UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Epidemiology of resistance to aminoglycosides and macrolidesin thermotolerant Campylobacter in livestock in Spain

Epidemiología de la resistencia a aminoglucósidos y macrólidos en Campylobacter termotolerantes en animales de abasto en España

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Vicente López Chavarrías

DIRIGIDA POR

Julio Álvarez Sánchez
María Ugarte Ruiz

Madrid

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MENCIÓN INTERNACIONAL

La presente Tesis Doctoral cumple con los requisitos requeridos exigidos por la Universidad Complutense de Madrid para obtener la mención de Doctor Internacional:

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  - Centro receptor: *Centre for Animal Health and Food Safety (CAHFS), College of Veterinary Medicine, University of Minessota (UMN), United States*
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  - Duración: 5 semanas (01/01/2022 – 06/02/2022)

2. Redacción y presentación (defensa) de una parte de la tesis doctoral, en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España. Esta tesis doctoral ha sido redactada integramente en inglés, y el resumen y las conclusiones también en castellano.
3. Evaluación de la tesis por un mínimo de dos expertos doctores pertenecientes a alguna institución de educación superior o instituto de investigación no española.
4. Tribunal evaluador de la tesis compuesto por al menos un experto perteneciente a alguna institución de educación superior o centro de investigación no española, con el título de doctor, y distinto del responsable de la estancia mencionado en el punto1.

INCLUSIVE LANGUAGE

The terms that in this document refer to people and appear in masculine or feminine gender do not necessarily relate to any specific gender.

LENGUAJE INCLUSIVO

Las denominaciones que en esta memoria hacen referencia a personas y que figuren en género masculino o femenino, no se relacionan necesariamente con ningún género en particular.

A mis padres, allá donde estén

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III. GLOSSARY OF ABBREVIATIONS

AFLP – Amplified Fragment Length Polymorphism

AMEs – Aminoglycoside Modifying Enzymes

AMG – Aminoglycoside

AMG-R – Aminoglycoside resistant

AMR – Antimicrobial resistance

ARGs – Antimicrobial resistance genes

CC – Clonal Complex

CCV – *Campylobacter* Containing Vacuole

CDT - Cytolethal Distending Toxin

CFA – Campy Food Agar

cgMLST – Core genome Multi-Locus Sequence Typing

DNA – Deoxyribonucleic acid

ECDC – European Centre for Disease Prevention and Control

EFSA – European Food Safety Authority

ERY-R – Erythromycin resistant

G+C – Guanine and Cytosine

GEN-R – Gentamicin resistant

GWAS – Genome Wide Association Studies

HCPC – Hierarchical Clustering of Principal Components

HGT – Horizontal Gene Transfer

LOS – Lipooligosaccharide

LPS – Lipopolysaccharide

MAC – Macrolide

MAC-R – Macrolide resistant

MALDI-TOF – Matrix-Assisted Laser Desorption Ionization - Time-of-Flight

MCA – Multiple Correspondence Analysis

mCCDA – modified Charcoal Cefoperazone Deoxycholate Agar

MDR – Multidrug resistance

MGEs – Mobile Genetic Elements

MLST – Multi-locus Sequence Typing

NGS – Next Generation Sequencing

PCR – Polymerase Chain Reaction

PDR – Pandrug resistance

PFGE – Pulsed Field Gel Electrophoresis

qPCR – Quantitative PCR

RAPD – Random Amplification of Polymorphic DNA

REA/RFLP – Restriction Endonuclease Analysis/ Restriction Fragment Length Polymorphism

RNA – Ribonucleotide acid

RTE – Ready-to-eat Foods

ST – Sequence Type

STR-R – Streptomycin resistant

SVR – Short Variable Region

T3SS – Type III Secretion System

VBNC – Viable But Non-Culturable

wgMLST – Whole genome Multi-Locus Sequence Typing

WGS – Whole Genome Sequencing

WHO – World Health Organization

IV. SUMMARY

Antimicrobial resistance is a major problem for the treatment of diseases caused by zoonotic bacteria such as thermotolerant *Campylobacter* (*C. coli* and *C. jejuni*). The mechanisms by which antimicrobial resistance spreads over bacterial populations, especially via horizontal gene transfer, can have broad consequences depending on the speed at which resistant phenotypes disseminate through food animal hosts, humans and the environment. Particularly relevant are genetic determinants capable of conferring resistance to three or more antimicrobial classes, making treatments more complicated when multi-drug resistant bacteria are involved.

In the case of *Campylobacter* infections, aminoglycosides and macrolides are the two antimicrobial classes most commonly used to treat clinical cases, when this is necessary. This thesis is focused on the characterization of the distribution and genetic basis of co-resistance mechanisms involving aminoglycosides and macrolides in *Campylobacter* that could explain possible ways of acquisition, maintenance and dissemination of these resistance phenotypes.

The national program for antimicrobial resistance monitoring in livestock in Spain served as the framework to conduct this research. Starting from all available data on antimicrobial resistance in thermotolerant *Campylobacter* from livestock, a 'top to bottom' approach of analysis was carried out. Isolates were grouped by phenotype, to assess the importance of those over time and per hosts, and then the genetic elements associated with a particular phenotype were identified using molecular methods. The use of phenotyping methods for microbial characterization is widespread and well developed, but the extent to which these techniques allow making inferences on the specific genetic mechanisms behind the antimicrobial resistance phenotypes observed is limited.

The material and methodology used were suited to achieve three Specific Objectives. The first objective was to evaluate the baseline level of phenotypic resistance to several antimicrobials, mainly aminoglycosides (represented by gentamicin and streptomycin) and macrolides (represented by erythromycin) in *Campylobacter* isolates retrieved from broilers, turkeys, pigs and cattle between 2002 and 2018, considering also their spatio-temporal distribution. The second objective was to assess the association between the

occurrence of phenotypic resistance to both antimicrobial classes. A third objective was to elucidate the genetic mechanisms implicated in the predominant resistance phenotypes observed, with the aim of identifying resistance determinants and mobile genetic elements that could favour their dispersion.

The database analyzed included information on the year, host, sample analyzed, results of the culture procedures and antimicrobial sensitivity testing for all samples analyzed in the context of the antimicrobial resistance surveillance programmes for *Campylobacter* in food animals during 2002-2018.

In Specific Objective 1, information on approximately 51,000 samples and approximately 11,000 *Campylobacter* isolates retrieved from those samples was analyzed across host and bacterial species. As expected, higher resistance levels were observed for *C. coli* compared with *C. jejuni*, and while very high levels of resistance to tetracycline, ciprofloxacin and nalidixic acid were observed regardless of host and bacterial species, a wider variability was observed between host species with regards to gentamicin, streptomycin and erythromycin.

When considering Specific Objective 2, a strong association between the occurrence of simultaneous resistance to aminoglycosides and erythromycin was observed for all hosts in both *C. coli* and *C. jejuni*. The analysis of relative synonymous codon usage in the sequence of the *flaA* gene, in a subset of 176 isolates selected based on their antimicrobial resistance profile, revealed the existence of clusters of isolates presenting co-resistant phenotypes, particularly for *C. coli*, what could be indicative of the presence of resistant lineages harboring specific resistance mechanisms.

In Specific Objective 3, 194 isolates selected to include representatives from both bacterial species, the four hosts, and a balance between co-resistant, single resistant or susceptible isolates with regards to their phenotype to aminoglycosides and macrolides was subjected to whole genome sequencing to characterize their resistance determinants and the presence of mobile genetic elements potentially associated with these. Overall, resistance to aminoglycosides was attributed to the presence of between one and six out of a total of 10 resistance genes identified. In contrast, resistance to macrolides was mostly due to the presence of A2075G point mutations in the *23S* rRNA gene of *Campylobacter*, with only four isolates carrying the *erm(B)* gene. Antimicrobial resistance genes were often linked to transposons, plasmids and insertion sequences such as *IS1595(ISCo2)*. A higher number of resistance determinants were found among

C. coli resistant isolates compared to *C. jejuni*, especially in the ones retrieved from pigs. This animal species was also the hosts in which more isolates harboring antimicrobial resistance genes on plasmids were found. A multiple correspondence analysis revealed certain host-specific and resistance-specific genetic patterns.

According to the results of this thesis, the occurrence of co-resistance phenotypes in thermotolerant *Campylobacter* is most commonly due to the simultaneous carriage of antimicrobial resistance genes conferring resistance to aminoglycosides and point mutations for macrolides, which would be transmitted following different dynamics. The phenotypic analyses of antimicrobial resistance data from thermotolerant *Campylobacter* alone would be insufficient to identify differences in the nature and distribution of the resistance mechanisms involved, often linked with specific bacteria and animal hosts.

V. *RESUMEN*

La resistencia a los antimicrobianos es un problema importante para el tratamiento de enfermedades causadas por bacterias zoonóticas como *Campylobacter* termotolerantes (*C. coli* y *C. jejuni*). Los mecanismos mediante los cuales la resistencia a los antimicrobianos se propaga en las poblaciones bacterianas, especialmente a través de transferencia horizontal de genes, pueden tener graves consecuencias dependiendo de la velocidad con que los fenotipos resistentes se diseminan entre animales de producción, los seres humanos y el medio ambiente. Son particularmente relevantes los determinantes genéticos capaces de conferir resistencia a tres o más clases de antimicrobianos, lo que complica los tratamientos en casos donde están involucradas bacterias multirresistentes.

Los aminoglucósidos y macrólidos son las dos clases de antimicrobianos más comúnmente utilizadas para tratar las infecciones por *Campylobacter* en caso de necesidad. Esta tesis se centra en la caracterización de la distribución y las bases genéticas de los mecanismos de co-resistencia que involucran aminoglucósidos y macrólidos en *Campylobacter*, los cuales podrían explicar posibles vías de adquisición, mantenimiento y diseminación de estos fenotipos de resistencia.

El programa nacional de vigilancia de resistencia a antimicrobianos en animales de abasto en España sirvió como marco para llevar a cabo estas investigaciones. A partir de todos los datos disponibles sobre resistencia a antimicrobianos en *Campylobacter* termotolerantes en estos animales, el trabajo se enfocó 'desde arriba hacia abajo', es decir, los aislados se agruparon inicialmente por fenotipo, para evaluar la importancia de estos a lo largo del tiempo, y según los diferentes hospedadores analizados para, posteriormente, identificar los elementos genéticos asociados a un fenotipo particular utilizando métodos moleculares. El uso de métodos de fenotipado para caracterización microbiana está ampliamente extendido y bien desarrollado, pero la capacidad que estas técnicas tienen para inferir mecanismos genéticos específicos asociados a los fenotipos de resistencia observados es limitada.

El material y la metodología utilizados sirvió para lograr tres Objetivos Específicos. El primer objetivo consistió en evaluar el nivel basal de resistencia fenotípica a varios antimicrobianos, principalmente aminoglucósidos (gentamicina y estreptomicina) y macrólidos (eritromicina) en aislados de *Campylobacter* obtenidos de *broilers*, pavos, cerdos y vacas entre 2002 y 2018, considerando también su distribución espacio-

temporal. El segundo objetivo consistió en evaluar la asociación entre la ocurrencia simultánea de resistencia fenotípica a ambas clases de antimicrobianos. Y el tercer objetivo pretendió identificar los mecanismos genéticos implicados en los fenotipos predominantes de resistencias observados, con el propósito de identificar determinantes de resistencia y elementos genéticos móviles que pudieran favorecer su dispersión.

La base de datos analizada incluyó información sobre año de recogida, hospedador, muestra analizada, resultado del cultivo microbiológico y perfil de resistencia a diferentes antimicrobianos para todas las muestras analizadas en el contexto del programa de vigilancia de resistencia a los antimicrobianos en *Campylobacter* en animales destinados al consumo entre 2002 y 2018.

En el Objetivo Específico 1 se analizó la información procedente de aproximadamente 51.000 muestras de las que se habían obtenido unos 11.000 aislados de *Campylobacter*, distribuidos según hospedador y especie bacteriana. Como era de esperar, se observaron mayores niveles de resistencia en *C. coli* en comparación con *C. jejuni*, y aunque se observaron niveles muy altos de resistencia a tetraciclina, ciprofloxacino y ácido nalidíxico, independientemente del hospedador y la especie bacteriana, se detectó una mayor variabilidad entre las especies hospedadoras analizadas en lo que respecta a gentamicina, estreptomicina y eritromicina.

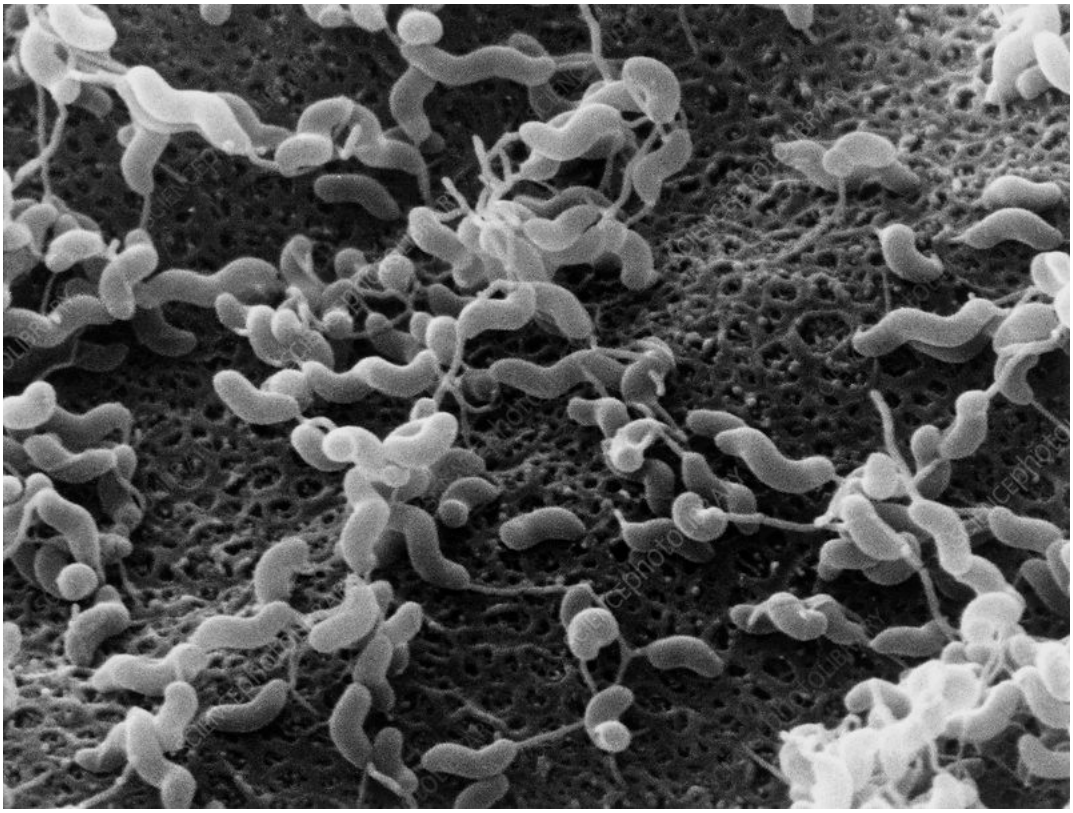
Al considerar el Objetivo Específico 2 se observó una fuerte asociación entre la ocurrencia de resistencia simultánea a aminoglucósidos y eritromicina en todos los hospedadores, tanto en *C. coli* como en *C. jejuni*. El análisis del uso relativo de codones sinónimos en la secuencia del gen *flaA*, en un subconjunto de 176 aislados seleccionados según su perfil de resistencia antimicrobiana, reveló la existencia de grupos de aislados con fenotipos co-resistentes, particularmente en *C. coli*, lo que podría ser indicativo de la presencia de linajes resistentes que albergarían mecanismos de resistencia específicos.

En el Objetivo Específico 3, 194 aislados fueron seleccionados para incluir representantes de ambas especies bacterianas, de los cuatro hospedadores, y manteniendo un equilibrio entre aislados co-resistentes, resistentes únicos o susceptibles a las clases de aminoglucósidos y macrólidos. Esta selección de aislados fue secuenciada para caracterizar sus determinantes de resistencia y la presencia de elementos genéticos móviles potencialmente asociados con estos.

En general, la resistencia a aminoglucósidos se atribuyó a la presencia de entre uno y seis genes de resistencia de un total de 10 genes identificados. En cambio, los determinantes de resistencia a macrólidos se debieron principalmente a la presencia de mutaciones puntuales (A2075G en el gen *23S* rRNA de *Campylobacter*), mientras que solo cuatro aislados presentaban el gen *erm(B)*. Los genes de resistencia a menudo estaban vinculados a transposones, plásmidos y secuencias de inserción, como por ejemplo la *IS1595 (ISCo2)*. Además, se encontró un mayor número de determinantes de resistencia en aislados resistentes de *C. coli* en comparación con *C. jejuni*, especialmente en los que se obtuvieron a partir de muestras de cerdos. Esta especie hospedadora fue también aquella en la que se encontraron más aislados con genes de resistencia a antimicrobianos en plásmidos. Por último, el análisis de correspondencia múltiple reveló ciertos patrones genéticos específicos de hospedador y resistencia.

Según los resultados de esta tesis, la aparición de fenotipos de co-resistencia en *Campylobacter* termotolerantes se debe principalmente a la presencia simultánea de genes de resistencia a aminoglucósidos y mutaciones puntuales que conferían resistencia a macrólidos, mecanismos sujetos a distintas dinámicas de transmisión. Los análisis fenotípicos de los datos de resistencia a antimicrobianos en *Campylobacter* termotolerantes por sí solos serían insuficientes para identificar diferencias en la naturaleza y distribución de los mecanismos de resistencia involucrados, que a menudo están vinculados con bacterias y hospedadores animales específicos.

INTRODUCTION



"There are many things in nature that are too small to be seen, yet they govern our lives."

Antoine van Leeuwenhoek

Merchant, often referred to as the "father of microbiology"

1. INTRODUCTION

1.1. *Campylobacter* spp. historical background

The first ever recorded account of bacteria belonging to what we know today as genus *Campylobacter* was made in 1886 by Theodor Escherich after observing spiral-shaped bacteria obtained from faecal samples of colons from children affected by what was then known as 'cholera infantum' or 'summer complaint' [1]. In his study, despite observing spiral bacteria in 35 of 72 children samples, Escherich did not suspect that the spiral agent was directly responsible for the enteric syndrome the children were suffering from.

In 1909, McFadyean and Stockman studied some vibrio-shaped bacteria collected from samples of aborted foetuses in ewes. Similar findings were observed in 1918 by Smith in abortions of bovines in the USA. He realized that his 'vibrio' was probably the same as those described by McFadyean & Stockman, and he confirmed it, along with Taylor, proposing the name *Vibrio foetus* (*V. foetus*) [2]. Additional authors further differentiated *V. foetus* analysing its biochemical and pathogenic factors, defining the sub-species *V. foetus venerealis* and *V. foetus intestinalis*. In 1931, Jones *et al*. described the species *V. jejuni* as the cause of 'winter dysentery' in calves and in 1944 a similar description was given by Doyle in pigs [3].

For decades, *Vibrio* was identified just in animals. The very first recorded report of cases in humans dates back to 1938 in Illinois, USA, when a milk-borne diarrhoea outbreak affected 355 people in state institutions. The broth culture of blood from 13 victims of the outbreak revealed growth of *V. jejuni*. In 1947, Vinzent *et al*. isolated *V. foetus* from the blood of three pregnant women with fever symptoms. Two of the three women aborted and large necrotic and inflammatory areas were found in their placenta. In 1957, King isolated similar organisms to those of Vinzent from blood samples but with markedly biochemical and antigenic variations and an optimal growth of 42°C, of patients with gastrointestinal disease. Therefore, despite the morphological similarity between the organisms described by Vinzent and King, in 1963 Sebald and Véron proposed the name "Campylobacter" (etymologically from Greek: *kampulos*, curved, and *bakteria*,

batton/cane) for the microorganisms described by King, which were recognized as the putative cause of gastrointestinal disease [2].

Some of the differences observed among the various agents identified during these decades were due to a lack of standardized faecal culture methods [2]. A relevant event in the history of *Campylobacter* occurred in 1968 when the agent was isolated for the first time from faeces of humans by Dekeyser, at the National Institute for Veterinary Research, and Butzler, at the St. Peter University Hospital, both institutions in Brussels, Belgium. In their work, published in 1972, these authors recovered what they described as a 'vibrio related' bacteria from a stool specimen of a 20-year old female using a special filter that allowed the bacteria to pass through and was further cultured using a selective medium. Since this agent was the only one isolated from this patient, the diarrhoea was deemed to be solely caused by it (later on identified as *Campylobacter jejuni*).

A year later, the same authors proved the association between the presence of the bacteria and clinical disease in an epidemiologic study, in which *Campylobacter* were isolated from 5.3% of 3,800 patients with diarrhoea, but only from 1.6% of 7,200 healthy individuals. In 1974 Butzler demonstrated the invasive ability of *C. jejuni* and proved the close relationship, or even identity, of *Campylobacter* from humans, poultry, sheep and pigs, in a truly one-health context [4].

The development of filtration techniques and selective media for *Campylobacter* made the faecal isolation of this agent an easier task in microbiology laboratories compared to previous detection and identification methods. Moreover, serotyping techniques were later described by several authors [5, 6] and these, along with biotyping, phage-typing and genotyping, are still common techniques for *Campylobacter* strain typing nowadays [1, 4].

1.2. Thermotolerant bacteria of the genus *Campylobacter*: *Campylobacter coli* and *Campylobacter jejuni*

1.2.1. *Campylobacter* spp. taxonomy

The genus *Campylobacter* belongs to filum *Proteobacteria*, class *Epsilonproteobacteria*, order *Campylobacterales* and family *Campylobacteraceae*.

Along with *Campylobacter*, two other genera are included in this family: *Arcobacter* and *Sulfurospirillum*. Bacteria in the genus *Campylobacter* and *Arcobacter* are recognized human and animal pathogens unlike those in genus *Sulfurospirillum* [7].

Véron and Chatelain published the first comprehensive taxonomy of *Vibrio*-like organisms (*Campylobacter* spp.) in 1963 [8]. They initially proposed four distinctive species: *C. fetus* (the prototype species), *C. coli* (isolated from pigs), *C. jejuni* (isolated from cattle, human and sheep) and *C. sputorum* (retrieved from humans, cattle, pigs and sheep) [9]. The improvement of isolation techniques in the 1980's saw the incorporation and re-classification of many species within the genus *Campylobacter*, including, but not limited to, *C. concisus*, catalase-negative *C. mucosalis* (demonstrated by DNA homology studies), *C. lari* and *C. hyointestinalis*. *Wolinella recta* and *Wolinella curva* were re-classified as *C. rectus* and *C. curvus*, respectively, and *Bacteroides corrodens* was renamed *Bacteroides ureolyticus* and finally became *C. ureolyticus* [10]. Furthermore, *C. pyloridis*, *C. mustelae*, *C. cinaedi* and *C. fennelliae* were later moved to the genus *Helicobacter*, while *C. pyloridis* and *C. mustelae* became *Helicobacter pylori* and *Helicobacter mustelae*, respectively. *Campylobacter nitrofigilis* and *C. cryaerophilus* were later moved to the genus *Arcobacter* [8, 11] (Bacterio.net website: www.bacterio.net, last accessed September 2024).

The first RNA-based phylogenetic study of *Campylobacter* was published by Thompson *et al.* [12] after comparing partial 16S rRNA sequences and grouping them into three separate ribosomal RNA sequence homology groups: 'Homology Group I' (including the prototype species *C. fetus*, *C. coli*, *C. jejuni*, *C. lari*, *C. hyointestinalis*, *C. concisus*, *C. mucosalis*, *C. sputorum* and *C. upsaliensis*), 'Homology Group II' (including *C. cinaedi*, *C. fennelliae*, *C. pylori* and *W. succinogenes*) and 'Homology Group III' (including *C. cryaerophila* and *C. nitrofigilis*). A few years later, Vandamme and De Ley [11] performed a series of immunotyping analyses that led to grouping all *Campylobacter* and related taxa in the same taxonomic group, which they called 'rRNA superfamily VI'. This group currently encompasses the Class 'Epsilon Proteobacteria' [8, 13] (Integrated Taxonomic Information System (ITIS) website: www.gbif.org, last accessed October 2024).

As of November 2024, the genus *Campylobacter* consists of 57 confirmed taxa (44 species and 13 subspecies). These can be subdivided into different groups according to their phylogenetic relations (Figure 1 and Table 1).

