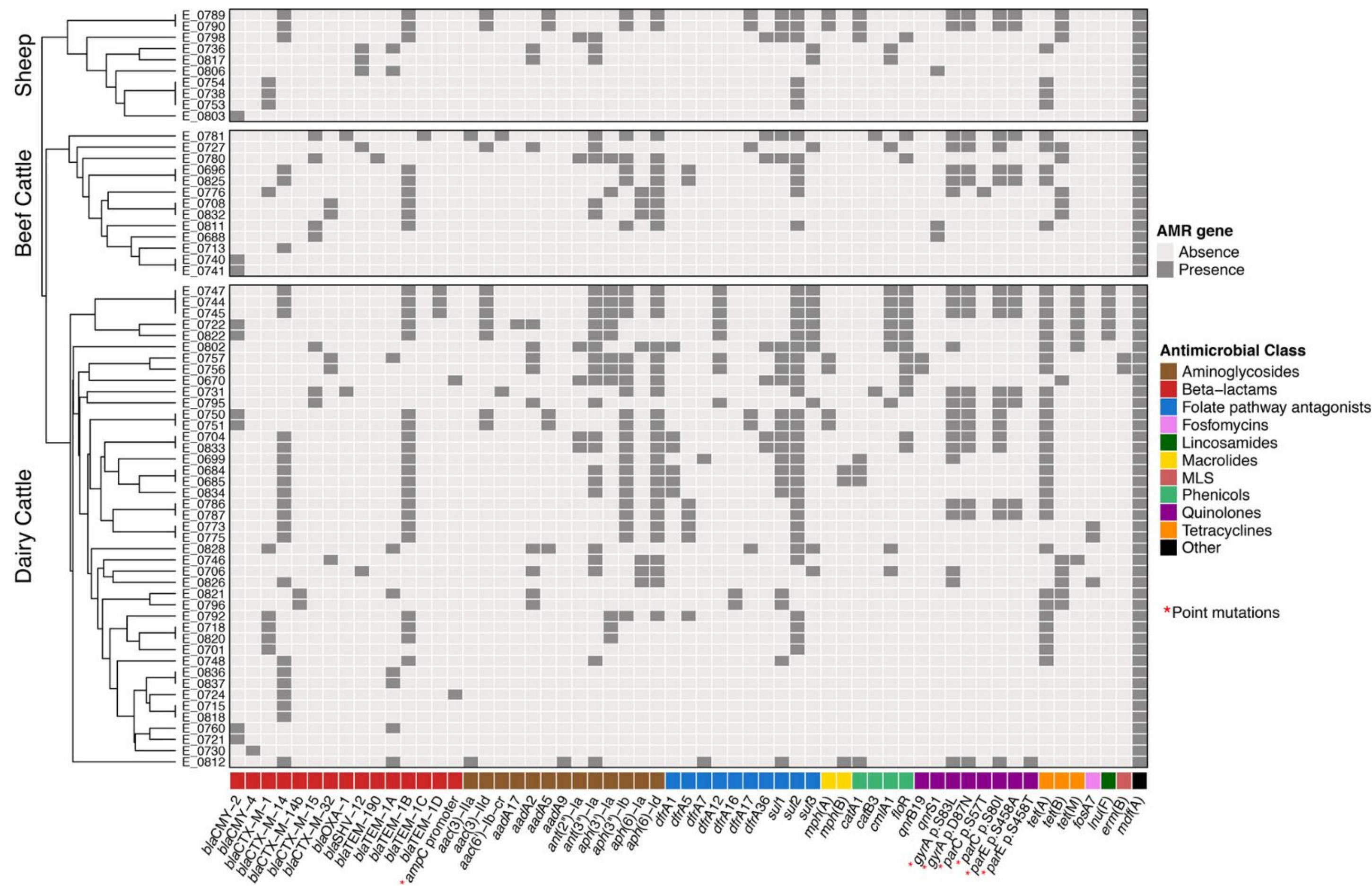

Figure 5. Heatmap showing the distribution of AMR genes detected by WGS in each isolate stratified by production system. Within each production system, samples were grouped based on their antimicrobial resistance pattern according to the result of the hierarchical clustering using the average linkage method (UPGMA) on the Euclidean distance matrix. Genetic determinants of resistance are grouped according to their corresponding antimicrobial classes, which are colour coded. Point mutations are indicated by red asterisks

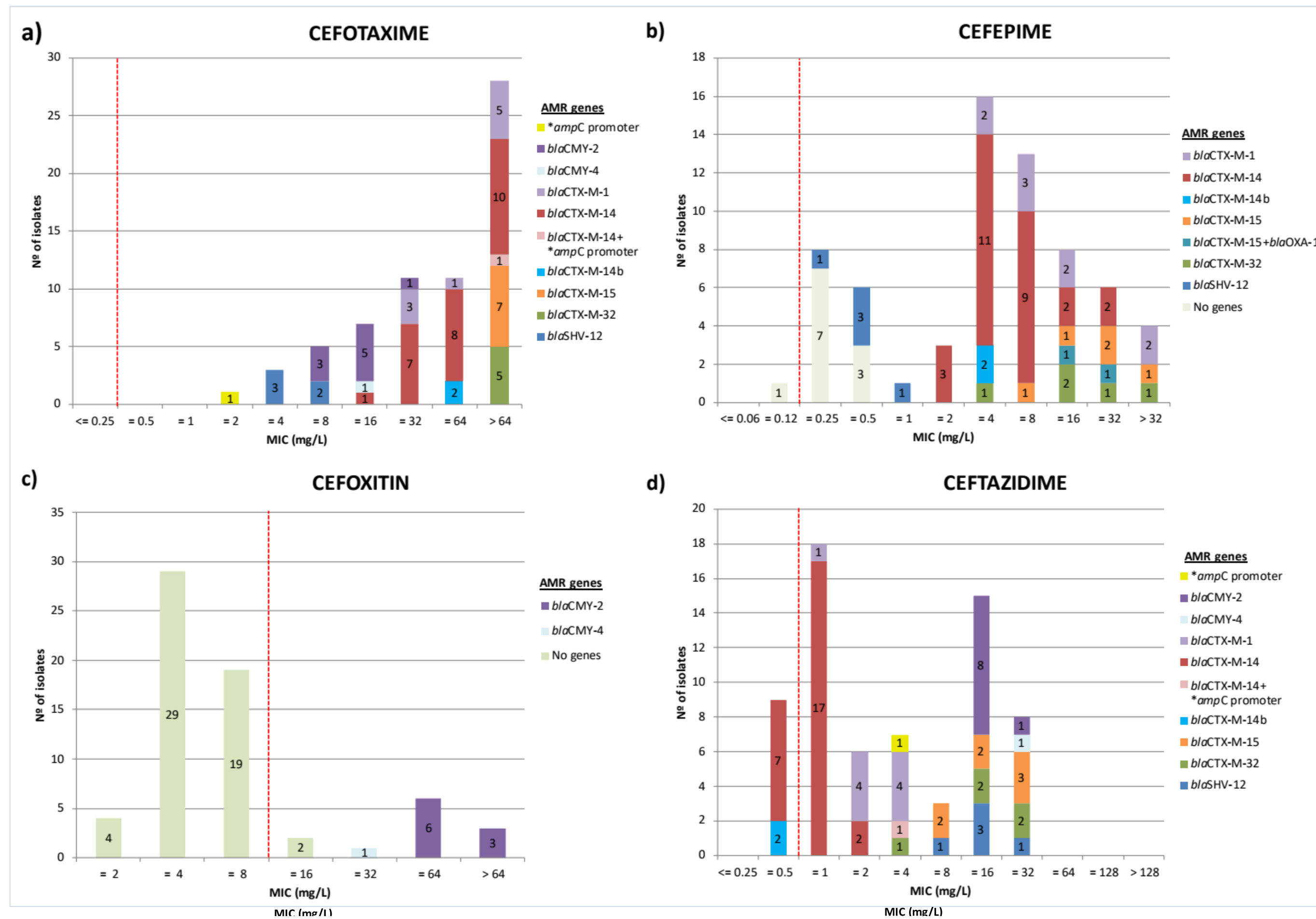

Figure 6. Bar plots illustrating the phenotypic and genotypic characterization of resistance to the β-lactams cefotaxime (a), cefepime (b), cefoxitin (c), and ceftazidime (d). Numbers within the stacked bar plots indicate the number of isolates observed with a particular MIC and genotype. ECOFF values are indicated with red dashed lines. Point mutations are indicated by asterisks. “No genes” refers to those isolates lacking any genetic determinant of resistance for the corresponding antimicrobial

Other genetic determinants of AMR found in the isolates included those associated with resistance to tetracyclines, aminoglycosides, phenicols, quinolones, sulfamethoxazole, trimethoprim, macrolides, lincosamide, and fosfomycin, many of them located in plasmids (Supplementary Table S2). Briefly, all tetracycline-resistant isolates (n=48) carried a tetracycline efflux gene, *tet*(A) being the most prevalent (37/48), although *tet*(B) and *tet*(M) were also detected in 14 and 7 isolates, respectively. Thirteen genes associated to resistance to aminoglycosides were detected, including genes encoding aminoglycoside acetyltransferases (*aac*), nucleotidyltransferases (*ant*), phosphotransferases (*aph*), and adenylyltransferases (*aad*). The distribution of those associated with gentamicin-resistance in relation to their MIC values is shown in Fig. 7a. Resistance to phenicols was coded by four genes, mainly those that activate efflux of phenicols (*floR* and *cmlA*), and also genes that mediate the enzymatic inactivation by chloramphenicol acetyltransferases (*catA* and *catB*) (Fig. 7b). Resistance to (fluoro)quinolones was associated to mutations in the gyrase (*gyrA*) gene alone and mostly in combination with different types of mutations in the topoisomerase genes, *parC* (codons 57 and 80) and *parE* (codons 355 and 458) (Fig. 7c). In addition, two isolates harboured the *aac(6´)-Ib-cr* acetyltransferase gene that confers resistance to ciprofloxacin. Another five isolates carried *qnr* genes (*qnr*B19 and *qnrS1*) that conferred resistance to ciprofloxacin (MIC = 0.25 mg/L, n=1 and MIC = 0.5 mg/L, n=4). Resistance to sulfamethoxazole was in all cases (49 isolates from which 35 were also resistant to trimethoprim) mediated by one or different combinations of the three *sul* genes (*sul1*, 42.9%; *sul2*, 81.6%; *sul*3, 24.5%), whereas resistance to trimethoprim was in all cases coded by different *dfr* genes encoding dihydrofolate reductases. However, four isolates with reduced susceptibility to trimethoprim did not present any genes coding for a phenotype of trimethoprim resistance when the ResFinder database was searched. Nevertheless, they all carried a gene that showed 100% homology with *dfrA36* (GenBank accession number CP038791), which was also found in another three isolates that also carried *dfrA1* gene.

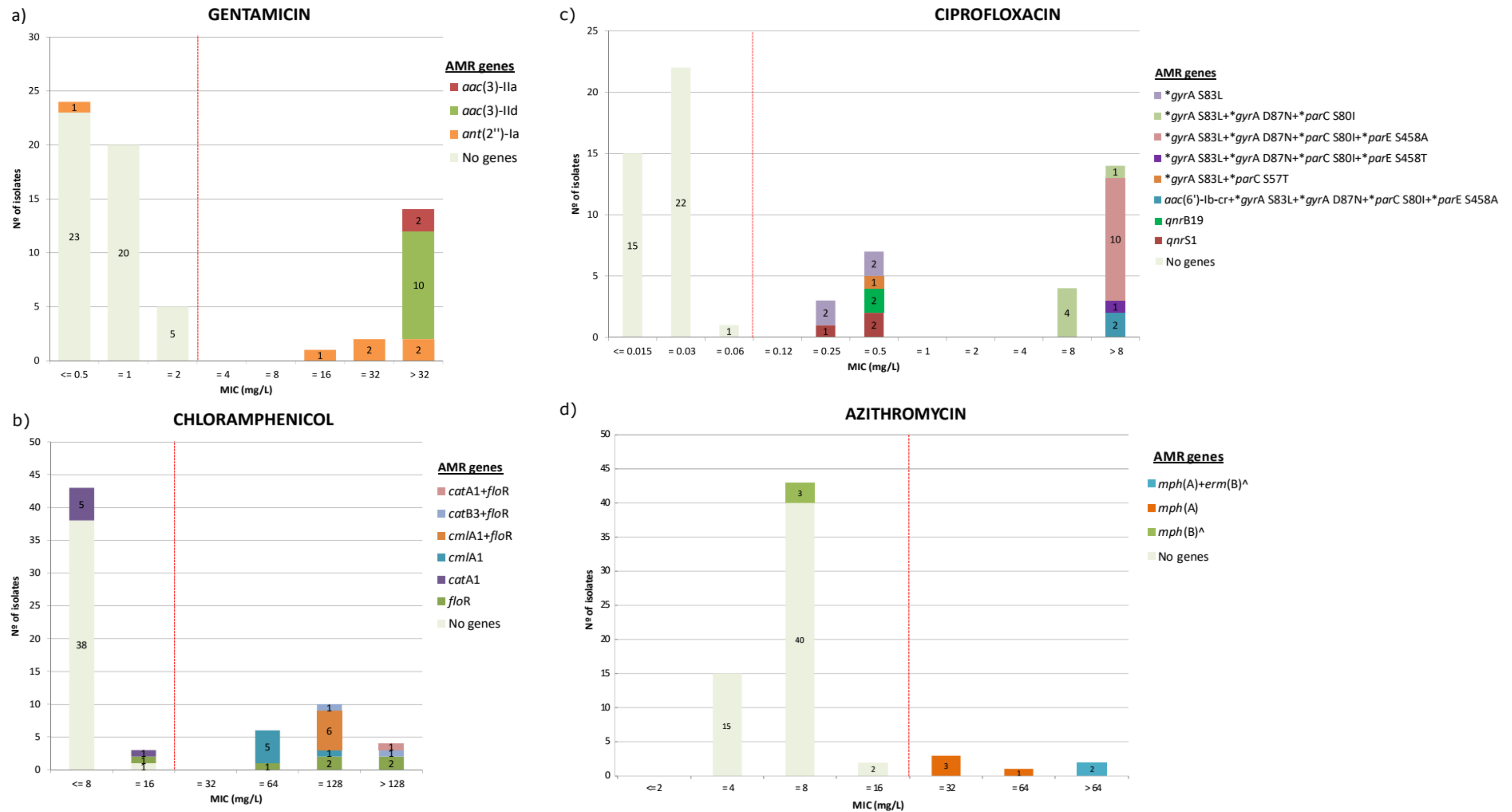

Figure 7. Bar plots illustrating the phenotypic and genotypic characterization of resistance to gentamicin (a), chloramphenicol (b), ciprofloxacin (c), and azithromycin (d). Numbers within the stacked bar plots indicate the number of isolates observed with a particular MIC and genotype. ECOFF values are indicated with red dashed lines. "No genes" refers to those isolates lacking any GDRs for the corresponding antimicrobial. Point mutations associated with ciprofloxacin resistance are indicated by asterisks. Genes marked with a caret symbol are not specific genetic determinants for azithromycin resistance but are associated with resistance to other macrolides.

Macrolide resistance encoding genes were sporadically identified, and included *mph*(A) (n=6), *mph*(B) (n=3), and also *erm*(B) (n=2). All six azithromycin isolates with MIC >16 mg/L carried the *mph*(A) gene, and the two with the highest MIC value (>64 mg/L) also carried *erm*(B) (Fig. 7d). The gene *lnu*(F), which confers resistance to lincomycin, was found in five isolates, and *fosA7*, which codes for fosfomycin resistance, was detected in three isolates, all obtained from dairy cattle. Finally, *mdf*(A) was present in all isolates.

Among the plasmids found, IncI1, IncQ1, and IncFIC were the ones that carried the greatest variety of AMR genes. Thus, besides several ESBL/AmpC genes, IncI1 plasmid harboured other AMR coding genes such as *aadA2*, ant(3'')-Ia, *cmlA*, *dfr*A16, *sul1*, *sul2*, *sul3*, and *tet*(A); IncQ1 carried *ant*(*3'')-Ia*, *ap*h(3''*)-Ib*, *aph(6)-Id*, *mph*(B), *dfrA1*, *sul1*, *sul2*, and *tet*(A), as well as bla_{CMY-4}; and, IncFIC carried *aph(6)-Ia*, *aph(6)-Id*, *tet*(A), and bla_{TEM-1B}.

In general, a strong agreement between gene presence and phenotypic susceptibility was observed as supported by kappa scores (Table 6), the only exception being cefepime, which presented the lowest agreement value. This was due to 10 isolates with reduced susceptibility to cefepime (MIC = 0.25 mg/L, n=7; MIC = 0.5 mg/L, n=3) that did not carry any genes described to confer resistance to this antimicrobial (Fig. 6b). However, these isolates carried bla_{CMY-2} (n=9) and bla_{CMY-4} (n=1) genes and displayed an AmpC phenotype.

Table 6. Concordance tests between phenotypic antimicrobial susceptibility testing and WGS-based predicted antimicrobial resistance.

Antimicrobial [a]	Sensitivity		Specificity		PPV[b]		NPV[c]		Cohen's kappa coefficient			
	%	95% CI	%	95% CI	%	95% CI	%	95% CI	κ	95% CI	*p* value	Interpretation
Tetracycline	100.0	92.6-100.0	100.0	82.4-100.0	100.0	92.6-100.0	100.0	82.4-100.0	1.00	1.00-1.00	<0.001	Very Good
Ciprofloxacin	100.0	87.9-100.0	100.0	90.8-100.0	100.0	87.9-100.0	100.0	90.8-100.0	1.00	1.00-1.00	<0.001	Very Good
Nalidixic acid	100.0	85.7-100.0	100.0	91.8-100.0	100.0	85.7-100.0	100.0	91.8-100.0	1.00	1.00-1.00	<0.001	Very Good
Chloramphenicol	100.0	83.9-100.0	84.8	71.8-92.4	74.1	55.3-86.8	100.0	91.0-100.0	0.77	0.62-0.93	<0.001	Good
Azithromycin[d]	100.0	61.0-100.0	100.0	94.0-100.0	100.0	61.0-100.0	100.0	94.0-100.0	100.0	1.00-1.00	<0.001	Very Good
Trimethoprim	100.0	90.1-100.0	100.0	89.0-100.0	100.0	90.1-100.0	100.0	89.0-100.0	1.00	1.00-1.00	<0.001	Very Good
Sulfamethoxazole	100.0	92.7-100.0	100.0	81.6-100.0	100.0	92.7-100.0	100.0	81.6-100.0	1.00	1.00-1.00	<0.001	Very Good
Gentamicin	100.0	81.6-100.0	98.0	89.3-99.6	94.4	74.2-99.0	100.0	92.6-100.0	0.96	0.89-1.00	<0.001	Very Good
Cefoxitin	85.7	60.1-96.0	100.0	93.1-100.0	100.0	75.8-100.0	96.3	87.5-99.0	0.90	0.77-1.00	<0.001	Very Good
Cefepime	84.6	73.9-91.4	100.0	20.7-100.0	100.0	93.5-100.0	9.1	1.62-37.7	0.14	0.11-0.39	0.024	Poor

[a] No data are provided for cefotaxime and ampicillin since all isolates were phenotypically and genotypically resistant, or for ceftazidime, since all isolates carried at least one gene associated to ceftazidime resistance.
[b] PPV, Positive Predictive Value
[c] NPV, Negative Predictive Value
[d] No ECOFF value given by EUCAST; a MIC > 16 mg/L was used as resistance breakpoints reference as proposed (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015)

3.1.4. Discussion

In this cross-sectional survey, herd-level prevalence of ESBL-, AmpC- and CP-producing *E. coli* was estimated in beef cattle, dairy cattle, and sheep without clinical signs of disease in the Basque Country (Northern Spain). A large and representative number of herds was tested, and selective isolation medium were used to increase sensitivity. Using cefotaxime-containing medium, presumptive ESBL/AmpC-producers were isolated in 15% of the herds, prevalence being significantly higher in dairy cattle (32.9%) than in beef cattle (9.6%) and sheep (7.0%). Although differences in sampling strategies and isolation methods among studies hamper comparisons, prevalence rates of ESBL/AmpC-producing *E. coli* in food-producing animals has been reported to vary by country and animal species. In Europe, prevalence in individual veal calves under one-year of age ranged from 7.1% in Denmark to 89.0% in Italy (mean in EU, 44.5%) in 2017 (EFSA and ECDC, 2019). Herd-level prevalence of ESBL/AmpC-producing *E. coli* was reported to be 30.0% (3/10) in cattle farms in eastern England (Ludden et al., 2019), and 41.0% (41/100) in a cross-sectional survey carried in dairy cattle in The Netherlands (Gonggrijp et al., 2016). In Germany, cefotaxime-resistant *E. coli* were found in 70% (42/60) and 85% (44/52) of beef and dairy cattle units, respectively (Hille et al., 2017). Studies in sheep are scarce, and it is difficult to find herd-prevalence data. In Switzerland, ESBL-producing *E. coli* were isolated in 6.9% of 58 sheep samples (Geser et al., 2012), similar to what we found in the study at herd level.

A higher prevalence of ESBL-/AmpC-producing *E. coli* in dairy cattle compared to beef cattle has already been reported (Hille et al., 2017). This might be associated to the different antimicrobial treatments used in the different management systems. In beef cattle, antimicrobial treatments are mostly implemented in young animals to treat diarrhoea and respiratory diseases, while dairy cattle suffer from a wider diversity of pathologies during their longer lifespan that need to be treated with antimicrobials. In dairy cattle, β-lactams are used to treat mastitis (mainly penicillins, but also cephalosporins such as ceftiofur and cefquinome) and also during dry-off to control and prevent intramammary infections following the last milking of the lactation period (mostly penicillins) (Simjee et al., 2018). However, intramammary application might be

expected to have less effect than oral administration on spread of AMR in the intestinal microbiota. In any case, the relationship between antimicrobial use and AMR is a complex process that differs depending on the bacterial species and the AMR involved. Most studies support the association between the use of third- or fourth- generation cephalosporins and the occurrence of ESBL/AmpC-producing *E. coli*, but the occurrence and persistence of ESBL- and/or AmpC-producing *E. coli* in the apparent absence of extended-spectrum cephalosporins use has been reported (Seiffert et al., 2013). In the Basque Country, management of beef cattle and sheep is semi-intensive and animals graze in farmland or mountain pastures most of the year, while dairy cattle are mostly housed in pens. Less intensive farm management systems have been associated with lower prevalence of infection with cefotaxime-resistant *E. coli* (Hille et al., 2017), maybe due to reduced stress and lower infection pressure and probability of re-circulation of resistant isolates.

WGS provided an insight into ESBL/AmpC resistance genes in ruminants in the Basque Country and identified $bla_{\text{CTX-M-14}}$ as the most common ESBL gene, and $bla_{\text{CMY-2}}$ as the most common resistant determinant of the AmpC phenotype. Here, $bla_{\text{CTX-M-14}}$ was significantly more prevalent than other CTX-M type genes like $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$, which have been reported to be the most prevalent ones in cattle in other countries (Schmid et al., 2013; Day et al., 2016; Gonggrijp et al., 2016; Michael et al., 2017; Ludden et al., 2019). In The Netherlands, although $bla_{\text{CTX-M-1}}$ still prevails, an increasing trend in prevalence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ has been reported in recent years (Ceccarelli et al., 2019). In our study, AmpC-type β-lactamases were mostly associated to the presence of plasmid-encoded genes (mostly IncI1−$bla_{\text{CMY-2}}$ but also IncQ1−$bla_{\text{CMY-4}}$), whereas a promoter mutation at position -42 of the chromosomally encoded *ampC* gene was only detected in two isolates. Opposite results were found in Dutch cattle where point mutations were more prevalent, while $bla_{\text{CMY-2}}$ predominated in the avian hosts (Ceccarelli et al., 2019). In Europe, the three most frequent *bla* genes reported in extended-spectrum cephalosporin-resistant *E. coli* isolates from humans have been reported to be, in descending order, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CMY-2}}$ (Seiffert et al., 2013). In Spain, despite the increasing prevalence of $bla_{\text{CTX-M-15}}$ in human

clinical samples, *bla*$_{CTX\text{-}M\text{-}14}$ is still a very prevalent CTX-M-type (Díaz et al., 2010; Cantón et al., 2012; Fernández-Reyes et al., 2014). On the other hand, *E. coli* strains harbouring *bla*$_{SHV\text{-}12}$ are mostly isolated from poultry and have been sporadically isolated from cattle (Briñas et al., 2005; Smet et al., 2010; Geser et al., 2012; Michael et al., 2017; Ceccarelli et al., 2019), but are commonly found in community-acquired *E. coli* infections in Spain (Díaz et al., 2010; Fernández-Reyes et al., 2014). Here, the ESBL gene *bla*$_{SHV\text{-}12}$ was only sporadically detected in two bovine and three ovine isolates, but represented a high proportion of the ovine isolates tested (3/10).