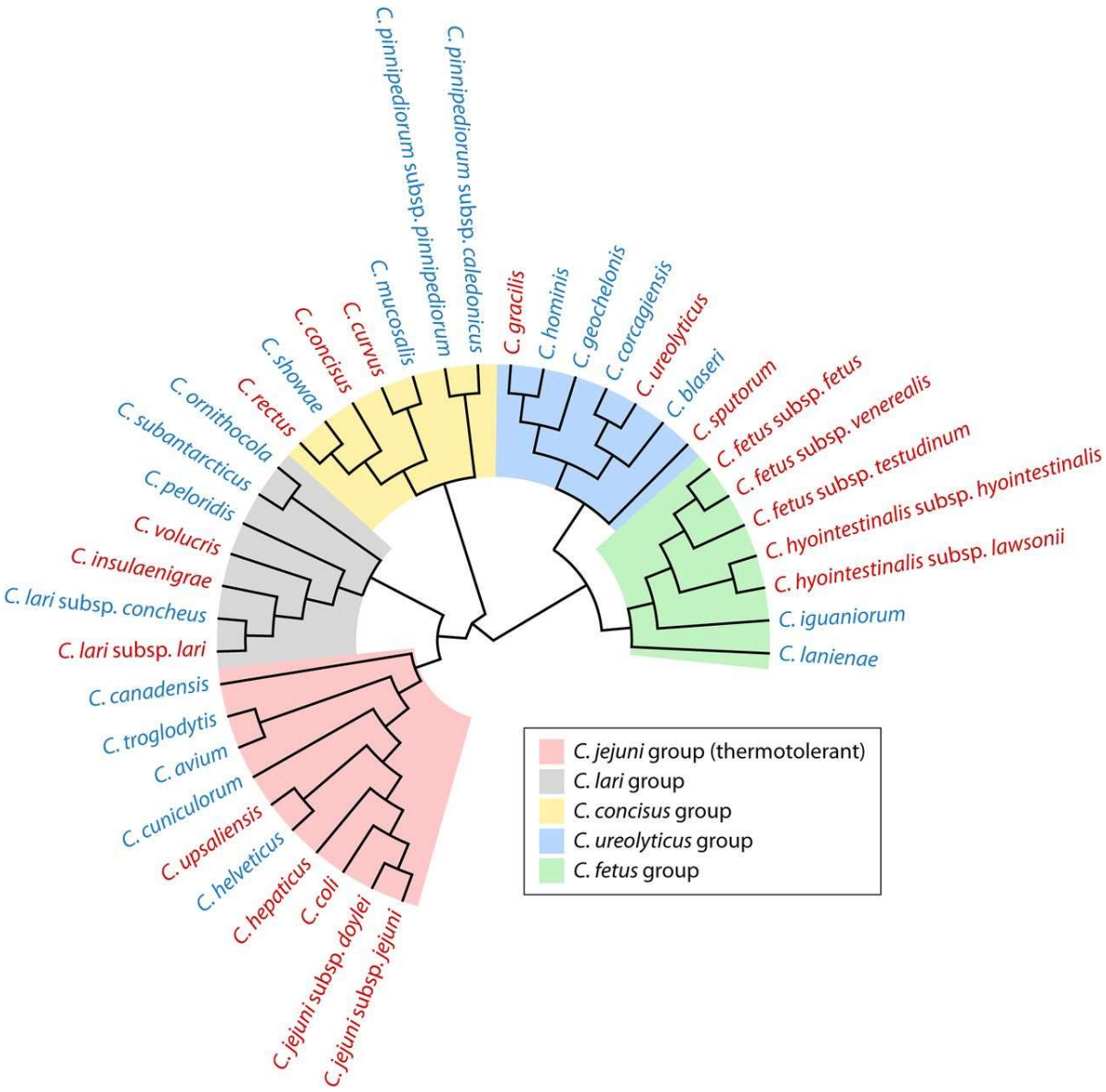


Figure 1. Phylogenetic relationships between described *Campylobacter* species as of 2018 (species in red cause infection/disease in animals/humans) (source: Costa & Iraola, 2019).

All species and subspecies within the genus *Campylobacter* spp., their hosts and chronologic references of their first isolation and further re-classifications up to November 2024 are listed on Table 1.

Table 1. List of *Campylobacter* spp. described in chronological order of first taxonomic identification up to November 2024 (extended from: Moore & Matsuda, 2002; Klein, 2017).

Taxa	Hosts	References
C. fetus subsp. *fetus*	Cattle Sheep	McFadyean & Stockman (1913); Smith (1918); Smith and Taylor (1919); Sebald and Véron (1963)
C. jejuni subsp. *jejuni*	Poultry Cattle Humans	Jones *et al.* (1931); Véron and Chatelain (1973)
C. sputorum, including: - *C. sputorum* subsp. *bubulus* - *C. sputorum* subsp. *sputorum*	Humans Cattle Pigs Sheep	Prévot (1940); Florent (1953); Véron and Chatelain (1973); Costas (1987); On *et al.* (1998, 2001)
C. coli	Pigs	Doyle (1948); Véron and Chatelain (1973)
C. ureolyticus	Humans	Eiken (1958); Jackson & Goodman (1978); Vandamme *et al.* (2010)
C. fetus subsp. *venerealis*	Cattle Sheep	Florent (1959); Véron and Chatelain (1973)
C. concisus	Humans	Tanner *et al.* (1981)
C. mucosalis	Pigs	Lawson *et al.* (1981); Roop *et al.* (1985); Costas (1987)
C. rectus	Humans	Tanner *et al.* (1981); Vandamme *et al.* (1991)
C. gracilis	Humans	Tanner *et al.* (1981); Vandamme *et al.* (1995)
C. lari subsp. lari	Seagulls Dogs Shellfish	Benjamin *et al.* (1983); Debruyne *et al.* (2009)
C. curvus	Humans	Tanner *et al.* (1984); Vandamme *et al.* (1991)

Table 1 (continued). List of *Campylobacter* spp. described in chronological order of first taxonomic identification up to November 2024 (extended from: Moore & Matsuda, 2002; Klein, 2017).

Taxa	Hosts	References
C. hyointestinalis subsp. *hyointestinalis*	Pigs, cattle, humans	Gebhart *et al.* (1985); On *et al.* (1995)
C. jejuni subsp. *doylei*	Humans	Steele and Owen (1988)
C. upsaliensis	Cats, dogs, monkeys	Sandstedt and Ursing (1991)
C. helveticus	Cats, dogs	Stanley *et al.* (1992)
C. showae	Humans	Etoh *et al.* (1993, 1998)
C. hyointestinalis subsp. *lawsonii*	Pigs	On *et al.* (1995)
C. lanienae	Pigs	Logan *et al.* (2000)
C. hominis	Humans	Lawson *et al.* (2001)
C. insulaenigrae	Seals, porpoises	Foster *et al.* (2004)
C. canadensis	Wild birds	Inglis *et al.* (2007)
C. lari subsp. *concheus*	Humans, shellfish	Debruyne *et al.* (2009)
C. cuniculorum	Rabbits	Zanoni *et al.* (2009)
C. peloridis	Humans, molluscs	Debruyne *et al.* (2009)
C. avium	Poultry	Rossi *et al.* (2009)
C. volucris	Black-headed gulls	Debruyne *et al.* (2010a)
C. subantarcticus	Wild birds	Debruyne *et al.* (2010b)
C. troglodytes	Chimpanzees	Kaur *et al.* (2011)
C. corcagiensis	Captive lion-tailed macaques	Koziel *et al.* (2014)
C. fetus subsp. *testudinum*	Reptiles, humans	Fitzgerald *et al.* (2014)
C. iguaniorum	Lizards, chelonians	Gilbert *et al.* (2015)
C. geochelonis	Tortoises	Piccirillo *et al.* (2016)
C. hepaticus	Chickens	Van *et al.* (2016); Gregory et al. (2018)
C. ornithocola	Wild birds	Cáceres *et al.* (2017)
C. pinnipediorum subsp. *pinnipediorum*	Otariid seals	Gilbert *et al.* (2017)

Table 1 (continued). List of *Campylobacter* spp. described in chronological order of first taxonomic identification up to November 2024 (extended from: Moore & Matsuda, 2002; Klein, 2017).

Taxa	Hosts	References
C. pinnipediorum subsp. *caledonicus*	Phocid seals	Gilbert *et al.* (2017)
C. blaseri	Common seals	Gilbert *et al.* (2018)
C. armoricus	Humans	Boukerb *et al.* (2019)
C. novaezeelandiae	Birds	Bloomfield *et al.* (2020)
C. aviculae	Zebra finches	Bryant *et al.* (2020)
C. estrildidarum	Zebra finches	Bryant *et al.* (2020)
C. taeniopygiae	Zebra finches	Bryant *et al.* (2020)
C. portucalensis	Bulls	Silva *et al.* (2020)
C. massiliensis	Humans	Antezack *et al.* (2021)
C. vulpis	Wild red foxes	Parisi *et al.* (2021)
C. anatolicus	Anatolian Ground Squirrel	Aydin *et al.* (2021)
C. bilis	Chickens	Phung *et al.* (2022)
C. majalis	Pigs	Lynch *et al.* (2022)
C. suis	Pigs	Lynch *et al.* (2022)
C. magnus	Pigs	Gruntar *et al.* (2023)
C. californiensis	Cattle, feral swine	Miller *et al.* (2024)
C. devanensis	Small ruminants	Miller *et al.* (2024b)
C. porcelli	Swine	Miller *et al.* (2024b)
C. vicugnae	Camelids	Miller *et al.* (2024b)

1.2.2. Biology of the genus *Campylobacter* spp.

Bacteria belonging to the genus *Campylobacter* are Gram-negative, non-saccharolytic microorganisms 0.2-0.8 μm wide by 0.5-5 μm long that have microaerobic growth requirements. Their cells are shaped as curved or spiral rods, or shaped like an 'S' when they are in a logarithmic growth phase [8], albeit they can form spherical or coccoid bodies in old cultures or when exposed to suboptimal temperature, starvation, osmotic stress or high O_2 concentrations [14]. These bacteria generally display a quick corkscrew movement thanks to the presence of an unsheathed flagellum at one or both of its poles [15] (Figure 2).

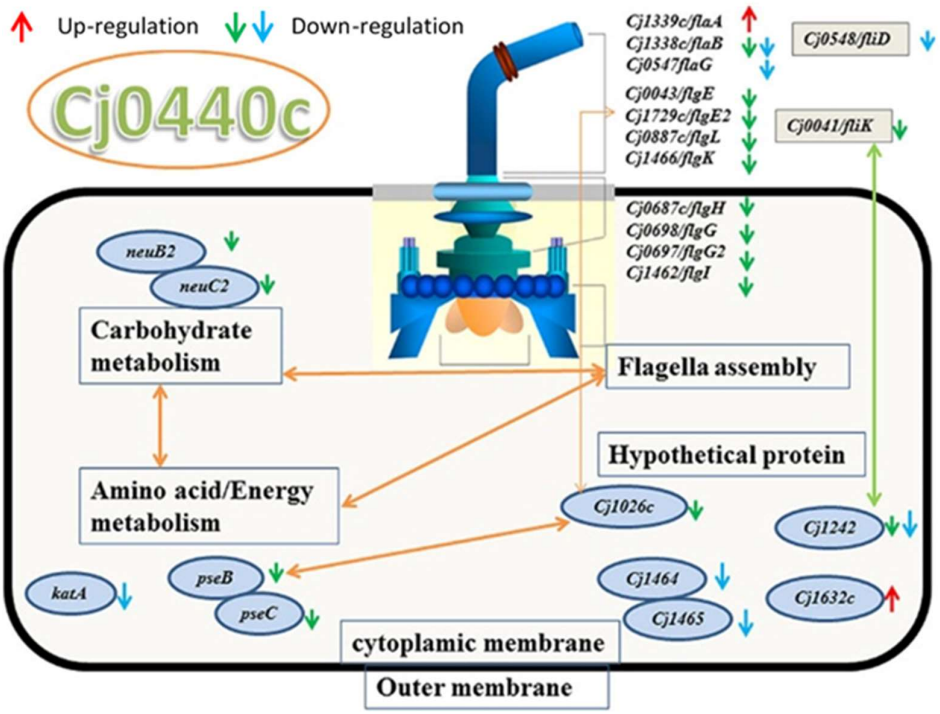


Figure 2. Location and structure of the flagellum apparatus in *Campylobacter* spp. Relationship between genes: orange double arrows=positive, green double arrows=negative (adapted from: Front. Microbiol., 25/04/2017 | 10.3389/fmicb.2017.00729).

This flagellum is not only vital for the movement of the bacteria but it is also related to pathogenicity and virulence roles, enabling the bacteria to penetrate the viscous mucin layer of the host intestine [16, 17]. However, *C. gracilis* [18], *C. hominis* [19], *C. ureolyticus* and *C. blaseri* [20] are non-motile species and strains from the species *C. showae* have

multiple flagella [7, 21] (Figure 3). The presence and number of flagella is one of the features that can aid in the identification of *Campylobacter* spp.

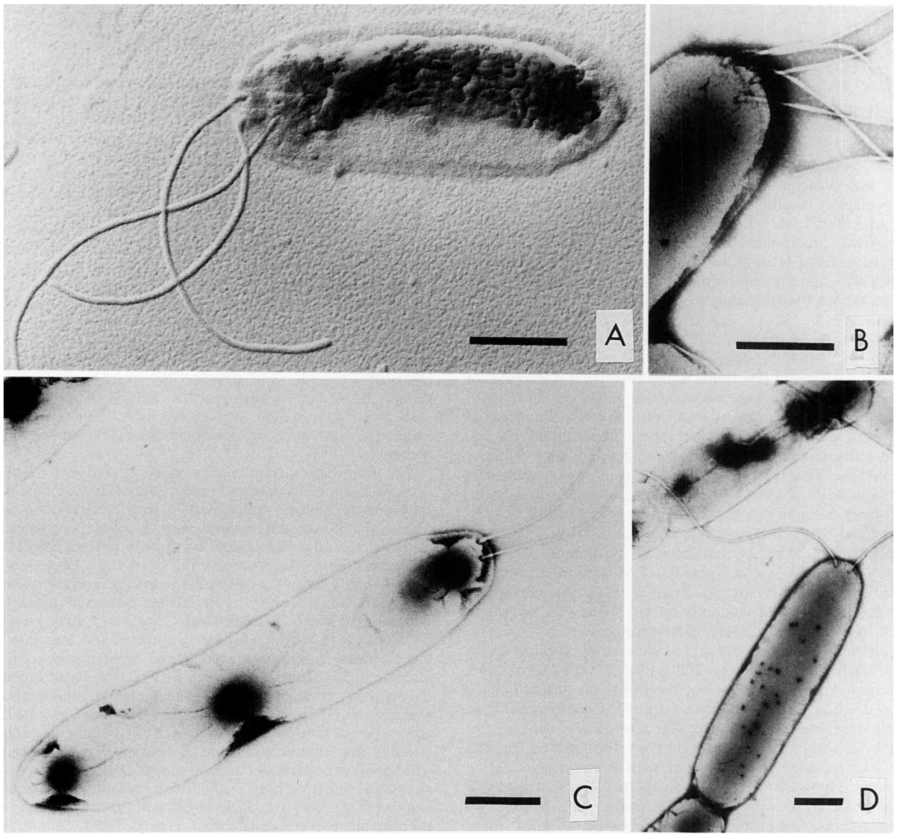


Figure 3. Transmission electron micrographs of *C. showae* showing multiple flagella. (A) Platinum-carbon shadowing. (B-D) Negatively stained preparations (source: Etoh. *et al.* 1993).

Campylobacter spp. are fastidious slow-growing microorganisms that can sometimes take up to four days to grow. These differences are reported as species-related [22], although for most species and most of the time the bulk of colonies grow in between 24 to 48 hours [23]. The two most important features with a major influence on *Campylobacter* spp. growth are a controlled atmosphere and a suitable temperature range.

Campylobacter spp. are microorganisms very sensitive to O_2 and free radicals and are considered microaerophilic bacteria, requiring a very specific gas mix in a microaerobic environment. Typically, the proportion of gas needed is 85% N_2, 3-5% O_2 and 5-10% CO_2 [24], but some *C. jejuni* strains also grow at higher O_2 levels (10%) or in aerobic conditions

even in starvation environments [25, 26]. Some strains thrive in anaerobiosis, like *C. canadensis* and *C. upsaliensis*. This explains their capacity to grow in a wide range of environments, making them one of the most ubiquitous bacterial species in the food chain [27].

There are several reasons why *Campylobacter* is unable to survive under aerobic conditions [28]. Among them, limited tolerance against reactive oxygen species (ROS), a inability to produce adequate antioxidant enzymes, low respiratory rate and presence of oxygen-labile essential enzymes [29], although some other enzymes, as a result of the expression of certain genes, are believed to play a critical role in protecting the cells from oxygen tension [30, 31]. Selective media used in the laboratory to culture *Campylobacter* generally contain neutralizers like blood, ferrous iron or charcoal (such as in mCCDA culture medium) [32].

In general, thermophilic microorganisms are able to grow at very high temperatures, sometimes higher than the boiling point of water [33], but it is more appropriate to describe thermophilic bacteria as those able to stand temperature ranges as wide as 30°C to 46°C [34]. However, the range for *Campylobacter* is narrower, growing better in microaerobic conditions at 37°C (human temperature) than at 42°C (chicken temperature), and thus 'thermotolerant' is preferred over 'thermophilic' to describe *Campylobacter*, although there are exceptions such as some *C. fetus* strains (not in the thermotolerant group) able to grow at temperatures lower than 30°C [7]. The thermotolerant group is the most important from a clinical perspective because *C. jejuni* and *C. coli*, the two species included in it, are the most frequently involved in human cases of campylobacteriosis [35]. For this reason, these are the two species on which this thesis is focused upon.

Host environment survival characteristics

Campylobacter spp. are very sensitive to external stress factors such as temperature (as already described), ultraviolet radiation, osmotic pressure and pH levels (they cannot survive NaCl concentrations over 2% or pH <4.9) [27]. There are many bacteria that compete with *Campylobacter*, hampering its isolation [36, 37], although some authors suggest this circumstance can be used with infection prevention purposes in the host [38]. In some culture protocols a sample enrichment step is necessary to maintain a trade-off between the inhibition of competitive bacteria and the specific recovery of

Campylobacter from different matrices/samples [39, 40]; however, some of the substances added to promote its culture may enhance biofilm formation as a secondary effect [41].

In spite of being a bacterium very sensitive to external factors, *Campylobacter* is able to colonize and grow in a variety of foods [37]. Sometimes it is difficult however to isolate just one colony from a *Campylobacter* spp. culture because bacteria tend to aggregate together in a 'swarming' process [42].

When *Campylobacter* is under environmental stress, and its survivability is compromised, it switches to a 'Viable But Non Culturable' (VBNC) state, in which the bacterium becomes inactive, not growing but retaining a minimum metabolic rate – and eventually its pathogenic potential – due to its ability to resuscitate under favorable conditions [43, 44]. Biofilm formation in the environment (generally along with other bacterial species) [45], the ability to adopt a VBNC state and the occurrence of interactions with other microorganisms are the main factors that contribute to its survival outside the host [8].

Biochemical characteristics

Campylobacter coli and *C. jejuni* do not obtain their energy from the metabolism of carbohydrates like other food-related pathogens such as *Salmonella* spp. or *Listeria* spp. [46]. Instead, they use amino acids or intermediate metabolites of the tricarboxylic acids cycle (acetate, aspartate, glutamate and pyruvate) as sources of energy [47]. Aspartate is classed as an amino acid promoting growth in most *C. jejuni* and *C. coli* strains because it is a precursor of the biosynthesis of lysine, methionine, threonine and isoleucine. Other amino acids promoting their growth are glutamate, proline and serine [48, 49]. Aspartate is de-aminated in *C. jejuni*, being converted to fumarate and this, in turn, onto oxaloacetate, which participates in a gluconeogenesis route to synthesise essential carbohydrates [8]. Although this route may also be common to *C. coli*, the metabolic route involving propionic acid is exclusive to *C.coli*, because *C. jejuni* lacks the genes needed to deploy it, therefore, this metabolic feature can be used to differentiate *C. coli* from *C. jejuni* [50].

The biochemical composition of *C. coli* and *C. jejuni*, in particular the surface structures (glycans, lipooligo- (LOS) and lipopoly-saccharides (LPS)) and protein glycosylation processes, explain their epidemiology, pathogenicity and virulence. Polysaccharides

play a major role in the host-bacteria interaction and are important for antigenicity. The post-translation glycosilation of proteins may be associated with immune evasion of *Campylobacter* in chickens [51], and glycosylation may play a major role in the intra-host conversion of *Campylobacter* from commensal to pathogenic [52].

Host-pathogen interaction: virulence and pathogenicity

C. jejuni has developed specific adaptations to survive and persist within intestinal host cells [17]. After invading the intestinal host cells, the bacterium survives in a specialized compartment called '*Campylobacter*-containing vacuole' (CCV) [17]. This compartment is not attacked by the host cell lysosome, but generally acts only as a temporary compartment.

These bacteria must not only evade the hosts' immune system and the harsh environmental conditions of the gastrointestinal tract, but also out-compete the native microbiota. *Campylobacter jejuni* is an efficient colonizer of both avian and mammalian hosts. In both these environments, *C. jejuni* is able to search for and metabolize the nutrients found within the mucus layer and out-compete commensal microbes [53].

The complexity of this bacterium in relation to growth and survival in different hosts is well recognized but not so well understood. The same strain can express virulence and cause disease in humans, whilst only establishing a commensal relationship with other hosts such as chickens [8]. It is argued that strains that can adapt to many host species, competing efficiently with the intestine microbiota are the ones that manage to adhere and infect intestinal epithelial cells causing clinical disease [54]. *C. jejuni*, for instance, resists well surviving inside protozoa when the environment is not favorable. This characteristic has been associated with its resistance to antimicrobials [55].

The most frequent virulence factors in *Campylobacter* are flagella and secretion systems. The polar and amphitrichous flagella of *Campylobacter* spp. are multifunctional organs which, besides motility, are crucial for pathogenesis, since they influence motility, chemotaxis, adhesion to host cells, secretion of virulence factors in the host cell, autoagglutination, microcolony and biofilm formation, and evasion of the innate immune system [16]. Chemotaxis is the controlled and directed movement of an organism, triggered by chemical stimuli [17].

The chemotactic system in *C. jejuni* is linked to the flagellum (and thus to motility), so through this system the bacteria can find a suitable ecological niche, avoiding its elimination through the normal peristaltic movement of the intestine [56].

Campylobacter secretion systems depend on a functional flagellar apparatus [57]. Type III secretion systems (T3SS) allow the specific delivery of effector proteins from the bacterial cytosol into the cytosol of the host cell. They have been described as 'molecular syringes' and are composed of a protein complex spanning both bacterial membranes [17]. The type VI secretion system (T6SS) is involved in defense against oxidative stress and adaptation to bile acids, and thus it enhances colonization of the host intestine, especially in chickens [58, 59].

Various genes, antigens, mechanisms of iron utilization and the response to oxidative and environmental stress are additional factors involved in successful colonization. The adhesion of *Campylobacter* to the host intestinal epithelium is vital for successful colonization. Several adhesion factors (adhesins) enable the host epithelial cell invasion and thus act as invasion factors. The best studied adhesin is CadF (*Campylobacter* adhesion protein to fibronectin) and the most important invasion factors are Cia (*Campylobacter* invasion antigen) and iamA (invasion-associated protein) [60, 61].

Campylobacter spp. are able to enter the basolateral side of the intestine via a phenomenon of 'transmigration', crossing the cellular barrier and the tight junctions, either by a transcellular and/or a paracellular pathway [62, 63]. The 'cytolethal distending toxin' (CDT) is the only toxin described in *Campylobacter* spp. so far. Its DNAse activity, inducing DNA damage, was first reported by Johnson and Lior in 1988. Neutralizing anti-CDT antibodies are produced in humans during infection, which indicates development of immunity during or after infection.

In contrast, a lack of neutralizing anti-CDT antibodies has been observed in chickens [64], being it another case of immune evasion by *Campylobacter*, this time in chickens, similar to the one described above in relation to the post-translation glycosilation of proteins in hosts suffering pathogenic consequences.

1.3. Identification and characterization methods for *C. coli* and *C. jejuni*

Identification and characterization methods are essential to fully describe species of microorganisms within a genus, to establish differences among species and to distinguish between strains in the frame of epidemiological studies. They can be classified into phenotypic and genotypic/molecular methods [65].

Phenotypic methods are based on visible or measurable characteristics expressed by bacteria, i.e. the gene expression. They can be based on the assessment of bacterial antigens (serotyping), biochemical features (biotypification), sensitivity to lysis by bacteriophages (phage typing) or antimicrobial resistance (antimicrobial susceptibility testing).

Genotypic/molecular methods are based on the bacterial genetic material and can identify genetic modifications such as mutations, insertions and deletions ('indels') or recombinations, providing a complementary discriminatory power [66]. They can either study individual *loci* (*flaA*, *porA* genes) and highly variable regions in a genome (Pulsed Field Gel Electrophoresis (PFGE), ribotyping, Amplified Fragment Length Polymosphism (AFLP), Random Amplification of Polymorphic DNA (RAPD)) at a specific point in time, or they can focus on small variations accumulated in genomes over a long time (Multi-Locus Sequence Typing (MLST), Whole Genome Sequencing (WGS)) [67-70].

The phenotypic and genotypic/molecular methods used in the identification and characterization of thermotolerant *C. coli* and *C. jejuni* are briefly described below:

1.3.1. Phenotypic identification and characterization methods

Serotyping

This method is based on the use of specific antibodies to identify bacterial surface antigens [71, 72]. They were developed for *Campylobacter* in Canada in the 1980s, using either heat-stable (capsule and LPS) [5] or heat-labile (flagellar proteins) [6] antigens. The 'Penner' method yields a higher percentage of typable strains than the 'Lior' method, although the combination of both schemes affords a greater degree of discrimination

than either system alone. These techniques have been superseded by more advanced molecular typing methods [65].

Phage typing

Bacteriophage viruses (phages) are viruses that parasite bacteria, first discovered in 1915-1917 [73]. There are two types: lysogenic cycle phages (pro-phage viruses whose genome is integrated in the bacterial genome) and lytic cycle phages (that destroy the bacterium after infection). Lysogenic *Campylobacter* phages are common in *C. jejuni* from chickens [74]. Lytic *Campylobacter* phages from *C. coli* and *C. jejuni* have been divided into three groups according to genome size and morphology [75]. Group I contains phages with large genomes (320–425 kb) [76], whereas Groups II (175–183 kb) and III (131–135 kb) have a low mean number of phages liberated per infected bacterium [77-79] and a very low G+C content (26-27 mol%) [80]. The scheme designed by Frost *et al*. in the UK in 1999 defined a 'shared phage type' as two or more epidemiologically unrelated isolates giving the same phage reaction pattern.

Matrix-Assisted Laser Desorption Ionization (MALDI) Time-Of-Flight (TOF) Mass Spectrometry (MS) (MALDI-TOF-MS)

MS is based in the principle of mass analysis, whereby an electron is taken from molecules to create single charged ions. It uses the ionization by fast atom bombardment (MS) and the association of gas chromatography [81]. The capability to register biomarker ions in a broad mass/charge range, that are unique and representative of individual microorganisms, forms the basis of current applications of mass spectrometry in microbiology, as it was demonstrated for the first time in 1975 [82, 83]. In these first experiments, the electron impact spectra contained biomarker ions tentatively assigned to diglycerides, ubiquinones, menaquinones, and other volatile metabolites. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization were first used in 1996, and provided spectra of protein biomarkers, already including a time-of-flight (TOF) spectrometer [84]. Since then, it has been used for bacterial identification in clinical mycrobiology [85, 86], whole cells analyses [87], identification of *Campylobacter* spp. [88], viruses [89] or even identification of antimicrobial resistance mechanisms [90]. MALDI-TOF methods are currently very frequently used.

1.3.2. Genotypic/molecular identification and characterization methods

Polymerase Chain Reaction (PCR) based techniques

These techniques are based in the use of specific oligonucleotides (PCR primers) binding to a specific DNA sequence target. The conventional method involves DNA replication of the target allowing for a selective amplification of several million fold of this target from a specific *Campylobacter* isolate in just a few hours. These amplified fragments can be then visualized in an agarose gel allowing for the detection of particular genes or organisms [91]. They take advantage of the genomic differences of a particular species to differentiate it from unrelated or related species [92]. PCR-based approaches have been successful for the detection of *Campylobacter* species or their genes [93], for example, genes encoding adhesins are used in PCR assays for identification of *C. jejuni.*

Normally, a central target for differential conventional PCR assays includes the *16S rDNA* gene. Other gene targets that exploit gene-specific differences between closely related *Campylobacter* species have also been used over the years (*porA*, *flaA*, *flaB* genes) [91]. The successful and specific detection of *Campylobacter* in mixed matrices however continued to be a challenge until the development of multiplex PCRs. This is a quick and reliable method for determining the presence or absence of multiple gene targets within a single sample, allowing for the rapid identification of several *Campylobacter* species [94].

An evolution in PCR technologies for the absolute detection and quantification of pathogens and nucleic acid sequences was the use of fluorescent dyes that could be detected with specialized laser-imaging systems. These dyes accumulate with each cycle with an intensity that is directly proportional to the amount of target template DNA. This technology, referred to as 'real-time PCR' or 'quantitative PCR' or 'qPCR' [95-97] can be used directly on samples and is replacing the use of conventional PCR. Other methods such as 'digital PCR' (dPCR) have been also applied for *Campylobacter* identification [91, 98].

Restriction Endonuclease Analysis/ Restriction Fragment Length Polymorphism - Polymerase Chain Reaction (REA/RFLP-PCR) of *flaA* and *flaB* genes

The genetic *locus* that encodes the *Campylobacter* flagellar protein (flagellin) consists of two tandem genes, *flaA* and *flaB*, whose DNA is 95% homologous to each other. Both genes have a central variable region flanked by well conserved regions. The *flaA* gene encodes the flagellin protein while the *flaB* gene acts like a variation 'genetic reserve' that can recombine with the *flaA* gene to lead to its variation, thus evading the host immune response [99].

The REA/RFLP-PCR technique, originally designed for *C. jejuni* [100], is based on the use of the conserved regions to design primers to amplify *flaA* and *flaB* gene fragments with the use of PCRs. The amplified products are digested with restriction enzymes, giving as a result fragments of different sizes according to the variable regions characteristic of each *Campylobacter* strain [101]. Typically, a *flaA* gene region of 1,728 base pairs (bp) is amplified and then digested with the restriction enzymes DdeI (delivers large fragments, less discriminative) or AluI (delivers small fragments, more discriminative). The fragments are separated by size using gel electrophoresis, creating a distinctive banding pattern for each strain based on the lengths of the restriction fragments. The resulting banding patterns are visualized and analyzed, serving as a fingerprint for each strain [102-104].

There may be variations in the *flaA* gene sequence that are not detected by the restriction enzymes, but since the *flaA* gene is highly variable this technique is still used due to easy processing, although generally jointly with other genetic methods [105, 106].

Analysis of the short variable region of the *flaA* gene (*flaA*-SVR)

This method was designed using just the *flaA* gene (whose role is associated with the formation of the flagellin A protein) in an attempt to overcome the problems encountered with the REA/RFLP-PCR method. The entire coding sequence of the *flaA* gene (1,764 nucleotides) of *C. coli* and *C. jejuni* contains two regions of high variability, one region from approximately base positions 700 to 1,450 and a 'short variable region' (SVR) from base positions 450 to 600, flanked by conserved regions [107, 108] (Figure 4).

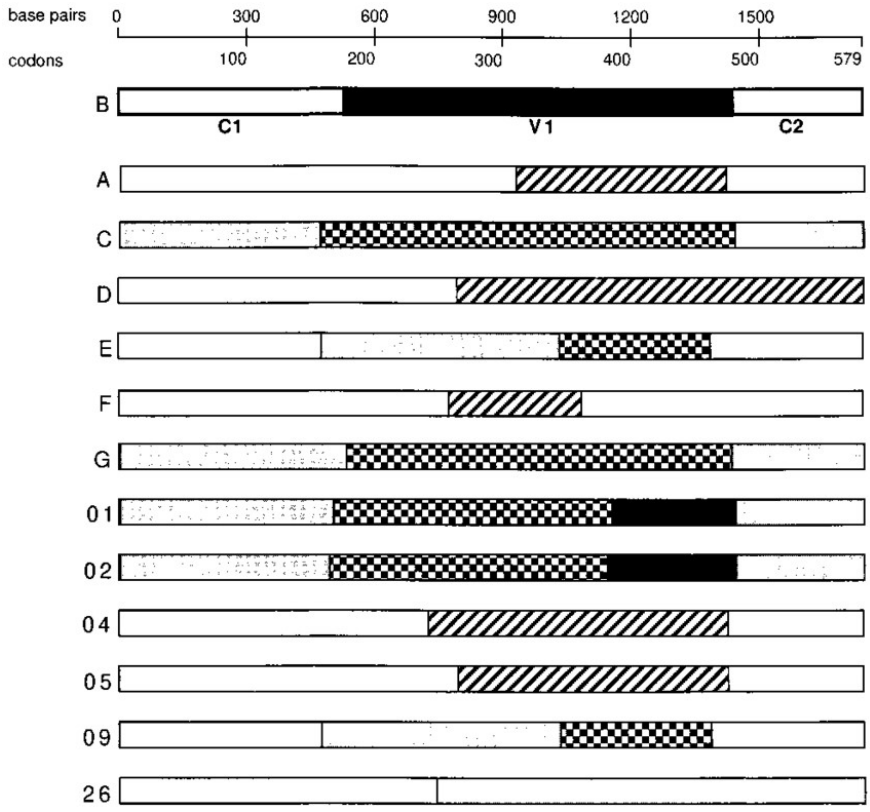


Figure 4. Genomic regions within the *flaA* gene of *Campylobacter* spp. showing the variable central region (source: www.researchgate.com).

These conserved regions are used as targets of primers for the amplification and sequencing of the target SVR sequences, which are included in a fragment of 321 bp, near the 5' end of the DNA strand [109, 110]. There is a free online repository of *flaA* allele sequences where new ones can be added and new numbers assigned (https://pubmlst.org/organisms/campylobacter-jejunicoli).

The sequence diversity in the variable region is useful for distinguishing between strains of bacteria and frequently used in molecular epidemiology and phylogenetic studies to track bacterial lineages, outbreaks and transmission patterns.

As with the REA/RFLP-PCR method, the *flaA* gene intrinsic variability precludes the use of the *flaA*-SVR method alone to investigate, for instance, common sources of infection. Some authors argue that it offers similar or higher discriminatory power than MLST and PFGE [111, 112].

Pulsed Field Gel Electrophoresis (PFGE)

This technique was developed in 1984 [113] to separate very large DNA fragments (of over 10 million bp) in common agarose gels [114]. This is possible thanks to the alternation of an electric field in more than one direction through a solid matrix to achieve the separation of the fragments. The method requires the preparation of untrimmed DNA, adding the bacteria to the agarose matrix, and the digestion of the DNA using restriction endonucleases, generating a reduced number of large size fragments. After that, the mix is loaded and the DNA fragments are separated in the agarose matrix according to their size, a process that can take up to 24 hours to complete. The banding patterns (pulse types) can be visualized and interpreted in a similar way to what is done in a normal electrophoresis. This technique randomly screens the bacterial whole genome, so the banding patterns obtained are specific to the microorganism under study [115].

In 1995, Tenover suggested categories of genetic/epidemiologic relatedness of strains subjected to PFGE based on the degree of differences between profiles [116]. For two strains to be considered epidemiologically related a minimum of 10 bands per pulse type were needed. In this way, strains of very different origin or not genetically related could not be compared, because the patterns obtained would be very different, though this ignores the fact that two very different strains could give very similar patterns only by chance.

The categories were based on the idea that a single genetic event in the genome could cause a difference of three bands in the pulse type, with four types of genetic events: point mutation with creation of a restriction site, point mutation with loss of a restriction site, and insertion or deletion of DNA into an existing restriction fragment resulting in a change in band sizes. Therefore, according to the number of different bands obtained, four categories of genetic and epidemiologic relatedness could be established: indistinguishable, closely related, possibly related and unrelated [116].

Some authors argued that the relatedness criterion was not always possible in the case of food-borne outbreaks, suggesting additional considerations with the aim of optimally interpreting the results such as the quality of the banding image, the intrinsic variability of some bacteria (for example, certain *Salmonella* spp. serotypes) and the lack of epidemiological data in some cases [42]. PFGE was used for *Campylobacter* spp. from the 1990s [117] and the protocol was refined for *C. jejuni* in 2001 [118].

Multi-Locus Sequence Typing (MLST)

MLST is a technique based on Multi-Locus Enzyme Electrophoresis (MLEE), which was one of the first methods used for genetic and epidemiological studies. MLEE is a non-DNA method that analyses the electrophoretic motility of different metabolic enzymes, assigning different alleles according to their motility profile. This technique rendered spurious results because different enzymes could have the same motility profile, thus MLST was designed to avoid these problems via replacing motility by the sequences of genes codifying for the enzymes [67].

MLST was developed for *C. jejuni* in 2001 and the genes (*loci*) to be sequenced for typing purposes were selected according to several criteria: they should be located far away from each other in the chromosome (minimum distance 70 kb), they should be amenable to design specific primers, their sequence should be variable enough, there should be absence of positive selection for each locus and it should be possible to find them in strains of different origins [68].

The gene selection for the MLST scheme must be adapted to each bacterial species; for example, the genes selected for *C. jejuni* were *aspA* (aspartase A), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *uncA* (ATP synthase a subunit). Each MLST profile is based on a specific seven *loci* combination of sequences that receives an allele number according to the PubMLST database (http://pubmlst.org/campylobacter). Each seven digit code containing the allele sequence of the seven *loci* is called 'Sequence Type' (ST). *Campylobacter jejuni* STs can be grouped into 'Clonal Complexes' (CCs), which can be defined as groups of two or more strains that share identical alleles in four or more of the seven *loci* at the central genotype (ST) [68]. Some studies have demonstrated that isolates belonging to the same CC generally share a common ancestor and show very similar phenotypic properties [119].

This scheme was adapted in 2005 for *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus* [120] and *C. fetus* [121]. The genes *adk* (adenylate kinase) and *pgi* (glucose-6-phosphate isomerase) replaced the genes *aspA* and *gltA* in *C. lari*, and *gltA* and *pgm* in *C. upsaliensis*, respectively. These genes (*loci*) were replaced because there was a lack of variability or because the genes did not produce suitable PCR amplification [120].

The selection of antimicrobial resistance (AMR) can also take place at clone or CC level [122], both in Gram-positive [123, 124] and Gram-negative [125-127] bacteria, the latter

including *Campylobacter* [128-130]. The use of MLST to compare STs and CCs between hosts and bacterial species has provided interesting findings, such as an association between *C. coli* clones in cattle [120, 131] or frequent recombination between *C. jejuni* and *C. coli* in some genes [120]. MLST is generally adequate in global epidemiological studies to investigate the structure of *Campylobacter* spp. populations [132-134]. The main advantage of MLST over Pulsed Field Gel Electrophoresis (PFGE) is that it can analyze strains that are not digested by some enzymes used in PFGE [135], although it is recommended to use MLST jointly with *flaA*-SVR, *flaB*-SVR or PFGE to increase its discriminatory power.

Next generation sequencing (NGS) techniques

Next-Generation Sequencing (NGS) techniques have revolutionized genomic studies, playing a crucial role in advancing our understanding of the biology, epidemiology and pathogenicity of the bacteria, including *Campylobacter*.

Whole genome sequencing involves sequencing the entire genomic DNA of an organism, providing comprehensive information about its genetic makeup. It has been used to analyze the complete genome of *Campylobacter* spp. strains, allowing the identification of genetic variations, including single nucleotide polymorphisms (SNPs), insertions, deletions, and structural variations, presence of virulence factors and potential antimicrobial resistance genes [136], aiding in surveillance and guiding treatment strategies [137-139]. Sequencing technologies can be classified based on the length of the reads obtained: short reads (e.g., Illumina) [140] or long reads (Oxford Nanopore Technologies, Pacific Biosciences) [141].

The pan genome of a species represents the entire set of genes found in representative of this species, and can be divided in a core genome (genes shared between all individuals of the species) and the accessory genome (the remaining genes) [142-144] (Figure 5). There are two WGS-based typing methods in *Campylobacter* spp. combining MLST with full bacterial gene sets: cgMLST (using the core genome) [145-147] and wgMLST (using the whole genome) [148-150].

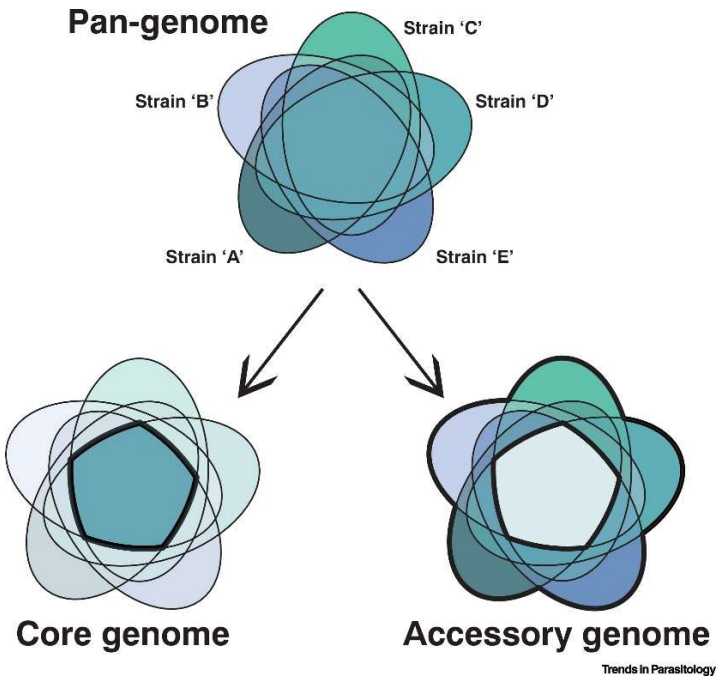


Figure 5. Graphical representation of the structure of eukaryote genomes showing the proportion of genes common to all strains compared with the proportion of genes specific to each strain (source: Sibbald, 2020, Trends in Parasitology).

Two additional ways of using NGS for microbial identification are by searching for genomic single nucleotide polymorphisms [151, 152] or looking at the 'k-mers' (all of a sequence's subsequences of different lengths) [153, 154].

Comparative genomics, based on the comparison of the genomes of different *Campylobacter* spp. strains to identify similarities and differences in genetic determinants associated with virulence, pathogenicity and adaptation to different environments, can be then used for the analysis of the outputs from WGS [155-158]. When combined with phylogenetic analyses they aid in the understanding of evolutionary relationships among strains, providing insights into the spread of particular lineages [159-162].

In addition, functional genomics, that involve studying the functions of genes and their roles in the biology of the organism, allow for the exploration of functional elements within the *Campylobacter* spp. genome, including genes involved in pathogenicity, metabolism and environmental adaptation [163].

1.4. Epidemology of campylobacteriosis

The most important sources of infection with thermotolerant *Campylobacter* are livestock, in particular poultry (mainly chicken), cattle and pigs, followed by contaminated water, milk and the environment. This variety of possible sources of disease in humans has been demonstrated by studies that have looked at several sources at once and found the same overall genotypic diversity [164-168].

Campylobacter infection in poultry flocks has been linked to certain risk factors, such as the increased temperature during the summer season, which promotes the presence of potential *Campylobacter*-carrying vectors like flies and rodents in poultry farms. Moreover, the likelihood of a chicken farm becoming colonized increases over time during rearing, resulting in an average of 60-80% of flocks becoming carriers of thermotolerant *Campylobacter* by the time of slaughter [8]. *Campylobacter* bacteria have evolved to readily colonise the avian gut, adapting to achieve high intestinal and faecal concentrations in broiler flocks [169].

A second important source of *Campylobacter* infections for humans is cattle/ruminants, including both dairy [170] and beef cattle [167, 171, 172]. In contrast, and although the prevalence of *C. coli* in pigs is high, previous studies found pigs associated with only 2% of all human cases [173]. A systematic review and meta-analysis carried out in the US and Canada to estimate the prevalence of *C. coli*, *C. jejuni* and *Campylobacter* spp. on beef cattle, dairy cattle, chickens, turkeys and pigs found that, with the exception of beef cattle, significant heterogeneity remained in the majority of studies [174].

In addition, increased rainfall can produce water puddles and ditch water reservoirs in which *Campylobacter* can accumulate, persist and transmit to new hosts [26, 175]. In European regions such as Scandinavia, waterborne outbreaks are very common [176]. Furthermore, some new sources of thermotolerant *Campylobacter* have emerged, including unpasteurized or poorly pasteurized milk [8].

Transmission to humans

Transmission of thermotolerant *Campylobacter* to humans occurs most commonly by consumption of various kinds of food of animal origin. Other transmission pathways include cross-contamination (involving handling and preparation of vegetables,

especially at barbecues), water consumption, milk consumption (typically raw or unpasteurized), environmental contamination, direct contact with animals and people or either of these sources combined.

The principal routes of thermotolerant *Campylobacter* infection involving the different habitats/hosts described above are displayed in Figure 6.

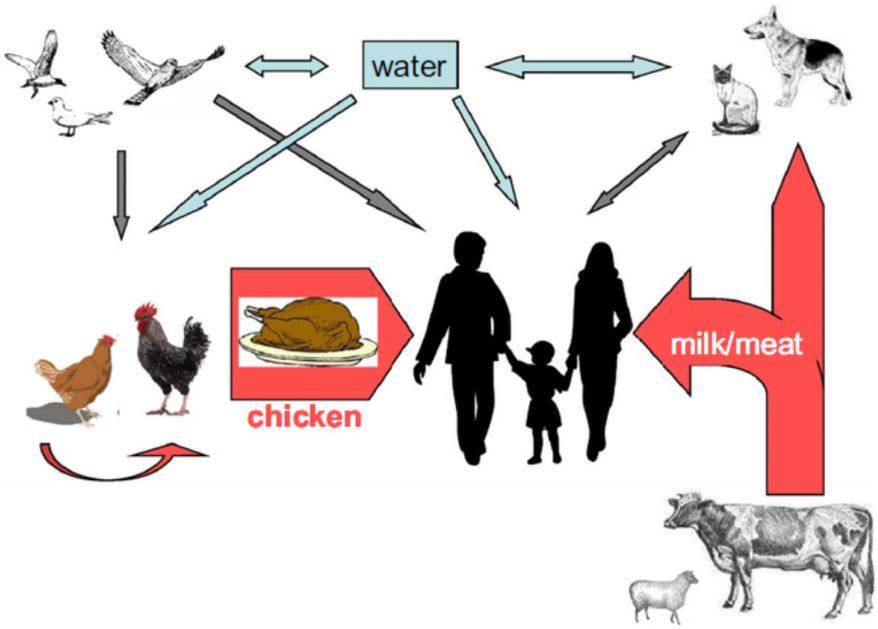


Figure 6. Most important routes for human infection by *Campylobacter* (source: Dasti, 2010).

In developed countries, poultry meat, whose carcasses are contaminated during slaughter and further processing [8, 26, 166, 167, 172, 177-179], constitute the main food vehicle at the origin of infections due to thermotolerant *Campylobacter*, which are predominantly sporadic and rarely traced back to the specific source of infection [8]. In addition, poultry products such as liver pâté have been identified as important sources of *Campylobacter* outbreaks in some countries [180]. Consumption of beef and cattle products [179] have been linked to 19% of human cases [171] and consumption of pig meat has often been linked to human cases caused by *C. coli* [166]. In developing countries, wild birds, pets and domestic animals (especially chickens), and

Campylobacter-contaminated foods as a consequence of poor sanitation, have all been identified as sources of human campylobacteriosis [181].

Some studies have identified ready-to-eat (RTE) foods, such as takeaways, as a risk for campylobacteriosis [166, 182], albeit many studies involving retail sale of RTE foods in Europe have failed to find *Campylobacter* spp. [183, 184], probably because the contamination or cross-contamination of such products occurs after selling, during preparation and cooking in the kitchen [185, 186].

Freshly harvested vegetables can become contaminated with thermotolerant *Campylobacter* directly at farms or during transport and processing in food factories [8, 186], and raw vegetables have been reported as the second most important risk factor after contaminated chicken meat in some studies [26]. Surface water has been identified as a source of transmission of campylobacteriosis [187, 188], although human illness due to infection via drinking water is highly unlikely in developed countries [189, 190]. The increase in the consumption of raw, unpasteurized milk, has led to an increase in milk-associated outbreaks of *Campylobacter* infection in recent years, particularly in developed countries [191].

Environmental contamination [26, 192], including contact with cattle manure [179] and wild birds [172, 193, 194], has also been described as a source of campylobacteriosis. The consumption of game meat and the use of swimming pools have also been identified as significant risk factors for infection with *Campylobacter* strains originating from the environment, especially during spring [8, 195]. Moreover, direct contact with animals (cattle, dogs, cats and horses) [167, 178, 196-199], in particular contact with puppies with diarrhea [26, 200] and animals at 'petting zoos' [201], contacts with travelling people [178], occupational exposure and rural living are additional factors linked to thermotolerant *Campylobacter* infection in humans. Multiple combinations of these sources can also be the origin of campylobacteriosis, such as contact with children previously contaminated by contact with wild birds [172, 202, 203] or environmental cross-contamination due to wild birds pecking milk bottle tops left at the doorstep of houses in the UK [204, 205].

Source attribution of campylobacteriosis

Champion *et al*. in 2005 [206] used genomotyping (whole-genome comparisons of bacteria using DNA microarrays) combined with Bayesian-based algorithms to model the

phylogeny of *Campylobacter* and elucidate sources of infection for humans. They observed that more than half of *C. jejuni* human infections may originate from non-livestock sources [206]. Another study compared outbreak and sporadic *Campylobacter* cases and found clusters of homogenous genotypes, including isolates without known epidemiological links, reflecting shared or related origins via food or the environment, but which went unrecognized [169].

Source attribution studies designed to disclose the most likely sources of human infection are based on either population genetics models such as STRUCTURE [207] or the asymmetric island model [208], with different genetic targets in terms of allele numbers, microsatellites, single nucleotide polymorphisms, etc. [164, 172, 195, 208, 209].

1.5. Clinical campylobacteriosis

The most important species within the thermotolerant group, based on their involvement on human cases of campylobacteriosis, are *C. jejuni* subsp. *jejuni* and to a lesser extent *C. coli*, but other *Campylobacter* spp. can also cause human infections [210]. Both species are characterized by their broad host range, as well as the spectrum of effects they induce [211].

The infective dose for campylobacteriosis in healthy humans is as low as 500-800 colony forming units (CFU) [211]. Besides, the higher the dose, the shorter incubation period and more severe the symptoms observed. After infection with *C. jejuni* in humans, the bacteria colonize and multiplies in the ileum and colon, interrupting the normal absorption and gastrointestinal secretion functions, and thus leading to intestinal campylobacteriosis (colitis) [8], with erythema and hemorrhage of the mucosa. Associated symptoms include fever, malaise, vomiting, abdominal pain and watery diarrhea, often bloody [212-214]. These symptoms commonly appear within 1-5 days post-exposure to the bacteria and generally last 10 days, being self-limited in healthy people, although relapse has been described, though 5-17% of cases are generally hospitalized, of which a small fraction die [215]. A mortality rate of 2.5 per 10,000 confirmed cases was reported in the EU in 2022 [216].

The median world burden of all hazards due to consumption of food of animal origin has been reported at 168 Disability Adjusted Life Years (DALYs) per 100,000 population and *Campylobacter* accounted for 16% (27 DALYs) of that global burden [217, 218]. In most

countries the true number of cases of campylobacteriosis is likely to be significantly higher than reported [219, 220], especially in children in developing countries [221]. The economic impact of campylobacteriosis is related to direct and indirect healthcare and non-healthcare costs of illness [8, 222], which in Europe were estimated at 30 million euros per year in 2003 [223].