Acquired CPs in *E. coli* have been rarely identified in food-producing animals (Kock et al., 2018) and prevalence of CP-producing *E. coli* among livestock seemed to be low (<1%) in European countries (EFSA and ECDC, 2019). Here, no *E. coli* isolates were recovered from the carbapenem-containing medium used to screen for CP-producing *E. coli*, but four isolates displayed a MIC value just above the ECOFF for ertapenem while being susceptible to imipenem and meropenem. WGS, however, did not identify any known CP-encoding gene in neither of these isolates, but they were all AmpC-producers (*bla*$_{CMY\text{-}2}$ gene carriers). In fact, AmpC β-lactamase production has been linked to ertapenem resistance due to loss or downregulation of outer membrane porins (Mammeri et al., 2008). In the present study, two isolates were phenotypically resistant to temocillin based on the recently set ECOFF for *E. coli* (MIC > 16 mg/L) but did not harbour any temocillin-resistance encoding gene. Still, this result was not unexpected, as MICs for temocillin in the range of 16 to 128 mg/L have been described in CTX-M-producing *E. coli* (Cavaco et al., 2019).

Comparison of WGS and phenotypic resistance profiles showed an overall very good agreement. However, presumptive discrepancies were also noticed in some instances. Thus, nine isolates that carried *bla*$_{CTX\text{-}M\text{-}14}$ were resistant to cefotaxime and cefepime, but tested susceptible to ceftazidime. This was, however, not unexpected since CTX-M enzymes, and specifically CTX-M-14, have been reported to confer higher levels of resistance to cefotaxime than to ceftazidime, whose MICs sometimes remain within the susceptible range (Bonnet, 2004; Williamson et al., 2012). Costa Ramos *et al.* (Costa Ramos et al., 2015) demonstrated that *E. coli bla*$_{CTX\text{-}M\text{-}14}$-bearing isolates

switched from ceftazidime-susceptible to ceftazidime-resistant phenotypes under selective pressure by mechanisms yet unknown. On the other hand, 10 isolates resistant to cefepime carried bla_{CMY-2} (n=9) or bla_{CMY-4} (n=1) genes but no ESBL-coding gene. Even though this phenomenon is rare, the potential development of cefepime resistance in CMY-2-producing *E. coli* isolates has already been reported (Dona et al., 2019).

Interestingly, four trimethoprim-resistant isolates did not harbour any of the genes coding for a phenotype of trimethoprim resistance included in ResFinder (updated on December 5, 2019). However, they all carried *dfrA36*, a dihydrofolate reductase gene which has been recently described in *E. coli* isolated from healthy Swiss fattening calves (Wuthrich et al., 2019). In addition, another three isolates carried *dfrA36* in combination with *dfrA1*. All seven *dfrA36*-carrying isolates also harboured *floR* and *sul2*, which are integrated along with *dfrA36* within the florfenicol/chloramphenicol-sulfonamide resistance ISCR2 element (Wuthrich et al., 2019). *E. coli* are typically intrinsically resistant to macrolides (attributable to natural low macrolide permeability and multidrug efflux systems), with azithromycin displaying certain activity against some Gram (-) bacteria (Gomes et al., 2017). Although no ECOFF for azithromycin resistance in *E. coli* has been established, MIC > 16 mg/L has been proposed as the azithromycin resistance breakpoints in some Enterobacteriaceae (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015). In the present study, all six isolates with MIC > 16 mg/L carried the *mph*(A) gene, whereas two isolates that solely harboured *mph*(B) gene had a low MIC (8 mg/L). These results confirm the relevant role of *mph*(A) in macrolide susceptibility previously reported (Nguyen et al., 2009; Gomes et al., 2019). The presence of *mph*(A) together with another gene (*erm*(A), *erm*(B) or *ere*(A)) has been reported to result in slightly higher MIC values (> 32 mg/L) (Gomes et al., 2019). Here, the two isolates that carried *mph*(A) in combination with *erm*(B) gene had the highest MIC value (> 64 mg/L). The increased MIC value observed in isolates harbouring *mph*(A) together with *erm*(B), suggests a slight contribution of 23S RNA methylation encoded by the *erm* gene to an increase in resistance.

The fact that the majority of ESBL/AmpC genes were plasmid located was not unexpected, and neither was the widespread distribution of IncI1 plasmids, since they are the most common plasmid type in *E. coli* isolated from animals in Europe (Rozwandowicz et al., 2018). In addition, IncI1 plasmids also carried the greatest variety of other AMR genes, including genes that code for resistance to aminoglycosides, chloramphenicol, trimethoprim and sulfamethoxazole. IncQ1, a mobilizable non-conjugative plasmid, also carried several AMR genes as well as bla_{CMY-4} (Kotsakis et al., 2015). This would explain the commonly observed co-resistance to extended spectrum cephalosporins and other antimicrobials.

Genetic determinants associated to ciprofloxacin resistance consisted mostly on mutations in the chromosomally encoded quinolone resistance-determining regions (QRDRs) of the DNA gyrase and DNA topoisomerase IV genes, whereas plasmid-mediated quinolone resistance (PMQR) markers were less common (*qnrS1*, n=3; *qnrB19*, n=2; *aac(6')-Ib-cr*, n=2). Interestingly, presence of *aac(6')-Ib-cr* was associated to the accumulation of mutations in *gyrA* (S83N and D87N), *parC* (S80I), and *parE* (S458A) genes as has been described by Poirel *et al.* (2012). Conversely, *qnr* genes, when present, were the only genetic determinants of fluoroquinolone resistance. A gene that codes for a lincosamide nucleotidyltransferase (*lnu*(F)) confering resistance to lincosamides was detected in five dairy cattle isolates. In cattle, lincosamides are used to treat mastitis caused by Gram (+) pathogens (Constable et al., 2008). Resistance to lincosamides is not routinely tested in *E. coli*, but considering that *lnu*(F) has been detected in *E. coli*, the potential risk for dissemination to other pathogens is worrisome. Presence of *fosA7* in the chromosome of three isolates was striking because it codes for resistance to fosfomycin, an antibiotic that is not used in cattle in Spain. In humans, fosfomycin is a first-line antimicrobial for the empirical treatment of uncomplicated urinary tract infections, currently being reconsidered as an alternative for the treatment of multidrug resistant Gram (-) pathogens (Meletis, 2016). Many Gram (-) species carry the *fosA* gene in the chromosome, but it is not frequently found in *E. coli* chromosome (Ito et al., 2017). In *E. coli*, *fosA3* is the most common plasmid-mediated FosA-coding gene, particularly in East Asia (Wang et al., 2017a). This gene was first

detected as a chromosomal gene in *Salmonella enterica* serovar Heidelberg isolated from chickens (Rehman et al., 2017), and later also found in *E. coli*. In *S.* Heidelberg, *fosA7* gene was demonstrated to confer a high level of resistance to fosfomycin and found to be potentially transferable by horizontal gene transfer (Rehman et al., 2017). This is a concern that requires surveillance to monitor for the spread of fosfomycin resistance in bacteria.

In conclusion, this study provided an insight into the prevalence of cefotaxime-resistant *E. coli* in ruminants in the Basque Country and the associated genetic determinants of AMR. Results in cattle were similar to those found in other European countries, whereas those in sheep constituted an important contribution to the limited repository of sheep data. Overall, these results showed that ruminants are reservoirs for MDR commensal *E. coli*. However, all isolates were susceptible to tigecycline, imipenem, meropenem, and colistin, which is reassuring because some of these compounds are last-line antimicrobial agents for the treatment of human infections. The results of this regional, short-term investigation highlighted the need to turn this investigation into a long run surveillance program to monitor trends over time. Antimicrobial susceptibility testing by phenotypic and molecular methods is key in surveillance programs to enhance early detection of resistance development, monitor resistance trends and provide guidance to clinicians in selecting the adequate therapy; all with the final aim of mitigating resistance spread.

Data availability: Sequencing data of the 66 genomes analysed in this study have been deposited at the NCBI Sequence Read Archive (SRA) database under accession numbers SRR11810138 to SRR11810203, associated with the BioProject accession number PRJNA633740.

3.2. Study II

Within-farm dynamics of ESBL-producing *Escherichia coli* in dairy cattle: resistance profiles and molecular characterization by long-read whole-genome sequencing

3.2.1. Background

Cephalosporins (third- and higher-generation) and carbapenems are critically important antimicrobials for human medicine since in some instances they are either the sole or one of the limited therapies available to treat multidrug-resistant (MDR) bacteria in human infections (WHO, 2019). *E. coli* strains can become resistant to these antimicrobials by the acquisition of antimicrobial resistance genes (ARGs) coding for enzymes like extended-spectrum β-lactamases (ESBL), AmpC cephalosporinases and carbapenemases (CP). ESBL and AmpC enzymes are capable of hydrolysing various β-lactam antibiotics such as penicillins, third- and higher-generation cephalosporins, and monobactams, while AmpC enzymes are additionally active against cephamycins and resistant to inhibition by clavulanate. CPs confer resistance to a broad spectrum of β-lactams, including carbapenems, a last resort for treating MDR Gram (-) bacterial infections.

ESBL/AmpC-producing *E. coli* are widely distributed in livestock (Poirel et al., 2018; Dantas Palmeira and Ferreira, 2020) but their contribution as a source of human infection remains controversial (Collis et al., 2019). On the other hand, CP-producing *E. coli* are still scarcely detected in cattle (Madec et al., 2017; Kock et al., 2018; EFSA and ECDC, 2021). The spread of ESBL-/AmpC-/CP-producing *E. coli* can be the result of the selection of resistance (usually at the intestinal level) under the pressure of antibiotic usage, and the dissemination of such resistant bacteria by cross-contamination of faecal material among animals (Seiffert et al., 2013). In Study I (see 3.1.) a higher prevalence was detected in dairy cattle compared with beef cattle and sheep. However, the association of animal age with the likelihood of ESBL-/AmpC-producing *E. coli* shedding was not investigated. Besides, cross-sectional studies do not provide information on the long-term dynamics of bacterial shedding, which is relevant for understanding their potential for spread and persistence within the farm. Longitudinal data on faecal shedding of ESBL-/AmpC-producing *E. coli* within farm animals remains limited. Other longitudinal studies performed on dairy cattle either focused on a single farm, were short time-framed, or applied different approaches and methodologies

(Hordijk et al., 2013, 2019; Horton et al., 2016; Gay et al., 2019; Plassard et al., 2021) but none combined long term monitoring with a detailed genomic analysis.

To further explore the epidemiology of ESBL-/AmpC-producing *E. coli* on dairy cattle farms, we studied the dynamics of faecal shedding in animals from different age groups in five dairy cattle farms in the Basque Country. To increase detection efficiency, selective pre-enrichment was used. Phenotypic antimicrobial susceptibility of isolates recovered from the five farms was tested, and in-depth genome characterization of isolates from two of the farms was performed using long-read sequencing (Oxford Nanopore Technologies, ONT) to investigate ARG transmission dynamics. Bacterial chromosomes and plasmids were reconstructed and typed.

3.2.2. Materials and methods

Study design. A longitudinal study was carried out in dairy cattle farms in the Basque Country (northern Spain) to monitor the occurrence of ESBL-/AmpC-/CP-producing *E. coli* in apparently healthy animals. Five commercial farms (designated F1, F2, F3, F4, and F5) representative of the style of farming in the region, were selected to be enrolled in the study. Farms were located in the three counties of the Basque Country, and the distance between farms ranged from 15-25 km for those located within the same county (*i.e.* F3-F4 and F1-F2, respectively) and up to 160 km (F4-F5). Before the study started, our team paid a visit to each farm and, in the presence of the farm veterinary clinicians, farmers were interviewed face to face using a questionnaire that addressed general information about farm characteristics, management practices, vaccine programs, and antimicrobial drug use. Farm size based on the combined number of lactating and dry cows, heifers, and calves, ranged between 140 and 320 animals (mean=240), with the number of lactating cows ranging from 75 (F5) to 200 cows (F1).

Monthly visits over a one year-period were planned for faecal sample collection. However, one of the farms (F5) dropped out after 5 samplings due to operational changes; samplings in the other four farms were interrupted midway through the study due to the COVID-19 pandemic and resumed at different times after

the lock-down to complete the 12 samplings scheduled. Overall, the collection of faecal samples commenced in February 2019 and ended in October 2020, and extended over 16-17 months within individual farms. Samples were collected from apparently healthy animals from different age groups defined according to the different management practices, *i.e.*, 1-5 month-old calves, 5-22 month-old heifers, and lactating cows. At each sampling time, rectal faecal samples (minimum of 5 g) were collected with a gloved hand from five animals randomly selected within each age group, and analysed in a single 25 g pool per age group (5 g per animal). In seven time points, heifers could not be sampled in the two farms (five sampling times in F2 and two in F4) that raised heifer replacements at a breeding centre. A total of 760 rectal faecal samples were collected and analysed in 152 pools. Additionally, environmental slurry samples were also collected from F3 and F4 (two samplings each).

Selective isolation of ESBL-/AmpC- and carbapenemase (CP)-producing *E. coli*. Upon arrival, samples were refrigerated at 4°C and sample processing was carried out within three days after collection, at the latest. Pooled faecal samples (25 g) were thoroughly mixed, diluted 1:10 in buffered peptone water (BPW, bioMérieux), and incubated at 37ºC for 20±2 h. For the isolation of ESBL-/AmpC-producing *E. coli*, two loops (20 µl) of BPW were subcultured onto MacConkey agar supplemented with 1 mg/L of cefotaxime and incubated at 37ºC for 20±2 h. Two morphologically different colonies per plate were harvested and confirmed as *E. coli* by species-specific real-time PCR detection of the *uidA* gene (Frahm and Obst, 2003).

For the isolation of CP-producing *E. coli*, two loops (20 µl) of BPW were subcultured onto MacConkey agar without antibiotics. A loopful of grown colonies was then harvested for DNA extraction and subjected to a real-time PCR amplification screening targeting the CP-coding genes bla_{NDM}, bla_{VIM}, bla_{KPC}, and $bla_{\text{OXA-48}}$ (Ellington et al., 2016). If any of these genes tested positive, a loopful of bacterial growth from the MacConkey agar was subcultured on ChromID® Carba Smart selective agar plates (bioMérieux), and isolated colonies were identified by *uidA* gene detection as above.

Antimicrobial susceptibility testing by broth microdilution. Between 1 and 3 isolates per plate were selected and tested to assess antimicrobial susceptibility.

Minimum inhibitory concentrations (MICs) were determined by broth microdilution using two Sensititre® MIC susceptibility plates (EUVSEC1 and EUVSEC2, Thermo Fisher Scientific) following the recommendations in Commission Implementing Decision 2013/652/EU (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri_CELEX:32013D0652&from_EN) concerning antimicrobials and dilution ranges, and the results were interpreted using epidemiological cut-off values (ECOFF). For antimicrobials with no ECOFFs assigned at the time, results were interpreted as follows: for temocillin ECOFF was fixed at 16 mg/L based on 2020/1729/EU; for azithromycin, 16 mg/L was used as reference based on the bibliography (Sjölund-Karlsson et al., 2011; Clinical and Laboratory Standards Institute, 2015).

Whole-genome sequencing (WGS) and bioinformatic analyses. Based on their phenotypic AMR profile, sampling time, and age group isolation source, 41 isolates (27 from F4, 11 from F1, and one each from F2, F3, and F5) were selected for WGS. For in-depth genome characterization, genomic DNA was extracted from pure cultures using NZY Microbial gDNA Isolation kit (NZYtech) and subjected to long-reads (Oxford Nanopore Technologies, ONT) WGS. For ONT sequencing, a library was prepared using the Ligation Sequencing Kit (SQK-LSK109). Native barcoding genomic DNA kits (EXP-NBD104 and EXP-NBD114) were used for sample multiplexing except for three isolates that were sequenced in singleplex. Libraries were run in FLO-MIN106 (R9.4.1) or FLO-MIN111 (R10.3) flow cells on a MinION Mk1C device (ONT). For validation purposes, five isolates also underwent short-reads (Illumina) WGS; genomic DNA was submitted to Eurofins Genomics, where libraries were prepared based on the NEBNext Ultra II FS DNA library prep kit (Illumina) and sequenced with Illumina NovaSeq 6000 (150-bp paired-end reads). The output files generated by ONT sequencing were basecalled in high accuracy mode (HAC) and quality-filtered using Guppy (Qscore > 7 in v4.2 and v4.3, and Qscore > 8 in v5.0). Then, reads were adapter-trimmed and filtered by length and quality as described in Study III (see 3.3.) and the resulting fastq reads were *de novo* assembled using Unicycler (Wick et al., 2017b). For one particular sample, Flye assembler (Kolmogorov et al., 2019) was used after retrieving inconsistent results in the draft genome generated with Unicycler, and the resultant assembly was the one

further used in this study. For isolates sequenced by both technologies, Illumina reads were pre-processed for assembly as described in Study I (see 3.1.) and the outputs were further used to generate hybrid Nanopore-Illumina assemblies with Unicycler (Wick et al., 2017b). As described In Study III (see 3.3.), isolates were subjected to *in silico* typing to determine their serogroup and phylogroup. MLST profiles were determined from unassembled long-reads using Krocus (Page and Keane, 2018). New sequence type (ST) assignations were obtained after submitting WGS reads to the Enterobase database (Zhou et al., 2020). Draft genomes were processed to predict plasmid- and chromosome-derived contigs using PlasFlow (v.1.1) (Krawczyk et al., 2018). Molecular characterization of the isolates, including screening of ARGs, chromosomal point mutations associated with AMR, virulence factors detection, and plasmid replicon identification were performed as in Study I (see 3.1.). Databases used for molecular characterization (ResFinder, PointFinder, PlasmidFinder, and ecoli_vf) were all updated on 20/10/2021. ResFinder hits were filtered at 90% coverage and identity and those with values below 100% were individually revised for frameshifts and amino acid changes, removing those considered not potentially functional. Virulence genes were filtered at 75% identity and 95% coverage, and the pattern of presence/absence of these genes was used as a typing scheme for genetic diversity. Genome annotations were carried out with Prokka (Seemann, 2014) and RAST (Aziz et al., 2008), and were graphically represented using SnapGene v.5.2.4 (http://www.snapgene.com/). Genome alignments were performed using MAUVE (Darling et al., 2010) in Geneious Prime v. 2020.2.4 (https://www.geneious.com) software. Blast Ring Image Generator (BRIG) v.0.95 was used for plasmid structural comparison (Alikhan et al., 2011).

Phenotypic resistance profiles and the genetic determinants of resistance (GDR) in each sequenced sample (chromosome and plasmids) were represented in heatmaps. The plasmid heatmap was graphed along with a dendrogram illustrating the similarity among plasmids based on their AMR pattern. The hierarchical clustering analysis for the dendrogram was performed with the unweighted pair-group method with arithmetic mean (UPGMA) based on the Jaccard distance matrix, using the function hclust (v.3.6.1) of the R statistical package v.3.6.3. To identify the shared and unique

phenotypic antimicrobial resistance profiles among the different age groups within each farm, Venn diagrams were constructed with the online tool InteractiVenn (Heberle et al., 2015).

Statistical analyses. To evaluate differences between age groups and farms in the shedding prevalence of cefotaxime-resistant *E. coli* and in the occurrence of phenotypic antimicrobial resistance for each antimicrobial, multivariate logistic regressions were performed including age group and farms as the explanatory variables. Adjusted odds ratios (OR_{adj}) were used as the measure of association between positivity and the explanatory variables and were expressed with their confidence interval at 95% (95% CI). Differences were considered statistically significant if $p < 0.05$. Simpson indices were estimated to calculate the diversity of phenotypic antimicrobial resistance profiles for each farm.

3.2.3. Results

Farms' descriptive data derived from the questionnaire. Following common practice in dairy farms in the Basque Country, all farms were closed production systems where replacement heifers originated from the same farm. Two of the farms (F2 and F4) raised their heifer replacements off-site in 2 different breeding centres. In both cases, animals leave the farm at 3-4 months of age and return already pregnant a few months before calving. A blanket antimicrobial treatment program was routinely used at dry-off that included the intramammary application of antimicrobials and teat sealant. The antimicrobials used for intramammary dry-cow therapy (DCT) were benzylpenicillin-benetamine/framycetin sulfate (Mamyzin) in F1 and F2, and cephapirin benzathine (Cefa-safe) in F3, F4, and F5. Farms participating in the study also used antimicrobials belonging to 12 antimicrobial drug classes for the treatment of disease in calves and cows. The antimicrobials most commonly used were third- and fourth-generation cephalosporins, followed by fluoroquinolones, and tetracyclines. Other antimicrobials used included penicillins, aminoglycosides, macrolides, and sulfonamides. Parenteral administration of fluoroquinolones was the most common treatment for mastitis during lactation in all except farm F2 where mastitis was not

treated with antimicrobials. Third- and fourth- generation cephalosporins were the most common drugs used to treat reproductive diseases, diarrhoea, and lameness that warranted systemic antimicrobial treatment. Reproductive diseases for which the producer opted to use antimicrobials included metritis, retained placenta, or other diseases related to reproduction.

Vaccination programs were quite different among farms. For example, vaccination against mastitis was only performed in F1. The vaccination program in F1 included vaccines against Infectious Bovine Rhinotracheitis (IBR), clostridia, and mastitis; IBR, *Leptospira,* and respiratory pathogens (Parainfluenza, bovine respiratory syncytial virus - BRS, *Mannheimia*) in F2; no vaccines at all were used in F3; IBR, Bovine viral diarrhoea virus and clostridia in F4; and, clostridia, diarrhoea in calves and respiratory pathogens (Parainfluenza, BRS, *Mannheimia*) in F5.

Cefotaxime-resistant *E. coli* isolates were frequently recovered in the five dairy cattle farms, but differences were found among age groups and farms. *E. coli* was isolated in cefotaxime-containing media in 92 of the 152 pooled faecal samples analysed (60.5 %) and in the 4 slurry samples collected from F3 and F4. These included samples collected from all farms and age groups, but differences in frequencies among age groups and farms were observed (Fig. 8). Overall, isolation frequency of cefotaxime-resistant *E. coli* was higher in lactating cows (OR_{adj}= 4.71 (1.76-12.64), p = 0.002) and calves (OR_{adj} = 4.21 (1.59-11.18), p = 0.004) compared with heifers, and lower in F1 and F2 compared with the other three farms (LR χ^2 = 21.55, $p < 0.001$).

The majority of cefotaxime-resistant *E. coli* isolates were also resistant to several other antimicrobials. When available, between 1 and 3 cefotaxime-resistant *E. coli* isolates per age group and sampling date were selected in each farm for antimicrobial susceptibility testing. Thus, 187 isolates from faecal samples (73 isolated from lactating cows, 40 from heifers, and 74 from calves) and 10 isolates from slurry were analysed. Since isolates had been obtained by selective isolation in a medium containing cefotaxime, they were all resistant to cefotaxime and ampicillin. Most isolates were also resistant to cefepime (99.0%) and ceftazidime (98.0%). Resistance to cefoxitin was detected in 36 isolates (18.3%) but 19 of them displayed a MIC value just

one dilution step above the ECOFF. All 197 isolates were susceptible to tigecycline and colistin. Two isolates obtained from the same pool of faeces collected from calves in F4 were resistant to all β-lactams tested, including temocillin and carbapenems (ertapenem, imipenem, and meropenem).

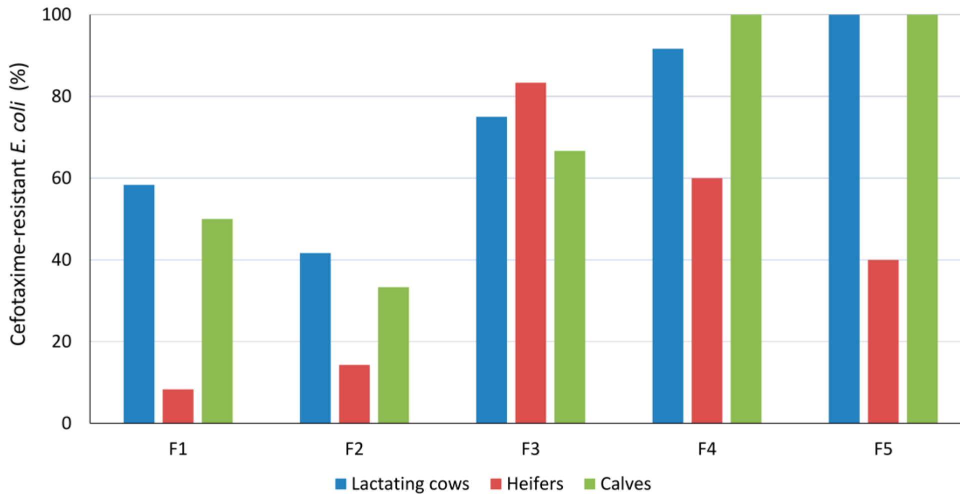

Figure 8. Isolation frequency of cefotaxime-resistant *E. coli* among age groups and farms. Results were based on 12 samplings per farm and age group, except for heifers in F2 and F4 where only 7 and 10 pool samples were collected, respectively, and F5, which dropped out from the study after 5 samplings due to operational changes.

Co-resistance to other antimicrobial classes was also observed in most isolates (161/197, 81.7%) and 72.1% (142/197) showed multidrug resistance (MDR, resistance to 3 or more antimicrobial classes). Overall, resistance to tetracycline (53.8%), nalidixic acid (45.7%), ciprofloxacin 66.5%), sulfamethoxazole (69.0%), trimethoprim (48.7%) and chloramphenicol (47.7%) was very frequent, while resistance to gentamicin (29.9%) and azithromycin (14.2%) was lower and mainly associated to F5. The prevalence of resistance to each antimicrobial tested did not differ between age groups. However, statistically significant differences between farms were observed in the occurrence of resistance to several antimicrobials. Compared to other farms, F1 and F5 presented a significantly higher prevalence of tetracycline, chloramphenicol, and trimethoprim (all with $p<0.001$). Resistance to gentamicin ($p<0.001$), azithromycin ($p<0.001$), ciprofloxacin ($p=0.002$), and nalidixic acid ($p=0.009$) were higher in F5 than in other farms, while resistance to cefoxitin was significantly higher in F1 and F2 ($p=0.003$).

The diversity of phenotypic resistance profiles varied among farms. A total of 45 different profiles of microbiological resistance (Table 7) resulting from the combination of antimicrobial agents that showed MICs above the ECOFF were observed in the study. Each phenotypic resistance profile was designated a letter of the Latin alphabet, and their distribution within each farm is represented in Figure 9. Within each farm, the number of different profiles ranged between 5 and 16 along the 12 samplings, the lowest diversity being found in F1 (Simpson index = 0.609) and the highest in F4 (Simpson index = 0.905). In F1, resistance to tetracycline, chloramphenicol, sulfamethoxazole, and trimethoprim remained stable during the entire study, whereas resistance to gentamicin, ciprofloxacin, and nalidixic acid was only observed in the second half of the study. This observation might reflect a shift in the circulating resistance profiles, where profile B, which dominated at the beginning of the study in all the age groups, was displaced by profile G in the second half of the study. On the contrary, the highest diversity in resistance profiles was observed in F4, where the three predominant profiles (A, C, and H) coexisted with 12 other profiles, with A and H dominating in the first half of the study, and profile C in the second half. Profile A only included resistance to ESBLs (penicillins and cephalosporins), whereas C and H included resistance to additional antimicrobials (Fig. 9, Table 7).

Table 7. List and abundance of the different AMR phenotypic profiles observed.

Profile Code	Phenotypic Resistance Profile	Isolates (n)
A	AMP-FOT-TAZ-FEP	27
B	AMP-FOT-TAZ-FEP-TET-CHL-SMX-TMP	15
C	AMP-FOT-TAZ-FEP-TET-CIP-SMX	13
D	AMP-FOT-TAZ-FEP-CIP	12
E	AMP-FOT-TAZ-FEP-GEN-TET-NAL-CIP-CHL-SMX-TMP	11
F	AMP-FOT-TAZ-FEP-GEN-AZI-TET-NAL-CIP-CHL-SMX-TMP	11
G	AMP-FOX-FOT-TAZ-FEP-GEN-TET-NAL-CIP-CHL-SMX-TMP	9
H	AMP-FOT-TAZ-FEP-GEN-NAL-CIP-CHL-SMX	8
I	AMP-FOT-TAZ-FEP-TET-NAL-CIP-SMX	7
J	AMP-FOX-FOT-TAZ-FEP	6
K	AMP-FOT-TAZ-FEP-CIP-TMP	6

L	AMP-FOT-TAZ-FEP-AZI-TET-NAL-CIP-CHL-SMX-TMP	6
M	AMP-FOT-TAZ-FEP-NAL-CIP-SMX	5
N	AMP-FOT-TAZ-FEP-TET-NAL-CIP-CHL-SMX-TMP	5
O	AMP-FOT-TAZ-FEP-TET-NAL-CIP	4
P	AMP-FOT-TAZ-FEP-GEN-TET-CIP-CHL-SMX-TMP	4
Q	AMP-FOT-TAZ-FEP-TET-SMX	3
R	AMP-FOX-FOT-TAZ-FEP-GEN-AZI-NAL-CIP-CHL-SMX-TMP	3
S	AMP-FOX-FOT-TAZ-FEP-NAL-CIP-SMX	3
T	AMP-FOT-TAZ-FEP-GEN-AZI-NAL-CIP-CHL-SMX-TMP	3
U	AMP-FOT-TAZ-FEP-TET-CHL-SMX	3
V	AMP-FOX-FOT-TAZ-FEP-ETP-SMX-TMP	3
W	AMP-FOT-TAZ-FEP-TET-NAL-CIP-SMX-TMP	3
X	AMP-FOT-TAZ-FEP-GEN-NAL-CIP-CHL-SMX-TMP	3
Y	AMP-FOT-FEP-TET-CIP-CHL-SMX-TMP	2
Z	AMP-TRM-FOX-FOT-TAZ-FEP-ETP-IMI-MERO-SMX-TMP	2
AA	AMP-FOT-TAZ-FEP-NAL-CIP-CHL-SMX-TMP	2
AB	AMP-FOX-FOT-TAZ-FEP-TET-SMX	1
AC	AMP-FOT-FEP-NAL-CIP-CHL-SMX-TMP	1
AD	AMP-FOX-FOT-TAZ-FEP-AZI-TET-NAL-CIP-CHL-SMX-TMP	1
AE	AMP-FOT-TAZ-FEP-IMI-GEN-AZI-TET-NAL-CIP-CHL-SMX-TMP	1
AF	AMP-FOX-FOT-TAZ-FEP-ETP-AZI-TET-NAL-CIP-CHL-SMX-TMP	1
AG	AMP-FOX-FOT-TAZ	1
AH	AMP-FOX-FOT-TAZ-FEP-TET-SMX-TMP	1
AI	AMP-FOT-TAZ-TET-SMX	1
AJ	AMP-FOT-TAZ-FEP-GEN-AZI-TET-CIP-CHL-SMX	1
AK	AMP-FOX-FOT-TAZ-FEP-CIP	1
AL	AMP-FOT-TAZ-FEP-GEN-TET-NAL-CIP-CHL-SMX	1
AM	AMP-FOX-FOT-TAZ-FEP-ETP-GEN-TET-NAL-CIP-CHL-SMX-TMP	1
AN	AMP-FOT-TAZ-FEP-GEN	1
AO	AMP-TRM-FOX-FOT-TAZ-FEP-GEN-AZI-NAL-CIP-CHL-SMX-TMP	1
AP	AMP-FOX-FOT-TAZ-FEP-CIP-TMP	1
AQ	AMP-FOT-FEP	1
AR	AMP-FOT-TAZ-FEP-GEN-TET-CIP-CHL-SMX	1
AS	AMP-TRM-FOX-FOT-TAZ-FEP	1

In F2, the prevalence of cefotaxime-resistant *E. coli* was the lowest, and fewer isolates were recovered and typed, particularly in heifers. Still, 11 different profiles were identified among 24 isolates, but profile D was the only one recovered in more than one sampling, in S7 in lactating cows and in S8 both in calves and lactating cows. In F3 high diversity in resistance profiles was observed, with a total of 16 different profiles, and a shift in the predominant resistance profiles occurred with time as happened in F4. Finally, the most outstanding feature of isolates recovered in F5 was the MDR pattern of all of them, with resistance to gentamicin and azithromycin being common in all age groups. On the other hand, isolates recovered from slurry samples shared their resistance profiles with isolates from faecal samples collected within the corresponding farms (Fig. 9 & Fig. 10).

Sequences generated by ONT sequencing successfully assembled into complete and circular chromosomes and plasmids. ONT sequencing provided a median of 60,729 reads per sample (IQR = 22,781–405,021) in a median of 631Mb per sample (IQR = 501-1,011 Mb) corresponding to a median coverage of 114X (IQR = 84X–182X) (Table 8). Upon assembly, the 5 isolates sequenced by both Illumina and ONT technologies, and 24 of the 36 ONT sequenced isolates resulted in circularised chromosomes. In all cases, the chromosome size of the assembled draft genome corresponded to the expected size of *E. coli* (median=4,999,307 bp; IQR=4,871,651 bp - 5,059,042 bp). Plasmid replicons were identified in a total of 125 contigs that in most cases (120/125, 96.0%) were assembled into complete circular plasmids. At least one plasmid replicon was identified in each isolate. IncF type plasmids were the most common (38/125, 30.4%), followed by IncB/O/K/Z (15/125, 12.0%), IncX1 (13/125, 10.4%), and IncY (13/125, 10.4%) along with 13 other replicon types. Screening for ARGs and SNPs associated with AMR identified 41 acquired ARGs and point mutations (9) in 4 other genes, coding for resistance to antimicrobials representing 9 different classes (Fig. 11). The combination of GDRs detected in each isolate resulted in 22 different genotypic profiles of resistance (Table 9). Sixty-two plasmids contained at least one ARG (Fig. 12). None of the IncL, IncP, IncX4, or Col plasmids carried ARG genes.

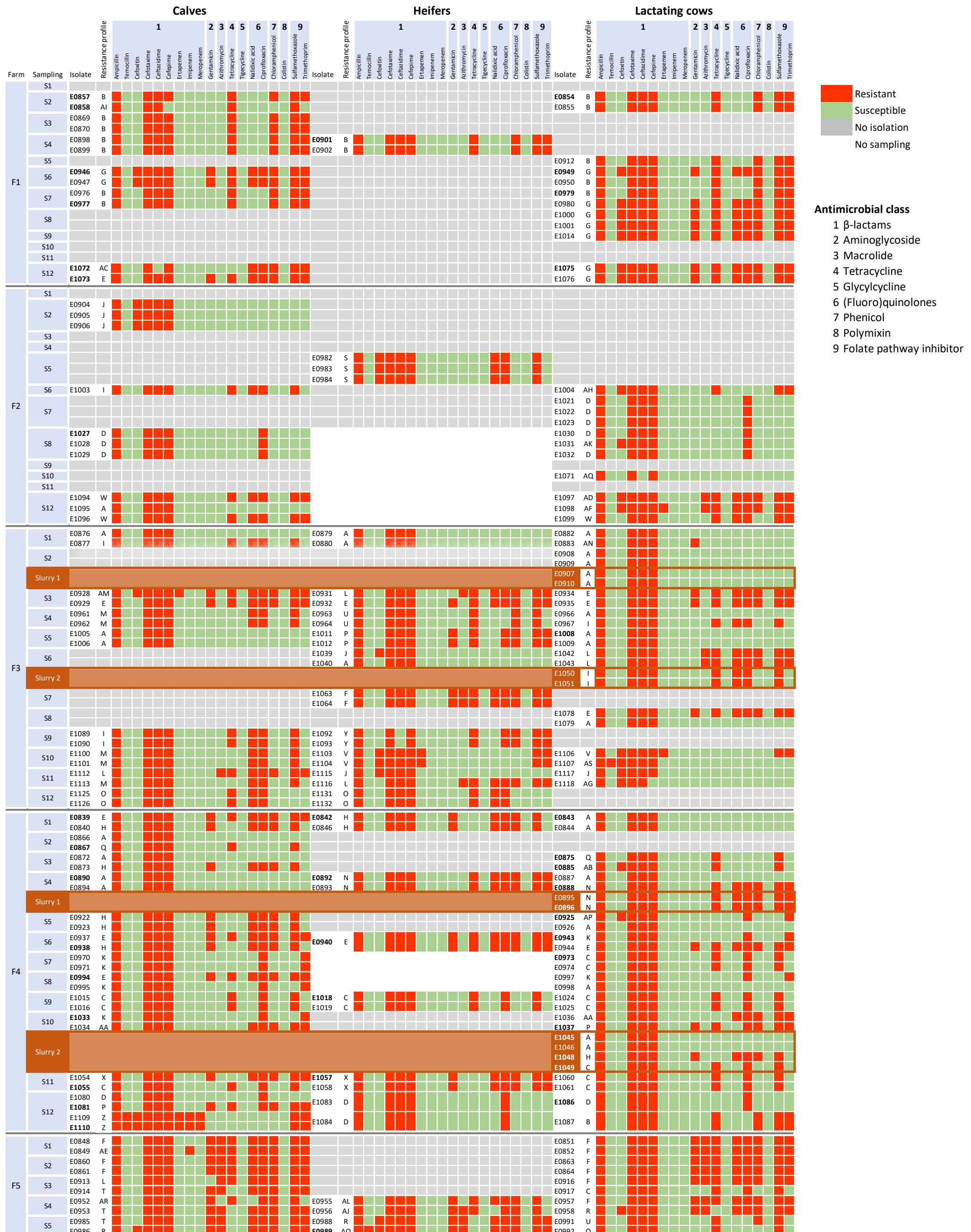

Figure 9. Distribution of AMR phenotypic profiles of the 197 *E. coli* isolates by farm, sampling, and age group. Each phenotypic profile is represented with a letter of the Latin alphabet as described in Table 7. Antimicrobial susceptibility, determined by the broth microdilution method and interpreted using epidemiological cut-off values (see text), is shown in green for susceptible, and in red for resistant isolates. Slurry samples are indicated with a different background colour (brown) and placed below lactating cows to save space. Antimicrobial classes are indicated with numbers: 1 = β-lactam, 2 = Aminoglycoside, 3 = Macrolide, 4 = Tetracycline, 5 = Glycylcycline, 6 = (Fluoro)quinolone, 7 = Phenicol, 8 = Polymyxin, 9 = Folate pathway inhibitor.

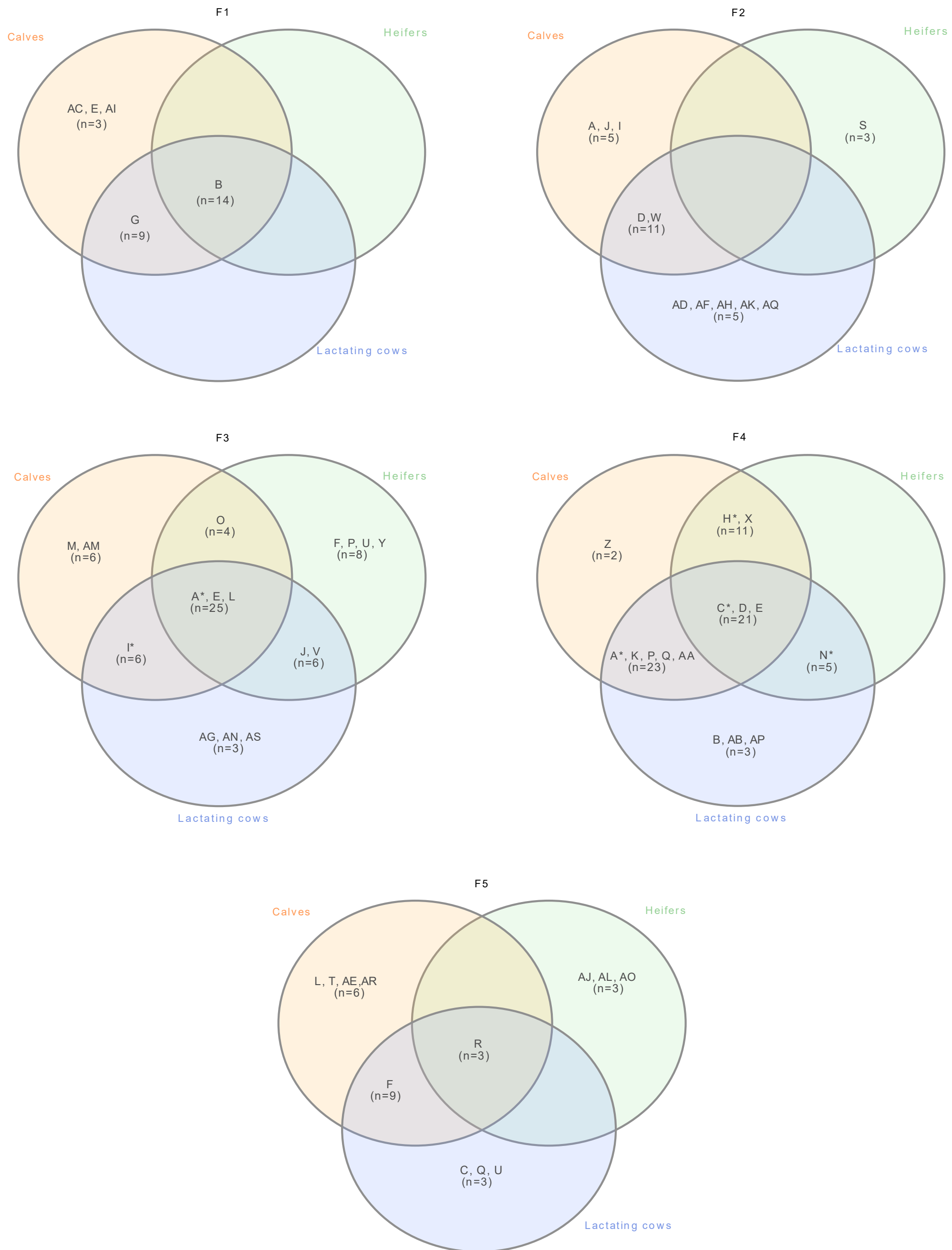

Figure 10. Venn diagrams showing the distribution of AMR phenotypic profiles for animal groups on each farm. AMR phenotypic profiles which are also detected in slurry samples are marked with an asterisk.

WGS confirmed the predominance of certain genomic subtypes of *E. coli* in F1 and great variability of strains in F4. A selection of isolates, mainly from farms F1 and

F4, were analysed by WGS to confirm whether the distribution of the different AMR profiles within the farms was due to different strains coexisting throughout the sampling period or to successive colonization by different strains. The 41 isolates were assigned to 18 MLST types, including two novel STs, *i.e.*, ST-11626 in F4 and ST-12870 in F3. In F1, the 11 isolates tested belonged to 4 ST types, and 2 of them (ST-69 and ST-2930) included more than one isolate (Fig. 11). Thus, ST-69 was represented by 5 isolates from the 3 age groups recovered at samplings S2, S4, and S7, which were identical in all other features, *i.e.*, phylogroup (D), serotype (O15:H18), phenotypic resistance profile (B), and genotypic profile (a).

Table 8. Overview of WGS read output data and assembly for each sample.

	ONT sequencing output data		Assemblies sequencing data						
Sample	**Total reads**	**Mb per sample**	**Assembly method**	**Total assembly size (bp) [chromosome + plasmids]**	**Chromosomic contigs (n)**	**Plasmidic contigs (n)**	**Unclassified contigs (n)**	**Chromosome size (bp)**	**Coverage (X)**
E0839	271,542	1,676	Unicycler-ONT	5,378,870	3	2	0	5,111,867	187.6
E0842	58,690	689	Unicycler-ONT	5,502,691	1	7	0	4,999,307	114.0
E0843	405,021	2,499	Unicycler-ONT	4,962,535	1	1	0	4,871,651	203.2
E0854	225,943	2,640	Unicycler-ONT	5,528,113	3	3	0	5,245,530	182.2
E0857	43,996	470	Unicycler-ONT	5,343,440	1	3	0	5,060,110	80.5
E0858	84,075	886	Unicycler-ONT	5,259,944	1	3	0	5,035,795	152.9
E0867	133,134	1,158	Unicycler-ONT	5,209,136	1	2	0	5,026,919	193.4
E0875	279,193	1,680	Flye-ONT	5,465,178	1	8	1	5,063,791	182.5
E0885	43,465	635	Unicycler-ONT	5,430,147	1	5	1	5,072,376	105.3
E0888	39,124	594	Unicycler-ONT	5,498,782	2	4	0	5,001,163	97.4
E0890	130,532	1,484	Unicycler-ONT	4,807,345	1	1	1	4,676,603	209.4
E0892	28,998	398	Unicycler-ONT	5,394,553	1	4	0	4,913,778	67.3
E0896	33,685	379	Unicycler-ONT	5,512,809	2	4	0	5,013,167	62.9
E0901	206,154	2,580	Unicycler-ONT	5,341,513	1	3	0	5,059,003	188.5
E0925	22,781	365	Unicycler-ONT	5,101,583	1	2	0	4,869,821	64.6
E0938	25,361	367	Unicycler-ONT	5,314,317	1	4	0	4,999,237	63.1
E0940	125,722	993	Unicycler-ONT	4,967,028	1	2	0	4,785,793	183.9
E0943	36,006	538	Unicycler-ONT	5,105,807	1	2	0	4,873,000	95.0

E0946	69,458	501	Unicycler-ONT	5,470,292	1	6	0	5,027,230	83.0
E0949	146,792	930	Unicycler-Hybrid ‡	5,488,241	1	10	0	5,026,163	157.0
E0973	52,722	647	Unicycler-ONT	5,040,759	1	1	0	4,955,174	115.5
E0977	58,621	479	Unicycler-ONT	5,348,237	1	4	0	5,062,028	81.5
E0979	74,990	670	Unicycler-ONT	5,341,560	1	3	0	5,059,042	112.6
E0989	51,190	522	Unicycler-Hybrid ‡	5,488,241	1	6	0	5,026,163	86.9
E0994	208,178	1,332	Unicycler-ONT	5,355,424	1	3	0	5,083,980	188.5
E1008	73,567	631	Unicycler-Hybrid ‡	4,920,105	1	2	0	4,811,098	116.9
E1018	44,444	563	Unicycler-ONT	5,040,851	1	1	0	4,955,262	116.6
E1027	75,618	625	Unicycler-Hybrid ‡	4,891,431	1	2	0	4,884,482	116.0
E1033	34,824	458	Unicycler-ONT	5,113,259	1	4	0	4,871,504	80.7
E1037	26,880	361	Unicycler-ONT	4,875,001	1	1	0	4,737,847	66.7
E1045	60,729	785	Unicycler-ONT	5,502,539	1	3	0	5,144,822	129.4
E1048	99,646	1,011	Unicycler-ONT	5,314,295	1	4	0	4,999,207	170.8
E1049	41,579	583	Unicycler-ONT	5,040,692	1	1	0	4,955,105	104.2
E1055	36,030	463	Unicycler-ONT	5,040,755	1	1	0	4,955,172	82.6
E1057	38,565	509	Unicycler-ONT	5,113,680	1	3	0	4,856,074	89.5
E1072	65,764	590	Unicycler-ONT	5,593,933	1	1	0	5,460,495	94.7
E1073	96,042	713	Unicycler-ONT	5,517,881	1	6	0	5,074,261	119.3
E1075	179,501	1,312	Unicycler-ONT	5,508,427	2	7	0	5,057,157	182.9
E1081	34,435	470	Unicycler-ONT	5,063,231	1	3	0	4,807,436	83.6
E1086	38,376	606	Unicycler-ONT	4,843,993	1	1	0	4,797,203	112.7
E1110	312,323	2,540	Unicycler-Hybrid ‡	4,961,728	1	1	0	4,816,563	201.5

ST-2930 included 4 isolates recovered in samplings S6 and S12 from calves and lactating cows that also shared all their genetic features, *i.e.*, they were all assigned to phylogroup A, serotype O100:H25, and genotypic profile c. However, they split into two phenotypic resistance profiles differing only in susceptibility to FOX (profile E, 1 susceptible isolate, MIC_{FOX}=2; and profile G, 3 resistant isolates, MIC_{FOX}=16). The remaining two isolates sequenced (E0858 and E1072) were recovered during the second and last samplings, and had unique features.