In non-immune humans and companion animals both *Campylobacter* species can cause severe gastrointestinal symptoms. However, farmed livestock, and especially poultry, generally carry these organisms as intestinal commensals, and humans can also become healthy asymptomatic carriers and shedders [224, 225]. These striking differences observed in the pathogenicity of the bacteria are most likely related to the various degrees of adaptation they have established with the different hosts. For instance, poultry adapted strains may cause severe disease in humans, which are classed as an accidental host. It is suggested that the commensalism observed in *Campylobacter* in poultry may be a sort of 'controlled parasitism', with the host able to control but not prevent colonization by the bacterium [169], and the latter benefiting from the body temperature of 42°C in poultry [31].

Although consumption of chicken meat products is a risk factor for campylobacteriosis, its frequent consumption can help develop partial immunity, conferring protection against *Campylobacter* [8, 226]. The symptoms, severity and disease duration depend on strain virulence and host immune response [7, 24, 227]. The phenotypic and genetic diversity observed between strains, along with gene expression capabilities, determine bacterial pathogenicity differences [60]. In addition, clinical disease may be more severe and prolonged when infection is caused by antimicrobial resistant strains [228-230], perhaps due to shared mechanisms such as TSSs or multi-drug efflux pumps.

Sequelae and death due to campylobacteriosis in humans

Some of the human cases developing clinical disease can develop post-infectious sequelae after recovery, affecting the nervous system (Guillain-Barré syndrome, GBS; Miller-Fisher syndrome, MFS; Bickerstaff encephalitis), joints (reactive arthritis) and/or intestine (inflammatory bowel disease, IBD; Crohn's disease; ulcerative colitis; irritable bowel syndrome, IBS) [8, 98, 214, 231-233]. The autoimmune reactions induced by *C. jejuni* in GBS and MFS are due to antibodies produced against LOSs which are also directed against ganglioside structures on host neurons [234]. Campylobacteriosis is fatal in some

cases, and mortality rates of 2.9 (95% CI: 1.9-4.0) deaths per 10,000 cases have been reported [235], but these situations generally occur in immunocompromised people with underlying disease, and in very young babies or old people (WHO, 2020).

Treatment of clinical campylobacteriosis

No specific treatment is required for most patients with *Campylobacter* enteritis other than oral replacement of fluid and electrolytes lost through diarrhea and vomiting [178]. Hence, antimicrobial therapy plays a limited role in most instances due to the short duration of symptoms [236]. In patients requiring treatment, macrolide antimicrobials, like erythromycin or azithromycin, and fluoroquinolones, like ciprofloxacin, are the drugs of choice [237]. When these drugs are ineffective, typically because of bacteriemia, the systemic administration of aminoglycosides, like gentamicin or streptomycin, is the preferred alternative option [238, 239].

Historically, macrolides such as erythromycin have been the first choice for campylobacteriosis treatment, followed by ciprofloxacin and other fluoroquinolones, but the use of fluoroquinolones has been reduced due to the ever-increasing appearance of resistant isolates against this antimicrobial class in many countries [35, 236]. Moreover, the World Health Organization (WHO) does not recommend its use in children because of its toxicity [240]. Chloramphenicol, amoxicillin-clavulanic acid and tetracycline have been used as alternative antibiotics instead of macrolides and fluoroquinolones, but high levels of resistant isolates to tetracycline have also been recorded [241]. Therefore, some authors suggest that macrolides should remain the drug of choice [242], complemented with aminoglycosides or imipenems to treat severe infections [236].

It is challenging to estimate the AMR burden in humans of bacteria in general, because of limited and unreliable information on geographic distribution [243], incidence and prevalence. Some authors have suggested using several dimensions to approach the challenge, like for example investigating the burden of different forms of sepsis as an intermediate pathway to death from AMR, or performing systematic reviews of published and unpublished clinical sources comparing the relative case-fatality rate of drug-resistant *versus* drug-sensitive infections [244]. The burden of AMR in humans due to AMR in livestock [245] or in humans-animals with a one-health perspective [246] have also been explored.

1.6. Surveillance and control of campylobacteriosis

Campylobacteriosis is the most commonly reported foodborne gastrointestinal infection in humans in the EU and has been so since 2007 [216, 247]. In 2022, the number of confirmed cases of human campylobacteriosis was 137,107 (notification rate of 43.1 per 100,000 population in 2022, compared to 41.1 per 100,000 population in 2021), either as outbreaks or as sporadic cases. Of all confirmed cases, 87.6% concerned *C. jejuni*, 10.7% *C. coli*, 0.26% *C. fetus*, 0.17% *C. upsaliensis*, 0.12% *C. lari* and 1.1% other *Campylobacter* spp. or undifferentiated. Spain reported 20,816 confirmed cases in 2022 [216]. Diverse epidemiological studies over the past 15 years have shown a steady increase in the overall prevalence of campylobacteriosis in developing and developed countries [178, 248]. A study from 2022 showed that over the period 2014-2022, the Czech Republic had the highest number of reported cases with 215 per 100,000, followed by Australia (147 per 100,000) and New Zealand (126 per 100,000) [249].

Human reported cases of campylobacteriosis are the 'tip of the iceberg' of all cases due to underreporting. Even in countries where it is a notifiable disease, for a case to be reported symptoms must be severe enough so the person goes to see a doctor, the doctor takes a sample that is transported in ideal conditions for *Campylobacter* spp. to survive, the sample is adequately and quickly processed and the case is included in the national registry [250].

Legal requirements for surveillance and control of campylobacteriosis in the EU and Spain

Both EU and Spanish legislation (in particular RD 1940/2004) establish two lists for zoonotic agents, and *Campylobacter* is included in 'List A' ('Zoonoses and zonotic agents that must be always under surveillance'). Besides, the EU Zoonoses Directive 2003/99/EC mandates that Member States monitor zoonoses, zoonotic agents and related antimicrobial resistance, and campylobacteriosis is included due to its significant public health impact. However, the reporting of numbers of confirmed human cases of campylobacteriosis to ECDC is currently mandatory in 22 EU MSs, as well as Iceland, Norway and Switzerland [247], where they have to keep a registry of clinical cases and report their cases also to other MSs.

In the remaining five EU countries (Belgium, France, Greece, Italy and the Netherlands) the notification is based on a voluntary system, which means that healthcare providers or laboratories report cases at their discretion, rather than under a legal mandate.

The surveillance of thermotolerant *Campylobacter* and campylobacteriosis in the EU (thus transposed to Spanish national legislation) is regulated through the following two pieces of legislation:

- Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC (EC, 2003).

- Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of Salmonella and other specified food-borne zoonotic agents (EC Zoonoses, 2003).

Under Spanish law, campylobacteriosis are regulated with the following "Real Decretos" (RDs), "Reglamentos" and "Ordenes Ministeriales" (OMs):

- 'RD 2210/ 1995, de 28 de diciembre, por el que se crea la red nacional de vigilancia epidemiológica.' (RD2210, 1995)

- 'RD 1940/2004, de 27 de septiembre, sobre la vigilancia de las zoonosis y los agentes zoonóticos.' (transposition of Directive 2003/99/EC) (RD1940, 2004)

- 'Reglamento (CE) 2073/2005, relativo a criterios microbiológicos aplicables a los productos alimenticios, que incluye unos criterios de higiene de proceso para valorar el buen funcionamiento de un proceso o fase de producción*' (Reglamento 2073, 2005) (*Modificado por: Reglamento (UE) 2017/1495, de 23 de agosto de 2017 que modifica el Reglamento (CE) Nº 2073/2005 por lo que se refiere a *Campylobacter* en canales de pollos de engorde)

- 'RD 526/2014, de 20 de junio, por el que se establece la lista de las enfermedades de los animales de declaración obligatoria y se regula su notificación.' (RD526, 2014)

- 'OM SSI/445/2015, de 9 de marzo, por la que se modifican los anexos I, II y III del Real Decreto 2210/1995, de 28 de diciembre, por el que se crea la Red Nacional de Vigilancia Epidemiológica, relativos a la lista de enfermedades de declaración obligatoria, modalidades de declaración y enfermedades endémicas de ámbito regional.' (OM SSI/445, 2015)

- 'Reglamento (UE) 2017/1495 de la Comisión de 23 de agosto de 2017 que modifica el Reglamento (CE) n.o 2073/2005 por lo que se refiere a *Campylobacter* en canales de pollos de engorde' (REGLAMENTO 1495, 2017)

1.7. Genetic characteristics of *C. coli* and *C. jejuni*

1.7.1. The genome of *Campylobacter* spp.

In 2000, Parkhill *et al*. published the first complete DNA sequence of a *Campylobacter* bacterium, the strain *C. jejuni* NCTC 11168, with a circular chromosome of 1,641,481 bp predicted to encode 1,654 proteins (Figure 7) [46]. The average G+C content of this strain was 30%, while the average genus G+C content is 46% (28-36% in the thermotolerant group)[251].

Its average gene length was 948 bp with genes typically not organized into operons and clusters, but with a broad repertoire of regulatory systems that may help bacteria to cope in diverse ranges of ecological niches [17, 55, 252, 253]. The genome lacked functional insertion and phage-associated sequences, transposons or retrons [46]. The genome of *C. jejuni* NCTC 11168 was re-analysed and re-annotated in 2007 [254].

The NCBI genome repository (https://www.ncbi.nlm.nih.gov/genome; last accessed 7 December 2024) now contains a collection of over 84,700 *C. jejuni* genomes and over 34,700 *C. coli* genomes. The reference for *C. jejuni* is *C. jejuni* subsp. *jejuni* NCTC 11168 (Sanger Institute): median genome length: 1.6 Mb; median gene length: 1,668 bp; median protein count: 1572; G+C: 30.5% (last accessed 7 December 2024) and the reference for *C. coli* is *C. coli* FDAARGOS_735 (US FDA): median genome length: 1.7 Mb; median gene length 1,775 bp; median protein count: 1688; G+C: 31.5% (last accessed 7 December 2024).

Hypervariable sequences in *C. jejuni*

The most striking feature of *C. jejuni* is the presence of hypervariable sequences consisting of homopolymeric (repetitive) nucleotides, such as poly-G tracts [255]. These sequences are involved in the biosynthesis or modification of surface structures (glycosilation) that could have a role in the survival strategy of the bacterium, including capsular polysaccharides (CPS), LOS and flagellum [256, 257], and are found at a much higher frequency than in other bacteria (Figure 7). *C. jejuni* has been called a "hyperglycemic bug" because it dedicates a large part of its small genome to the biosynthesis of several glycostructures [236], albeit it does not use carbohydrates as source of energy, as described in section 1.2.2. of this thesis. The rapid genetic changes and evolution observed in *C. jejuni*, similar to what has been described for *H. pylori*, suggest that *C. jejuni* may lack the DNA repair functions that are present in other bacteria.

As a result, the random, spontaneous and reversible errors that occur during DNA replication in a phenomenon called 'phase variation' lead to changes in certain phenotypic traits such as surface structures like flagella, LOS and capsule formation [17]. These surface structures play crucial roles in the bacterium's survival, host colonization and interactions with the host immune system. Moreover, it is argued that *C. jejuni* strains act like dynamic mutant groups in which mutating individuals have only a short-lived existence, similar to RNA viruses. The unusual dual protein glycosylation system of the bacterium [258] is essential for an effective 'phase variation', permanently changing to adapt to different hosts [1, 17, 259].

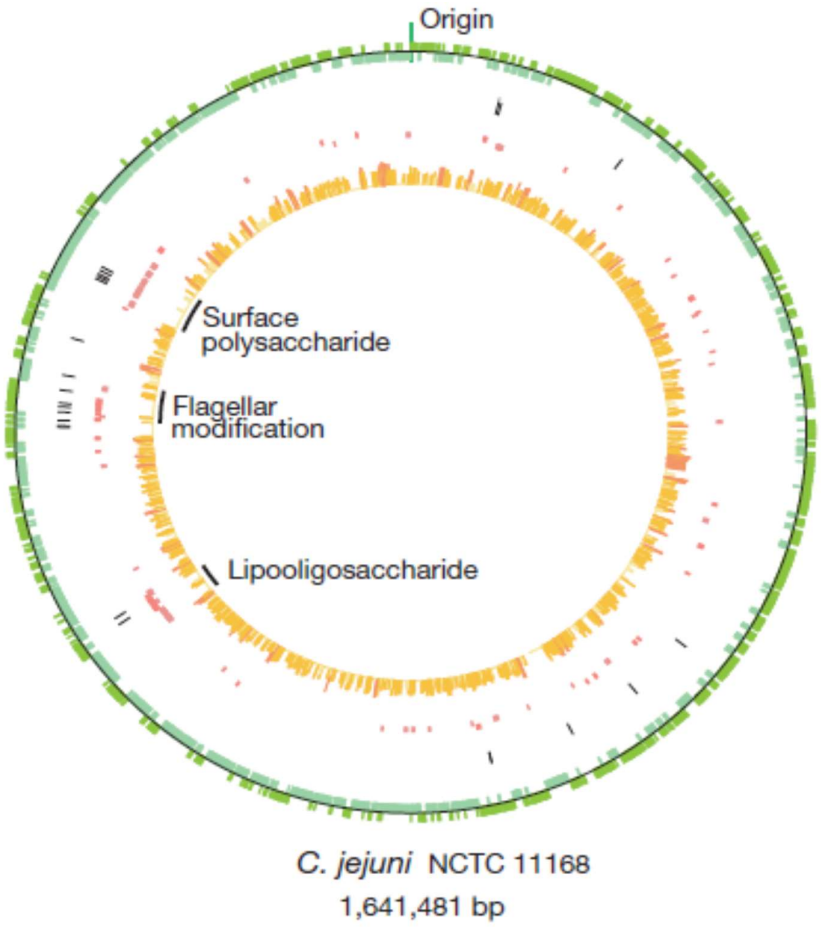


Figure 7. Circular representation of *C. jejuni* NCTC 11168 genome; Green circle: coding sequences; Black and red (partial) circles: hypervariable sequences coding for 3 surface structures (black) and genes involved in their codification (red); Yellow circle: genes similarity with *H. pylori*, where less similar regions coincide with hypervariable sequences in *C. jejuni* (source: Parkhill, 2000).

1.7.2. Genetic population structure of *C. coli* and *C. jejuni*

The genetic population structures of *C. coli* and *C. jejuni* are very different. *Campylobacter coli* are genetically highly structured into clusters of related isolates (CCs) whereas *C. jejuni* generally display a non-clonal population structure, showing evidence of high rates of HGT [260], also known as lateral gene transfer (LGT) (Figure 8A), the exchange of mobile genetic elements (MGEs) between bacteria belonging to the same or different species [261].

Data from the PubMLST database (https://pubmlst.org/organisms/campylobacter-jejunicoli; last accessed 7 December 2024) showed that 86% of *C. coli* isolates in the database (30,097 out of 35,014 isolates) belonged to just two CCs, ST-828 (29,012/30,097; 96%) and ST-1150 (1,085/30,097; 4%) whereas 52% of all *C. jejuni* isolates present in the database (52,335 out of 101,107 isolates) belonged to the five most common CCs (ST-21, ST-353, ST-45, ST-48 and ST-257), although the total number of STs reach thousands. The analysis of *C. coli* isolates from a broad range of sources showed that the two CCs are part of only one clade (clade 1) of three *C. coli* deep-branching clades (Figure 8B).

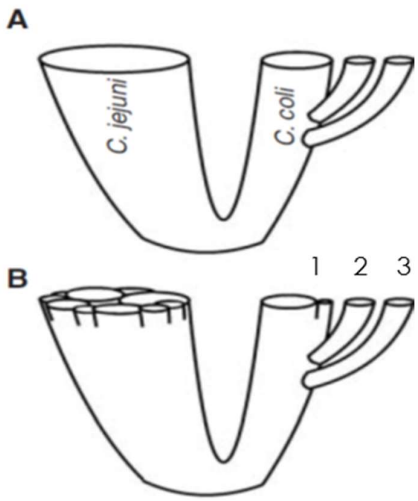


Figure 8. Population structure and evolution of *C. jejuni* and *C. coli*: two main human disease-associated species (A) and CC and clade structure in *C. jejuni* and *C. coli* (1, 2, 3) (B) (adapted from: Sheppard, 2015).

Moreover, the maintenance of this 3-clade *C. coli* population structure suggests the existence of different gene pools with HGT occurring preferentially among members of the same clade and being less frequent among members of different clades [1].

Campylobacter coli from Clade 1 are retrieved most commonly from clinical and farm animal samples and are ecologically separated from *C. coli* from Clade 2 and Clade 3, which are more abundant in waterfowl environments. Furthermore, the overwhelming presence of ST-828 CC in Clade 1 suggests relatively recent independent evolution (Figure 8B) [119, 262]. *Campylobacter jejuni* and *C. coli* are both found in chicken and cattle at a ratio of 9:1 and most of the isolates found in pigs are *C. coli* [263]. There is a strong host-genotype relationship at the MLST CC, ST and allele levels, particularly within *C. jejuni* [119, 263], which could be either a consequence or a cause of ancestral barriers to HGT among lineages [1].

Campylobacter hosts represent a complex combination of selective pressures characterized by distinct tissue immune responses or fluctuating concentrations of metabolites and cell by-products, so different thermotolerant *Campylobacter* genotypes could adapt to distinct ecological subniches within the same host, expressing different virulence phenotypes [264]. *Campylobacter jejuni* is a foodborne pathogen and the major cause of gastroenteritis in humans worldwide, where adhesion to, and invasion of, host epithelial cells have been investigated as important mechanisms in the infection process. Many genes have been identified and many mechanisms have been suggested to be involved in the interaction with host cells. *Campylobacter jejuni* seems to be an opportunistic pathogen in humans and its invasion potential varies among strains (where human clinical isolates are generally more invasive compared to non-clinical isolates) and hosts (*C. jejuni* is able to cause disease in certain host species and remains avirulent in others [169]).

1.7.3. MGEs in *C. coli* and *C. jejuni*

MGEs are elements that facilitate intracellular (between chromosome and plasmids or between plasmids) and intercellular (typically by plasmids) DNA mobility. Among the MGEs that thermotolerant *Campylobacter* can harbor there are plasmids, transposons, genomic islands, insertion sequences (ISs), integrons and bacteriophages, of which

plasmids are the best described and characterized group in the literature for these bacteria.

Plasmids

The term 'plasmid' was first coined in 1952 to refer to an extra-chromosomal 'hereditary determinant', now understood as a DNA extrachromosomal genetic element that does not encode capsids for transmission, as phages do [265]. Plasmids are very relevant MGEs in thermotolerant *Campylobacter*, as they are responsible for both bacterial diversity and adaptability, promoting these in three different ways: facilitating the increase in the number of copies of the genes they harbor, incrementing the chances of appearance of mutations in these genes and being able to transfer between bacteria most of the times. A significant number of plasmids can replicate in different species within a genus or even between species belonging to bacteria from different genera [238, 266], but they are generally independent of the bacterial chromosome and they are rarely found incorporated within it [267]. Moreover, the recombination between plasmid and chromosomal DNA material is uncommon because there are few regions of sequence homology between both to allow for this phenomenon to happen [268].

There were more plasmids initially described in *C. coli* than in *C. jejuni* [236] but that situation has been reversed (654 in *C. jejuni versus* 363 in *C. coli*; NCBI website: https://www.ncbi.nlm.nih.gov/genome; last accessed 7 December 2024). Some authors examined the phylogenetic relatedness of thermotolerant *Campylobacter* plasmid-bearing species from retail meats finding that they did not cluster according to the species of bacteria, suggesting intra-species dissemination of plasmids between *C. coli* and *C. jejuni* [269]. The primary phenotypic marker associated with *Campylobacter* plasmids is ARGs [236, 270], but they can carry other genes involved in pathogenicity [271].

In terms of plasmid structure, there are four elements generally present in plasmids, with few exceptions: mating pair formation ('MPF'), origin of transfer ('oriT'), 'relaxosome' and 'replicon'. The MPF is a plasmid element that functions as a secretion machinery for intercellular DNA transfer during bacterial conjugation [272]. The oriT is a region located within the plasmid sequence that is important for the control of replication and number of copies. The relaxosome is an assembly or complex formed by a series of proteins at the oriT necessary for replication. The replicon, one of the *loci* always present in plasmids, is

a highly conserved region harbouring genes encoding the replication initiation sequence (*rep*), a mobilisation region (*mob*) encoding specific relaxosome proteins and the oriT [273, 274].

Plasmid types in thermotolerant *Campylobacter*

Plasmids in thermotolerant *Campylobacter* may be classified according to five main features: mobility/transferability, size, relaxase families, incompatibility groups and similarity of replicons.

Plasmids can be classified into three categories according to their mobility or transferability: conjugative, mobilizable and non-mobilizable [275]. A plasmid that encodes for its own set of MPF genes is called conjugative or self-transmissible and if the MPF is not present but can be borrowed from another genetic element present in the cell (which provides the missing additional conjugative functions) the plasmid is called mobilizable (or plasmid bearing 'adapted conjugation') [272]. Plasmids that are neither conjugative nor mobilizable are non-mobilizable and spread by natural transformation or transduction but not by conjugation. Both conjugative and mobilizable plasmids carry the genetic information necessary for relaxosome formation and processing, but this is lacking in non-mobilizable plasmids [273].

Plasmids can be classified into three different groups in thermotolerant *Campylobacter*, according to their size: megaplasmids (>100 Kb and up to 180-200 Kb in size) [265, 276], small plasmids (>5 Kb and up to 100 Kb in size) with several genes that encode proteins involved in plasmid conjugation, and cryptic plasmids (<5 Kb in size) with genes that encode 1-2 replication proteins and 1-2 mobilization proteins [273, 277, 278].

In thermotolerant *Campylobacter*, megaplasmids (such as *pVIR*, *pCJDM202* and *pCJDM67L*) [265] and small plasmids (such as *pTet* and *pcc31*) [269, 279] may be involved in HGT of antimicrobial resistance and virulence factors (T4SS and T6SS secretion systems) [280]. However, cryptic plasmids (such as *pCJ01*, *pCC2228-1* and *pCJ419*) [238, 281] are extrachromosomal DNA elements that do not encode for recognizable phenotypes apart from their own replication functions [282] and their precise role is unclear [283].

The classification system based on the similarity of relaxases, focusing on motifs I, II and III of the catalytic centre of the relaxase [273], defines four groups in Gram-negative

bacteria like *Campylobacter* spp.: the *MOBq* family, the *CloDF13* family, the *ColE1* superfamily and the *pMV158* superfamily (the last two are also present in Gram-positive bacteria). This classification system increases the discriminatory power in plasmids with relaxase genes, but it misses plasmids without them [284].

Up to nine incompatibility (Inc) groups have been described in thermotolerant *Campylobacter*, all in cryptic plasmids [281], and they cannot co-exist in the same bacteria. This classification includes all the variability of plasmids obtained after bacterial replication (especially in *C. jejuni*), comprising also the mutations they undergo in the replicon regions [273, 284], making it cumbersome to use for practical purposes.

The similarity of plasmid-encoded replication initiation regions (*rep*) may be a better criterion for classification of plasmids present in thermotolerant *Campylobacter*. However, plasmids may contain multiple or even recombinant *rep* regions in their replicon machinery, and only plasmids previously included in the typing scheme can be detected [236].

Transposons

Transposons (or transposable elements) are DNA sequences that can change their position within the genome so they are capable of translocation (transposition) from one replicon (plasmid or chromosome) to another. They do not share extensive regions of homology with the recipient replicon. They are bounded by DNA sequences called direct or inverted repeats of approximately 40 kb in length which provide highly specific recognition sites for transposase enzymes that catalyse the transposition [268]. In addition, they can use insertion sequences to facilitate this movement. They can transfer antimicrobial resistance genes (ARGs) between plasmids and between different bacteria or in/out of the chromosome. Their genetic structure contains transposase and resolvase genes, encoding for mobilization functions, but they are non-conjugative. There have been few descriptions of transposons in *Campylobacter* [236, 285].

Tn5405, originally described as transposon Tn5405 in *Staphylococcus aureus* by Lambert in 1985 and by Derbise in 1997 [286, 287], can mediate in the transfer of genes encoding resistance to gentamicin, streptomycin and streptothricin.

Genomic islands

Genomic islands are regions of the bacterial genome that are normally acquired through HGT. Genomic islands can harbor genes for pathogenicity, metabolism, antibiotic resistance and symbiosis [288, 289] and are a common way of disseminating ARGs in *Campylobacter* [290, 291].

Insertion sequences

ISs are small (<2.5 kb) and genetically compacted isolated DNA sequences delimited by short terminal inverted repeats that contain one (or sometimes two) open reading frames that encode for 'transposases', which are proteins required for their mobility [292]. These ISs can add to both ends of a gene or assembly of genes (gene cassettes) converting it into a transposon. Theoretically, all replicons are accessible to transposition and all genes are potentially transposable. Transposons and ISs play a vital role in plasmid evolution [268]. ISs are transposable elements responsible for the mobilization and transfer of ARGs [274, 293] and have been well described in *C. coli* [294], which is more prone to acquire ARGs via MGEs than *C. jejuni* [295]. Although several of these MGE mechanisms were first discovered in *C. coli*, evidence of HGT to *C. jejuni* was shown thereafter [296].

Insertion sequences IS1595 [297], IS6 [298] and IS30 [299] belong to the 'DDE' superfamily [300, 301], which includes a transposase and a protein involved in regulation.