In F4, 27 isolates were sequenced and assigned to 4 phylogroups (A, B1, C, and D) and 13 different ST types. Eight of the ST types were identified in more than one isolate (n=2-4) and 5 were represented by a single isolate (Fig. 11). As in F1, the most

prevalent MLST type was ST-69. It included isolates recovered from lactating cows (S7), heifers (S9), calves (S11), and slurry. They all shared the same phylogroup (D), serotype (O15:H18), and genotypic (b) and phenotypic AMR profiles (C), suggesting that the same clone spread after sampling 7 in all animal groups and was also detected in slurry. As described in F1, differences associated with FOX among otherwise similar isolates were also observed in F4 within ST-23 (profiles AP and K) and ST-109 (profiles AB and Q). Other identical clones infecting several animals in F4 were those with MLST types ST-88 (n=2), and ST-448 (n=3).

On the other hand, several isolates with identical ST differed in other features (Fig. 11). These included differences in resistance due to the carriage of ARG-harbouring plasmids (*i.e.*, ST-4981 and ST-69) and occasionally also in chromosomally-encoded features (*i.e.*, ST-58 isolates, which differed in ARG-harbouring plasmids as well as serotype and chromosomally-encoded ARGs). Finally, the isolates from F2, F3, and F5 were unique in all their features. When virulence genes were examined, patterns of presence/absence were highly conserved within ST types, with ST-58 and ST-69 being the only exceptions (Supplementary Table S3). Thus, for these epidemiologically related isolates, the typing scheme based on the presence/absence of virulence genes confirmed the genetic diversity profile inferred from the combination of all other features (phylogroup, serotype, and GDR profile).

Resistance to cephalosporins was mainly due to plasmid-encoded *bla*$_{\text{CTX-M}}$ genes. F1 differs from F4 regarding the diversity and location of cephalosporin resistance genes. ARG-harbouring plasmids were present in all but two of the isolates (E1072 and E1027); 16 isolates carried a single plasmid and 23 carried 2 types of plasmids with ARGs. Overall, ARGs were present in 9 different types of plasmids, and 7 of them harboured ESBL-encoding genes, alone or in combination with several other ARGs (Fig. 12).

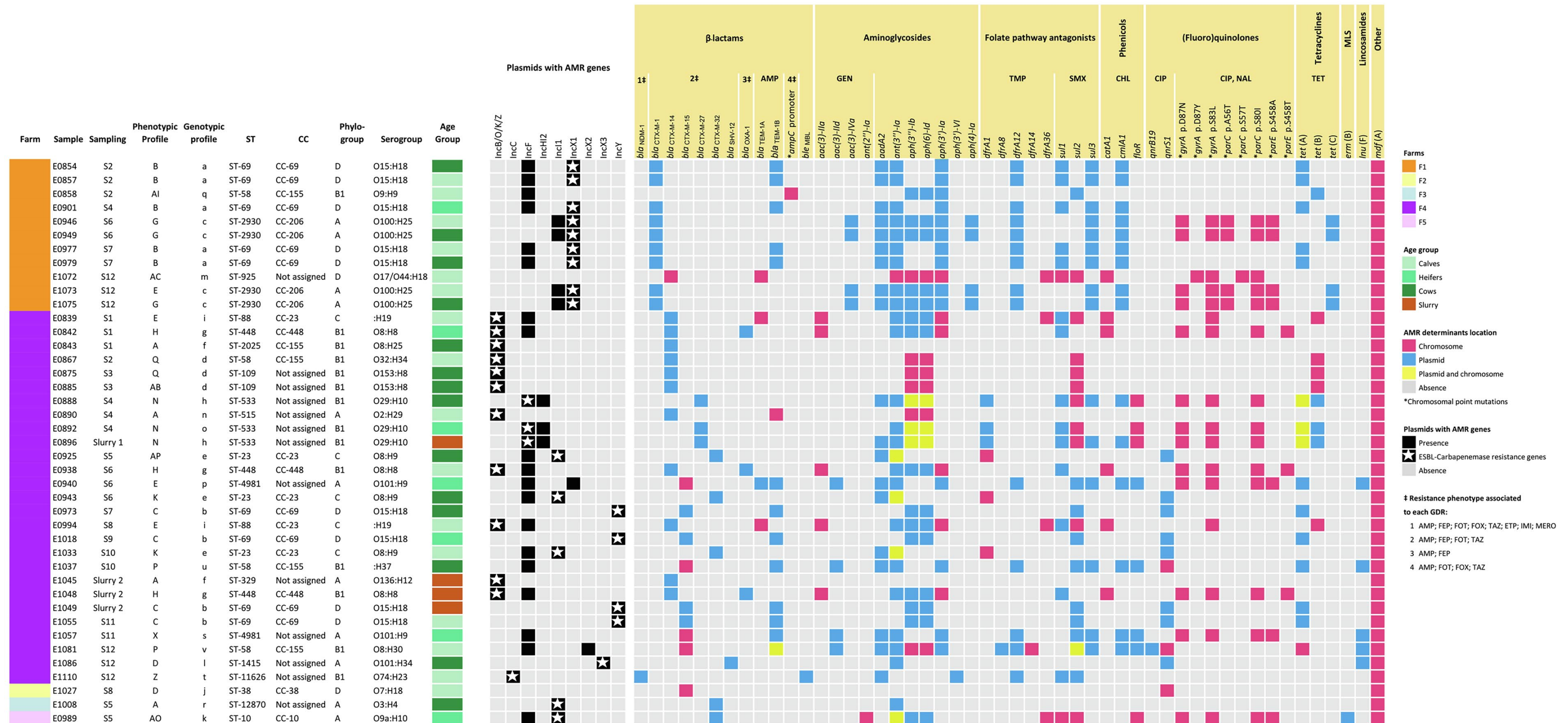

Figure 11. Heatmap showing the distribution of the AMR genes and plasmids detected by WGS (presence or absence and location are indicated as per the legend). Isolates are arranged per farm, sampling, and source (age group or slurry). Additional information including MLST type (ST and CC), phylogroup and serogroup are included. AMR phenotypic resistance profiles are as indicated in Figure 9 and described in Table 7. Each AMR genotypic profile resulting from an identical combination of GDR is represented with a letter of the Latin alphabet in lower case. The resistance phenotypes associated with each GDR are indicated for those antimicrobials tested, which were abbreviated as follows: ampicillin (AMP), cefepime (FEP), cefotaxime (FOT), cefoxitin (FOX), ceftazidime (TAZ), ertapemen (ETP), imipenem (IMI), meropenem (MERO), gentamicin (GEN), trimethoprim (TMP), sulfamethoxazole (SMX), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), tetracycline (TET). MLS, macrolide-lincosamide-streptogramin.

Table 9. List and abundance of the different AMR genotypic profiles observed.

Profile Code	Profile of Genetic Determinants of Resistance (GDR)	Isolates (n)
a	*bla*$_{\text{CTX-M-1}}$ /*bla*$_{\text{TEM-1B}}$/*aadA2/ant(3'')-Ia/aph(3')-Ia/dfrA12/sul1/sul3/cmlA1/tet*(A)/*mdfA*	5
b	*bla*$_{\text{CTX-M-15}}$/*bla*$_{\text{TEM-1B}}$/*aph(3'')-Ib/aph(6)-Id/sul2/qnrS1/tet*(A)/*mdfA*	4
c	*bla*$_{\text{CTX-M-1}}$ /*aac(3)-IVa/aadA2/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/aph(4)-Ia/dfrA12/sul3/cmlA1/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.A56T/**parC* p.S80I/**parE* p.S458A/*tet*(C)/*mdfA*	4
d	*bla*$_{\text{CTX-M-14}}$ /*aph(3'')-Ib/aph(6)-Id/sul2/tet*(B)/*mdfA*	3
e	*bla*$_{\text{CTX-M-32}}$/*aadA2/ant(3'')-Ia/dfrA1/qnrS1/mdfA*	3
f	*bla*$_{\text{CTX-M-14}}$/*mdfA*	2
g	*bla*$_{\text{CTX-M-14}}$/*bla*$_{\text{OXA-1}}$/*aac(3)-IIa/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/sul1/catA1/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/**parE* p.S458T/*mdfA*	3
h	*bla*$_{\text{CTX-M-27}}$/*aadA2/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/dfrA1/sul1/sul2/sul3/cmlA1/floR/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/*tet*(A)/*tet*(B)/*mdfA*	2
i	*bla*$_{\text{CTX-M-14}}$/*bla*$_{\text{TEM-1A}}$/*aac(3)-IIa/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/dfrA36/sul1/sul2/catA1/***gyrA* p.S83L /*tet*(B)/*mdfA*	2
j	*bla*$_{\text{CTX-M-15}}$/*qnrS1/mdfA*	1
k	*bla*$_{\text{CTX-M-32}}$/*ant(2'')-Ia/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/dfrA36/sul1/sul2/floR/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/**parE* p.S458A/*ermB/mdfA*	1
l	*bla*$_{\text{SHV-12}}$/*ant(3'')-Ia/qnrS1/lnuF/mdfA*	1
m	*bla*$_{\text{CTX-M-14}}$/*bla*$_{\text{TEM-1A}}$/*ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/dfrA36/sul1/sul2/catA1/***gyrA* p.D87Y /**gyrA* p.S83L /**parC* p.S57T/**parC* p.S80I/*mdfA*	1
n	*bla*$_{\text{CTX-M-14}}$ /*bla*$_{\text{TEM-1B}}$/*aph(3'')-Ib/aph(6)-Id/mdfA*	1
o	*bla*$_{\text{CTX-M-27}}$/*ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/dfrA1/sul1/sul2/floR/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/*tet*(A)/*tet*(B)/*mdfA*	1
p	*bla*$_{\text{CTX-M-15}}$/*bla*T$_{\text{EM-1A}}$/*bla*$_{\text{TEM-1B}}$/*aac(3)-IId/aadA2/ant(3'')-Ia/aph(3')-Ia/dfrA12/sul2/sul3/cmlA1/floR/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/**parE* p.S458A/*tet*(A)/*lnuF/mdfA*	1
q	**ampC* promoter/*aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/sul2/tet(B)/mdfA*	1
r	*bla*$_{\text{CTX-M-32}}$/*ant(3'')-Ia/mdfA*	1
s	*bla*$_{\text{CTX-M-15}}$/*bla*$_{\text{TEM-1B}}$/*aac(3)-IId/aadA2/ant(3'')-Ia/aph(3')-Ia/dfrA12/sul2/sul3/cmlA1/floR/***gyrA* p.D87N/**gyrA* p.S83L /**parC* p.S80I/**parE* p.S458A/*lnuF/mdfA*	1
t	*bla*$_{\text{NDM-1}}$/*ble*$_{\text{MBL}}$/*aadA2/aph(3')-VI/dfrA12/sul1/sul2/mdfA*	1
u	*bla*$_{\text{CTX-M-15}}$/*bla*$_{\text{TEM-1B}}$/*aac(3)-IId/aadA2/ant(3'')-Ia/aph(3')-Ia/dfrA12/sul2/sul3/cmlA1/floR/qnrS1/tet*(A)/*lnuF/ mdfA*	1
v	*bla*$_{\text{CTX-M-15}}$/*bla*$_{\text{TEM-1B}}$/*aac(3)-IId/aadA2/ant(3'')-Ia/aph(3'')-Ib/aph(6)-Id/aph(3')-Ia/dfrA8/dfrA12/dfrA14/sul2/ sul3/cmlA1/floR/qnrB19/qnrS1/tet*(A)/*lnuF/mdfA*	1

GDRs associated with ESBL production were only sporadically located in the chromosome. These included *bla*$_{CTX-M-14}$ (n=1), *bla*$_{CTX-M-15}$ (n=5), and the point mutation (nt 42 C → T) in the *ampC* promoter (n=1). However, differences between farms were found concerning the diversity and location of these genes.

In F1, the most prevalent ESBL-encoding gene was *bla*$_{CTX-M-1}$ (9/11 isolates), present in isolates recovered from all animal groups and at different sampling times along the study (Fig. 11). This gene was located in IncX1 type plasmids which always carried the same repertoire of ARGs. These plasmids were structurally compared using MAUVE and showed a high degree of similarity demonstrating the presence of largely conserved collinear coding blocks (data not shown). Hence, in addition to *bla*$_{CTX-M-1}$, IncX1 plasmids harboured the aminoglycoside resistance genes *aadA2*, *ant(3'')-Ia*, and *aph(3')-Ia*, a trimethoprim resistance gene (*dfrA12*), a sulfamethoxazole resistance gene (*sul3*), and a chloramphenicol resistance gene (*cmlA1*; Fig. 12). This plasmid was present in all isolates assigned to ST-69 and ST-2930.

In F4, a higher diversity of ESBL-encoding genes was observed (Fig. 11). These included *bla*$_{CTX-M-14}$ (n=11), *bla*$_{CTX-M-15}$ (n=8), *bla*$_{CTX-M-27}$ (n=3), *bla*$_{CTX-M-32}$ (n=3), *bla*$_{SHV-12}$ (n=1). In addition, *bla*$_{NDM-1}$ was detected in one isolate (E1110). All were located in plasmids except for 4 chromosomally-encoded *bla*$_{CTX-M-15}$. The *bla*$_{CTX-M-14}$ gene was always located in IncB/O/K/Z type plasmids that did not carry any additional ARGs (Fig. 12). This plasmid was found in *E. coli* of different ST, genotypic and phenotypic profiles, isolated from slurry and animals of all age groups throughout the study. The *bla*$_{CTX-M-15}$ gene, which was also detected in all animal groups and environmental samples, was the predominant ESBL-encoding gene in isolates recovered in the second half of the study. This gene was located in IncY plasmids (all 4 ST-69 isolates) or in the chromosome (ST-4981 and ST-58). Besides *bla*$_{CTX-M-15}$, IncY plasmids harboured 6 other ARGs (Fig. 12). The *bla*$_{CTX-M-27}$ gene was detected in the IncF plasmid of 3 ST-533 isolates, along with 5 other identical ARGs. Three isolates (ST-23) carried the *bla*$_{CTX-M-32}$ gene in an Incl1 plasmid, and the *bla*$_{SHV}$ gene was present in an IncX3 plasmid in one isolate recovered from lactating cows in the last sampling. The isolation in F4 of a carbapenem-resistant

E. coli harbouring the *bla*$_{NDM-1}$ gene in an IncC plasmid was a significant finding extensively reported in Study III (see 3.3.).

Cephalosporin-resistant *E. coli* isolates carried additional plasmids with ARGs and exhibited other chromosomally encoded GDR. In F1, ST-2930 isolates, besides IncX1, also carried an IncI1 plasmid that harboured another 5 ARGs (*aac(3)-IVa*, *aph(3'')-Ib*, *aph(6)-Id*, *aph(4)-Ia*, and *tet*(C)), while ST-69 isolates carried an IncF plasmid that harboured another 4 different ARGs (*bla*$_{TEM-1B}$, *ant(3'')-Ia*, *sul1* and *tet*(A)). A different IncF plasmid with a different collection of ARGs was present in one isolate (E0858). This isolate also carried a chromosomally encoded mutation in the *ampC* promoter. No plasmids were detected in the remaining isolate of F1 sequenced (ST-925), which harboured several GDRs in its chromosome, including *bla*$_{CTX-M-14}$ (Fig. 11). Resistance to (fluoro)quinolones was always associated with point mutations in the gyrase and topoisomerase genes (*gyrA*, *parC*, and *parE*) and only observed in *E. coli* strains assigned to ST-2930 and ST-925 (Fig. 11).

In F4, the 3 ST-23 isolates, which carried an IncI1 plasmid harbouring the *bla*$_{CTX-M-32}$ gene, also carried an IncF plasmid, resulting in an identical genotypic profile. Instead, ST-533 isolates (E0888, E0892, and E0896) carried a second plasmid (IncHI2) with a different repertoire of ARGs (Fig. 12). These IncHI2 plasmids were structurally compared (Fig. 13) and showed extensive sequence similarity, but E0896 lacked an 11.000 bp fragment that included *aadA2*, *cmlA1*, *ant(3'')-Ia*, and *sul3* genes which was present in E0888 and E0892.

Other genes coding only for resistance to narrow-spectrum β-lactamases like *bla*$_{OXA-1}$ (n=3), *bla*$_{TEM-1A}$ (n=4), and *bla*$_{TEM-1B}$ (n=14) were mostly located in IncF plasmids, and less frequently in the chromosome or other type of plasmids such as IncX2 and IncY (Fig. 11 & Fig. 12). Resistance to (fluoro)quinolones in F4 was associated with point mutations in the gyrase and topoisomerase genes (*gyrA*, *parC*, and *parE*) in 10 isolates, and with the *qnrS1* gene in another 10 (along with *qnrB19* in one of them). Interestingly, the gene that codes for resistance to lincosamides, *lnuF*, was present in 5 isolates recovered from all age groups in the second half of the study. *lnuF* was always located in IncF and IncX3 plasmids (Fig. 12).

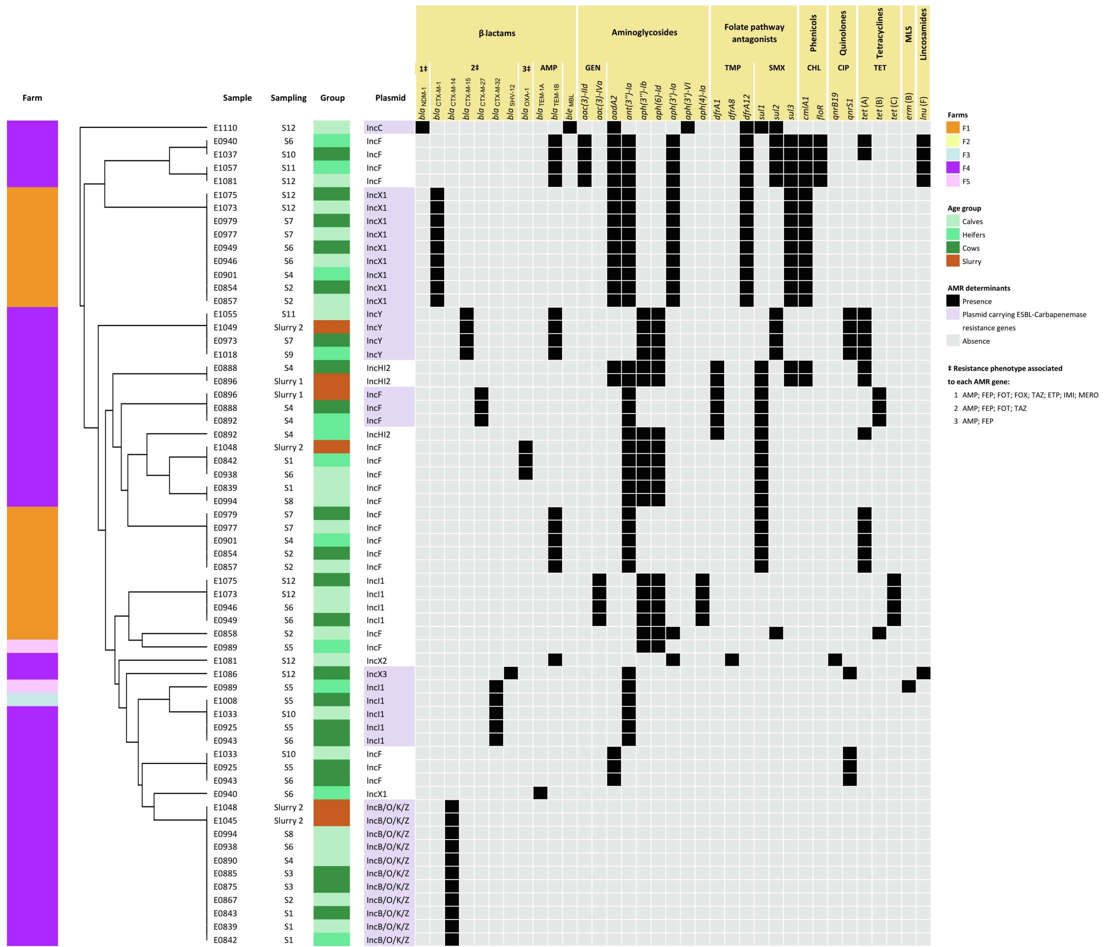

Figure 12. Heatmap showing ARG-harbouring plasmids along with a dendrogram illustrating the similarity among plasmids based on their AMR pattern. Plasmids were grouped based on their antimicrobial resistance pattern (ARGs) according to the result of the hierarchical clustering using the average linkage method (UPGMA) on the Jaccard distance matrix. Further information including sampling and source (age group or slurry) is also included.

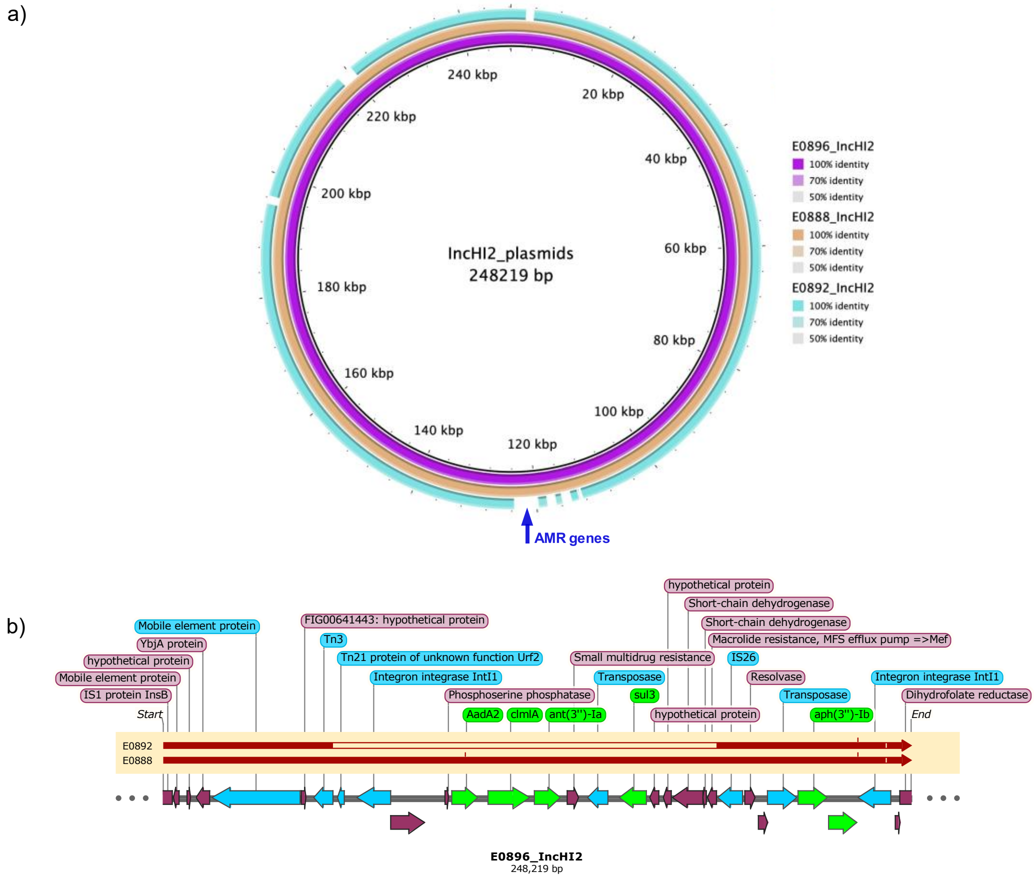

Figure 13. Structural comparison of IncHI2 plasmid in isolates E0888, E0892, and E0896. (a) Circular comparison of plasmids using Blast Ring Image Generator (BRIG), using E0896 IncHI2 plasmid as a reference. The location of ARGs is indicated with an arrow. (b) Comparison of ARG region in IncHI2 plasmids of E0888 and E0892. Coding sequences (CDS), represented by arrows indicating the translational direction, are named above and colored as follows: blue, insertion sequences (IS); green, AMR genes; brown, genes with other functions; and grey, hypothetical proteins. IS designations are followed by the family name in brackets. Annotations were graphically depicted using SnapGene (v.5.2.4) (http://www.snapgene.com/).

3.2.4. Discussion

This longitudinal study was designed to monitor the occurrence of ESBL-/AmpC-/CP-producing *E. coli* and their antimicrobial resistance profiles in apparently healthy animals in dairy cattle farms for over 16 months. Longitudinal surveillance allows the assessment of the bacterial population dynamics throughout time, enabling the detection of emerging genotypes and changes in the AMR profiles over time. The longitudinal survey presented here encompassed five farms that represented the style

of farming in the Basque Country, and therefore, might provide a useful understanding of the regional situation regarding cephalosporin-resistant *E. coli* distributions and AMR transmission dynamics.

In Study I (see 3.1.), ESBL/AmpC producers were isolated in 32.9% of the 82 dairy cattle herds tested. Here, cephalosporin-resistant *E. coli* were detected in all the five investigated dairy cattle farms, surely due to the more intensive longitudinal sampling strategy used that comprised 12 samplings and three age groups. Isolation frequency varied along time, as well as among farms and age groups. Both calves and lactating cows had a higher prevalence of cephalosporin-resistant *E. coli* than heifers but no difference was observed between them. This could be associated with age-related differences in management practices. Pregnant heifers and dry cows had access to the outside pastures whereas lactating cows were permanently housed indoors, where increased infection pressure and a higher probability of recirculation of resistant isolates occur. As observed in Study I (see 3.1.) ruminants raised under less intensive management systems have been associated with a lower prevalence of infection with cefotaxime-resistant *E. coli*, *e.g.* beef cattle and sheep in the Basque Country and elsewhere (Hille et al., 2017; Collis et al., 2019). The higher incidence found in lactating cows compared with heifers could also be explained by the continuous and prolonged exposure of older cows to antimicrobials used to treat intramammary and other infections during their lifespan. These treatments include the commonly used cephalosporins, which do not require a withdrawal period for milk. On the other hand, calf management practices differed from those in heifers and lactating cows. Calves are kept in different housing facilities and are administered a different diet. Moreover, calves are susceptible to different diseases such as neonatal diarrhoea and pneumonia, which are the main reasons for antimicrobial treatment in this age group. Besides, young calves rapidly acquire antibiotic-resistant *E. coli*, which are often multiresistant (Hordijk et al., 2013; Gay et al., 2019), and their resistome has been reported to be more diverse than that of adult cattle (Noyes et al., 2016).

Antimicrobial use (AMU) in food animals has been linked to an increased prevalence of resistant bacteria, but this relation depends on the antimicrobial class,

microorganism, and sector (ECDC et al., 2021). Here, in the absence of detailed records of AMU, differences extracted from the questionnaires were related to mastitis treatments and DCT. Fluoroquinolones were the antibiotics of choice for mastitis treatment in F1, F3, and F5, the combination of parenteral enrofloxacin with an intramammary ointment containing cefquinome was common practice in F4, and no antimicrobials were used to treat mastitis in F2. On the other hand, the antimicrobials used for blanket DCT to control mastitis were penicillins and aminoglycosides in F1 and F2, and a first-generation cephalosporin in F3, F4, and F5. This could somehow explain the higher prevalence of cephalosporin-resistant *E. coli* found in F3, F4, and F5 compared to F1 and F2. Differences in farm infrastructure and management practices (e.g., vaccine programs and hygiene) may impact animal disease incidence and, consequently, influence the use of antimicrobials and the subsequent increase in AMR prevalence.

Antimicrobial susceptibility testing of 197 cefotaxime-resistant *E. coli* isolates identified 72.1% of them as MDR. This is not unexpected since ESBL-producing *E. coli* are commonly co-resistant to other classes of antimicrobials (Seiffert et al., 2013). However, the diversity of phenotypic resistance profiles varied among farms. Therefore, to thoroughly compare the relationship of the circulating strains, a selection of isolates from the two farms that showed the lowest (F1) and largest (F4) AMR profile diversity were further whole-genome characterized. This analysis identified certain isolates with phenotypic AMR profiles that differed only in their susceptibility to FOX; the FOX-resistant isolates showed a MIC value of just a single two-fold dilution above the ECOFF (MIC_{FOX}=16 mg/L), and, therefore, within the widely accepted margin of error of the microdilution method. These isolates did not carry any GDR associated with AmpC production, and based on WGS results (ST, phylogroup, serotype, GDR, and virulence genes) these isolates could be considered the same strains as their FOX-susceptible counterparts within the same ST type. The opposite situation, *i.e.*, isolates with the same phenotypic profile but clearly different ARGs was also observed. This occurred in F4 and was due to changes in the chromosome and the carriage of different

plasmids (ST-58) or the loss of a fragment within an otherwise similar plasmid (ST-533). Isolates with different ST and serotypes that shared the same ARGs were also found.

Even though the genomic data provided in this study represents only two farms, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-14}$, and *bla*$_{CTX-M-15}$ were the most common ESBL-encoding genes as reported in Study I (see 3.1.). Noteworthy was the detection of a gene coding for CP production in F4. The identification, in the frame of this study, of a *bla*$_{NDM-1}$-carrying *E. coli* was described in more detail in Study III (see 3.3.). Previous to this study, CP-producing *E. coli* had not been detected in food-producing animals in the Basque Country, and *bla*$_{NDM-1}$-carrying *E. coli* had never been isolated from cattle neither in the Basque Country nor elsewhere.

ESBL-/CP-encoding genes were mostly located in plasmids, with an apparent association of each gene with certain types of plasmids. IncB/O/K/Z plasmids are frequently found in *E. coli* from animal sources and have been associated with the spread of *bla*$_{CTX-M-14}$ in Europe, especially in Spain and the UK (Rozwandowicz et al., 2018). IncF is the most frequently described plasmid type from human and animal sources (Rozwandowicz et al., 2018) and encodes different *bla*$_{CTX-M}$ variants. Here, IncF was the most abundant plasmid but only sporadically carried ARG coding for resistance to ESBL, specifically *bla*$_{CTX-M-27}$ gene, an association already described in cattle (Tadesse et al., 2018). Other ESBL-encoding gene and plasmid associations found here, such as *bla*$_{CTX-M-1}$ in IncX1, *bla*$_{CTX-M-15}$ in IncY, *bla*$_{CTX-M-32}$ in IncI1, and *bla*$_{SHV}$ in IncX3 plasmids, are not so frequently described (Rozwandowicz et al., 2018). In Study I (see 3.1.) most of the ESBL/AmpC gene-carrying plasmids were identified as IncI1, but since Illumina was the sequencing technology then used, the type of many of the plasmids could not be assigned. Here, using long-read ONT sequencing, most of the genomes (both the chromosomes and plasmids) were completely sequenced and circularised, allowing a better characterization of plasmids, which is one of the main advantages of this technique (Wick et al., 2017a).

Genome sequencing and characterization of this selection of isolates allowed elucidation of whether transmission of ESBL genes was the result of the persistence of certain strains or multiple source contamination. In F1, only 4 different strains were

identified, two of them being recovered multiple times and from all age groups. One predominated during the first half of the study and was then replaced by a very different strain. Their chromosomally-encoded features (all 7 ST alleles, CC, phylogroup, serotype, virulence genes profile, and point mutations associated with quinolone resistance) were completely different but both carried the same ESBL-encoding gene ($bla_{CTX\text{-}M\text{-}1}$) harboured by an identical IncX1 plasmid. Further differences between both strains were due to genes present in different additional plasmids. These results may reflect an endemic situation where, due to clonal expansion, just a few strains persisted in the farm over a long time thus giving the opportunity for plasmid transfer. Conversely, the situation in F4 was completely different. Although a few genotypes persisted for some time, there was a large diversity of genotypes carrying multiple and diverse GDRs both in the chromosome and in different plasmids, likely due to multiple source contamination events. Yet, different *E. coli* isolates containing the same type of plasmids that carry the same repertoire of ARGs were also identified (*e.g.* IncB/O/K/Z in 7 different STs). This strongly suggested that horizontal transfer of ESBL-carrying plasmids occurred within the farm.

In conclusion, this study illustrates the within-farm diversity and dynamics of cefotaxime-resistant *E. coli* over time in dairy cattle, and shows the power of genomic surveillance in deciphering the complex epidemiology underlying multidrug resistance dissemination within a farm. Despite the differences observed between both farms, the presence of certain plasmid types with the same repertoire of ARGs in different *E. coli* STs might be indicative of the occurrence of horizontal transfer of such plasmids among strains circulating within the farms. AMU, environmental selection pressure, or co-selection with other advantageous genes might drive these events. Although we cannot rule out the existence of certain niche-specific clones that are better adapted to the calf intestinal environment, we found that the more widespread clones could readily infect animals of all age groups. Recommendations for the implementation of biosecurity measures to prevent the introduction of ESBL-producing *E. coli* and management protocols that limit contact between animals of different age groups were made to farmers to avoid cross-contamination and the spread of resistant bacteria.

Considering the public health importance of ESBL-producing *E. coli* both as pathogens and as vectors for resistance mechanisms, the presence of β-lactamase- and other AMR-encoding genes in plasmids that can be readily transferred between bacteria is a concern that highlights the need for One Health surveillance.

Data Availability: The raw sequencing data presented in this study can be found online at the NCBI Sequence Read Archive (SRA) database, associated with the BioProjects PRJNA833969 and PRJNA680938. The accession number(s) can be found in Supplementary Table S1.

3.3. Study III

Characterization of a carbapenem-resistant *Escherichia coli* from dairy cattle harbouring bla_{NDM-1} in an IncC plasmid

3.3.1. Background

Antimicrobial resistance to carbapenems is an ongoing global public-health problem that can result from the activity of carbapenemases (CPs), a heterogeneous group of enzymes that hydrolyse most β-lactams including carbapenems. One of the most widespread CPs within the Enterobacteriaceae is the New Delhi metallo-β-lactamase (NDM), which belongs to the amber class B of β-lactamases and is capable of hydrolysing almost all β-lactams except for monobactam (Nordmann et al., 2011). NDM was firstly reported in a Swedish patient who travelled to India (Yong et al., 2009). Since then, the number of NDM-producing isolates has been rising across different countries (Dortet et al., 2014; Kazmierczak et al., 2020), prevalence being higher in South Asia, the Balkans, North Africa, and the Middle East (Wu et al., 2019). In Spain, the earliest case of NDM producing Enterobacteriaceae was reported in 2011 from a patient with a recorded travel history to India (Solé et al., 2011). Afterwards, NDM-producing isolates disseminated across different Spanish regions (Pérez-Vázquez et al., 2019). A similar increase in human clinical cases associated with CP-producing Enterobacteriaceae has been reported across Europe (Kazmierczak et al., 2020), partly attributed to an increase of NDM-like enzymes.

Enterobacteriaceae seem to be the major host of *bla*$_{NDM}$, *Klebsiella pneumoniae* being the most common species, followed by *E. coli* and the *Enterobacter cloacae* complex (Wu et al., 2019). *bla*$_{NDM}$ genes can be chromosomally located but are more often carried by different types of transferable plasmids, thus favouring dissemination among isolates. In particular, IncX3 appears to be the most common type of *bla*$_{NDM}$-carrying plasmid, followed by IncFII and IncC plasmids (Wu et al., 2019). Some of these plasmids usually carry additional antimicrobial resistance (AMR) genes (Harmer and Hall, 2015; Rozwandowicz et al., 2018).

In food-producing animals, the detection of CP-producing isolates is still rare (Madec et al., 2017; Kock et al., 2018; EFSA and ECDC, 2021), and, for instance, in Europe, only a few studies have described NDM genes in wild and food animals (Fischer et al., 2013; Diaconu et al., 2020). So far, in cattle, NDM-producing Enterobacteriaceae were detected in India, Algeria and China, *bla*$_{NDM-5}$ gene being the only isolated variant

(Purkait et al., 2016; Yaici et al., 2016; He et al., 2017b, 2017a). In this study, we report, to the best of our knowledge, the first detection of an NDM-1-producing *E. coli* in cattle (dairy calves), that carried the *bla*$_{NDM-1}$ gene in an IncC plasmid of 145,165 bp which was fully reconstructed using a hybrid Illumina–Oxford Nanopore sequencing and assembly approach.

3.3.2. Materials and methods

Bacteria isolation and antibiotic susceptibility testing. In July 2020, two presumptive CP-producing *E. coli* isolates were recovered from a pool of rectal faeces collected from calves in a dairy cattle farm in the Basque Country, northern Spain. Samples had been collected in the frame of Study II (see 3.2.). The July sampling was the last of 12 samplings carried out in the farm from March 2019. Briefly, faeces from 5 calves (5 g each) were thoroughly mixed and 1:10 diluted in buffered peptone water (bioMérieux), incubated at 37ºC for 20±2 h and subcultured onto MacConkey agar. Grown colonies were harvested for DNA extraction and subjected to a real-time PCR amplification screening targeting the CP-coding genes, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{KPC}$, and *bla*$_{OXA-48}$ (Ellington et al., 2016), that tested positive for *bla*$_{NDM}$. To isolate the *bla*$_{NDM}$-harbouring strains, a loopful of colonies grown on MacConkey agar were subcultured in MacConkey broth supplemented with 1 mg/L of cefotaxime at 37ºC for 20±2 h, and then cultured on ChromID® Carba Smart selective agar plates (bioMérieux). A species-specific real-time PCR targeting the *uid*A gene was conducted to confirm colonies with morphology compatible with *E. coli* (Frahm and Obst, 2003). In February 2021, three of the five animals whose faeces comprised the positive pool were sampled again and analysed individually as described above.

To assess antimicrobial susceptibility, minimum inhibitory concentrations (MICs) of the isolates were determined by broth microdilution using two Sensititre MIC susceptibility plates (EUVSEC1 and EUVSEC2, Thermo Fisher Scientific) following the recommendations in Commission Decision 2013/652/EU (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri CELEX:32013D0652&from EN) with regard to antimicrobials and dilution ranges. Results were interpreted using

epidemiological cut-off values (ECOFF) as developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org).

Whole-genome sequencing (WGS) and analyses. For in-depth genome characterization, genomic DNA was then extracted from a single colony pure culture (NZY Microbial gDNA Isolation kit, NZYtech) and subjected to short-reads (Illumina) and long-reads (Oxford Nanopore Technologies, ONT) whole-genome sequencing (WGS). For Illumina sequencing, genomic DNA was submitted to Eurofins Genomics, where libraries were prepared based on the NEBNext Ultra II FS DNA library prep kit (Illumina), and the genome was sequenced with Illumina NovaSeq 6000 (150-bp paired-end reads). For ONT sequencing, a library was prepared using the Ligation Sequencing Kit (SQK-LSK109) and run in a FLO-MIN106 (R9.4.1) flow cell on a MinION Mk1C device (ONT).

Illumina reads were subjected to quality control using FastQC v.0.11.9 (Andrews, 2010). After quality filtering with Trimmomatic (Bolger et al., 2014) and PRINSEQ (Schmieder and Edwards, 2011) the reads were *de novo* assembled using SPAdes (Bankevich et al., 2012). The quality of the assemblies was assessed with QUAST (Gurevich et al., 2013), discarding contigs below 200 bp with PRINSEQ. On the other hand, the raw fast5 files generated by ONT sequencing were base called on Guppy (v4.2.3) using the High Accuracy Calling mode. The resulting Fastq read's adapters were removed using Porechop (Wick et al., 2017a) and then filtered by Filtlong (https://github.com/rrwick/Filtlong), keeping reads longer than 1,000 bp and discarding the worst 10% of those remaining reads. Finally, the lowest quality reads from the resulting pool were also filtered out, retaining only the best 1,000 Mbp to carry out the assembly.

A hybrid assembly (Illumina-Oxford Nanopore) was generated with Unicycler (Wick et al., 2017b). Assembled reads were screened for acquired AMR genes using BLASTn v.2.11.0 (Zhang et al., 2000) and ABRicate v.1.0.1 (T. Seemann, https://github.com/tseemann/abricate) against the following databases (last updated in November 2020): ResFinder 4.0 (Bortolaia et al., 2020), PointFinder (for chromosomal point mutations associated with antimicrobial resistance) (Zankari et al.,

2017), and Virulence Factors Database (VFDB) (Chen et al., 2005). Plasmid replicons were identified using PlasmidFinder (Carattoli and Hasman, 2020), and plasmid- and chromosome-derived contigs were predicted with PlasFlow (Krawczyk et al., 2018). In silico FimH typing was achieved using FimTyper (Roer et al., 2017), and *Escherichia* genus strain phylotyping was performed with the Mash genome clustering tool (ClermonTyper) (Beghain et al., 2018). Multilocus sequence types (MLSTs, Achtman scheme) were queried against the *E. coli* MLST database PubMLST (Jolley and Maiden, 2010) using mlst, and the core-genome MLST (cgST) was assigned using cgMLSTFinder, following the EnteroBase *E. coli* cgMLST scheme (Zhou et al., 2020). Plasmid Multilocus sequence typing (pMLST) was carried out on the pMLST server (https://cge.cbs.dtu.dk/services/pMLST/). The assembled genome was annotated with Prokka (chromosome) (Seemann, 2014) and RAST (plasmid) (Aziz et al., 2008), and then manually edited, in particular for the insertion sequences (ISs) by using the ISfinder database (Siguier et al., 2006). Annotations were graphically depicted using SnapGene (v.5.2.4) (http://www.snapgene.com/). The plasmid sequence was further compared with sequences available in public repositories (GenBank) using the Nucleotide collection (nr/nt) and Microbial Nucleotide complete plasmid databases. A progressive alignment of the related complete plasmids was performed with progressiveMAUVE (Darling et al., 2010), and genetic structures were compared and visualized in Easyfig (Sullivan et al., 2011).

3.3.3. Results and discussion

Resistance profile and whole genome sequencing output. The two presumptive CP-producing *E. coli* isolates recovered from healthy dairy calves showed the same profile of microbiological multidrug resistance, *i.e.* resistance to all β-lactams tested, including temocillin and carbapenems (ertapenem, imipenem, and meropenem), along with sulfamethoxazole and trimethoprim (Table 10). Illumina sequencing of one of the two isolates, strain *E. coli* EC1110, resulted in 10,996,380 reads (335X coverage) that were assembled into 118 contigs, whereas ONT sequencing of the same strain generated 312,323 reads of a mean read length of 8,399 bp, N50 value of 15,491 bp,

and 2.54 Gb of total yield, with an average Q-score of 12.4. The hybrid (Illumina-Oxford Nanopore) assembly produced two large contigs, one corresponding to the chromosome (4,816,563 bp), and another identified as an IncC plasmid (145,165 bp) that contained the *bla*$_{NDM-1}$ gene along with another nine AMR genes.

Table 10. Antimicrobial resistance profile (Minimum Inhibitory Concentrations – MICs) of *E. coli* EC1110 and genetic determinants of resistance in *E. coli* plasmid pEC1110_NDM-1.

Class	Antimicrobial	MIC (mg/L)	Acquired AMR genes [a]
β-lactams (penicillins)	Ampicillin (AMP)	>64	*bla*$_{NDM-1}$ (Δ*bla*$_{DHA-1}$)
	Temocillin (TRM)	128	*bla*$_{NDM-1}$
β-lactams (2nd gen cephalosporins)	Cefoxitin (FOX)	>64	*bla*$_{NDM-1}$ (Δ*bla*$_{DHA-1}$)
β-lactams (3rd gen cephalosporins)	Cefotaxime (FOT)	>64	
	Ceftazidime (TAZ)	>128	
β-lactams (4th gen cephalosporins)	Cefepime (FEP)	32	*bla*$_{NDM-1}$
β-lactams (carbapenems)	Ertapenem (ETP)	>2	
	Imipenem (IMI)	2	
	Meropenem (MERO)	8	
Aminoglycosides [b]	Gentamicin (GEN)	1	-
Macrolides	Azithromycin (AZI)	4	-
Tetracyclines	Tetracycline (TET)	$\leq$2	-
Glycilcyclines	Tigecycline (TGC)	$\leq$0.25	-
(Fluoro)quinolones	Nalidixic acid (NAL)	$\leq$4	-
	Ciprofloxacin (CIP)	$\leq$0.015	-
Phenicols	Chloramphenicol (CHL)	$\leq$8	-
Polymyxins	Colistin (COL)	$\leq$1	-
Folate pathway inhibitors	Sulfamethoxazole (SMX)	>1024	*sul1* (2 copies); *sul2*
	Trimethoprim (TMP)	>32	*dfrA12*

[a] Δ*bla*$_{DHA-1}$, the plasmid-mediated AmpC gene *bla*$_{DHA-1}$ that confers resistance to broad spectrum cephalosporins was truncated and non-functional
[b] genes conferring resistance to other aminoglycosides other tan gentamicin were detected, *i.e.*, *aph(3')-VI* (amikacin-resistance encoding gene) and *aadA2* (streptomycin-resistance encoding gene)

Molecular characterization of *E. coli* EC1110. The chromosome had a G+C content of 51% and contained 4,634 genes, of which 4,556 were protein-coding, 78 pseudogenes, and 118 RNA genes (86 tRNA, 22 rRNA, 10 ncRNA). The isolate was predicted to belong to phylogroup B1, serogroup O74:H23 and was assigned to a novel MLST sequence type of the Achtman scheme, ST-11626 and cgST-151275. It carries a

fimH60 fimbrial adhesion allele, and harbours virulence-associated genes related to adhesion (including CFA/I fimbria and the Type 1 fimbriae operon), iron uptake by the siderophore enterobactin (*ent* and *fep* genes), invasion of brain endothelial cells (*ompA*, *ibeB*, and *ibeC* genes), and the pore-forming toxin hemolysin E encoding gene (*hlyE*). Resistance genes identified on the chromosome included several multidrug efflux pump associates genes, such as *mdf(A)*, *emrA*, and *emrB* of the multidrug resistance efflux complex EmrAB-TolC, and other TolC-associated efflux pumps genes (*acrB*, *acrD*, *emrB*, *emrY*, *mdtC*, *mdtF*, and *acrEF*). The intrinsic β-lactamase genes of *E. coli*, *ampC1*, *ampC2*, and *ampH*, were also present.

Genetic structure of the *bla*$_{\text{NDM-1}}$-carrying plasmid pEC1110_NDM-1. The plasmid, named pEC1110_NDM-1, was 145,165 bp in length, had a GC content of 52%, 176 coding regions (CDS), and belonged to the C incompatibility group (IncC plasmid, formerly known as IncA/C_2). Plasmid multilocus sequence typing assigned it to pST-3. The plasmid backbone (76% of the total pEC1110_NDM-1 plasmid size) included genes required for the initiation of replication (*repA* gene), conjugative transfer (*tra*) and plasmid partitioning (*stb* and *par*) (Fig. 14A). Additionally, it contained accessory modules of antimicrobial resistance islands ARI-A and ARI-B where AMR genes were located. The *bla*$_{\text{NDM-1}}$ gene was located in the ARI-A region (Fig. 14B) flanked upstream by *ISAba*125 and downstream by the bleomycin resistance gene *ble*$_{\text{MBL}}$, followed by a truncated Δ*bla*$_{\text{DHA-1}}$ gene. Besides these genes, the ARI-A multi-resistance region also contained some other AMR determinants that coded for the AMR phenotype observed, including two copies of the sulfonamide resistance gene *sul1*, the dihydrofolate reductase gene *dfrA12* associated to resistance to trimethoprim, two genes encoding aminoglycoside-modifying enzymes, *i.e.*, *aph(3')-VI* and *aadA2*, conferring resistance to amikacin and streptomycin respectively, and the *qacE*Δ*1* gene (resistance to quaternary ammonium compounds). In addition, a mercury resistance operon (*merDACPTR*) was found. The ARI-B region only contained the sulfonamide resistance gene *sul2* (Fig. 12B).