Integrons

Integrons, often involved in the transmission of ARGs, are special molecular structures that can enable the formation of new combinations of ARGs within a bacteria (on plasmids or on chromosomic or plasmidic transposons) in response to selection pressures [302]. They consist of two conserved segments of DNA flanking inserted genes such as ARGs. Individual ARGs can be inserted or removed between the conserved sequences in different orders or combinations forming 'cassettes' [303], or associated to plasmids, transposons and the chromosome with large gene 'cassettes' [122], forming chromosomal integrons or super-integrons. Class 1 integrons in *Campylobacter* are often associated with antibiotic resistance [236, 304, 305]. Integrons can also facilitate the expression of ARG 'cassettes' from unrelated bacteria by means of supplying a promoter for their transcription [268, 306].

Phages

Phages do not need a plasmid or transposon to transfer genetic material between bacteria [255, 268] but encode for the formation of capsids for transmission [265]. Almost all phages described so far in thermotolerant *Campylobacter* are lytic and belong to the family Myoviridae. According to their genome size, these phages can be divided into group I (320-425 Kb), group II (175-183 Kb) and group III (131-135 Kb). Lytic thermotolerant *Campylobacter* phages have been studied for more than 30 years and can be used to combat this pathogen in animals and food products [80].

1.7.4. Horizontal gene transfer in thermotolerant *Campylobacter*

In thermotolerant *Campylobacter*, HGT occurs by three different mechanisms: transformation (bacteria uptake plasmids or chromosomal DNA), conjugation (transfer of plasmid DNA via pilli or adhesins) and transduction (by phages) [274, 293, 307].

Transposition (or illegitimate recombination) is an observed type of transformation in which the genes pass or translocate from one (plasmidic or chromosomic) bacterial transposon (replicon) to another [270]. This mechanism is more typical of *C. coli* than *C. jejuni* because *C. coli* offers less barriers to HGT than *C. jejuni* [308]. The occurrence of transposition explains the great variety of betalactamases present in bacteria in general [309] and also in *Campylobacter* [310, 311]. Most of the time, transposons consist of individual ARGs or groups or genes that confer resistance to many different antibiotics [268, 312].

It has been estimated that HGT of ARGs occur twice as much as mutations leading to resistance in thermotolerant *Campylobacter* [313], on multiple occasions [314] and expands locally, forming clusters [1, 315, 316].

Recombination is a strong evolutionary force in thermotolerant *Campylobacter*, leading to the emergence of new lineages and even large-scale genome-wide interspecies introgression between *C. jejuni* and *C. coli* [317] (Figure 9), which can be studied and analysed using GWAS or MLST CC-derived methods [1].

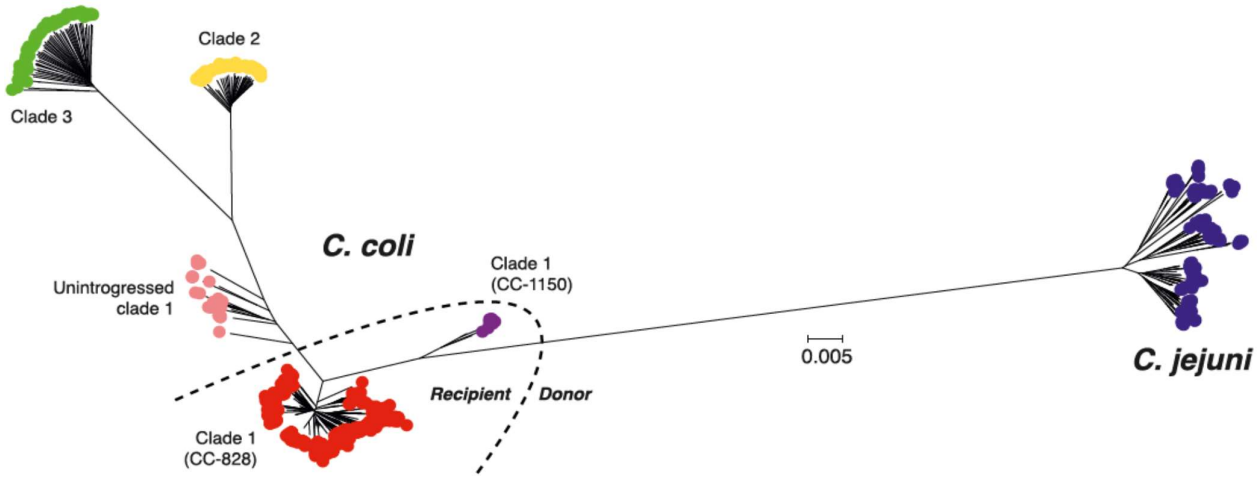


Figure 9. Phylogenetic tree reconstructed by the Neighbour-Joining (NJ) method on a whole-genome alignment of *C. jejuni* and *C. coli* isolates. Red: introgressed *C. coli* clade 1 (CC-828); purple: introgressed *C. coli* clade 1 (CC-1150); pink: un-introgressed *C. coli* clade 1; yellow: clade 2; green: clade 3; violet: *C. jejuni* genomes; dash line: separation between recipient and donor populations (source: Taylor, 2023).

High levels of interspecies HGT (up to 25% of their genome) between *C. jejuni* and *C. coli* have been described [318], even though they are 12% divergent at the nucleotide sequence level [1]. Although interspecies HGT has been challenged [319, 320], subsequent studies using WGS analyses are consistent with an evolutionary introgression scenario in which a single *C. coli* lineage (Clade 1 in Figure 9) has been progressively accumulating *C. jejuni* DNA, replacing 10% and 23% of the *C. coli* core genome by novel DNA in CCs ST-828 and ST-1150, respectively [321]. The authors of these studies argued that, if maintained over time across the genome, this level of interspecies HGT would lead to merging of the species within the agricultural niche in a process described as merging or 'despeciation' (Figure 10), while much of the diversity within these two *Campylobacter* species is still found in wild-bird populations, due to ancient barriers to HGT [1].

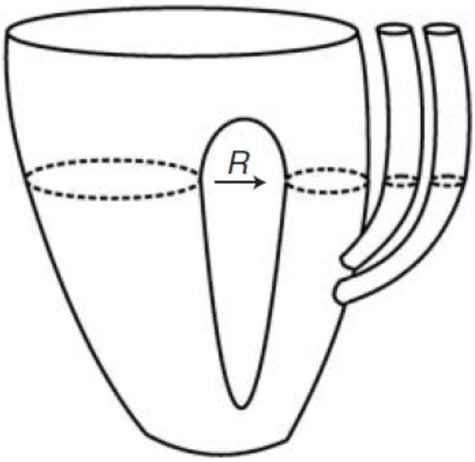


Figure 10. 'Despeciation' evolutionary scenario, resulting from high recombination (R: arrow from *C. jejuni* to *C. coli*) among the species, whereby up to 25% of *C. jejuni* genetic material is transferred onto *C. coli* strains (source: Sheppard, 2015).

Recombination by HGT is more commonly detected in *C. coli* from Clade 1 than in *C. coli* from Clades 2 and 3 [169], and this asymmetry is due to the proliferation of the *C. coli* three-clade in an environment where *C. jejuni* outnumbers *C. coli* [318] and to the adaptability of *C. jejuni* to gene flows. A comparison of gene sequences from related non-thermotolerant *Campylobacter* species showed that *C. jejuni* may have evolved within the last 12,000 years in response to changes in agricultural practices and animal domestication brought about by the Neolithic revolution [322].

An MLST study carried out in 149 *C. coli* strains recovered from pigs, cattle, chickens and turkeys looked at host-associated alleles and found that pig strains were the most diverse, cattle strains were the most clonal and the majority of ST types were found in pigs and poultry [263]. The introgression and progressive hybridization of *C. coli* species with *C. jejuni* genetic material, as described above, can produce as a result hybrid *C. coli* lineages that proliferate in agricultural animals, emerging as the major cause of *C. coli* infection in humans [169, 323, 324].

1.8. Antimicrobial resistance in thermotolerant *Campylobacter*

1.8.1. Molecular mechanisms of antimicrobial resistance in thermotolerant *Campylobacter*

Antimicrobial resistance genes (ARGs) existed before the introduction of antimicrobials in human and veterinary medicine. It is widely assumed that the emergence and survival of resistant bacteria is a result of selective pressure, allowing the fittest strains to become predominant [325]. This phenomenon is associated with the widespread use of antimicrobials from the second third of the XX century [268], in both humans [326, 327] and animals [328, 329].

Without selective pressure, AMR is usually considered a handicap rather than an asset to a bacterium since it often carries a metabolic penalty (also called fitness cost) for the cell [268], which has been observed both in *C. coli* [330] and *C. jejuni* [331]. For example, in macrolides like erythromycin, resistance is generally mediated by mutations in the *23S* ribosomal RNA (rRNA) gene, which consist of a substitution of A by G at position 2075 ('A2075G' substitutions). Some authors suggest that in *C. jejuni* this modification causes a reduction in bacterial fitness which in turn translates into a reduced ability to colonize the chicken host [239]. However, sometimes the AMR trait may increase bacterial fitness, for example in the case of accumulation of mutations [332]. Once the selective pressure is removed, the susceptibility to an antimicrobial is typically resumed [333], although in some occasions the AMR phenotype is maintained, for instance, by plasmids, integrative and conjugative elements [334] even in the absence of antibiotic selection pressure [335].

Critically Important Antimicrobials

In 2017, the World Health Organization (WHO) published a document with the purpose of formulating and prioritizing risk assessment and risk management strategies for the containment of AMR, mainly due to the use of antimicrobials in food producing animals. This document includes three lists of antimicrobials, classified based on two criteria. Criterion 1 (C1) specifies that 'the antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people'. Criterion 2 (C2) specifies that

'the antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources' [240]. According to these criteria, WHO established 'Critically Important Antimicrobials' (meeting C1 and C2), 'Highly Important Antimicrobials' (meeting either C1 or C2) and 'Important Antimicrobials' (not meeting C1 neither C2). Five out of the six antimicrobials (ciprofloxacin, nalidixic acid, gentamicin, streptomycin and erythromycin) included in the EU AMR monitoring programme from 2013 to 2020, which are also the study framework of this thesis, are included on the 'Critically Important Antimicrobials' list, with tetracycline included on the 'Highly Important Antimicrobials' list [240].

Generic processes and types of AMR in thermotolerant *Campylobacter*

AMR can initially be classified into intrinsic and acquired. 'Intrinsic' AMR is natural resistance possessed by bacteria usually encoded by chromosomal genes. On the other hand, 'acquired' AMR is the type of resistance that occurs in naturally susceptible bacteria that become resistant either by alterations of their existing genome (mutational resistance) or by transfer of genetic information between bacteria (transmissible resistance) [268].

Several types of bacterial AMR processes can be described: co-resistance, cross-resistance, pleitropic resistance and multiple resistance [122]. Co-resistance involves transfer of several genes into the same bacteria and/or the acquisition of mutations in different genetic loci affecting different antimicrobials, whereas cross-resistance is produced by mutations or by acquisition of resistance genes affecting antimicrobials from the same antimicrobial class, generally sharing the same AMR mechanism [336]. Pleiotropic resistance implies the same mutation or acquisition of a resistance gene affecting several antimicrobial classes. Finally, multiple resistance (or multiresistance) is produced by mutations or by acquisition of resistance genes affecting antimicrobials from different antimicrobial classes, hence, with different AMR mechanisms [122, 268]. Multiple resistance includes three subtypes: MDR (acquired non-susceptibility to at least one antimicrobial in three or more antimicrobial classes), extensive drug resistance (XDR, non-susceptibility to at least one antimicrobial in all except a maximum of two antimicrobial classes) and pan-drug resistance (PDR, non-susceptibility to all agents in all antimicrobial categories) [337].

Mutations are random and spontaneous 'mistakes' (deletion, substitution and/or addition of genetic material) generally occurring at a low frequency (10^{-4} – 10^{-10} per cell division). When an antimicrobial selects for a spontaneous antimicrobial resistant mutant, this mutant survives and proliferates to become the predominant type whenever in the presence of such antimicrobial [268]. If the antimicrobial is used at the right concentration, the mutations that occur are typically single-step mutations that translate into large increases in the MICs. However, if the antimicrobial is used at low or suboptimal concentrations, the mutations occur in a multi-step manner, and this translates into cumulative gradual increases in the MICs every time the antimicrobial is used [268].

Genomic islands are large chromosomal segments present in certain bacteria that represent DNA regions which have been previously transferred by other mobile genetic elements, thus, they belong to the bacterial pangenome [338]. They can carry one or more genes associated to virulence, metabolism or AMR [288] (Figure 11).

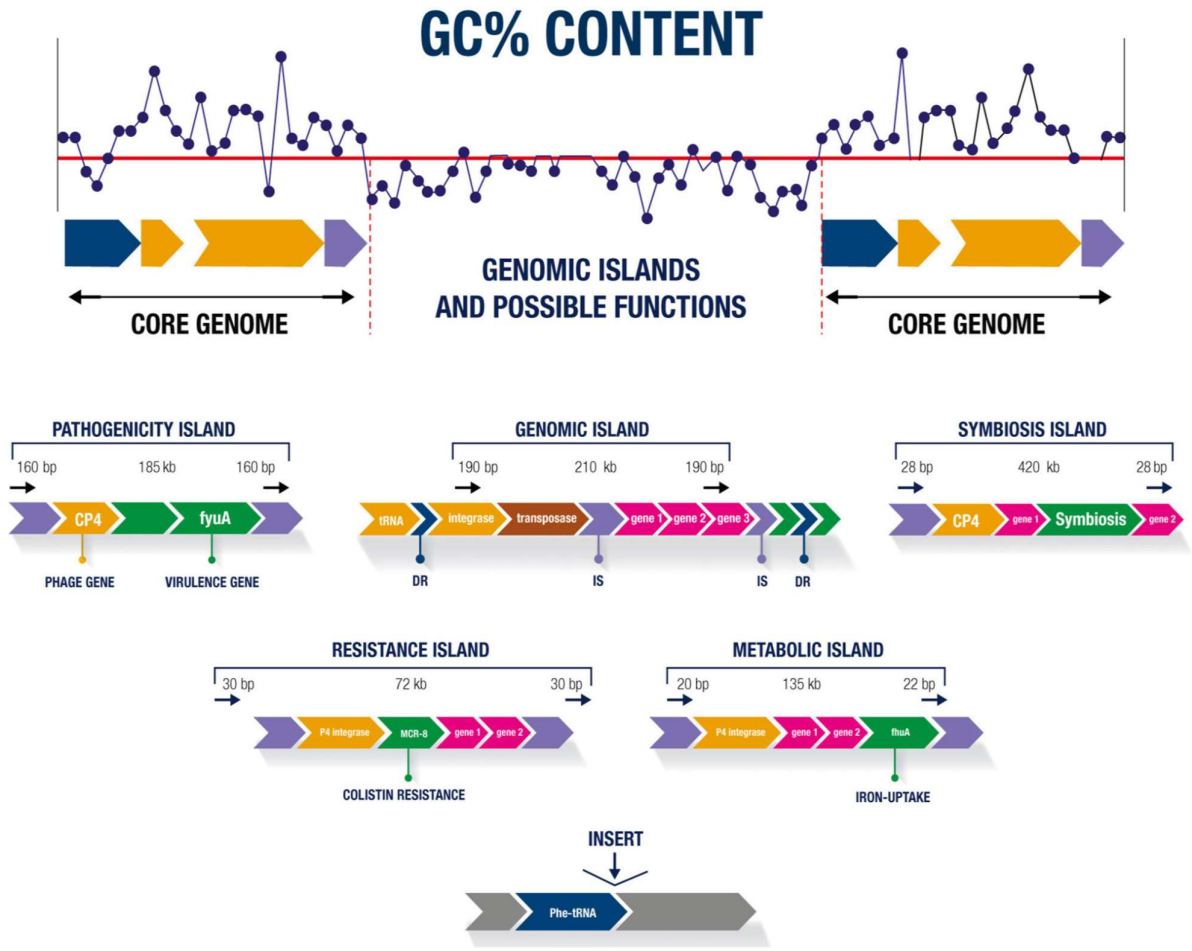


Figure 11. Main characteristics of genomic islands with functions (source: da Silva Filho *et al.*, 2018).

Campylobacter is considered a high-risk pathogen in terms of antimicrobial resistance due to the high levels of HGT (mainly in *C. coli*) and the association of antimicrobial resistance genes in multi-drug resistance genomic islands (MDRGIs) [239, 287, 308, 329, 339-341]. The presence of genes conferring resistance to gentamicin and erythromycin in the same genomic island in a specific *Campylobacter* isolate could expand or disseminate such combined resistance by HGT to other *Campylobacter* bacteria or to other species [122, 342].

Some authors argue that the transfer of MDRGIs is likely to lead to co-selection phenomena after their genetic mobilization, by which a single antimicrobial can select for different MDR isolates and different antimicrobials can select for the same MDR

phenotype in the same isolate [122]. This could explain why *Campylobacter* adapts so quickly in its interaction with the host, constantly obtaining improved phenotypes [1].

General mechanisms of AMR in thermotolerant *Campylobacter*

There are four main mechanisms by which bacteria in general, and *Campylobacter* specifically, interact with antimicrobials to challenge or contain their action [268]:

1. Inactivation or modification of the antimicrobial (beta-lactams, cloramphenicol and aminoglycosides; in aminoglycosides this action translates into reduced antimicrobial uptake).

2. Alteration of the antimicrobial target (beta-lactams, streptomycin, chloramphenicol, erythromycin, fusidic acid, quinolones, rifampicin and glycopeptides; in erythromycin and glycopeptides this is due to enzymatic modification, and is typical of cases where high levels of resistance are acquired in a single step [268].

3. Reduced permeability to the access of the antimicrobial (tetracycline, beta-lactams, chloramphenicol, quinolones & aminoglycosides; in tetracycline and quinolones this action translates into increased efflux).

4. Metabolic by-pass (trimethoprim and sulphonamides).

The specific AMR mechanisms used by thermotolerant *Campylobacter* with the different antimicrobial classes, with an emphasis on aminoglycosides and macrolides, are described below and displayed in Figure 12.

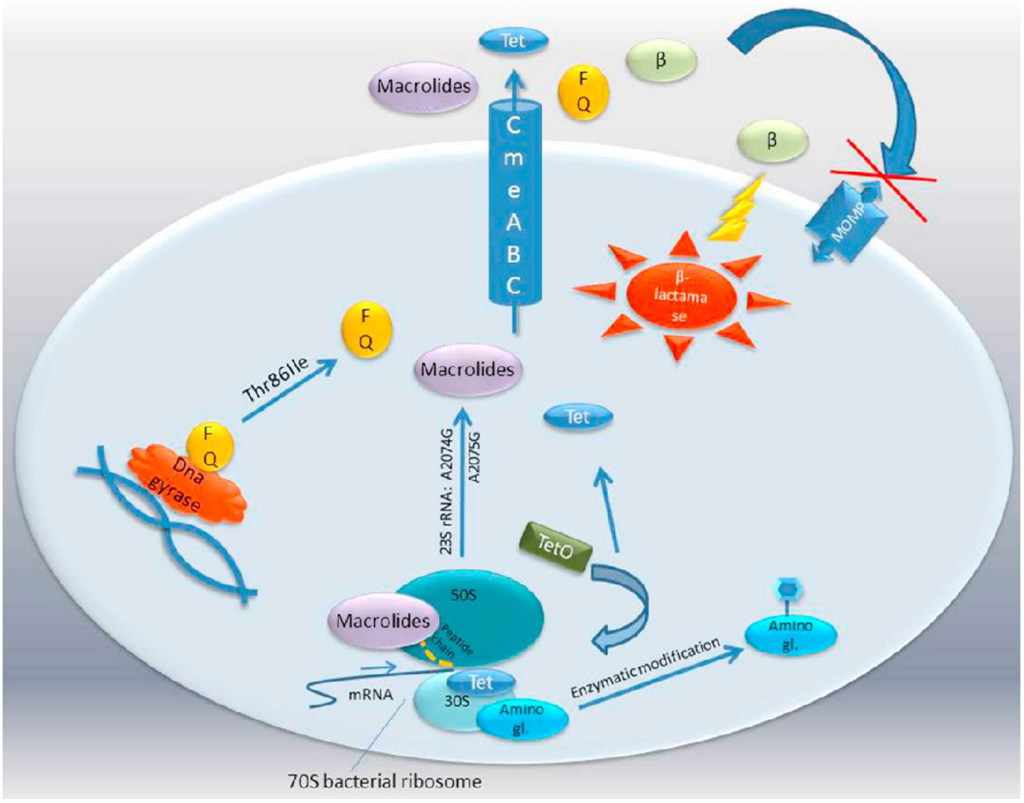


Figure 12. Schematic presentation of cellular targets of antimicrobial activities; FQ=fluoroquinolones, β=beta-lactams, MOMP=Major Outer Membrane Protein (source: Antimicrobial resistance and Food Safety; Chin-Yi Chen *et al.* 2015).

Aminoglycosides resistance mechanisms

Aminoglycoside antimicrobials (gentamicin, streptomycin, kanamycin, tobramycin, neomycin, amikacin) act by binding to the A-site of the bacterial 30S subunit of the ribosome [343, 344]. The resistance to aminoglycosides in *Campylobacter* is achieved by reduced uptake of the antimicrobial, mutational modifications of the *16S* rRNA and of ribosomal proteins, and most importantly, by enzymatic modification of the *16S* rRNA [268]. This resistance mechanism is induced by aminoglycoside-modifying enzymes (AMEs) modifying the binding site, therefore reducing the affinity of the antimicrobial for the rRNA A-site [345].

These enzymes are encoded by genes with chromosomic mutations [346] or encoded in conjugative plasmids and transferred (mainly in *C. coli*) via integrons and transposons [237, 304, 344, 347]. There are three types of AMEs: aminoglycoside acetilating enzymes

or acetyl transferases (AACs), nucleotidyltransferase enzymes or adenyl transferases (ANTs), and phosphorylating enzymes or phosphotransferases (APHs) [344].

There are multiple AMEs identified and described within each of the three groups, and all are likewise encoded in multiple genes [348-350], located either in the chromosome or transferred via plasmids.

AACs catalyse the transfer of acetate from acetyl coenzyme A to an amino group on those aminoglycoside molecules containing deoxystreptamine. Since streptomycin and spectinomycin do not contain deoxystreptamine, AAC enzymes do not have effect on these two antimicrobials. ANTs and APHs modify exposed hydroxyl groups of the aminoglycoside by attaching ATP and other nucleotides, or phosphate molecules, respectively [268]. In this case, the active transport of aminoglycosides into the cell is blocked – causing membrane alterations – as soon as these antimicrobials start to be modified by the enzymes. These AMEs are placed near the inner cytoplasmic membrane to easily access acetyl coenzyme A and ATP (Figure 12).

The result of the action of the three enzymes is the production of a 30-O-aminoglycoside phosphotransferase, which is the most common enzyme found in *C. jejuni* and *C. coli* [344]. Some aminoglycosides like kanamycin A can be modified by the three types of enzymes, whereas other antimicrobials are more enzyme-specific.

Specifically, streptomycin binds to the protein 'S12', which plays an important role in the genetic structure of the decoding site in the *30S* ribosomal subunit of the bacteria [351], in a way that a single aminoacid change in the structure of 'S12' is enough to confer high level of AMR to streptomycin, but gentamicin is not affected by this change [268]. This mutation is normally encoded in the *rpsL* gene and several rRNA (*rrn*) operons [352] as well as in the 16S rRNA *rrs* gene in the chromosome.

Resistance to kanamycin in *Campylobacter* is normally mediated by a conjugative plasmid also encoding resistance to tetracycline [353].

Macrolides resistance mechanisms

AMR to macrolides (erythromycin, clarithromycin, azithromycin) in *Campylobacter* is mediated by modification of the antimicrobial target in the 23S rRNA subunit of the ribosome [354-356], by modification of the ribosomal proteins L4 and L22 [357] and by efflux pump systems [358, 359].

Erythromycin produces conformational changes in the 50S ribosomal subunit, binding at positions 2058 and 2059 [360], thus inhibiting the bacterial RNA-dependent protein synthesis [361]. Resistance to this antimicrobial is produced by methylation of the antimicrobial target in the 23S rRNA subunit of the ribosome by erythromycin-inducible plasmid-encoded enzymes, called ribosome methyl transferases (RMT) or ribosome methylases (RmtB, ArmA) [329, 362]. These enzymes lead to a reduced binding of erythromycin to the ribosome, which in turn translates into cross-resistance to other macrolides, lincosamides (clindamycin) and streptogramins [344] in a phenomenon called 'disociated resistance' [268].

These enzymes are sometimes encoded in ribosomal RNA methylase *erm(B)* genes [291, 308, 355, 363, 364] which on many occasions appear along other ARGs in MDR isolates [308]. These *erm(B)* genes normally confer very high levels of erythromycin resistance (MIC>=512 mg/L), and this resistance can be easily transferred via plasmids.

The chromosome of *Campylobacter* contains three *23S rRNA* gene copies [255], and ERY-resistant (ERY-R) strains commonly have mutations in all three copies. Some authors argue that this is necessary to confer resistance to erythromycin [360], while some others did not find differences in resistance levels when one or more wild-type alleles were not mutated [353].

Base substitutions at positions 2074 and 2075 of the adenine residues in all three copies of the *23S rRNA* gene (*rrnB* operon) in the chromosome of *Campylobacter* are the most common point mutations conveying erythromycin AMR [365]. Although mutation rates for the acquisition of erythromycin resistance are low [237], A2074C, A2074G, and A2075G mutations confer high levels of erythromycin resistance (MIC > 128 mg/L) in both *C. coli* and *C. jejuni*, and the A2074T mutation has been associated with very high levels of erythromycin resistance (MIC >= 512 mg/L) [366].