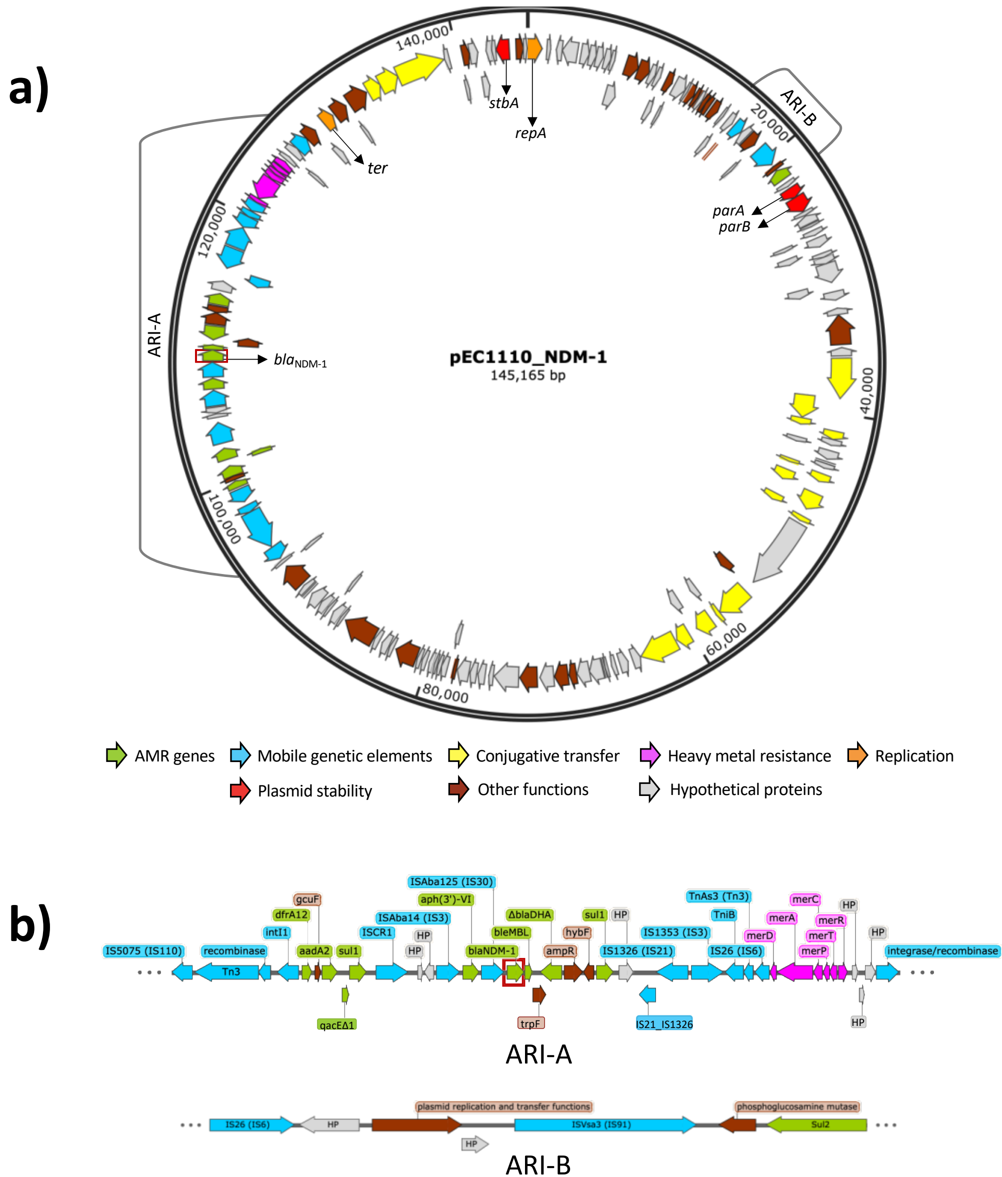

Figure 14. Circular representation of pEC1110_NDM-1 IncC plasmid from *E. coli* strain EC1110 isolated from dairy cattle. Schematic representation of the genetic context of the *bla*$_{NDM-1}$ gene in the ARI-A region of pEC1110_NDM-1 plasmid and *sul2* gene in the ARI-B region. *bla*$_{NDM-1}$ gene position is highlighted in a red square. Coding sequences (CDS), represented by arrows indicating the translational direction, are named above and coloured according to the key. Insertion sequence (IS) designations are followed by the family name in by brackets. Annotations were graphically depicted using SnapGene (v.5.2.4) (http://www.snapgene.com/).

The location of the *bla*$_{NDM-1}$ gene in an IncC plasmid has been reported before (Poirel et al., 2011; Harmer and Hall, 2015; Ambrose et al., 2018; Wu et al., 2019). IncC plasmids are associated with multidrug resistance and can encode ESBLs (*bla*$_{TEM}$, *bla*$_{SHV}$,

but rarely *bla*$_{CTX-M}$), AmpC (*bla*$_{CMY}$, *bla*$_{DHA}$), CPs (*bla*$_{OXA}$, *bla*$_{NDM}$, *bla*$_{IMP}$), and genes conferring resistance to several other antibiotics (Rozwandowicz et al., 2018). IncC plasmids have a broad host range as well as a worldwide distribution (Rozwandowicz et al., 2018; Wu et al., 2019). However, this type of plasmids is not so common in Europe, neither in animals nor in humans. IncC is the dominant plasmid type in animals from North and South America but animals in Europe are mainly colonized by *E.coli*-carrying IncI plasmids (Rozwandowicz et al., 2018), in agreement with the results we found in ruminant *E. coli* in our region in Study I (see 3.1.). Plasmids from human sources in European isolates are more diverse, but IncC is not the most prevalent type (Rozwandowicz et al., 2018). Nevertheless, an IncC plasmid containing a *bla*$_{NDM-1}$ gene in an *E. coli* isolate was already described in a hospital in Madrid, Spain (Pérez-Vázquez et al., 2019).

A BLAST search in GenBank identified several complete IncC plasmids from bacteria within the Morganellaceae and Enterobacteriaceae families that shared high sequence identity and similar backbone structures to the pEC1110_NDM-1 plasmid (Fig. 15). pEC1110_NDM-1 plasmid was most closely related (94% coverage and 99.9% identity) to a 178,277 bp *Providencia stuartii* plasmid pMR0211 (accession number: JN687470.1) isolated from a human patient in 2011 in Afghanistan, to a 177,190 bp *Salmonella enterica* subsp. *enterica* serovar Corvallis pSE12-01738-2 plasmid (accession number: CP027679.1) isolated from a wild bird (*Milvus migrans*) in Germany (92% coverage and 99.9% identity), and to *E. coli* pM214_AC2 plasmid isolated from a human patient in Myanmar in 2015 (accession number: AP018143) (88% coverage and 99.9% identity), all harbouring a *bla*$_{NDM-1}$ gene. Furthermore, the pEC1110_NDM-1 plasmid shared a highly similar structure compared with IncC plasmids isolated from Enterobacteriaceace within food-producing animals that do not carry *bla*$_{NDM}$ genes: A *Salmonella enterica* subsp. *enterica* serovar Newport plasmid (p34530-1, CP051319.1) isolated in North Carolina (USA) from cattle (92% coverage and 99.9% identity) and an *E. coli* plasmid (pEC3-1/2a, MT559986.1) from chicken in China (91% coverage and 99.9% identity). The pEC1110_NDM-1 plasmid also shared homologous regions (89% coverage and 99.8% identity) with the first IncC plasmid described in detail, *i.e.*,

pRMH760 plasmid from *K. pneumoniae* (accession number: KF976462) isolated from a hospitalized patient in Sydney, Australia.

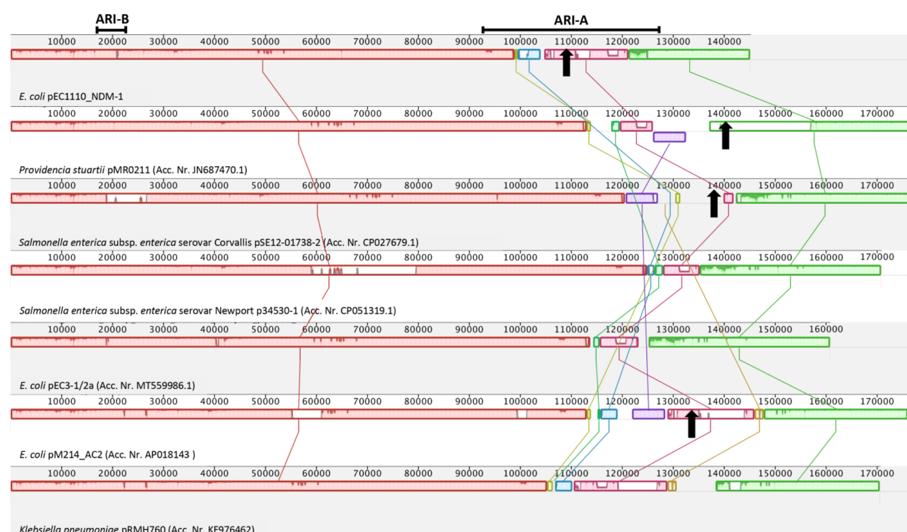

Figure 15. MAUVE alignment of colinear blocks of *E. coli* pEC1110_NDM-1, *Providencia stuartii* plasmid pMR0211, *Salmonella enterica* subsp. *enterica* serovar Corvallis pSE12-01738-2, Salmonella enterica subsp. *enterica* serovar Newport p34530-1, *Escherichia coli* pEC3-1/2a, *Escherichia coli* pM214_AC2 and *Klebsiella pneumoniae* pRMH760 complete plasmids. Lines connecting blocks with identical colours represent the regions aligned and show synteny or gene rearrangements. The start nucleotides of each complete plasmid were shifted to the *repA* gene. Antimicrobial resistance islands ARI-A and ARI-B are indicated on pEC1110_NDM-1 plasmid. bla_{NDM-1} gene position is indicated with a black vertical arrow.

When specifically comparing the ARI-A multi-resistance region, the gene synteny in pEC1110_NDM-1 plasmid was highly conserved compared with the same region in the p34530-1 plasmid of *S. enterica* subsp. *enterica* serovar Newport (accession number: CP027679.1). Despite lacking the 9,639 Kb fragment from the IS91 element to the *ampR* gene where the bla_{NDM-1} gene is located, the p34530-1 plasmid displays a backbone similar to that found in pEC1110_NDM-1 (Fig. 15), and shares a *sul1*-type class 1 integron structure (intI1-*dfrA12-gcuF-aadA2-qacEΔ1-sul1*) in the ARI-A island (Fig. 16) suggesting a common origin. On the other hand, the region containing the bla_{NDM-1} gene in pEC1110_NDM-1 plasmid has features that appear in most bla_{NDM-1}-harbouring strains, *i.e.*, the insertion sequence IS*Aba125* upstream of bla_{NDM} (which

provides the – 35 promoter region for *bla*$_{NDM-1}$ expression) (Poirel et al., 2010) and a bleomycin resistance gene, *ble*$_{MBL}$, located downstream (Wu et al., 2019). In fact, the IS*Aba*125-*bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF*-Δ*bla*$_{DHA-1}$-*ampR* region was also conserved in the partial sequence of the plasmid of an *E. coli* (strain DVR22, accession number: JF922606; 4036 bp) recovered from the stool samples of a patient returning from India that represented the first description of an NDM-1 CP-producing *E. coli* in Spain in 2011 (Solé et al., 2011). Homology with the PGI1-*Pm*PEL genomic island integrated into a *Proteus mirabilis* chromosome (Girlich et al., 2015) was nearly 100% in the segment that extended from the IS*CR*1 upstream *bla*$_{NDM-1}$ to the *ampR* gene (Fig. 16). Insertion of *bla*$_{NDM-1}$ from a circular molecule mediated by IS*CR*1 has been proposed (Bonnin et al., 2013), and IS*CR*1 has also been associated with the acquisition of the *bla*$_{DHA}$-*ampR* gene region as part of a class 1 integron (Hennequin et al., 2018), an structure observed in pEC1110_NDM-1.

Regarding the ARI-B region, the pEC1110_NDM-1 plasmid has an IS26-mediated deletion of 12,451 bp located upstream the *parA* and *parB* genes that removed part of the plasmid backbone. Usually, the most commonly seen configuration of ARI-B is associated with a 10,984 bp IS26-mediated deletion of the backbone (Harmer and Hall, 2014), but the size of these deletions, which are potentially useful as evolutionary and epidemiological markers, is variable (Harmer and Hall, 2015; Ambrose et al., 2018). Although the ARI-B region usually contains some other AMR genes such as *floR*, *strA*, *strB*, and *tet*(A) (Harmer and Hall, 2015), pEC1110_NDM-1 plasmid only contained the *sul*2 gene.

First description of a *bla*$_{NDM-1}$-carrying *E. coli* in cattle. Despite the increase in human clinical cases associated with CP-producing Enterobacteriaceae, the detection of CP-producing isolates and NDM-producing Enterobacteriaceae in wild and food-producing animals is still rare (Madec et al., 2017; Kock et al., 2018)

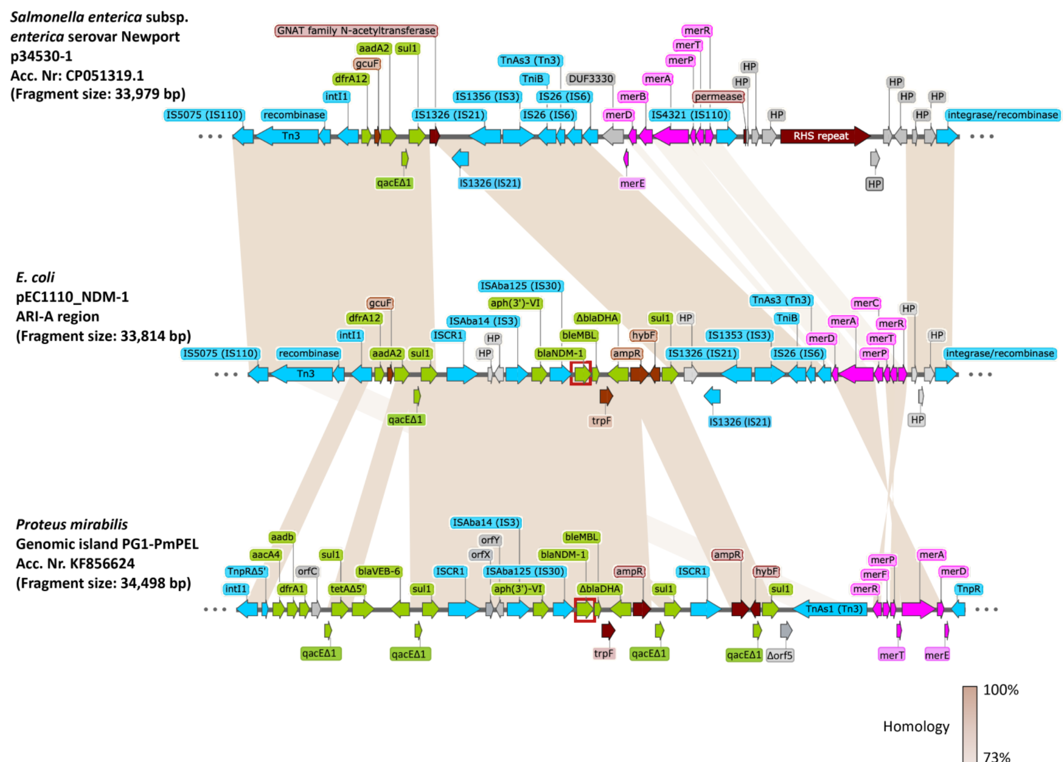

Figure 16. Comparison of ARI-A region and $bla_{\text{NDM-1}}$ gene environments in *E. coli* pEC1110_NDM-1 plasmid, *Salmonella enterica* subsp. *enterica* serovar Newport p34530-1 plasmid (Acc. Nr. CP051319.1), and the chromosomally located *Proteus mirabilis* genomic island PG1-PmPEL (Acc. Nr. KF856624). Common features are highlighted with different shades of brown according to homology. Coding sequences (CDS), represented by arrows indicating the translational direction, are named above and coloured as follows: blue, insertion sequences (IS); green, AMR genes; pink, heavy metal resistance genes; brown, genes with other functions; and grey, hypothetical proteins. IS designations are followed by the family name in brackets.

In Europe, NDM-producing Enterobacteriaceae have been isolated in a pig in Italy (*E. coli* with $bla_{\text{NDM-4}}$ in an IncFII plasmid) and in a migratory bird in Germany (*S. enterica* subsp. *enterica* serovar Corvallis with $bla_{\text{NDM-1}}$ in an IncA/C plasmid) (Fischer et al., 2013; Diaconu et al., 2020). In China, NDM-1 producing *E. coli* are more widespread and have been isolated from poultry and swine (Wang et al., 2017b; Zhao et al., 2019; Sapugahawatte et al., 2020; Zhai et al., 2020). The only NDM CP type described in cattle so far was NDM-5, and included *E. coli* isolated from mastitic cows in India and healthy cattle in Algeria, as well as *K. pneumoniae* from animals presenting clinical mastitis in China (Purkait et al., 2016; Yaici et al., 2016; He et al., 2017b, 2017a). In those studies where the plasmid type was characterized, the $bla_{\text{NDM-5}}$ gene was carried by IncX

plasmids. Previous to this study, NDM CPs had never been detected in *E. coli* in the Basque Country; they have not been reported in humans in this region, and they were not found in Study I (see 3.1.). To the best of our knowledge, the study herein represents the first description of a bla_{NDM-1}-carrying *E. coli* isolated from cattle in the Basque Country and elsewhere.

NDM-1 has been identified in a wide diversity of Enterobacteriaceae sequence types (Poirel et al., 2011) and there are no predominant STs associated with NDM-1-producing *E. coli* (Pérez-Vázquez et al., 2019; Wu et al., 2019). The isolate described here, *E. coli* strain EC1110, belonged to serogroup O74:H23 and was assigned to a novel MLST type (ST-11626 and cgST-151275) not reported before, and therefore different from previously described NDM-carrying *E. coli* sequence types. Among the chromosome encoded virulence factors present in *E. coli* EC1110 there were some associated to the diarrheagenic enterotoxigenic *E. coli* (ETEC) pathovar, but also to the extraintestinal pathovars of *E. coli* uropathogenic (UPEC) and neonatal meningitis *E. coli* (NMEC) (Croxen and Finlay, 2010).

E. coli EC1110 was isolated from a pool of rectal faeces collected from 5 apparently healthy dairy calves. However, when they were individually analysed 6 months later (only 3 animals remaining), none of the animals included in the initial faecal pool shed CP-producing *E. coli*. Similarly, no CP-producing *E. coli* were isolated from rectal faeces collected from lactating cows, heifers, or calves during the monthly samplings carried out during the previous year or in slurry samples collected in Study II (see 3.2.). This suggested a sporadic occurrence of CP-producing *E. coli* in the farm. NDM and among them, NDM-1, has become one of the most commonly isolated and distributed CPs worldwide. Since carbapenems are not used in food-producing animals, there is no selective pressure associated with their use. Therefore, animal exposure to NDM-1-producing bacteria may be environmental and occurrence in animals might reflect the types of CPs known to be the most prevalent in human isolates. Alternatively, the use of other β-lactams like penicillins and cephalosporins, which were widely used in the study herd, may pose a selective pressure.

In conclusion, to the best of our knowledge, this study reports for the first time an NDM-1-producing *E. coli* isolated from cattle. Besides the carbapenem resistance gene $bla_{NDM\text{-}1}$, the fully characterized pST-3 IncC-type plasmid carried AMR genes associated to resistance to aminoglycosides, sulfonamide and trimethoprim. The occurrence of NDM-1 plasmid-mediated carbapenem resistance in *E. coli* from cattle is worrisome since it might pose a risk for resistance spread in food-producing animals. However, this was the only positive sample after monitoring the herd for over a 2-year period, which suggests it was a sporadic event. Nevertheless, One Health surveillance programs are needed to early monitor emergence and prevent farm animals from becoming an important source of such bacteria for humans.

Data Availability: The nucleotide sequences of *E. coli* EC1110 strain chromosome and pEC1110_NDM-1 plasmid were deposited under GenBank accession number JADWPF000000000, BioProject accession number PRJNA680938, and BioSample accession number SAMN16926619.

3.4. Study IV

Draft genome sequence of *Escherichia marmotae* E690, isolated from beef cattle

3.4.1. Background

The genus *Escherichia* (family Enterobacteriaceae) includes four species, *i.e.*, *Escherichia coli* (Castellani and Chalmers, 1919), *Escherichia fergusonii* (Farmer et al., 1985), *Escherichia albertii* (Huys et al., 2003), and the recently described *Escherichia marmotae* (Liu et al., 2015, 2019) along with several cryptic clades (Clermont et al., 2011). *E. marmotae* was first isolated in Qinghai-Tibet plateau in 2012 from the faeces of Himalayan marmot and was described as a novel species in 2015 (Liu et al., 2015). When this PhD Thesis was proposed, *E. marmotae* had not been described in humans or animals other than the abovementioned Himalayan marmot. In the frame of Study I (see 3.1) an ESBL-producing *Escherichia* spp. isolate (E690) was recovered from rectal faeces collected from beef cattle. Here we present the draft genome of the strain and describe the main features that allowed its identification as a member of the newly described species *E. marmotae*.

3.4.2. Materials and methods

Faeces (25 g) diluted 1:10 in modified Tryptic Soy Broth (bioMérieux) supplemented with novobiocine (Biolife) were incubated at 41±1°C (6-7h), pre-enriched in MacConkey broth with cefotaxime 1 mg/L (37±1ºC, 24h) and subcultured onto MacConkey agar with cefotaxime (1 mg/L). DNA extracted from pure culture (Wizard genomic DNA purification kit, Promega) was submitted to Eurofins Genomics where libraries were prepared using the NEBNext Ultra™ II FS DNA Library Prep Kit (Illumina). The genome was sequenced using Illumina NovaSeq6000 (150 bp pair-ended reads) resulting in 23,934,128 reads (718X coverage). Quality control was assessed using FastQC v.0.11.9 (Andrews, 2010), and then analysed via TORMES v.1.0 (Quijada et al., 2019). Briefly, reads were quality filtered using Trimmomatic (Bolger et al., 2014) and PRINSEQ (Schmieder and Edwards, 2011), and *de novo* assembled using SPAdes (Bankevich et al., 2012). The quality of the assemblies was assessed with QUAST (Gurevich et al., 2013), discarding contigs below 200 bp with PRINSEQ. BLASTn v.2.9.1+

(Zhang et al., 2000) and ABRicate were used to screen for acquired antimicrobial resistance genes in ResFinder (Zankari et al., 2012), chromosomal point mutations associated with antimicrobial resistance in PointFinder (Zankari et al., 2017), and virulence genes in the Virulence Factors Database (VFDB) (Chen et al., 2005). Plasmid replicons were identified using PlasmidFinder (Carattoli and Hasman, 2020). PlasFlow (Krawczyk et al., 2018) predicted plasmid- and chromosome-derived contigs. *In-silico* FimH typing was achieved using FimTyper (Roer et al., 2017). Multilocus sequence types (MLSTs) (Achtman scheme) were queried against the *E. coli* MLST database PubMLST (Jolley and Maiden, 2010) using mlst, and core-genome MLST (cgST) was assigned using cgMLSTFinder following the EnteroBase *E. coli* cgMLST scheme (Zhou et al., 2020). Gene identification and annotation were retrieved from NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Zhao et al., 2012). See Table 11 for specific tools' versions/commands used for specific tools.

Table 11. List of bioinformatic tools used in the study along with the versions, scripts and references.

Tool	Version	Scripts ran on command line	Reference
trimmomatic	v.0.38	trimmomatic PE -phred33 Raw_reads/Sample_R1.fastq.gz Raw_reads/Sample_R2.fastq.gz /cleaned_reads/Sample_noadapt.R1.fastq.gz /dev/null cleaned_reads/Sample_noadapt.R2.fastq.gz /dev/null ILLUMINACLIP: adapters.fa:1:30:1	(Bolger et al., 2014)
prinseq	v.0.20.4	1. raw data quality filtering: perl prinseq-lite.pl -verbose -fastq cleaned_reads/Sample_noadapt.R1.fastq -fastq2 cleaned_reads/Sample_noadapt.R2.fastq -out_good cleaned_reads/Sample_ok -out_format 3 -out_bad null -min_len 125 -min_qual_mean 25 -trim_qual_right 25 -trim_qual_window 15 -trim_qual_type mean 2. contigs filtering: perl prinseq-lite.pl -fasta assembly/Sample_assembly/contigs.fasta -min_len 200 -out_good genomes/Sample -out_bad null	(Schmieder and Edwards, 2011)

SPAdes	v.3.13.0	python spades.py --careful -1 cleaned_reads/Sample_ok_1.fastq.gz -2 cleaned_reads/Sample_ok_2.fastq.gz -o assembly/Sample_assembly -t 8	(Bankevich et al., 2012)
QUAST	v.5.0.2	quast genomes/Sample.fasta -o /genome_stats/Sample_genome_stats -t 16 --min-contig 200 --no-icarus --silent --no-sv	(Gurevich et al., 2013)
ABRicate	v.0.8.10	abricate genomes/Sample.fasta --db db* –nopath (in the curation of the results table, all hits with coverage below 60% and identity below 90% were removed) *db = Resfinder, PlasmidFinder, VFDB	https://github.com/tseemann/abricate
PointFinder	v.3.1.0	python PointFinder.py -i genomes/Sample.fasta -p pointfinder_db -m blastn -m_p blastn -s ecoli -o point_mutations/Sample	(Zankari et al., 2017)
PlasFlow	v.1.1	PlasFlow.py --input genomes/Sample.fasta --output PlasFlow/ Sample.predictions --threshold 0.7	(Krawczyk et al., 2018)
FimTyper	v.1.1	perl fimtyper.pl -d fimtyper_db -b ncbi-blast-2.9.0+/ -i genomes/Sample.fasta -o /fimH_typing/Sample -k 95.00 -l 0.80	(Roer et al., 2017)
mlst	v.2.16.1	mlst genomes/Sample.fasta --nopath --quiet > mlst/mlst.tab	https://github.com/tseemann/mlst
cgMLSTFinder	v.1.1	docker run --rm -it -v $(workdir):/workdir cgmlstfinder -o output -s ecoli -db cgmlstfinder_db -t temp cleaned_reads/Sample.fastq.gz	https://bitbucket.org/genomicepidemiology/cgmlstfinder/src/master/
PGAP	v.4.11 2020-03-30.build4489	./pgap.py -r -o Sample_results genomes/Sample/input.yaml	(Zhao et al., 2012)

3.4.3. Results

The chromosome sequence length was 4,303,797 bp (63 contigs, N50=175,886 bases), with a 50.4% G+C content. The chromosome contains 4,094 genes (3,951 protein-coding, 74 pseudogenes, and 69 RNA genes). Moreover, two plasmid incompatibility group-determining sequences were identified, IncFI and IncI1. Pairwise comparisons of the E690 genome versus closely related strain genomes performed at the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker, 2019), identified

the genome of *E. marmotae* HT073016^T as the closest match. Intergenomic comparison of E690 and *E. marmotae* HT073016^T by digital DNA-DNA hybridization (d_4 = 94.6 [93.0 - 95.9]) and G+C content (0.18 difference) identified strain E690 as *E. marmotae*.

MICs were determined by broth microdilution following recommendations in Commission Decision 2013/652/EU using two Sensititre® MIC Susceptibility plates (EUVSEC1 and EUVSEC2, ThermoFisher Scientific). *E. marmotae* E690 exhibits microbiological resistance to tetracycline (MIC > 64 mg/L) and the β-lactams ampicillin (MIC > 64 mg/L), cefotaxime (MIC = 8 mg/L), ceftazidime (MIC = 16 mg/L), and cefepime (MIC = 1 mg/L), and carries the *tet*(A) and bla_{SHV-12} genes in an IncI1 plasmid. It carries mutations in the topoisomerase genes, *par*C (p.S57T), and *par*E (p.I355T), but is susceptible to nalidixic acid (MIC < 4 mg/L) and ciprofloxacin (MIC = 0.03 mg/L). It belongs to MLST type ST-6495 and cgST-141216 and carries a *fim*H160 allele. *E. marmotae* E690 harbours virulence factors related to extraintestinal pathogenic *E. coli* (ExPEC) like F1 fimbriae, K1 capsule, and OmpA protein (chromosomally encoded) and to animal enterotoxigenic *E. coli* (ETEC), like F4 fimbriae and a heat-stable enterotoxin EAST1 (plasmid-encoded).

Data availability: The genome sequence was deposited under GenBank accession number JABXGM000000000, BioProject accession number PRJNA632731, and BioSample accession number SAMN14918579.

4. GENERAL DISCUSSION

AMR is an urgent global health threat that requires a One Health approach to be effectively tackled. Food-producing animals are a relevant reservoir of resistant bacteria that can be disseminated to humans either by direct contact or indirectly via food or the environment (Laxminarayan et al., 2013; Poirel et al., 2018). The most important resistance mechanism in Enterobacteriaceae is the production of ESBL, AmpC and CP enzymes, whose encoding genes can be located on MGEs that can be transferred among bacterial species. Among the aims of this PhD Thesis was to generate knowledge on the prevalence and epidemiology of ESBL, AmpC and CP-producing commensal *E. coli* in domestic ruminants. The cross-sectional study carried out in this Thesis (Study I) provided data on herd-prevalence of cefotaxime-resistant commensal *E. coli* in dairy cattle, beef cattle, and sheep in the Basque Country, filling the lack of data in the region at the time. Comparison with other similar studies is sometimes difficult due to differences in methodology (for instance isolation methods). Nevertheless, prevalence levels of cefotaxime-resistant *E. coli* described in cattle in this study were similar to those reported in England and The Netherlands (Gonggrijp et al., 2016; Ludden et al., 2019) and lower compared to a cross-sectional study carried out in Germany (Hille et al., 2017). Despite the strong variation observed among European countries, the prevalence levels in cattle observed here in the Basque Country were below the mean values reported in the EU surveillance studies conducted by the ECDC and EFSA in 2017 (EFSA and ECDC, 2019) and 2019 (EFSA and ECDC, 2022) in calves under 1 year. In reference to sheep, this Thesis added information to the limited prevalence data on ESBL-, AmpC- and CP-producing *E. coli* available. Our results were in line with a previous study conducted in Switzerland (Geser et al., 2012), but prevalence was lower compared to other studies. This is the case of two studies conducted in North Carolina (USA) and China using non-selective isolation (Atlaw et al., 2021; Zhao et al., 2021) or a study carried out in the south of Portugal using selective isolation media, where ESBL-producing *E. coli* were detected in almost all faecal samples collected from sheep in one farm (Palmeira et al., 2021b).

The results presented in Study I evidenced a higher prevalence of cefotaxime-resistance *E. coli* in dairy cattle in comparison with beef cattle and sheep. A higher

prevalence of cefotaxime-resistant *E. coli* in dairy cattle compared to beef cattle had already been observed (Hille et al., 2017). This variation could be related to the different management systems. A higher incidence of coliform mastitis was reported in confined cows, where conditions for cross-infection within animals are favoured, compared to pasture-based cows (Collis et al., 2019). In the Basque Country, dairy cattle are mostly housed in pens under intensive production, whereas beef cattle management system is semi-intensive; animals graze in farmland pastures in spring and part of the summer, in communal mountain pastures from the middle of July until the end of November and are housed in winter. Sheep flocks from the Latxa breed, native from the Basque Country, are also managed under a semi-intensive production system; housed during lambing and grazing on communal mountain pastures in summer and autumn. Nevertheless, complex and multiple factors are involved in the development and transmission of AMR in farm environments that need to be further investigated.

Phenotypic characterization of the isolates was carried out using broth microdilution, a gold standard method recommended by the EUCAST. Results reflected that most of the cefotaxime-resistant *E. coli* isolates recovered from ruminants presented an ESBL phenotype, followed by an AmpC phenotype, as further confirmed by WGS analyses. These findings are consistent with those observed in a cross-sectional study conducted in dairy cattle farms in the Netherlands (Gonggrijp et al., 2016) and with the last summary report conducted by the ECDC and EFSA (EFSA and ECDC, 2022). Co-resistance to other antimicrobial classes was commonly observed in most of the isolates, namely for tetracycline, nalidixic acid, ciprofloxacin, sulfamethoxazole, trimethoprim and chloramphenicol, which is not unusual in ESBL/AmpC-producing isolates (Seiffert et al., 2013; Michael et al., 2017; Collis et al., 2022). In fact, co-selection with antibiotics other than extended-spectrum cephalosporins is also an important driver in resistance in ESBL/AmpC-producing bacteria, which are often MDR (Madec et al., 2017). Resistance to colistin and tigecycline (glycylcycline), two last-resort antimicrobials that are currently used to treat infections caused by MDR Gram (-) bacteria, was not detected. In the case of colistin resistance, the co-existence of cephalosporin and colistin ARGs in the same strain has only been sporadically identified

in food-producing animals, including an epidemic clone in calves in France (Haenni et al., 2018; Madec and Haenni, 2018; Hassen et al., 2019). Moreover, in Spain, colistin-resistance in Enterobacteriaceae in food-producing animals has been mainly detected in pigs, but resistance rates showed a decreasing trend in the last few years as a result of a reduced use of this antimicrobial (Miguela-Villoldo et al., 2019). Resistance to tigecycline (glycylcycline) is not commonly detected in food-producing animals as this antimicrobial is not authorised in veterinary medicine in the EU (EMA/AEMEG, 2019).

In Study I, short-read Illumina WGS provided an insight into the GDRs in ruminants in the Basque Country. Concerning the ESBL resistance genes, *bla*$_{CTX-M-14}$ was the most frequently detected in our study. Such gene, albeit not being predominant, showed an increasing trend in prevalence in livestock in The Netherlands (Ceccarelli et al., 2019). Genes *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$ were also detected here, but did not prevail in cattle as in other European countries (Schmid et al., 2013; Day et al., 2016; Gonggrijp et al., 2016; Michael et al., 2017; Ludden et al., 2019; Giufrè et al., 2021). Other genes encoding ESBL enzymes such as SHV-12 were sporadically detected. In other studies, *E. coli* strains harbouring *bla*$_{SHV-12}$ genes were also occasionally isolated from cattle but poultry seemed to be the major host (Briñas et al., 2005; Smet et al., 2010; Geser et al., 2012; Michael et al., 2017; Ceccarelli et al., 2019). On the other hand, AmpC-type β-lactamases were mostly associated with the presence of plasmid-borne *bla*$_{CMY}$ genes (specifically the *bla*$_{CMY-2}$ variant) rather than being associated to point mutations in the promoter of the chromosomal *bla*$_{ampC}$ gene. These results differed from those presented in other studies conducted in dairy and beef cattle where chromosomal mediated AmpC-hyperproducing *E. coli* were more frequently detected than plasmid mediated AmpC (Schmid et al., 2013; Burgess et al., 2021). Nevertheless, *bla*$_{CMY-2}$ genes have also been identified in cattle (Hordijk et al., 2013; Seiffert et al., 2013; Ceccarelli et al., 2019), but are mostly detected in poultry (Ceccarelli et al., 2019; Giufrè et al., 2021). Overall, the obtained results for ESBL and AmpC resistance genes in ruminants in the Basque Country were in line with those observed in humans in Europe. In fact, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$ ESBL-encoding genes are very commonly reported, also in Spain (Díaz et al., 2010; Cantón et al., 2012; Fernández-Reyes et al., 2014). Most of the

ESBL and AmpC genes detected in this study were located in plasmids but in many cases the specific types of plasmids could not be identified. This was due to the short length of Illumina reads, which results in highly fragmented genomes (Goodwin et al., 2016). Nevertheless, in some cases, the plasmid replicon and the AMR gene were located in the same contig and we were able to observe some associations. For instance, $bla_{\text{CMY-2}}$ genes were associated with IncI1 plasmids, a very common plasmid-type in food-producing animals in Europe (Rozwandowicz et al., 2018), or IncQ with $bla_{\text{CMY-4}}$, a plasmid/gene combination that has already been observed (Kotsakis et al., 2015). These two plasmid types also carried the greatest variety of other ARGs coding for resistance to aminoglycosides, chloramphenicol, trimethoprim, and sulfamethoxazole.

An important and worrisome finding was the presence of genes coding for resistance to lincosamide (*lnu*(F)) and fosfomycin (*fosA7*) in dairy cattle isolates. Lincosamides are used in cattle to treat mastitis caused by Gram (+) pathogens (Constable et al., 2008) and fosfomycin is not authorised as veterinary medicine in Spain, though it has been reintroduced as a first-line antimicrobial for the treatment of uncomplicated urinary tract infections in humans caused by multidrug-resistant Gram (-) pathogens, including ESBL- and CP-producing Enterobacteriaceae (Meletis, 2016). Among the *fosA*-like genes, *fosA3* is widely distributed among Enterobacteriaceae. However, the *fosA7* gene-variant has been increasingly reported since its first identification on the chromosome of *Salmonella enterica* serovar Heidelberg from chickens in 2017 (Rehman et al., 2017). In fact, a recent study conducted in China has also reported a high prevalence of *fosA7* genes in several animal species and in other Enterobacteria besides *Salmonella*, including *E. coli* (Zhang et al., 2022). In our study, the *fosA7* gene was located on the chromosome, which is uncommon in *E. coli* (Ito et al., 2017). The genetic environment of the detected *fosA7* genes was not addressed in our study but according to other authors these gene is often associated with MGE along with other AMR genes, enabling rapid dissemination and co-selection of fosfomycin resistance under the selective pressure of other antimicrobials (Tseng et al., 2015; Zhang et al., 2022). Thus, the spread of fosfomycin resistance in food-producing animals should be closely monitored.

The higher prevalence of cefotaxime-resistant *E. coli* in dairy cattle in comparison to beef cattle and sheep in the cross-sectional Study I, highlighted the need to conduct a long-run surveillance study to better understand the dynamics of cefotaxime-resistant *E. coli* within dairy cattle farms. In Study II, five dairy cattle farms that represented the style of farming in the Basque Country were monitored to determine the prevalence and transmission dynamics of ESBL-, AmpC- and CP-producing *E. coli* during 12 samplings. Cefotaxime-resistant *E. coli* were recovered in the five dairy cattle farms, but the isolation frequency varied among farms and animal age groups. A higher prevalence of cephalosporin-resistant *E. coli* was observed in lactating cows and calves compared to heifers. These results can be somehow explained by the fact that calves and lactating cows were permanently housed indoors, whereas pregnant heifers and dry-cows had access to outdoor pastures. Indeed, in Study I, we had already observed that less intensive management systems were associated with a lower herd prevalence of cefotaxime-resistant *E. coli* infection. Another possible explanation for the higher prevalence of cefotaxime-resistant *E. coli* in lactating cows compared to heifers is that older animals have been longer exposed to antimicrobials. In the case of calves, other studies reported colonization of young animals with ESBL- and AmpC-producing *E. coli,* sometimes as early as 1 day of age (Donaldson et al., 2006; Hordijk et al., 2013; Gay et al., 2019) with ESBL carriage decreasing along the fattening process (Hordijk et al., 2013; Gay et al., 2019). Furthermore, it has been reported that the resistome is more diverse in younger calves compared to adult animals (Noyes et al., 2016) and that antimicrobial resistant *E. coli* isolates could be better adapted to the calf intestinal environment (Khachatryan et al., 2004).

The variation on the prevalence of cefotaxime-resistant *E. coli* between animals from different age groups and farms may be also attributed to antimicrobial consumption. Here, the absence of a detailed record of the AMU in the monitored farms impeded drawing solid conclusions implying causality. However, it is worth mentioning that different antimicrobials were used for mastitis treatment and control in lactating cows in each farm. Interestingly, the lowest prevalence of cefotaxime-

resistant *E. coli* was observed in F2, where mastitis was not treated with antimicrobials. The use of third- and fourth-generation cephalosporins in dairy cattle has been associated with significantly higher odds of having a positive ESBL/AmpC herd status (Gonggrijp et al., 2016). In our study, the five dairy cattle farms applied a blanket dry-cow therapy (DCT) treatment, using either penicillins and aminoglycosides or first-generation cephalosporins. It has been suggested that this practice may contribute to the development of antimicrobial resistant bacteria (Collis et al., 2019; McCubbin et al., 2022). In fact, in the farms where first-generation cephalosporins were used for blanket DCT (F3, F4 and F5), higher prevalence of cephalosporin-resistant *E. coli* was detected. In order to reduce AMU in dairy cattle farms, blanket DCT should be replaced with selective DCT, in which only cows with high risk of intramammary infection receive antimicrobial treatment. It has been shown that the risk of intramammary infection incidence along the dry period and after calving did not differ between selective DCT or blanket DCT as long as an internal teat sealant was used at dry-off (Kabera et al., 2021; Rajala-Schultz et al., 2021; McCubbin et al., 2022). Selective DCT, widely implemented in some northern European countries such as Denmark, Finland, Norway and Sweden or The Netherlands (Rajala-Schultz et al., 2021) is not yet largely used in Spain.

Phenotypic characterization of the isolates derived from Study II was performed using the broth microdilution method as in Study I and the results were comparable, i.e., the majority of cefotaxime-resistant *E. coli* isolates were also resistant to several other antimicrobials, such as tetracycline, nalidixic acid, ciprofloxacin, sulfamethoxazole, trimethoprim and chloramphenicol. Furthermore, in accordance with Study I, all the isolates were susceptible to tigecycline and colistin. One interesting finding was that the diversity of the phenotypic resistance profiles varied among farms and sampling time. To further explore these differences and to better understand the genetic diversity and the relationship of the circulating strains, a selection of isolates from the two farms that showed the lowest (F1) and largest (F4) AMR profile diversity were subjected to WGS. However, in this case we opted for long-read ONT sequencing technology in an attempt to overcome the limitations experienced in Study I when using short-read Illumina sequencing. Due to its low error rate, Illumina is a very useful

technology for the accurate detection of novel GDRs and new variants of AMR genes and has been the most widely used WGS for microbial genomics. However, short-read sequencing is often insufficient for assessing the genomic structure. As previously stated, in Study I we obtained very fragmented genome assemblies and, in many cases, we were unable to determine the genetic location of the AMR genes. In Study II, long-read ONT sequencing successfully overcame this limitation, permitting us to completely resolve and circularise most of the genomes (both chromosomes and plasmids) and thus reveal the genomic locations of the majority of the AMR genes, which is an important aspect for epidemiological purposes. Changing the sequencing technology demanded adaptations in the bioinformatic analysis, including the need for specific bioinformatic tools for ONT data analysis. One concerning downside of using the ONT platform is the higher error rates compared to Illumina sequencing (Delahaye and Nicolas, 2021); despite the constant improvement over the last years in read accuracy, ONT sequencing still faces limitations to determine single-nucleotide variations (SNVs). This can affect MLST type assignation and the identification of SNP mutations associated to AMR when calling SNVs from ONT-derived assembled genomes. In an assembly, all coverage at a given locus is collapsed into a single base, whereas in raw reads the depth of coverage to each allelic combination can be quantified, providing a greater resolution. Calling SNVs directly from unassembled long reads rather than from assembled genomes provide advantages in terms of accuracy and efficiency, which is critical to overcome the higher error rate of ONT sequencing. In this sense, the analysis of ONT-derived raw reads with Krocus, which allows MLST type assignation from unassembled long-reads (Page and Keane, 2018) successfully resolved the problem. Furthermore, *de novo* assembly is one of the most time-consuming and resource-intensive parts of the sequence analysis workflow. Predicting ST before this step provides results within minutes, as read data is produced. On the other hand, for the correct determination of resistance mechanisms associated with point mutations, such as those occurring in the *ampC* promoter or in gyrase and topoisomerase genes, obtained results were manually revised keeping only known mutations included in the PointFinder database. When properly solving these limitations, ONT sequencing can be

considered a promising technology as the chemistry and basecallers are quickly progressing (Taylor et al., 2019; Amarasinghe et al., 2020). Additionally, ONT devices, especially the MinION platform, are relatively cheap and highly portable. As we have observed, there are different strategies to decrease the price of completing a genome, such as sample multiplexing. Moreover, real-time data streaming permits stopping sequencing when sufficient depth is achieved. In this PhD Thesis ONT sequencing was applied for WGS, but ONT has also demonstrated its usefulness in metagenomic, epigenomic and transcriptomic research (Wang et al., 2021).

In Study II, genome analyses revealed two different patterns in the occurrence of ESBL genes. In F1, two different strains predominated and were recovered multiple times in animals from all the age groups indicating persistence over time. On the other hand, in F4, likely due to multiple source contamination events, a large diversity of genotypes carrying multiple and diverse GDRs (both chromosome and plasmid located) was observed and only few genotypes persisted for some time. A more in-depth investigation of the location of the ESBL-coding genes showed that ESBL gene spread was mainly plasmid-mediated in both farms sometimes with an apparent ESBL-gene/plasmid association. In F1, the most frequently detected ESBL gene, *bla*$_{CTX-M-1}$, was located in IncX1 plasmids which were dispersed in the two predominant strains. In contrast, in F4, IncB/O/K/Z plasmids were associated with the widespread presence of *bla*$_{CTX-M-14}$ gene in *E. coli* isolates belonging to different STs. Other studies have also described this ESBL-gene/plasmid association, in particular for IncB/O/K/Z - *bla*$_{CTX-M-14}$ which is frequently found in *E. coli* from animal sources (Rozwandowicz et al., 2018). A common phenomenon observed in both farms was that different STs harboured the same type of plasmids with an identical ARG repertoire, suggesting possible horizontal transfer of ESBL-carrying plasmids among circulating strains.

The spread and dynamics of ESBL and AmpC genes has been studied on several occasions in dairy cattle farms (Hordijk et al., 2013, 2019; Horton et al., 2016; Gay et al., 2019). In a longitudinal study conducted in The Netherlands in veal calves during the fattening process, in two of the three monitored farms, the occurrence of ESBL genes was mainly due to the clonal spread of *E. coli* strains with the same ST, ESBL genes

and plasmids (*E. coli* ST57 and *bla*$_{CTX-M-14}$ genes located on an IncF plasmid) (Hordijk et al., 2013). In another recent longitudinal study also conducted at veal fattening farms, a high diversity of *E. coli* clones and *bla*$_{CTX-M}$ carrying plasmids was observed but few *E. coli* strains with the same ST, *bla*$_{CTX-M}$ genes and plasmids dominated in the long-term (Massot et al., 2021). On the other hand, other longitudinal studies found that the spread of ESBL genes in dairy cattle farms could also occur due to a horizontal transfer of ESBL and AmpC genes carrying plasmids between unrelated strains (Horton et al., 2016; Gay et al., 2019; Hordijk et al., 2019), as was observed in F4. However, the results derived from some of these longitudinal studies need to be interpreted with caution as different approaches or methodologies were applied and, in some occasions, the genetic location of ESBL and AmpC genes was not resolved.