AMR to erythromycin in *Campylobacter* is also caused by modifications (mutations, insertions, deletions) of the genes encoding the L4 and L22 ribosomal proteins [367-370], but their exact role remains to be elucidated.

Up to eight different efflux pump systems have been identified in *Campylobacter* [344], although some are implicated in functions other than antimicrobial resistance, for example, in pathogenesis [371]. The *CmeABC* multidrug efflux pump is the most relevant efflux mechanism responsible for AMR to several antimicrobials in bacteria [372], including macrolides and fluoroquinolones [239, 329, 373]. It consists of a periplasmic

fusion protein, an inner membrane drug transporter and an outer membrane protein, encoded by the operon genes *cmeA*, *cmeB* and *cmeC*, respectively [344]. The pump is negatively regulated by the CmeR regulator [374] and plays a very important role in intrinsic and acquired AMR in *Campylobacter* and its inactivation leads to increased susceptibility to different antimicrobials [375]. Some variants of the *CmeABC* efflux pump have been reported, such as the resistance-enhanced variant *RE-CmeABC* [376], in addition to other putative efflux pumps in *C. jejuni* [377].

The *CmeABC* efflux pump has been proved to work synergystically with specific mutations [378, 379], conferring high macrolide AMR levels [380]. In isolates with high erythromycin resistance levels (MIC>128 mg/L) and harbouring *23S rRNA* gene point mutations, the inactivation of the *CmeABC* efflux pump reduced erythromycin resistance levels, whereas in isolates with low erythromycin resistance levels (MIC=8-16mg/L) without *23S rRNA* gene point mutations, the inactivation of the *CmeABC* efflux pump brings back the susceptibility to erythromycin, suggesting the involvement of this efflux system in resistance of *Campylobacter* [381]. The *CmeABC* efflux pump acts in a synergic manner with L4 and L22 ribosomal gene mutations to confer AMR to erythromycin in thermotolerant *Campylobacter* [382].

The European Food Safety Authority (EFSA) recommends investigating the molecular mechanisms of macrolide resistance, especially in isolates resistant to high erythromycin resistance levels, with the purpose of detecting concomitant chromosomal *23S rRNA* gene point mutations and/or the presence of transferable *erm(B)* genes [383]. The same guidelines recommend searching for ERY-R genes, not only in resistant isolates presenting phenotypic co-resistance to aminoglycosides or a MDR phenotype [384, 385] (Figure 13) but also in susceptible isolates [383].

Furthermore, since *C. coli* is as prevalent as *C. jejuni* in broilers in some countries [344], monitoring of *C. coli* and *C. jejuni* AMR levels, particularly to macrolides and aminoglycosides, is equally relevant and important [383, 386]. Additionally, *C. coli* may contain and transfer resistance genes that could be exchanged with *C. jejuni*, further justifying the monitoring of AMR levels in *C. coli* [387, 388].

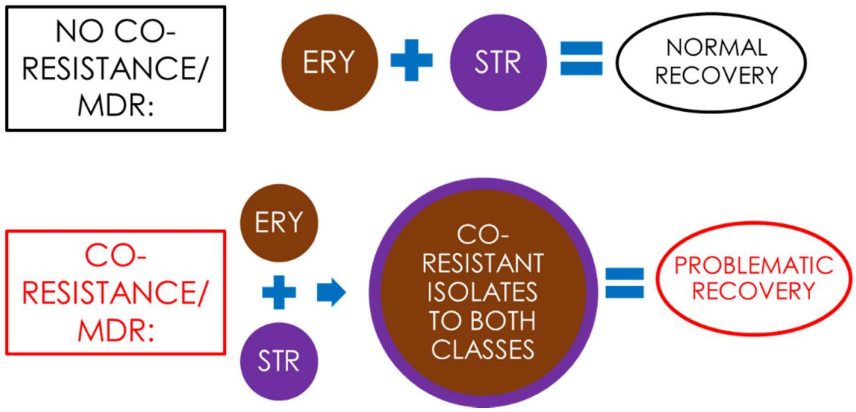


Figure 13. Graphical scheme showing possible recovery prospects in patients with and without co-resistance/MDR to aminoglycosides (STR) and macrolides (ERY).

Quinolones resistance mechanisms

In *Campylobacter* AMR to quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin) is acquired by inhibition of bacterial DNA synthesis [389], by reduction of the outer membrane permeability, and by an efflux pump system [375]. The quinolones cause inhibition of the synthesis of bacterial DNA by targeting two large enzymes, the DNA gyrase (as primary target) and the topoisomerase IV (as secondary target) [390]. The AMR level conferred by changes on the DNA encoding the gyrase enzyme is increased when the topoisomerase IV is also targeted [237].

The enzymatic activity of the DNA gyrase and topoisomerase IV is associated with their respective subunits, GyrA-GyrB and ParC-ParE. AMR to fluoroquinolones arises primarily from mutations in the *gyrA* gene, which result in amino acid substitutions within the quinolone resistance-determining region (QRDR) of the DNA gyrase. Similar mutations in the QRDR of the *parC* gene, encoding a subunit of topoisomerase IV, can also contribute to resistance [391].

The amino-acid substitutions Thr86Ile, Asp90Asn, Thr86Lys, Thr86Ala, Thr86Val and Asp90Tyr have all been described in *Campylobacter*, but the most commonly observed substitution is the Thr86Ile, resulting from the C257T mutation, which translates into high AMR levels [392] and only one mutation is enough to confer phenotypic resistance to quinolones. The Thr86Ala substitution is responsible for high AMR to nalidixic acid and low AMR to ciprofloxacin [377]. Other less common and less relevant reported *gyrA* mutations include T86K, A70T and D90N.

In addition to mutations, resistance to quinolones in thermotolerant *Campylobacter* is also confered by *qnr* genes [393], which can be transferred between bacteria via plasmids. As in the case for macrolides, the *CmeABC* multidrug efflux pump works in synergy with *gyrA* mutations to confer fluoroquinolone resistance, but as opposed to macrolides, a blockage of the efflux pump in the presence of *gyrA* mutations results in a reduction in ciprofloxacin AMR to the levels of susceptible strains [394]. Fluoroquinolone AMR can impose a fitness cost in *Campylobacter* in certain conditions [330].

Tetracyclines resistance mechanisms

AMR to tetracycline in *Campylobacter* is conferred mainly by the *tet(O)* gene [395, 396] but several other tetracycline AMR genes encoding an efflux pump, ribosomal protection, enzymatic inactivation and a membrane-associated efflux system have been described to date [397]. Once tetracycline passes through the cell membrane it binds to the A site on the 30S ribosomal subunit, inhibiting peptide elongation, interfering with the transport system causing accumulation of the antimicrobial [268]. To prevent this accumulation, the *tet(O)* gene encodes for ribosomal protection proteins (RPPs) that bind to the A site on the ribosome, hence displacing the antimicrobial.

The *tet(O)* gene is commonly located either on a self-transmissible plasmid [398], or is transposon mediated [380], although a chromosomic location of this gene has also been reported [399]. The *tet(O)* gene has been shown to confer very high AMR levels to tetracycline (512mg/L) [353]. Apart from plasmids or transposons, the presence of other MGEs like insertion sequences alongside tetracycline AMR genes have also been described in *Campylobacter* [397].

Resistance mechanisms to other antimicrobial classes

Most thermotolerant *Campylobacter* strains are resistant to many betalactam antimicrobials (penicillin, oxacillin, amoxicillin, ampicillin, carbpenicillin, carbapenems, monobactams and cephalosporins) [344, 400] and there are several mechanisms inducing this resistance, including: direct disruption of the peptidoglycan cross linkings during bacterial cell wall formation due to a big variety of betalactamases, membrane permeability alterations encoded on plasmidic genes [344] and *CmeABC* efflux pump systems [373, 401]. AMR levels to betalactam antimicrobials have been increasing since

the 1990s when *Campylobacter* spp. was susceptible to amoxicillin/ampicillin to the current situation where there are myriads of beta-lactamases reported and scarce compared effectivity from only a few drugs [402]. Beta-lactamase genes associated with ampicillin resistance, such as OXA-61 [238], have been demonstrated to be acquired by HGT via plasmids or via other MGEs [403].

Among the betalactams, carbapenems are especially relevant because resistance levels to this antibiotic keep increasing, to the point that in 2020 ertapenem was included in the official panel of AMR monitoring in the EU [404]. Carbapenems are used in clinical cases of campylobacteriosis not responding to macrolides, and the mechanism of resistance to this antibiotic remains unclear, but is likely to originate as a consequence of prolonged selective pressures [405], and hence resistance to this drug in humans is worrisome.

The antimicrobial chloramphenicol inhibits protein biosynthesis in bacteria by binding in a reversible manner to the *50S* ribosomal subunit. Chloramphenicol resistance in *Campylobacter* is conferred by a plasmidic gene called *cat* that encodes for an acetyl transferase enzyme that modifies the antimicrobial molecule [406]. Resistance to this antimicrobial in *Campylobacter* is rare in the EU since it was banned in 1994 in food-producing animals due to its toxicity [407], however, it keeps being reported as it develops cross-resistance with florfenicol, which is still used in the EU. Resistance to chloramphenicol is also reported in countries where this antimicrobial is still used [408].

Sulphonamide resistance in *C. jejuni* is caused by an interruption of the folic acid metabolism of the bacteria, and it is either due to a chromosomally encoded mutational substitution of amino-acid residues in the enzyme dihydropteroate synthetase (DHPS) or due to plasmid borne genes that encode alternative drug-resistant variants of the enzyme [409].

1.8.2. Protocols for determination of antimicrobial resistance

There are two main protocols generally used for antimicrobial susceptibility testing (AST): the two-fold broth micro-dilution reference method and the disk diffusion technique.

The two-fold broth micro-dilution method allows the determination of the minimum inhibitory concentration (MIC) for a given antimicrobial, which is the lowest concentration of an antimicrobial agent that inhibits the appearance of visible growth of an isolated bacteria. Bacterial strains are tested for their ability to produce visible growth using serial dilutions of the antimicrobial agent [410].

The disk diffusion method uses an agar plate where a bacterial suspension is streaked and different antimicrobial disks are added on. The inhibition zone diametre (IZD) is identified measuring the zone that inhibits the growth of the inoculated bacteria around each disk, which represents the level of susceptibility of a particular bacteria to a specific antimicrobial, so the larger the diameter, the more susceptible the bacteria is to the antimicrobial tested [411].

A microorganism that is likely to be inhibited by an antimicrobial at concentrations achievable in human or animal tissues at standard dosing is referred to as 'suceptible' and if it is not likely to be inhibited in those same conditions is referred to as 'non susceptible'. The cut-off point is determined by clinical breakpoints, which are established based on pharmacokinetics and pharmacodynamics of the antimicrobial drug, clinical efficacy and epidemiological data, and intermediate categories may also be considered. A microorganism is classified as 'wild type' (WT) if it does not have any resistance (mutational or acquired by HGT) to an antimicrobial and if that resistance exists then the microrganism is termed 'non wild type' (NWT). In this case the cut-off point is determined by epidemiological cut-off values (ECOFFs) where WT bacteria exhibit MICs ≤ ECOFF. The ECOFFs do not account for pharmacokinetics or pharmacodynamics of the drug or clinical outcomes, focusing purely on the presence or absence of resistance mechanisms [412]. WT and NWT isolates are from here on in this thesis referred to as 'susceptible' and 'resistant', respectively.

These concepts follow the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; https://www.eucast.org; last accessed 8 August 2024). Additional guidance is available at the 'Clinical and Laboratory Standards Institute' (CLSI) (https://clsi.org; last accessed 10 August 2024) in the US, the 'Comité de l'Antibiogramme de la Société Française de Microbiologie' (CA-SFM) (www.sfm-microbiologie.org/presentation-de-la-sfm/sections-et-groupes-de-travail/comite-de-lantibiogramme; last accessed 10 August 2024) in France and the 'British Society for Antimicrobial Chemotherapy' (BSAC) (https://bsac.org.uk; last accessed 10 August 2024) in the UK.

1.8.3. Antimicrobial resistance surveillance in C. *coli* and C. *jejuni*

Antimicrobial resistance surveillance in the EU

Antimicrobial resistance monitoring in animals and food from animal origin in EU countries is enforced by an EU Decision from 2013 [413] recently amended in another Decision in 2020 [404]. Although the monitoring of antimicrobial resistance for *Campylobacter* in animals and food is implemented on a national level, it is also internationally supervised by EFSA and published annually in a report that also includes data from human reported cases [35]. The AMR surveillance in humans is not an active process like in animals, but rather a passive analysis of the cases presented in primary care or in hospitals.

This European decision specifies that for both *C. coli* and *C. jejuni*, surveillance samples of caecal content from broilers, pigs, turkeys and cattle under one year should be taken at slaughterhouse (turkeys and cattle are sampled only in countries producing more than 10,000 tonnes per year). In addition, samples from meat and meat products of all four animal species should be collected at retail and border inspection posts. In terms of frequency, samples of pigs and cattle and their products should be carried out in odd years and poultry samples (broilers and turkeys) in even years.

Commission Decision 2013/652/EU listed the following six antimicrobials to be monitored in *Campylobacter* surveillance programmes: ciprofloxacin (CIP), tetracycline (TET), nalidixic acid (NAL), streptomycin (STR), erythromycin (ERY) and gentamicin (GEN). Moreover, Commission Decision 2020/1729/EU included some changes with regards to the antimicrobials monitored (where nalidixic acid and streptomycin were replaced by chloramphenicol and ertapenem).

Spain was one of the pioneers in the surveillance and monitoring of AMR in animals as it started in 1996 (the same year as the Denmark DANMAP and the US NARMS programmes) with the creation of the VAV network ('Red de Vigilancia de Resistencias Antibióticas en Bacterias de Origen Veterinario') [414], including healthy and sick animals, and food.

In addition, Spain implemented a 'National plan for control of AMR' in animals and humans (PRAN: https://www.resistenciaantibioticos.es/es) in 2014 with a One Health strategy that also included *Campylobacter* among its targets, and coordinated by the 'Agencia Española de Medicamentos y Productos Sanitarios' (AEMPS).

Antimicrobial resistance surveillance outside Europe

At the global level, the 68th World Health Assembly endorsed the Global Action Plan (GAP) on antimicrobial resistance in 2015, with five objectives: improve awareness through education and training, increase intelligence through surveillance and research, prevent infections and improve sanitation, optimize antimicrobial use and develop the economic case for sustainable investment (WHO, 2015). Most antimicrobial surveillance programs implemented in the world include thermotolerant *Campylobacter*.

The 'US National Action Plan' on antimicrobial resistance in the United States aims at accelerating the country's response to antimicrobial resistance, improving the health of its citizens. This plan is implemented through the 'National Antimicrobial Resistance Monitoring System for Enteric Bacteria' (NARMS) surveillance system (https://www.cdc.gov/narms/index.html), last accessed 09 September 2024), whose aim is to track changes in antimicrobial susceptibility of enteric bacteria from sick people (managed by the Centers for Disease Control and Prevention - CDC), retail meats (managed by the Food and Drugs Administration - FDA) and food animals (managed by the United States Department of Agriculture - USDA).

In Canada, the 'Canadian Antimicrobial resistance Surveillance System' (CARSS), implements the 'Canadian Integrated Program for Antimicrobial Resistance Surveillance' (CIPARS) (https://www.canada.ca/en/public-health/services/surveillance/canadian-integrated-program-antimicrobial-resistance-surveillance-cipars.html, accessed 12 Feb 2023) oversaw by the Public Health Agency of Canada (PHAC), collecting, analyzing and communicating trends in antimicrobial use and resistance from humans, animals, and retail meat across the country.

In Asia, there are national AMR monitoring programmes implemented in various countries such as Nepal (through their National Public Health Laboratory), the Republic of Korea (through the Korea Centers for Disease Control and Prevention), China (National Action Plan administered by their National Health and Family Planning Commission and China CDC) and Japan (National Action Plan implemented by the Clinical Laboratory Division of the Japan Nosocomial Infections Surveillance and the Ministry of Agriculture (MAFF)). In Oceania, AMR monitoring are managed by several key agencies and programmes, including the Australian Government and New Zealand's Ministry of Health and Ministry of Primary Industries.

The African Union established the Africa Centres for Disease Control and Prevention (Africa CDC) in 2017, in charge of supervising, among others, the AMR situation in the continent, along with the South African National Institute for Communicable Diseases (NICD), the Kenya Medical Research Institute (KEMRI), the Institute Pasteur at Dakar, the Uganda National Health Laboratory Services (UNHLS) and the National Public Health Institutes (NPHIs).

In Central and South America, several agencies and institutions are in charge of monitoring AMR, often under the auspices of the Pan-American Health Organization (PAHO), such as the Agência Nacional de Vigilância Sanitária (ANVISA) in Brazil, the Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) in Argentina, the Instituto Nacional de Salud (INS) in Colombia, the Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) in Mexico, the Instituto de Salud Pública (ISP) in Chile and the Instituto Nacional de Salud del Perú (INS) in Perú.

Approaches to analysis of AMR data

Traditionally AMR data has been analyzed separately for each antimicrobial, reporting the total number of isolates resistant to a given antimicrobial out of the total number of isolates tested. An alternative way is to consider the data of multiple antimicrobials at the bacterial isolate level, allowing for a more in-depth analysis of resistance including occurrence of co-resistance and multi-drug resistance (MDR) involving different antimicrobial classes, thus potentially leading to hypothesis on the distribution of specific resistance mechanisms or the potential clonal spreading of bacterial strains. The study of isolate-based phenotypic data *versus* aggregated data has proven to be a reliable means of gaining insight into such mechanisms [415, 416] while guiding the genetic analysis in a 'top-down' approach [1, 417, 418].

AMR situation in the EU and outside the EU

In the EU, EFSA reports the proportion of *Campylobacter* AMR isolates in the following categories: extremely low (<2%), very low (2-5%), low (5-25%), medium (25-30%), high (30-50%), very high (50-80%) and extremely high (>80%) [35, 383]. According to EFSA's reports on 'Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food' for the period 2020-22, *Campylobacter* AMR levels were overall higher in

animals than humans, and higher in *C. coli* than in *C. jejuni* [35, 419] (Table 2). Resistance levels to ciprofloxacin in both *C. coli* and *C. jejuni* were similar in animals (poultry, pigs and calves; 50-80%) and humans (60-70%). Nalidixic acid resistance levels in both *C. coli* and *C. jejuni* were high to very high in pigs and calves (50-80%) and very high in poultry (60-70%). In 2019, tetracycline resistance levels were very high in all species (including humans), particularly in *C. coli* in cattle (94%), and that finding was observed in *C. coli* in turkeys (89%) in 2020. Erythromycin resistance levels were higher in *C. coli* (humans, pigs, poultry) (4-25%) than in *C. jejuni* (humans, poultry, calves) (<2%), except for Spain (14.2%) (Table 2).

In broiler meat, AMR levels to tetracycline and nalidixic acid were high to very high (40-70%), and resistance to gentamicin was not observed in *C. jejuni*. With regards to erythromycin and streptomycin, the AMR levels detected ranged from extremely low to low (0-11%) and low (7-24%), respectively [388, 419] (Table 2).

In both North American and Asian countries, *C. coli* isolates exhibited higher levels of resistance to all antimicrobials than *C. jejuni*, except for tetracycline in humans in Canada and ciprofloxacin, tetracycline and gentamicin in broilers in the Republic of Korea (Table 3). Resistance levels to all antimicrobials were lower in the United States and Canada than in Asian countries, particularly for quinolones (ciprofloxacin/nalidixic acid) and erythromycin. Moreover, resistance to erythromycin in both bacterial species was higher for humans and pigs, especially in China and Nepal (Table 3).

Table 2. Humans, animals and food AMR occurrence (%) in thermotolerant *Campylobacter* in the EU for the period 2019-21 (EFSA-ECDC, 2022, 2023).

Hosts	Bacteria	CIP	NAL	TET	ERY	GEN	STR
Humans	C. *coli*	60-70%		Very high: 70%	8.5%	2.4%	
	C. *jejuni*	64.5%		High: 45%	1.1% (Spain: 14.2%)	0.7%	
Animals - broilers	C. *coli*	50-80%	60-70%	High-very high	4.4%		11.4-16.5%
	C. *jejuni*	50-80%	60-70%	High-very high	0.8-1.7%		11.4-16.5%
Animals - turkeys	C. *coli*	50-80%	60-80%	Extremely high: 89%	21.5%		11.4-16.5%
	C. *jejuni*	50-80%	60-70%	High-very high	0.8-1.7%		11.4-16.5%
Animals - calves	C. *coli*	50-80%	50-80%	Extremely high: 90-94%	35.7%	12.4%	65%
	C. *jejuni*	50-80%	50-80%	High-very high	0.8-1.7%	0.5%	
Animals - pigs	C. *coli*	50-80%	50-80%	High-very high	12.3%	2.6%	70%
	C. *jejuni*	50-80%	50-80%	High-very high	0.8-1.7%	1.7%	
Food	C. *coli*	52-100%	52-100%	43-100%	40%	2.6%	18%
	C. *jejuni*	52-100%	52-100%	43-100%	12.5%		24%

Table 3. AMR occurrence (%) in thermotolerant *Campylobacter* in selected North American and Asian countries (* latest year retrieved).

Country	Hosts	Bacteria	Period*	Humans	Broilers	Turkeys	Cattle	Pigs	References
United States	Humans	*C. coli*	2019 2020	ERY(8%) CIP(45%)					NARMS, 2019
		C. jejuni	2019 2020	ERY(3%) CIP(34%)					
	Animals	*C. coli*	2019 2020		ERY (4%)		CIP (62%)	ERY (26%)	
		C. jejuni	2019-2020		CIP(26%) ERY(3%)				
Canada	Humans	*C. coli*	2020	ERY(13.2%) CIP(15.5%)					Otto, 2020; Inglis, 2021
		C. jejuni	2020	ERY(0.5%) CIP(9.4%) TET(65%)					
	Animals	*C. coli*	2020		CIP (29%)	TET (50%)			Inglis, 2006; Varga, 2019: CIPARS, 2020
		C. jejuni	2020		CIP(29%) TET(75%)	TET (100%)			
Nepal	Humans	Thermotolerant *Campylobacter*	2019	CIP-NAL(100%) ERY(73%) GEN-TET(50%)					Acharia & Wilson, 2019
	Animals	Thermotolerant *Campylobacter*	2012 2013					ERY(93%) TET(61%) CIP(31%) GEN(6%)	Ghimire, 2014
China	Humans	*C. coli*	2019	CIP(100%) TET(96%) GEN(60%) ERY(60%)					Gao, 2023
		C. jejuni	2019	CIP(96%) TET(90%) GEN(6.5%) ERY(2.5%)					

JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

2. JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

2.1. Justification of the work

There is a debate in the scientific community over to what extent the phenotyping of microorganisms is able to sufficiently characterize them, thus adequately informing policy makers on their decision making or if, on the other hand, genotyping techniques are needed to guide those decisions in spite of the costs associated with these approaches.

2.2. Hypothesis

The overall hypothesis of this PhD is that the phenotypic analysis of antimicrobial resistance data from thermotolerant *Campylobacter* (*Campylobacter coli* and *Campylobacter jejuni*) obtained through national monitoring programmes on livestock allows for the detection and characterization of predominant and/or emergent strains carrying antimicrobial resistance determinants, with a focus on aminoglycosides and macrolides. In order to test this hypothesis, the following objectives were formulated, taking into consideration the assessment of the possible role of the different hosts on the distribution of thermotolerant *Campylobacter* strains with emerging resistance patterns.

2.3. Objectives

2.3.1. General Objective 1

To analyze data from the AMR monitoring programme on *Campylobacter* spp. from livestock in Spain including frequency of isolation, resistance phenotypes and levels of resistance with two Specific Objectives:

Specific Objective 1

To evaluate the baseline level of the phenotypic resistance to several antimicrobials (with a focus on aminoglycosides and macrolides) in thermotolerant *Campylobacter* isolates retrieved from food animals (broilers, turkeys, pigs and cattle) between 2002 and 2018, considering their spatial and temporal distribution.

Specific Objective 2

To assess the association between the occurrence of phenotypic resistance to antimicrobials in the aminoglycoside and macrolide antimicrobial classes and its possible relationship with genetic variants as determined by PCR-based typing techniques.

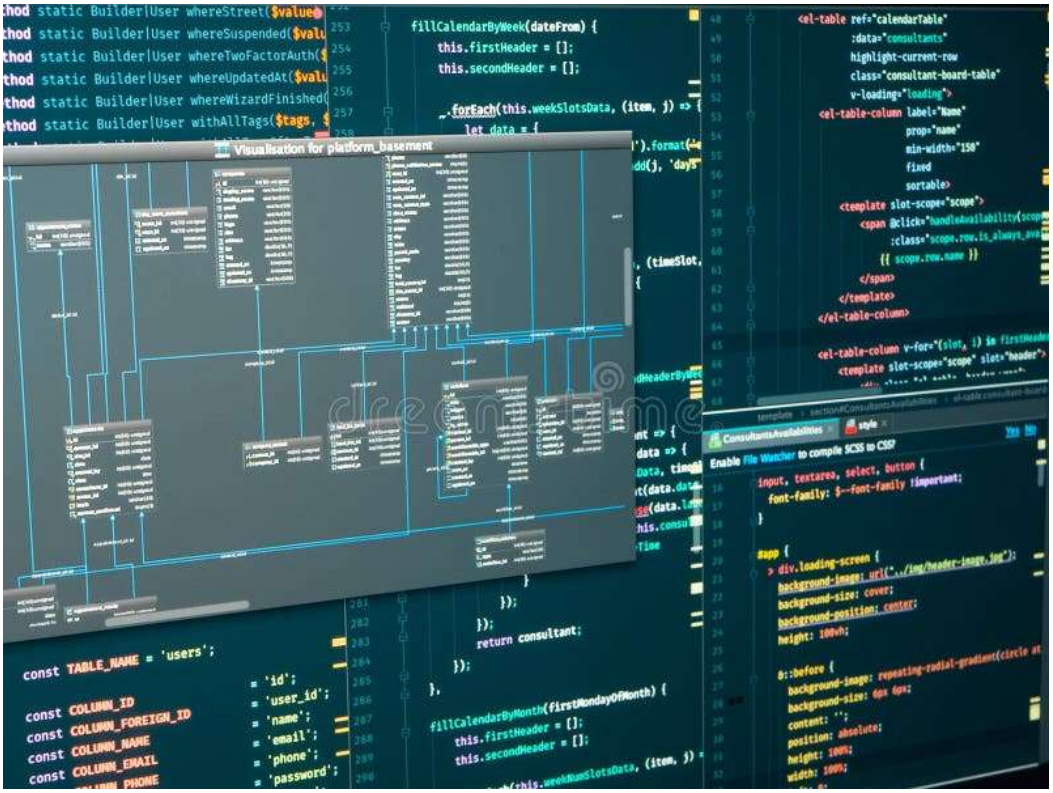
2.3.2. General Objective 2

To perform a complete genetic characterization of a subset of the thermotolerant *Campylobacter* isolates obtained through the AMR monitoring programme in livestock with the following Specific Objective:

Specific Objective 3

To elucidate the genetic mechanisms implicated in the predominant resistance phenotypes observed in food animals in Spain with a focus on aminoglycosides and macrolides, and to identify resistance determinants and mobile genetic elements that could favor their dispersion.

MATERIAL AND METHODS



"Each protein sequence that is established, each evolutionary mechanism that is illuminated, each major innovation in phylogenetic history that is revealed will improve our understanding of the history of life."

Margaret Oakley Dayhoff

Physical chemist, often referred to as the "mother of bioinformatics"

3. MATERIAL AND METHODS

3.1. Material

The material used in this thesis was obtained from three sources:

- The original database with the culture results and antimicrobial susceptibility testing (AST) data from 50,939 livestock samples analyzed for the detection of *Campylobacter* spp. in Spain from 2002 to 2018. This database was the main data source for the study and analyses carried out to answer Specific Objective 1.

- A collection of *Campylobacter* spp. isolates from the same period re-cultured from storage at -80°C and used for Sanger and WGS sequencing and posterior epidemiological and bioinformatic analyses. The information obtained from the analyses of these isolates was used to answer Specific Objectives 2 and 3.

- The raw reads (.FASTQ format) of isolates subjected to whole genome sequencing and readily available for analysis

3.1.1. *Campylobacter* spp. main original database

All the isolates whose information was used to create this database were retrieved from 2002 to 2018 through the National Veterinary AMR Surveillance Programme in Spain (VAV network, *Red de Vigilancia Veterinaria de Resistencia a Antimicrobianos*) coordinated by the Spanish Ministry of Agriculture, Fisheries and Food (MAPA, *Ministerio de Agricultura, Pesca y Alimentación*). According to EU legislation, poultry (broilers and turkeys), pigs and cattle samples were analyzed for *Campylobacter* spp. detection and all the isolates obtained were kept at frozen storage at -80°C in VISAVET Health Surveillance Centre.

Although the sampling, initial culture and AST testing were not conducted as part of this thesis, these protocols are explained to provide context on how the isolates whose

data/metadata were used to conform the main database used in Specific Objective 1 (Figure 14) were retrieved.

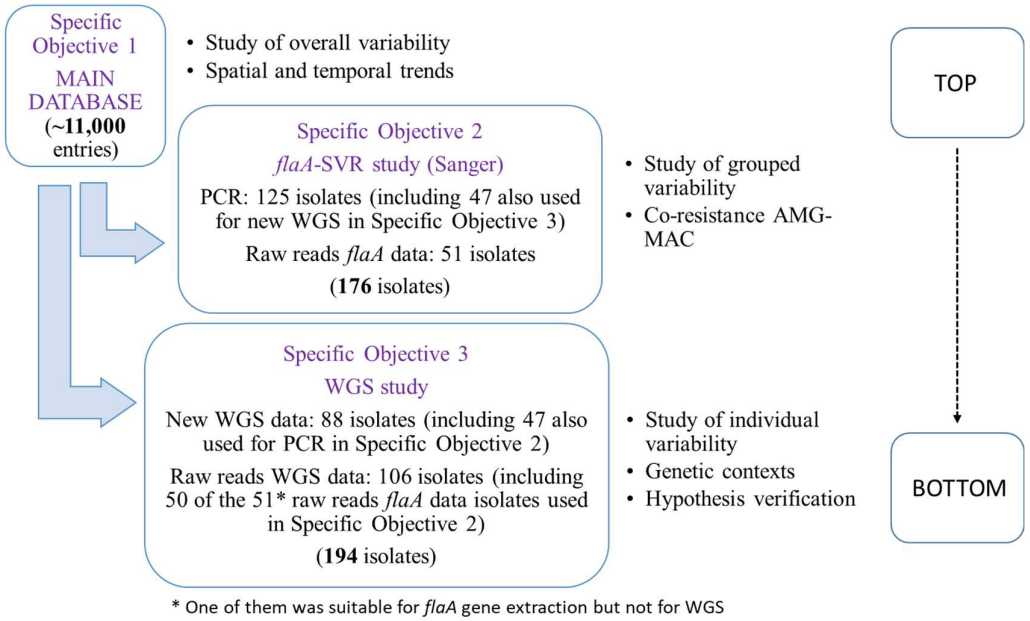


Figure 14. Summary of material and methodology used in the three Specific Objectives of this thesis.

Samples from each animal species originating from different farms were collected at slaughterhouses covering 60% of the national throughput (Table 4).

Broiler samples were retrieved every year from 2002 to 2014 and every two years thereafter, turkey samples were collected every two years from 2014 to 2018, pig samples were collected every year from 2002 to 2013 and every two years thereafter, and cattle samples were collected every year from 2007 to 2013 and every two years thereafter. Altogether, from 2014 onwards broiler and turkey samples were collected in even years and pig and cattle samples in odd years.

The number of farms sampled per year ranged between 76 and 500 (mean=228) for broilers, between 467 and 500 (mean=485) for turkeys, between 171 and 384 (mean=268) for pigs, and between 163 and 384 (mean=261) for cattle (Table 4).

Table 4. VAV Network sampling plan for *Campylobacter* spp. (samples/farms/abattoirs) from pigs, broilers, cattle and turkeys (2002-2018) (AST = Antimicrobial Susceptibility Testing) (one farm = one pool).

Year	Host species	Sampling Period (No. of months sampled/year)	No. of abattoirs	Average No. of farms/ abattoir	No. of individual samples	Average No. of farms/ month-year	No. of samples/farm (No. of samples/pool)	No. of farms (No. of pools)	No. of isolates (C. spp)	No. of AST isolates (C. coli/ C. jejuni)
2002	Pigs	Sep-Dec (4)	10	29	580	72.5	2	290	97	94
	Broilers	Feb-Jul (6)	4	21.2	170	14.2	3	85	33	29
2003	Pigs	Jan-Nov (11)	10	31.9	638	29	2	319	125	113
	Broilers	Mar-Oct (8)	9	17.8	480	20	3	160	42	32
2004	Pigs	Feb-Nov (10)	8	22.9	366	18.3	2	183	116	87
	Broilers	Feb-Jun (5)	8	19.9	477	31.8	3	159	52	33
2005	Pigs	Feb-Dec (11)	8	24.4	390	17.7	2	195	156	140
	Broilers	Nov-Dec (2)	6	12.7	228	38	3	76	36	31
2006	Pigs	Mar-Sep (7)	8	24.5	392	28	2	196	145	100
	Broilers	May-Sep (5)	6	16.3	294	19.6	3	98	49	46
2007	Pigs	Mar-Sep (7)	8	38.7	620	44.3	2	310	165	143
	Broilers	May-Nov (7)	7	12.7	267	12.7	3	89	41	40
	Cattle	Jun-Nov (6)	8	20.4	326	27.2	2	163	75	69
2008	Pigs	Mar-Dec (10)	7	24.4	342	17.1	2	171	112	93
	Broilers	Jun-Nov (6)	7	16.1	339	18.8	3	113	69	69
	Cattle	Jun-Oct (5)	8	21	334	33.6	2	168	63	61
2009	Pigs	Apr-Dec (9)	11	25.8	568	31.5	2	284	192	167
	Broilers	Apr-Dec (9)	9	22	594	22	3	198	118	115
	Cattle	Apr-Dec (9)	10	25.8	516	28.7	2	258	107	105
2010	Pigs	May-Nov (7)	13	16.5	428	30.6	2	214	135	105
	Broilers	May-Nov (7)	13	15.5	2,020	28.8	10	202	131	127
	Cattle	May-Nov (7)	13	15.4	400	28.6	2	200	132	130

Table 4 (continued). VAV Network sampling plan for *Campylobacter* spp. (samples/farms/abattoirs) from pigs, broilers, cattle and turkeys (2002-2018) (AST = Antimicrobial Susceptibility Testing) (one farm = one pool).

Year	Host species	Sampling Period (No. of months sampled/year)	No. of abattoirs	Average No. of farms/ abattoir	No. of individual samples	Average No. of farms/ month-year	No. of samples/farm (No. of samples/pool)	No. of farms (No. of pools)	No. of isolates (*C. spp*)	No. of AST isolates (*C. coli*/ *C. jejuni*)
2011	Pigs	May-Dec (8)	15	17.1	514	32.1	2	257	167	129
	Broilers	May-Dec (8)	15	15.8	2,370	29.6	10	237	162	156
	Cattle	May-Dec (8)	15	15.9	478	29.9	2	239	142	132
2012	Pigs	Jun-Oct (5)	17	9.6	326	32.6	2	236	163	73
	Broilers	Jun-Oct (5)	15	10.2	1,530	30.6	10	239	182	86
	Cattle	Jun-Oct (5)	16	9.1	292	29.2	2	221	151	75
2013	Pigs	Apr-Oct (7)	19	12.1	460	32.8	2	338	252	108
	Broilers	Apr-Oct (7)	15	15.3	2,300	32.8	10	370	284	140
	Cattle	Apr-Oct (7)	19	12.2	464	33.1	2	347	232	115
2014	Broilers	Mar-Oct (8)	15	33.3	5,000	62.5	10	500	268	170
	Turkeys	Mar-Oct (8)	7	71.4	5,000	62.5	10	500	370	170
2015	Pigs	May-Nov (7)	20	18.6	746	53.3	2	373	193	170
	Cattle	May-Nov (7)	17	21.6	734	52.4	2	367	136	120
2016	Broilers	Apr-Nov (8)	17	25.1	4,270	53.4	10	427	186	162
	Turkeys	Apr-Dec (9)	6	81.3	4,880	54.2	10	488	204	170
2017	Pigs	Jan-Dec (12)	19	20.2	768	32	2	384	200	170
	Cattle	Jan-Dec (12)	24	16	768	32	2	384	228	170
2018	Broilers	Jan-Dec (12)	21	21.9	4,600	38.3	10	460	276	170
	Turkeys	Jan-Dec (12)	5	93.4	4,670	38.9	10	467	401	170

Samples from poultry (broilers and turkeys) consisted of sections of the caecum of 10 animals belonging to the same farm, which were pooled in the lab in order to obtain a single sample. Samples from pigs and cattle consisted of a pool of faeces (caecum content) of two animals belonging to the same farm obtained and mixed at the abattoir to form a single sample.

Samples from poultry, pigs and cattle were transported refrigerated to the laboratory immediately after collection, and were analyzed within the first 24 hours using direct plating onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, Oxoid, Hampshire, UK) or CampyFood Agar (CFA, bioMérieux, Lyon, France) selective media to isolate *Campylobacter* spp.

A sample was considered positive when at least one *Campylobacter* isolate from that sample was positive. Single colonies with morphology compatible with *Campylobacter* spp. were identified as '*C. coli*', '*C. jejuni*' or '*C.* spp.' using API strips (up to 2010) and a multiplex PCR (from 2010 onwards) [40]. In the 17 years of study period covering this thesis, less than 50 *C. jejuni* were isolated from pigs, thus, this category was excluded from any analyses on this thesis.

With regards to ASTs, the AMR phenotype of the *Campylobacter* spp. isolates was determined using the two-fold broth micro-dilution reference method according to ISO 20776-1:2006 so that the Minimum Inhibitory Concentrations (MICs) for each antimicrobial were obtained. The disk diffusion method was used in some isolates (as explained below) yielding Inhibition Zone Diameters (IZDs).

The AST results for the six antimicrobials listed on AMR surveillance programmes for *Campylobacter* spp. in the EU [413], compiled in the main database, included ciprofloxacin, tetracycline, nalidixic acid, streptomycin, erythromycin and gentamicin.

For erythromycin in broilers, IZDs were used up to 2004 (included) and MICs were used from 2005 onwards. For streptomycin in broilers and pigs, IZDs were used up to 2005 (included) and MICs were used from 2006 onwards.

In 2012 and 2013 there were also data on AST for several antimicrobials other than the six included in the EC compulsory panel but they were not considered because comparisons across the whole study period (2002-2018) would not have been possible.

Quantitative values (MIC/IZD) for all antimicrobials were transformed into qualitative results using the Epidemiological Cut-off Values (ECOFFs) (Table 5) listed on Commission

Decision 2013/652/EU [413] and based on EUCAST guidance (https://www.eucast.org/mic_and_zone_distributions_and_ecoffs; last accessed 20 November 2024).

These ECOFFs were used to differentiate between 'wild type' (WT) and 'non-wild type' (NWT) isolates, named in this thesis as 'susceptible' and 'resistant' isolates, respectively, as explained in the Introduction section.

Table 5. ECOFFs used for interpretation of MICs (source: EUCAST and EC, 2013) and IZDs (source: EUCAST) in *Campylobacter* spp. as of 2019.

Antimicrobial	*C. coli* MIC (mg/L)	*C. coli* IZD (mm)	*C. jejuni* MIC (mg/L)	*C. jejuni* IZD (mm)
Gentamicin (GEN)	>2	ND*	>2	<20
Streptomycin (STR)	>4	<13	>4	<13
Erythromycin (ERY)	>8	<24	>4	<22
Ciprofloxacin (CIP)	>0.5	<26	>0.5	<26
Nalidixic Acid (NAL)	>16	ND	>16	ND
Tetracycline (TET)	>2	<30	>1	<30

* ND: values not yet defined by EUCAST as of 2019 (not available when these data were used)

3.1.2. *Campylobacter* spp. isolates re-cultured for further analyses

A total of 166 isolates kept frozen were recultured for sequencing and further analyses to answer Specific Objective 2 (125 isolates subjected to Sanger sequencing) and 3 (88 isolates subjected to whole genome sequencing) (Appendix V) (Figure 14).

3.1.3. Raw reads from previously sequenced *Campylobacter* spp. (.FASTQ format)

Whole genome sequences of 106 isolates (50 for Specific Objective 2 and 56 for Specific Objective 3) from the main original database were readily available (from previous projects) in .FASTQ format along with their corresponding metadata to be used for both Objective 2 and 3 (Appendix V) (Figure 14).

3.2. Methods

As explained in the Introduction section, the methodology used in this thesis was designed with a 'top to bottom' perspective, starting from the data gathered in the main database and ending with an analysis of individual isolates.

3.2.1. Laboratory methodology

Molecular characterization of *flaA* gene (Sanger) (Specific Objective 2)

A subset of 176 isolates from the main database including all combinations of hosts, bacterial species, year of recovery and AMR phenotype, chosen within each category, was used to assess their genetic relatedness by comparing the flagellin *flaA* SVR gene sequence as described by other authors [420, 421].

This was carried out in two steps. First, all combinations of resistance phenotypes to the three antimicrobials (gentamicin, streptomycin and erythromycin) were identified for both *C. coli* and *C. jejuni*, and thereafter, all isolates were classified into two categories: those with at least simultaneous phenotypic resistance to gentamicin-erythromycin, streptomycin-erythromycin or both (aminoglycoside-macrolide (AMG-MAC) co-resistant group, n=61) and all the rest (non-co-resistant group, n=115) (Table 6). As many isolates as it was possible to grow were selected from each category attending at their phenotypic characteristics, and this ended up with a maximum of 6 and 18 isolates, for the resistant and all-susceptible category, respectively. For 125 out of 176 isolates, the *flaA* gene was determined by PCR and Sanger sequencing, and for the remaining 51 isolates the *flaA* gene was extracted from whole genome sequences readily available in .FASTQ format.

Table 6. Numbers and categories of *Campylobacter* isolates selected for the *flaA*-SVR study.

Resistotype	Bacterial species	Broilers PCR*	Broilers WGS**	Cattle PCR	Cattle WGS	Pigs PCR	Pigs WGS	Turkeys PCR	Turkeys WGS	Total
ERY	*C. coli*	4	1	1		3		6	1	16
ERY	*C. jejuni*	3	2	2				2		9
ERY/STR	*C. coli*	4	2	5		4	1	6		22
ERY/STR	*C. jejuni*			4				2		6
GEN	*C. coli*	1				2		1		4
GEN	*C. jejuni*	2		1						3
GEN/ERY	*C. coli*	1				3		1		5
GEN/ERY/STR	*C. coli*	6	3	3		4		6	1	23
GEN/ERY/STR	*C. jejuni*	1		3			1			5
STR	*C. coli*	6		6	1	3		6		22
STR	*C. jejuni*	3	6	5	4			4		22
SUSCEPTIBLE	*C. coli*	2		2		2		2		8
SUSCEPTIBLE	*C. jejuni*	1	10	1	18			1		31
Total		34	24	33	23	21	2	37	2	176

Resistotype in red font: isolates with co-resistant AMG-MAC phenotype

* PCR: *flaA* gene sequence determined by PCR and Sanger sequencing

** WGS: *flaA* gene sequence extracted from WGS sequences

The 125 isolates from which the *flaA* gene was extracted by PCR and Sanger sequencing were sourced from frozen storage, thawed and recultured initially on Columbia Blood Agar 5% medium (bioMérieux) and for the more fastidiously growing isolates on a selective medium (mCCDA, Oxoid). They were then incubated in microaerophilic conditions on incubator at 37°C during 48h or 42°C during 24h. When the growth of each isolate was evident, an inoculum from a single colony was taken and suspended afterwards in tubes with 600 µl sterile double distilled water. The extraction of DNA was carried out by boiling the tubes with the isolate suspension for 10 minutes at 100°C to lyse the cells, followed by centrifugation at 3,000 rpm for 5 minutes. The amplification of the

*fla*A gene was performed from the supernatant fraction of the boiled isolate extract, which contained the DNA.

Conventional PCR was used for amplification of the short variable region (SVR) of the *flaA* gene following the protocol from Ugarte-Ruiz *et al.* [421], using the primer pair Fla1 (AAGTCCTGTTCCAACTGAAGT) and Fla2 (AATGGTAATGATGCTTTAGGTA). The PCR amplification of the 444-bp long product was performed in 40 µl containing 2.1 µl of lysed cell supernatant, 20 µl of a PCR master mix (QIAGEN Multiplex PCR kit; Qiagen, Hilden, Germany) and 0.19 µmol l^{-1} of each primer (Invitrogen, Life Technologies, Paisley, UK). The amplification protocol was carried out in T100TM and C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) thermocyclers, starting with denaturation for 15 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 90 seconds at 57°C and 1 minute at 72°C, and a final 10 min extension at 72°C [421].

The detection of PCR products (amplicons) was carried out by gel electrophoresis in 2% agarose gels (Biotools MB agarose) containing 10 mg/ml of SYBR green stain (Invitrogen, Life Technologies) for detection, running at 70 mV and 400 mA for 20 min. A DNA molecular weight marker (100-bp low ladder; Biotools, B&M Labs, Madrid, Spain) was included for reference. The *flaA* bands were visualized under UV light and the gel images were taken with a UV Bio-Rad Molecular Imager (Bio-Rad).

All *flaA*-derived amplicons were purified using ExoProStar PCR clean up technology (Cytiva, Danaher, Washington DC, USA). The purified amplicons were sequenced by Stabvida (Caparica, Portugal). Allele numbers were assigned by sequence comparisons against the existing *flaA*-SVR public sequences deposited on the *Campylobacter* PubMLST database (https://pubmlst.org/organisms/campylobacter-jejunicoli/; last accessed 21 November 2024), hosted at the University of Oxford.

Characterization of AMR determinants (WGS) (Specific Objective 3)

The 194 isolates included in the study to answer Specific Objective 3 were selected to include both susceptible and resistant phenotypes to aminoglycosides (gentamicin, streptomycin) and macrolides (erythromycin), ensuring inclusion of the seven possible bacterial/host species combinations (*C. coli* from broilers, turkeys, pigs and cattle and *C. jejuni* from broilers, turkeys and cattle) (Table 7) and spanning as much of the spectrum of MIC values as possible (Tables 8-11). Among the selected resistotypes (combination of

resistance phenotypes to each antimicrobial), 94 isolates were susceptible to all three antimicrobials, four, 15 and 43 isolates were only resistant to gentamicin, erythromycin and streptomycin respectively, two isolates were resistant to gentamicin and streptomycin, 17 to streptomycin and erythromycin and 19 were resistant to all three antimicrobials.

Table 7. Resistance phenotypes to AMG and MAC for 194 isolates selected for Specific Objective 3 across host species for *C. coli* and *C. jejuni* together.

GEN-ERY									
	Broilers		**Cattle**		**Pigs**		**Turkeys**		**Total**
	GEN-S	GEN-R	GEN-S	GEN-R	GEN-S	GEN-R	GEN-S	GEN-R	
ERY-S	81	2	42		2	2	12	2	143
ERY-R	11	4	2	4	5	9	14	2	51
Total	92	6	44	4	7	11	26	4	194

STR-ERY									
	Broilers		**Cattle**		**Pigs**		**Turkeys**		**Total**
	STR-S	STR-R	STR-S	STR-R	STR-S	STR-R	STR-S	STR-R	
ERY-S	61	22	30	12	2	2	5	9	143
ERY-R	6	9	1	5	1	13	7	9	51
Total	67	31	31	17	3	15	12	18	194

Table 8. Number of *C. coli isolates* selected for whole genome sequencing across gentamicin-erythromycin MIC spectrum in all hosts species.

***C. coli* all hosts (GEN-ERY) (n=72)**											
		GEN-S						GEN-R			
		0.06	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**
ERY-S	**0.06**										
	0.12										
	0.25										
	0.5					1					
	1			2	15	2	1			1	2
	2				3	1					
	4										
	8					1	1				1
ERY-R	**16**					1					1
	32				3	1					
	64				3	1	1				
	128					2	1				7
	>128		1		7	3			1	1	7

Table 9. Number of *C. coli isolates* selected for whole genome sequencing across streptomycin-erythromycin MIC spectrum in all hosts species.

***C. coli* all hosts (STR-ERY) (n=72)**											
		STR-S							STR-R		
		0.06	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**
ERY-S	**0.06**										
	0.12										
	0.25										
	0.5										1
	1					7	4	1			11
	2							1			3
	4										
	8						1				2
ERY-R	**16**								1		1
	32					1	2				1
	64								1		4
	128							1			9
	>128					1	2	2		1	14

Table 10. Number of *C. jejuni isolates* selected for whole genome sequencing across gentamicin-erythromycin MIC spectrum in all hosts species.

***C. jejuni* all hosts (GEN-ERY) (n=122)**											
		GEN-S						GEN-R			
		0.06	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**
ERY-S	**0.06**										
	0.12										
	0.25										
	0.5		35	4	7	28	9				
	1		2	8	6	3			1		1
	2		2		1	1					
	4		1	1			2				
ERY-R	**8**										
	16										
	32										
	64				1	2					1
	128			1							
	>128		1	1	2						1

Table 11. Number of *C. jejuni isolates* selected for whole genome sequencing across streptomycin-erythromycin MIC spectrum in all hosts species.

***C. jejuni* all hosts (STR-ERY) (n=122)**											
		STR-S							STR-R		
		0.06	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**>16**
ERY-S	**0.06**										
	0.12										
	0.25										
	0.5					58	5	8	1		11
	1			1	2	4			1	2	11
	2				2	1					1
	4				2			1		1	
ERY-R	**8**										
	16										
	32										
	64					3					1
	128					1					
	>128				2						3

According to this, 106 isolates (50 of which were used for Specific Objective 2) which had already been whole genome sequenced for other projects were initially selected (14 *C.coli* – eight from broilers, two from turkeys, three from pigs and one from cattle, and 92 *C.jejuni* – 62 from broilers and 30 from cattle).

In order to complete the dataset and taking into consideration the host, year, bacterial species and resistance phenotype of the already sequenced isolates, 88 additional isolates (58 *C. coli* – 13 from broilers, 20 from turkeys, 15 from pigs and 10 from cattle – and 30 *C. jejuni* – 15 from broilers, eight from turkeys and seven from cattle) were selected for whole genome sequencing (47 of which were used for Specific Objective 2), reaching the final number of 194 isolates (Figure 14).

The frozen stock of the 88 isolates selected for whole genome sequencing was thawed and recultured as explained in the previous section (*fla*A gene study). Once single colonies were obtained in pure culture, as much of this material as possible was harvested from a single colony per isolate in small tubes containing sterile double distilled water, stirred and centrifuged for 5 minutes at 3,000 rpm. Then, as much as possible supernatant was removed in order to obtain a dry pellet in the bottom of the tubes, which were sent refrigerated as early as possible to an external laboratory (Itacyl, Castilla y León) for whole genome sequencing.