In this PhD Thesis the potential of WGS in AMR gene detection and surveillance was demonstrated. As observed in Study I and Study II, both Illumina and ONT technologies achieved very good overall results in identifying GDRs. In both cases, genotypic (WGS-derived) and phenotypic (MIC-derived) resistance profiles showed a strong agreement, and only in few cases discrepancies were noticed, namely associated to cefoxitin (FOX), ertapenem (ETP), cefepime (FEP) and temocillin (TRM). Most of them concerned isolates that were phenotypically resistant without any associated GDR being detected. Many of these isolates showed a MIC value of just a single two-fold dilution above the ECOFF, which is the widely accepted margin of error of the microdilution method, suggesting that these isolates were most likely susceptible as determined by WGS. This was evident in two of the three FOX resistant isolates from Study I and in six FOX resistant isolates from Study II that showed a presumptive ESBL+AmpC phenotypic profile but only carried an ESBL-encoding gene. Different was the case of four ETP resistant isolates from Study I (10 isolates) that only carried the AmpC *bla*$_{CMY-2}$ gene without any known CP-encoding gene. Resistance to FEP while only carrying *bla*$_{CMY-2}$ or *bla*$_{CMY-4}$ has already been reported (Dona et al., 2019) and might be due to the AmpC β-lactamase production, which has been associated to loss or downregulation of outer membrane porins (Mammeri et al., 2008). In addition, in Study I, two TRM resistant isolates were detected without any know CP-encoding gene. In line

with our results, other studies have demonstrated that CTX-M-producing *E. coli* isolates also seem to express low level of TRM resistance (Cavaco et al., 2019). Nevertheless, we cannot exclude the possibility of unknown AMR mechanisms. Occasionally, apparent genotype–phenotype inconsistences were resolved when AMR genes that were not included in the AMR gene databases at the time of the analysis were considered. This was the case of four trimethoprim-resistant isolates from Study I for which no resistance determinant was initially detected. However, after manual revision, we found that all those isolates carried the *dfrA36* gene, a trimethoprim resistance gene (Wuthrich et al., 2019) not yet included in the ResFinder database at the moment of the analysis. In contrast, only in few cases genotype–phenotype discordances were due to the presence of the ARG in phenotypically susceptible isolates. This occurred in nine isolates from Study I that carried the $bla_{\text{CTX-M-14}}$ gene but tested susceptible to ceftazidime. This phenomenon has already been observed in other studies (Williamson et al., 2012). In fact, by mechanisms yet unknown and under selective pressure, $bla_{\text{CTX-M-14}}$-bearing *E. coli* isolates could switch from ceftazidime-susceptibility to ceftazidime-resistant phenotypes (Costa Ramos et al., 2015).

WGS is becoming a common tool in molecular epidemiological research and surveillance studies, and it has begun to completely replace other, lower-resolution, molecular methods (WHO, 2020). Furthermore, the EFSA proposed to follow a gradual integration of WGS within the harmonised AMR monitoring (EFSA et al., 2019). In line with the results presented in this PhD Thesis, other studies also showed a high concordance between WGS-predicted resistance and expression of phenotypic resistance (Zankari et al., 2013; Hendriksen et al., 2019; Stubberfield et al., 2019; Hesp et al., 2021; Rebelo et al., 2022). Moreover, studies evaluating the sensitivity and specificity of both techniques have concluded that WGS is just as suitable for monitoring AMR in commensal *E. coli* from livestock as culture-based AST (Hesp et al., 2021). However, inferring antimicrobial susceptibility using WGS alone has a number of limitations. In fact, in contrast to broth microdilution, WGS cannot be used to quantify the level of microbiological resistance (WHO, 2020). On the other hand, genetic susceptibility alone based on gene search on available databases will not be able to

detect new and unknown mechanisms of resistance that might emerge (Rebelo et al., 2022). Furthermore, there is still a need for standardization of pipelines and AMR gene databases (Hendriksen et al., 2019; Hesp et al., 2021). In fact, the results obtained from WGS (usually outputs for gene length and percentage of similarity) may not be fully comparable among different databases, as the nomenclature, availability of genes and frequency of curation may vary (for instance, ARG-ANNOT is not currently actively updated) (Hendriksen et al., 2019; Papp and Solymosi, 2022). Consequently, the choice of the AMR gene database can somehow influence the sensitivity and specificity of WGS-based susceptibility testing. It is unlikely that broad implementation of WGS will happen in a short period of time as data handling is still challenging (it requires considerable computing resources and efficiency) and there is a lack of bioinformatics expertise and standard operating procedures (Goodwin et al., 2016; WHO, 2020; Rebelo et al., 2022). All in all, considering the existing limitations, phenotypic methods could only be partially replaced by WGS depending on the purpose of the study. WGS alone has proven its efficacy in AMR surveillance and monitoring programs, but as for clinical decision-making or as a warning that new mechanisms might be emerging, phenotypic methods will be still needed (Rebelo et al., 2022).

Apart from the detection of AMR determinants of resistance, the usefulness of WGS for bacterial identification and characterization was further proven in this PhD Thesis by the in-depth genomic profiling of two particular isolates of interest derived from the samplings carried out in Studies I and II. On the one hand (Study III), WGS was used to fully characterize a carbapenem-resistant isolate (E1110) that was detected in one of the farms monitored in Study II. On the other hand (Study IV), WGS was employed in the accurate identification and the in-depth genomic profiling of an isolate (E690) recovered from beef cattle in the frame of Study I. The recovery of a carbapenem-resistant *E. coli* (E1110) from a dairy cattle farm was a significant finding considering that carbapenems are one of the sole limited therapies in the treatment of severe infections due to ESBL or AmpC producing Enterobacteria in humans. Furthermore, CP-producing *E. coli* are still rare in livestock (Madec et al., 2017; Kock et al., 2018; EFSA and ECDC, 2021). Noteworthy, no CP-producing *E. coli* had been isolated

in any of the rectal faeces collected from the 300 herds sampled in Study I. The detection of a CP-producing *E. coli* in the frame of Study II may be rather striking, as a remarkable lower number of farms was sampled in comparison to Study I. Nevertheless, there are some possible explanations for this result. For example, 12 periodical samplings were performed in each farm in the frame of Study II (only one sampling per farm in Study I), thus increasing the chances to detect low prevalent phenotypes. On the other hand, the isolation strategy was also more intense in Study II compared to Study I and included a non-selective pre-enrichment step, followed by inoculation on MacConkey agar, and a real-time PCR amplification screening targeting the CP-coding genes, bla_{NDM}, bla_{VIM}, bla_{KPC}, and bla_{OXA-48} (Ellington et al., 2016) on the culture growth. Upon detection of the bla_{NDM} gene during this screening step, a selective enrichment using MacConkey broth supplemented with cefotaxime was performed followed by a subsequent culture on CP selective agar plates. A combination of short-read Illumina and long-read ONT WGS was used to produce high-quality complete genomes of this isolate. The hybrid assembly proved essential to completely resolve and circularise the bacterial chromosome and the IncC plasmid that contained the CP-producing bla_{NDM-1} gene along with another nine AMR genes. The location of the bla_{NDM-1} gene in an IncC plasmid had been reported before (Poirel et al., 2011; Harmer and Hall, 2015; Ambrose et al., 2018; Wu et al., 2019) but, to the best of our knowledge, this was the first time this gene-plasmid association has been described in cattle. In fact, in Europe, NDM-producing Enterobacteriaceae have been occasionally isolated in other food-producing animals such as pigs or wild animals (Fischer et al., 2013; Diaconu et al., 2020). It is in China where NDM-1 producing *E. coli* are more widespread in food-producing animals (Wang et al., 2017b; Zhao et al., 2019; Sapugahawatte et al., 2020; Zhai et al., 2020). But so far, the only NDM variant described in cattle was NDM-5 and in studies where the plasmid type was characterized it was located in IncX plasmids (Purkait et al., 2016; Yaici et al., 2016; He et al., 2017b, 2017a). Further comparisons of the structure of the IncC plasmid of E1110 revealed that it shared a high degree of backbone similarity with other bla_{NDM}-harbouring IncC plasmids, but also with plasmids from Enterobacteriaceace isolated from food-producing animals that did not contain

CP-producing genes. Moreover, the variable region where the *bla*$_{NDM-1}$ gene was located (ARI-A region) has common features that appear in other *bla*$_{NDM-1}$-harbouring strains, such as the insertion sequence IS*Aba125* upstream of *bla*$_{NDM}$ (Poirel et al., 2010) and a bleomycin resistance gene, *ble*$_{MBL}$ (Wu et al., 2019). This region was conserved in the first NDM-1 CP-producing *E. coli* described in Spain in 2011 (Solé et al., 2011) and has high homology with the PGI1-*Pm*PEL genomic island integrated into a *Proteus mirabilis* chromosome (Girlich et al., 2015). Interestingly, despite lacking the region where *bla*$_{NDM-1}$ gene is located, the ARI-A multi-resistance region is very similar to the same region of a plasmid isolated from cattle, indicating a possible common origin. Previous studies evaluating the acquisition of *bla*$_{NDM-1}$ gene proposed that this gene could have been inserted by rolling-circle transposition mediated by IS*CR1* (Bonnin et al., 2013), a structure observed in our study that is also associated with the acquisition of the *bla*$_{DHA}$-*ampR* gene region as part of a class 1 integron (Hennequin et al., 2018).

As CP-producing *E. coli* E1110 was detected in a pool of faeces collected from calves during the last of the 12 samplings carried out in the farm, we decided to sample individually the animals whose faeces comprised the positive pool (only 3 remaining in the farm by then) and collect another slurry sample. Despite using the same bacterial isolation methodology, no other *bla*$_{NDM}$-harbouring strain was detected. Furthermore, considering that no CP-producing *E. coli* were isolated in the slurry samples collected throughout the study in the dairy cattle farm, it can be assumed that prevalence was low and that the persistence on the farm environment was improbable. As for its origin, since carbapenems are not used in food-producing animals, it is possible that the use of disinfectants in the farm may have had an impact in the co-selection of carbapenem resistant bacteria. This CP-producing *E. coli* carried in the same IncC plasmid where the *bla*$_{NDM}$ gene was encoded the *qacEΔ1* gene that confers resistance to quaternary ammonium compounds, which are present in some disinfectants. It has been reported that the prolonged and improper use and discard of disinfectants (including quaternary ammonium compounds) may select for bacterial cross-resistant to antimicrobials (Davies and Wales, 2019; Merchel Piovesan Pereira et al., 2021). In fact, these compounds tend to bind to organic matter and soil, persisting in the environment

(Hegstad et al., 2010). Alternatively, animal exposure to NDM-1-producing bacteria may be environmental. All in all, considering that further attempts to isolate other CP-producing *E. coli* in the herd were unsuccessful, it can be suggested that the circulation within the herd was improbable and that this was a sporadic finding. Nonetheless, the possibility of food animals acting as reservoirs and source for the re-circulation of CP-producers back to the human population cannot be completely dismissed. Therefore, a One Health approach is essential to early monitor AMR emergence in both humans and animals.

In the frame of Study I, an isolate (E690) recovered from beef cattle and originally identified as *E. coli* based on the selective isolation methods, morphology compatibility, biochemical profile, and PCR-based detection of the *uidA* gene was later found unusual when its genome was sequenced (Study IV). WGS analysis showed a relatively unusual low level of similarity with the *E. coli* type species genome. In fact, pairwise comparisons of the E690 genome versus closely related *Escherichia* type strain genomes identified an *E. marmotae* genome as the closest match. Further intergenomic comparison of these two genomes confirmed that strain E690 belonged to the species *E. marmotae.* This was a rather interesting outcome, as this novel *Escherichia* species had been recently described. In fact, *E. marmotae* was first isolated in Qinghai-Tibet plateau in 2012 from the faeces of Himalayan marmot and was described as a novel species in 2015 (Liu et al., 2015). It is worth mentioning that when this PhD Thesis was proposed, *E. marmotae* had not been described in humans or animals other than the abovementioned Himalayan marmot. One study carried out later, isolated *E. marmotae* from human-invasive infections and proposed that this species could be a relatively common human pathogen probably misidentified as *E. coli* (Sivertsen et al., 2022). In fact, *in vitro* studies for virulence testing had determined its potential as a human pathogen (Liu et al., 2019) as was confirmed later (Sivertsen et al., 2022). The isolate recovered from beef cattle in Study I carried virulence factors related to extraintestinal pathogenic *E. coli* (ExPEC) and to animal enterotoxigenic *E. coli* (ETEC). In addition, this isolate harboured a $bla_{\text{SHV-12}}$ ESBL gene, which may not be unusual since other recently characterized *E. marmotae* isolates also carried acquired

β-lactam resistance genes, including ESBL and CP encoding genes (Sivertsen et al., 2022). Its potential as a human pathogen and its ability to carry ARGs claim for more efforts aimed at the identification and characterization of this species. WGS demonstrated its usefulness to distinguish between these two closely related *Escherichia* species that were phenotypically indistinguishable.

In conclusion, this PhD Thesis has broadened knowledge about cefotaxime-resistant *E. coli* in domestic ruminants. Updated herd-level prevalence of ESBL-, AmpC- and CP-producing *E. coli* in the Basque Country as well as phenotypic and genotypic data has been provided, information that was compared to other prevalence studies. Furthermore, the diversity and dynamics of cefotaxime-resistant *E. coli* in dairy cattle farms was investigated, providing valuable information about the complex epidemiology underling AMR dissemination within farms. This PhD Thesis has also demonstrated the power of WGS, not only for the identification of GDRs, but also for the identification and the in-depth genomic profiling of AMR bacteria. Finally, considering the parallel occurrence of ESBL/AmpC genes in humans and animals and the possibility of food animals acting as reservoirs of CP-producing *E. coli*, further research based in a One Health approach would be highly recommended. Future coordinated initiatives focusing on the genomic epidemiological investigation of ESBL, AmpC and CP-producing *E. coli* recovered from livestock, humans and the environment should be undertaken in our region.

5. CONCLUSIONS

1. Domestic ruminants in the Basque Country are important reservoirs of commensal ESBL-/AmpC-producing *E. coli*, herd prevalence being significantly higher in dairy cattle compared to less intensive management systems like beef cattle and sheep.
2. In dairy cattle, ESBL-/AmpC-producing *E. coli* are more prevalent in lactating cows and calves compared to heifers.
3. Most of the cefotaxime-resistant *E. coli* isolates recovered from domestic ruminants showed an ESBL phenotype. The AmpC phenotype was occasionally identified and the ESBL+AmpC phenotype was nearly absent.
4. A large proportion of the ESBL-/AmpC-producing *E. coli* isolates exhibited co-resistance to other antimicrobial classes besides β-lactams, including critically important antimicrobials like fluoroquinolones. All isolates were susceptible to last-resort antimicrobials currently used to treat human infections caused by multi-drug resistant Gram (-) bacteria like imipenem, meropenem, colistin, and tigecycline.
5. $bla_{\text{CTX-M-14}}$ was the most common gene responsible for the ESBL phenotype, whereas $bla_{\text{CMY-2}}$ was the prevailing resistance determinant of the AmpC phenotype. Most ESBL/AmpC genes were located in plasmids, IncI1 being the most prevalent type that harboured the greatest variety of AMRs.
6. CP-producing *E. coli* do not seem to be circulating in ruminant herds in the Basque Country. Still, one strain was isolated from dairy calves and its genome characterized. The CP was encoded by a $bla_{\text{NDM-1}}$ gene located in an IncC-type plasmid, this being the first time that an NDM-1-producing *E. coli* has been reported in cattle.
7. The within-farm transmission dynamics of ESBL-producing *E. coli* vary between farms with either the persistent spread of a small number of predominant clones or the intermittent presence of a large diversity of genotypes, probably as the result of multiple source contamination events. However, in both cases there is a risk of ARGs dissemination through horizontal transfer of circulating plasmids to different *E. coli* MLST types.

8. There was a strong agreement between phenotype and genotype-based inference of AMR, proving that WGS (both Illumina and ONT) is a powerful tool for AMR detection and surveillance studies. WGS was also shown fundamental for the accurate identification and the in-depth genomic characterization of closely related *Escherichia* species.

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6. CONCLUSIONES

1. Los rumiantes domésticos en el País Vasco son importantes reservorios de *E. coli* comensal productor de BLEE/AmpC. Sin embargo, la prevalencia a nivel de rebaño es significativamente mayor en el ganado bovino lechero que en los sistemas de manejo menos intensivos como el ganado bovino de carne y el ganado ovino.
2. En el ganado bovino lechero, *E. coli* productor de BLEE/AmpC es más frecuente en vacas en lactación y en terneros que en novillas.
3. La mayoría de los aislados de *E. coli* resistentes a cefotaxima aislados en rumiantes domésticos mostraron un fenotipo BLEE. El fenotipo AmpC se identificó ocasionalmente y el fenotipo BLEE+AmpC de manera muy puntual.
4. Una gran proporción de los aislados de *E. coli* productor de BLEE/AmpC mostró co-resistencia a otras clases de antimicrobianos además de a los β-lactámicos, incluidos antimicrobianos de importancia crítica como las fluoroquinolonas. Todos los aislados fueron susceptibles a los antimicrobianos de último recurso que se utilizan actualmente para tratar infecciones humanas causadas por bacterias Gram (-) multi-resistentes como imipenem, meropenem, colistina y tigeciclina.
5. El gen más común en los aislados con fenotipo BLEE fue $bla_{CTX\text{-}M\text{-}14}$, mientras que el determinante génico de resistencia predominante del fenotipo AmpC fue $bla_{CMY\text{-}2}$. La mayoría de los genes BLEE/AmpC se localizan en plásmidos, siendo IncI1 el tipo de plásmido más prevalente y el que alberga la mayor variedad de genes de resistencia.
6. *E. coli* productor de carbapenemasas no parece estar circulando en los rebaños de rumiantes del País Vasco. Aun así, en terneros de bovino lechero se ha aislado y caracterizado el genoma completo de una cepa de *E. coli* productor de carbapenemasas. Dicha carbapenemasa estaba codificada por un gen $bla_{NDM\text{-}1}$ localizado en un plásmido tipo IncC. Este hallazgo supone la primera descripción de un *E. coli* productor de NDM-1 en ganado bovino.
7. La dinámica de diseminación de *E. coli* productor de BLEE en los rebaños de bovino de leche difiere entre granjas, caracterizándose en ocasiones por la

persistencia y propagación de un pequeño número de clones predominantes y en otras por la presencia de una gran diversidad de genotipos, probablemente como resultado de eventos de contaminación con múltiples orígenes. Sin embargo, en ambos casos existe el riesgo de diseminación de los genes de resistencia a través de la transferencia horizontal de plásmidos entre cepas de *E. coli* pertenecientes a distintos tipos MLST.

8. Existe un alto grado de concordancia entre la inferencia de las resistencias basada en el fenotipo (mediante CMI) y el genotipo (mediante WGS), lo que demuestra que la secuenciación de genomas completos (tanto con la tecnología Illumina como con ONT) es una herramienta muy útil para los estudios de vigilancia y caracterización de bacterias resistentes. WGS también demostró ser clave para la identificación precisa y la caracterización genómica de especies de *Escherichia* filogenéticamente próximas y difíciles de diferenciar por pruebas fenotípicas.

7. APPENDICES

Appendix 1: Supplementary figures and tables

Supplementary table S1: List of NCBI accession numbers associated to uploaded raw data.

Sample Name	Sequencing Technology	SRA	BioProject	BioSample
E1110	Illumina	SRR13172075	PRJNA680938	SAMN16926619
E1110	Nanopore	SRR14916398	PRJNA680938	SAMN16926619
E0839	Nanopore	SRR19090885	PRJNA833969	SAMN28053238
E0842	Nanopore	SRR19090884	PRJNA833969	SAMN28053239
E0843	Nanopore	SRR19090873	PRJNA833969	SAMN28053240
E0854	Nanopore	SRR19090862	PRJNA833969	SAMN28053241
E0857	Nanopore	SRR19090851	PRJNA833969	SAMN28053242
E0858	Nanopore	SRR19090846	PRJNA833969	SAMN28053243
E0867	Nanopore	SRR19090845	PRJNA833969	SAMN28053244
E0875	Nanopore	SRR19090844	PRJNA833969	SAMN28053245
E0885	Nanopore	SRR19090843	PRJNA833969	SAMN28053246
E0888	Nanopore	SRR19090842	PRJNA833969	SAMN28053247
E0890	Nanopore	SRR19090883	PRJNA833969	SAMN28053248
E0892	Nanopore	SRR19090882	PRJNA833969	SAMN28053249
E0896	Nanopore	SRR19090881	PRJNA833969	SAMN28053250
E0901	Nanopore	SRR19090880	PRJNA833969	SAMN28053251
E0925	Nanopore	SRR19090879	PRJNA833969	SAMN28053252
E0938	Nanopore	SRR19090878	PRJNA833969	SAMN28053253
E0940	Nanopore	SRR19090877	PRJNA833969	SAMN28053254
E0943	Nanopore	SRR19090876	PRJNA833969	SAMN28053255
E0946	Nanopore	SRR19090875	PRJNA833969	SAMN28053256
E0949	Nanopore	SRR19090872	PRJNA833969	SAMN28053257
E0949	Illumina	SRR19090874	PRJNA833969	SAMN28053257
E0973	Nanopore	SRR19090871	PRJNA833969	SAMN28053258
E0977	Nanopore	SRR19090870	PRJNA833969	SAMN28053259
E0979	Nanopore	SRR19090869	PRJNA833969	SAMN28053260
E0989	Nanopore	SRR19090867	PRJNA833969	SAMN28053261
E0989	Illumina	SRR19090868	PRJNA833969	SAMN28053261

E0994	Nanopore	SRR19090866	PRJNA833969	SAMN28053262
E1008	Nanopore	SRR19090864	PRJNA833969	SAMN28053263
E1008	Illumina	SRR19090865	PRJNA833969	SAMN28053263
E1018	Nanopore	SRR19090863	PRJNA833969	SAMN28053264
E1027	Nanopore	SRR19090860	PRJNA833969	SAMN28053265
E1027	Illumina	SRR19090861	PRJNA833969	SAMN28053265
E1033	Nanopore	SRR19090859	PRJNA833969	SAMN28053266
E1037	Nanopore	SRR19090858	PRJNA833969	SAMN28053267
E1045	Nanopore	SRR19090857	PRJNA833969	SAMN28053268
E1048	Nanopore	SRR19090856	PRJNA833969	SAMN28053269
E1049	Nanopore	SRR19090855	PRJNA833969	SAMN28053270
E1055	Nanopore	SRR19090854	PRJNA833969	SAMN28053271
E1057	Nanopore	SRR19090853	PRJNA833969	SAMN28053272
E1072	Nanopore	SRR19090852	PRJNA833969	SAMN28053273
E1073	Nanopore	SRR19090850	PRJNA833969	SAMN28053274
E1075	Nanopore	SRR19090849	PRJNA833969	SAMN28053275
E1081	Nanopore	SRR19090848	PRJNA833969	SAMN28053276
E1086	Nanopore	SRR19090847	PRJNA833969	SAMN28053277

Supplementary table S2:

List of genes detected by WGS (acquired resistance genes – ResFinder, and chromosomal point mutations – PointFinder) and chromosomal/plasmid location. This table is attached separately as an Excel file in electronic supplementary material, available at https://journals.asm.org/doi/10.1128/AEM.00742-20 as Supplementary Data Set S1.

Supplementary table S3:

Heatmap showing the distribution of virulence factors detected by WGS in each isolate. this table is attached separately as an Excel file in electronic supplementary material, available at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.936843/full#supplementary-material as Supplementary Table S4.

Appendix 2: Ethics statement

In these studies, ethical review and approval by the Ethics Committee for Animal Experimentation was not required for the animal study because sample collection was carried out by veterinary practitioners strictly following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013) as part of their routine veterinary practice. Informed consent was obtained from the farm owners at the time of sample collection. Written informed consent was obtained from the owners for the participation of their animals.

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CONTRIBUTION TO CONFERENCES

Tello, M., Oporto, B., Lavín, J. L., Ocejo, M., Hurtado, A. Poster publication. Characterization of a carbapenem-resistant *Escherichia coli* from dairy cattle harbouring *bla*$_{NDM-1}$ in an IncC plasmid. EAVLD Virtual meeting 2021, 17 November 2021

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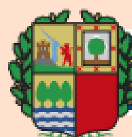

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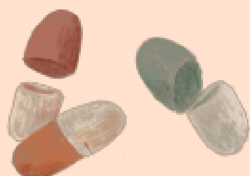

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