Once these pellets were received at the laboratory, each isolate was grown in BHI liquid medium at 37ºC and DNA purification was carried out with the Qiagen DNA Blood & Tissue kit. The extracted DNA was quantified fluorometrically using the Qubit fluorimeter and the DNA was stored at -20ºC until library preparation using the Nextera XT DNA Library Preparation kit (Illumina, San Diego, MA, USA). One nanogramme/genome was used to generate the library. Each library was quantified by fluorimetry (Qubit), adjusting the concentrations to 4 nM so that each library reached an equimolar concentration in a single pool of 48 samples. The normalized libraries were mixed in a pool and were denatured with 0.2 N NaOH and loaded into the sequencer with PhiX as a control. The genomes were sequenced using parallel synthesis technology on a MiSeq platform using the 2×300 cycle V3 Kit following Illumina sequencing protocols.

3.2.2. Bioinformatic analyses

Molecular characterization of *flaA* gene (Specific Objective 2)

Eigth out of the 176 *flaA*-SVR sequences obtained in this study were discarded due to poor quality and the resulting 168 sequences from all hosts with different resistotypes were used, first, to build a Neighbor-Joining phylogenetic tree, and second, to assess the existence of groups by means of a 'relative synonymous codon usage' (RSCU) analysis.

Neighbour-Joining (NJ) tree

MEGA-X [422] and DnaSP6 [423] were used on imported *flaA* gene DNA sequences for the preparation and analysis of sequence alignments once sequences were aligned using the MUSCLE algorithm [424].

The Neighbour-Joining (NJ) phylogenetic tree with 1,000 bootstraps was built to evaluate the phylogenetic relationship between isolates. The NJ method constructs phylogenetic trees by iteratively joining pairs of nodes with the smallest corrected distance, creating new internal nodes and adjusting branch lengths based on pairwise genetic distances. This process continues until all nodes are connected, resulting in a finalized tree that represents the evolutionary relationships between the sequences. The *flaA* gene of the NCTC 1168 *C. jejuni* subsp. *jejuni* strain (1719 nucleotides-long, bacterial chromosome positions 1269232 to 1270950) was used as the external reference.

RSCU analysis

The RSCU analysis is a method used in molecular biology and bioinformatics to study codon usage patterns in a gene or genome. It provides insights into the preference for synonymous codons (codons that encode the same amino acid) across coding sequences [425]. The RSCU is the ratio of the observed frequency of a codon to its expected frequency assuming all synonymous codons for an amino acid are used equally. Its value ranges between 0 and infinity with RSCU = 1 indicating that the codon usage matches the expected frequency under the assumption of no selection between synonymous codons [110].

This analysis was performed to detect differences in codon usage between thermotolerant *Campylobacter* in the *flaA* gene depending on their AMG-MAC

resistance phenotype. In this study, the RSCU values were calculated using an in-house built R script, based on the total number of synonymous codons for each aminoacid in the *flaA* gene and their observed frequencies in the whole dataset of 168 isolates finally included in the study. The R packages 'BiocManager' ('coRdon') [426] and 'seqinr' [427] were used for the calculation of RSCU values.

Characterization of whole genome sequenced isolates (Specific Objective 3)

Analysis of AMR determinants

The raw reads in .FASTQ format from the 194 isolates included in Specific Objective 3 were subjected to bioinformatic analyses using an in-house built script in Python. The flow of the script with detailed steps is shown in Figure 15.

The quality of pre-trimmed and post-trimmed raw reads was checked with FastQC [428] and adaptors, low quality reads and duplicates were removed with Trimmomatic [429], with the values 30, 30, 4.20 and 40 for the parametres leading, trailing, sliding window and minlen, respectively. Good quality reads were taxonomically identified using Kraken2 [430] followed by Bracken [431] in order to keep only *C. coli* and *C. jejuni* isolates for further analyses. Kraken2 estimates the origin of kmers from a taxonomic database whereas Bracken uses a Bayesian algorithm to estimate the abundance of species within a sample. The reads thus filtered were assembled using a reference using the tool BWA [432] and assembly statistics and quality were evaluated with Samtools Flagstat [433] and Qualimap [434], respectively. Assemblies of isolates with a read depth threshold of <20 were discarded. The reference used for the assembly was *C. jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) chromosome (as described in the Introduction section).

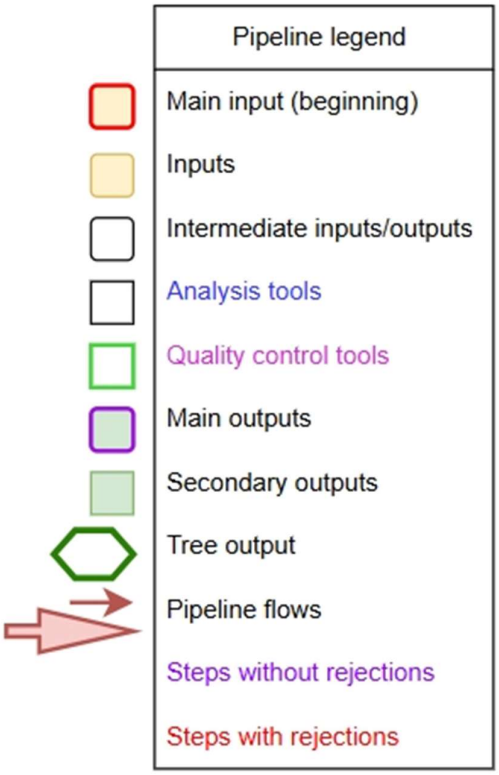
The search for AMR determinants (genes and point-mutations) was performed using AMRFinder [435] and RGI-CARD [436, 437], with 90% and 95% identity thresholds, respectively. By default, the coverage was 50% and >50% for AMRFinder and RGI-CARD, respectively. The search for point-mutations was complemented with Pointfinder [438].

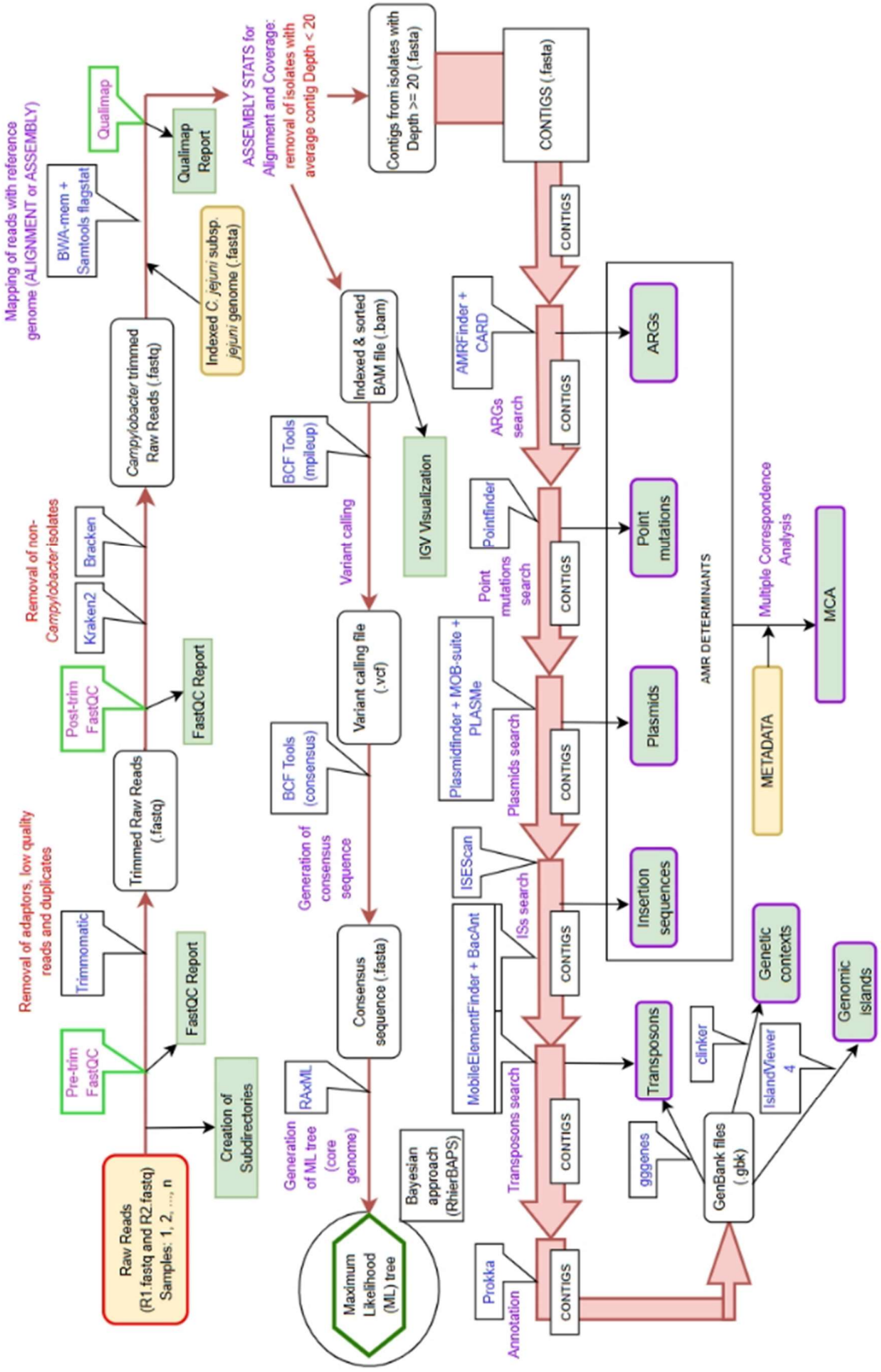
With regards to MGEs, the assemblies were screened for the presence of plasmid replicons using PlasmidFinder [439], the plasmid prediction was analyzed using PLASMe [440] to predict their likely source as either plasmidic or chromosomic, and the predictable transferability of the plasmids was explored with MOB-SUITE [441]. Insertion

sequences were searched for with ISEScan [442], and MobileElementFinder [443] and Bacant [444] were used for confirming insertion sequences and for searching for transposons.

The annotation of assemblies was carried out with Prokka [445]. The .GBK files generated by Prokka served to search for MDRGIs (groups of genes with evidence of having been acquired by HGT) in the genes using IslandViewer 4 [446] by means of its prediction tools IslandPath-DIMOB and SIGI-HMM, and taking *C. jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) chromosome as the reference.

Figure 15 (next page). Complete analysis flow used for Specific Objective 3 (in-house built Python script used for the bioinformatic analysis); pipeline legend below.




Raw Reads (R1.fastq and R2.fastq) Samples: 1, 2, ..., n
Creation of Subdirectories
Pre-trim FastQC
FastQC Report
Removal of adaptors, low quality reads and duplicates
Trimmomatic
Trimmed Raw Reads (.fastq)
Post-trim FastQC
FastQC Report
Removal of non-Campylobacter isolates
Kraken2
Bracken
Campylobacter trimmed Raw Reads (.fastq)
Mapping of reads with reference genome (ALIGNMENT or ASSEMBLY)
BWA-mem + Samtools flagstat
Qualimap
Qualimap Report
Indexed C. jejuni subsp. jejuni genome (.fasta)
ASSEMBLY STATS for Alignment and Coverage: removal of isolates with average contig Depth < 20
Contigs from isolates with Depth >= 20 (.fasta)
CONTIGS (.fasta)
CONTIGS
Indexed & sorted BAM file (.bam)
IGV Visualization
BCF Tools (mpileup)
Variant calling
Variant calling file (.vcf)
BCF Tools (consensus)
Generation of consensus sequence
Consensus sequence (.fasta)
RAxML
Generation of ML tree (core genome)
Maximum Likelihood (ML) tree
Bayesian approach (RhierBAPS)
AMRFinder + CARD
ARGs search
ARGs
Pointfinder
Point mutations search
Point mutations
Plasmidfinder + MOB-suite + PLASMe
Plasmids search
Plasmids
ISEScan
MobileElementFinder + BacAnt
ISs search
Insertion sequences
Transposons search
Transposons
clinker
gggenes
GenBank files (.gbk)
IslandViewer 4
Genetic contexts
Genomic islands
Prokka
Annotation
CONTIGS
AMR DETERMINANTS
METADATA
Multiple Correspondence Analysis
MCA

For the analysis of the genetic context of AMR determinants, the contigs (generally one per strain) containing AMR determinants for aminoglycosides and macrolides were identified, extracted from the Prokka generated .GBK files and used in the web-based software 'clinker' [447] for their graphic visualization and comparison. Moreover, the visualization of the distribution and coverage of the transposons found by MobileElementFinder and Bacant was explore using the 'gggenes' [448] R package.

The 'co-occur' R package [449] was used to explore the co-occurrence of all possible pairs of determinants including resistance genes (aminoglycosides, erythromycin, tetracycline, streptothricin and lincomycin) and mobile genetic elements. The 'co-occur' algorithm calculates the frequency of co-occurrence between each pair of determinants and compares it with the expected frequency if these were randomly distributed, assigning negative values to observed values with a frequency below the expected and positive values to observed values with a frequency above the expected.

A network analysis was carried out to better characterize the co-occurrence of gentamicin and streptomycin resistance genes and mobile genetic elements. For this purpose a grid-layout network using the 'igraph' R package was built [450].

Maximum likelihood (ML) tree

To assess the presence of genetic clusters and their phylogenetic relatedness a ML phylogenetic tree was built using the consensus sequences of the core-genome obtained from all strains (*C. coli* and *C. jejuni*) bearing AMR determinants and MGEs of interest associated with aminoglycosides and erythromycin resistance determinants. These consensus sequences were obtained with BCFTools [451] mpileup and consensus. Single nucleotide polymorphisms (SNPs) were extracted from the concatenated consensus sequences in .FASTA format and their SNP distances were calculated, followed by the use of RAxML Version 8 [452] with the GTR + Gamma model to perform a rapid bootstrapping (1000 replicates) in order to find the best-scoring ML tree, and no reference was used.

In the ML tree method, an initial tree is first built using a fast but suboptimal method such as NJ, and its branch lengths are adjusted to maximize the likelihood of the data set for that tree topology under the desired model of evolution. Then variants of the topology are created with the aim of searching for topologies that fit the data better. ML branch lengths are computed for these variant tree topologies and the greatest likelihood is

retained as the best choice so far. The search continues until no greater likelihoods are found (https://www.megasoftware.net/web_help_11/Maximum_Likelihood_ML.htm).

The phylogenetic tree was visualized using the web software 'iTOL' version 6.9.1 [453]. At the end of the process, the R tool RhierBAPS [454] was used to identify the genetic population structure by clustering the sequences based on their SNPs applying Bayesian analysis (Bayesian Analysis of Population Structure – BAPS).

3.2.3. Statistical analyses

Specific Objective 1

Data collation and categorization

After ordering and cleaning the main original database the metadata available for all samples was ID number, abattoir province, abattoir region, farm province, farm region and year of sampling. The data/metadata available for the isolates included host species, *Campylobacter* culture result (positive/negative), *Campylobacter* species ID and AST test results for the six antimicrobials in the panel, including MIC and IZD values as well as their dichotomization into 'susceptible' or 'resistant' categories. Samples with missing information on culture result, molecular identification and/or AST typing were discarded from the analyses. For descriptive purposes the proportions of resistant isolates for each bacterial and host species per period were defined as very low (<1%), low (1.1-10%), moderate (10.1-20%), high (20.1-50%), very high (50.1-70%) and extremely high (70.1-100%), as recommended by EFSA [35, 383].

Statistical tests

Proportions of *C. coli* and *C. jejuni* isolates from the different hosts resistant to each antimicrobial were compared using Z-tests adjusted for multiple comparisons by the Holm-method. Cochrane-Armitage logistic regressions were used to test for trends of AMR phenotypic resistance in *C. coli* and *C. jejuni* per antimicrobial and host species, and the relative change in the proportion of resistant isolates per year was computed along with its 95% confidence interval.

The association between the simultaneous presentation of phenotypic resistance to streptomycin/erythromycin, gentamicin/erythromycin and gentamicin/streptomycin over the whole study period and in different time periods (2002-2006, 2007-2012 and 2013-2018) was further evaluated for each bacterial and host species using relative risk (RR), chi-squared and Fisher's exact tests.

In order to evaluate differences in the distribution of MICs values in *C. coli* and *C. jejuni* from the four host species, these were displayed as squashtograms (Appendices I-IV). The existence of statistical differences in MICs distributions depending on bacterial species (for a given host) or on host (for a given bacterial species) was evaluated separately for susceptible and resistant isolates using Mann-Whitney or Kruskal-Wallis tests followed by Dunn's post-hoc tests correcting for multiple comparisons by the Benjamini-Hochberg method.

Microsoft Access was used for data handling and database initial analyses. Data was further handled with Microsoft Excel and imported into 'R' version 3.6.3 [455]. The R packages 'FSA' [456], 'plyr' [457] and 'ggplot2' [458], 'dplyr' [459], 'reshape2' [460] and 'tidyr' [457] were used for the analysis and visual representation of the data.

Specific Objective 2

Multiple Correspondence Analysis of *flaA* sequences

MCA and HCPC (Hierarchical Clustering on Principal Components) analyses were carried out to assess the existence of groups or clusters among the 168 selected isolates based on the relative synonymous codon usage (RSCU) of their *flaA* gene. Three dimensions were considered in the HCPC in the search for clusters. The variable 'RSCU categories' was transformed into a dichotomous variable as described in the Bioinformatic analyses section: >1 (positive bias) or <1 (negative bias). In addition to the RSCU values of variable codons, the MCA included other available covariates such as bacterial species (*C. coli versus C. jejuni*), host species (broilers, turkeys, pigs and cattle), resistance to gentamicin, streptomycin and erythromycin (yes/no), and clade as determined visually in the previously constructed NJ phylogenetic tree. The software packages 'Corrplot' [461], 'FactoMineR' [462], 'factoextra' [463] and 'ggthemes' [464] were used for the MCA and HCPC analyses.

Specific Objective 3

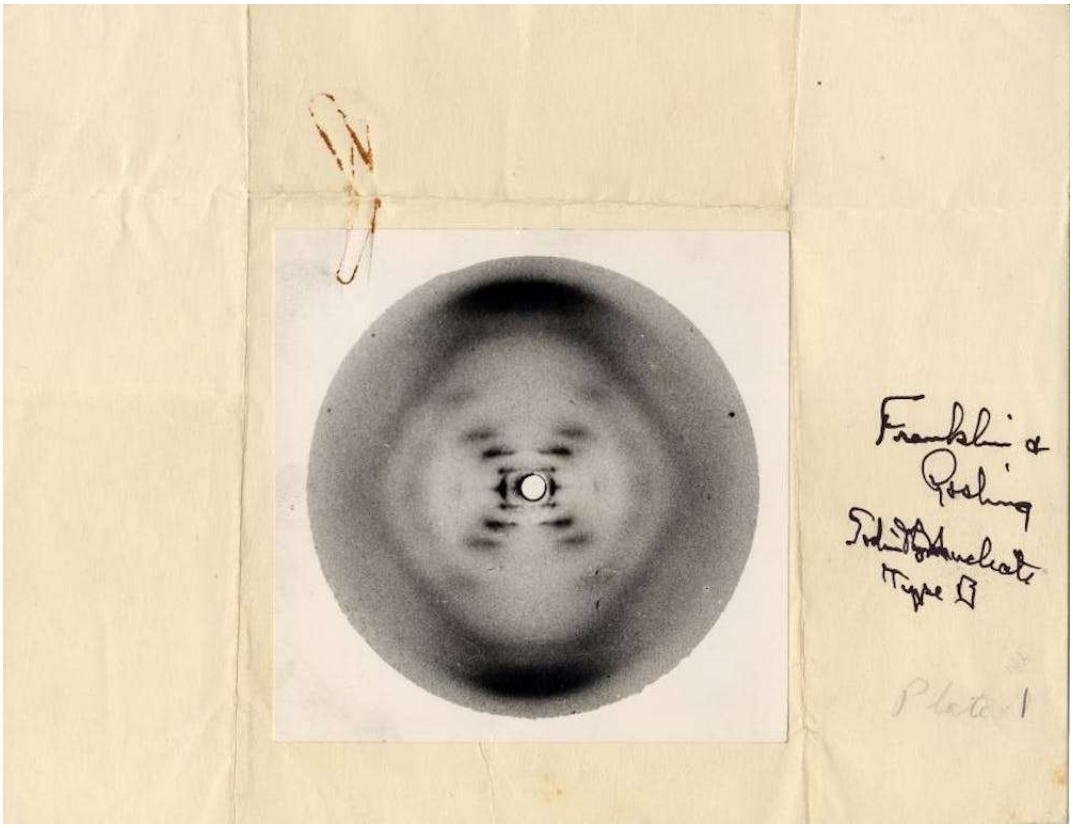
The overall data management and visual mapping of AMR determinants was carried out with the R packages [455] 'ggplot2' [458], 'reshape2' [460], 'forcats' [465] and 'tidyr' [457]. AMR genes associated with aminoglycosides and macrolides along with other AMR determinants and mobile genetic elements (plasmids, insertion sequences and transposons) found in the same contigs were considered as targets of interest. We compared the distribution of AMR determinants among bacterial species and hosts by performing Mann-Whitney and Kruskal-Wallis tests, respectively, adjusting post-hoc Dunn's comparison tests by the Bonferroni method in Kruskal-Wallis tests, if necessary.

The accuracy of prediction of the resistance phenotype for aminoglycosides and macrolides based on the presence/absence of AMR determinants was evaluated considering the phenotype as the gold standard. The level of agreement between phenotypic and genetic (i.e., presence of AMR determinants) resistance profiles for both antimicrobial classes was evaluated using Cohen's kappa agreement coefficients through the R 'irr' package [466]. We visually assessed the relationship between the distribution of log-transformed gentamicin and streptomycin MIC values and the presence/absence of different AMR determinants depending on host and bacterial species by means of scatterplots using the 'ggplot2' [458], 'ggpubr' [467] and 'patchwork' [468] R packages.

Multiple correspondence analysis of whole genome sequences

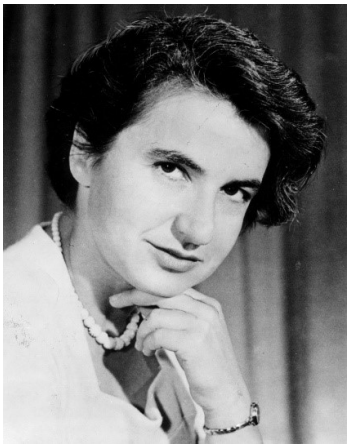
The presence of clusters involving AMR determinants was explored by means of MCA and HCPC analyses taking into consideration the following variables: bacterial species (*C. coli versus C. jejuni*), host species (broilers, turkeys, pigs and cattle), MGE categories, presence or absence of each ARG encoding resistance to gentamicin, streptomycin and erythromycin, presence or absence of each resistance-associated mutation present in the database, and resistance phenotype for gentamicin, streptomycin and erythromycin. Three dimensions were considered in the HCPC in the search for clusters. The 'corrplot' [461], 'FactoMineR' [462], 'factoextra' [463] and 'ggthemes' [455] R packages were used to carry out the MCA and analyse the clusters resulting from the HCPC, complemented with the R packages 'ggrepel' [469] and 'patchwork' [468].

RESULTS



"The results suggest a helical structure (which must be very closely packed) containing probably 2, 3 or 4 coaxial nucleic acid chains per helical unit and having the phosphate groups near the outside."

Rosalind Franklin
Physical chemist, discoverer of the molecular structure of DNA



4. RESULTS

4.1. Specific Objective 1

Overall, 3,413 independent fecal samples from broilers, 1,455 from turkeys, 3,750 from pigs and 2,347 from cattle were included in the analysis, of which 2,000 (58.6%), 1,090 (74.9%), 2,218 (59.2%) and 1,273 (54.3%) yielded isolations of *Campylobacter* spp., respectively (Table 12).

Table 12. Collection period (years), number of faecal samples, positives samples to *Campylobacter* and isolates retrieved from those from each host species included in the study.

Host species	Broilers	Turkeys	Pigs	Cattle
Years	2002-2018	2014-2018	2002-2017	2007-2017
Sample size	3,413	1,455	3,750	2,347
Samples positive for *Campylobacter*	2,000	1,090	2,218	1,273
Positive isolates *C. coli*	1,023 (51.2%)	815 (74.8%)	1,968 (88.7%)	183 (14.4%)
Positive isolates *C. jejuni*	957 (47.8%)	273 (25.1%)	33 (1.5%)	1,074 (84.4%)
Positive isolates *Campylobacter* spp.*	20 (1.0%)	2 (0.1%)	217 (9.8%)	16 (1.2%)

* *Campylobacter* species other than *C. coli* and *C. jejuni*

Over the whole study period (2002-2018), *C. coli* was the most frequently identified species in pig (88.7%; 1,968/2,218) and turkey (74.8%; 815/1,090) isolates, whereas *C. jejuni* was the most frequent species in cattle (84.4%; 1,074/1,273). In broilers, the proportion of *C. coli* and *C. jejuni* was very similar (51.2%; 1,023/2,000 and 47.8%; 957/2,000, respectively) (Table 12).

Although there were differences across years, the proportion of positive samples to *C. coli* and *C. jejuni* remained relatively constant over the entire study period in pigs, cattle and turkeys, with one bacterial species being more prevalent than the other one, while the situation was more variable in broilers (Figure 16).

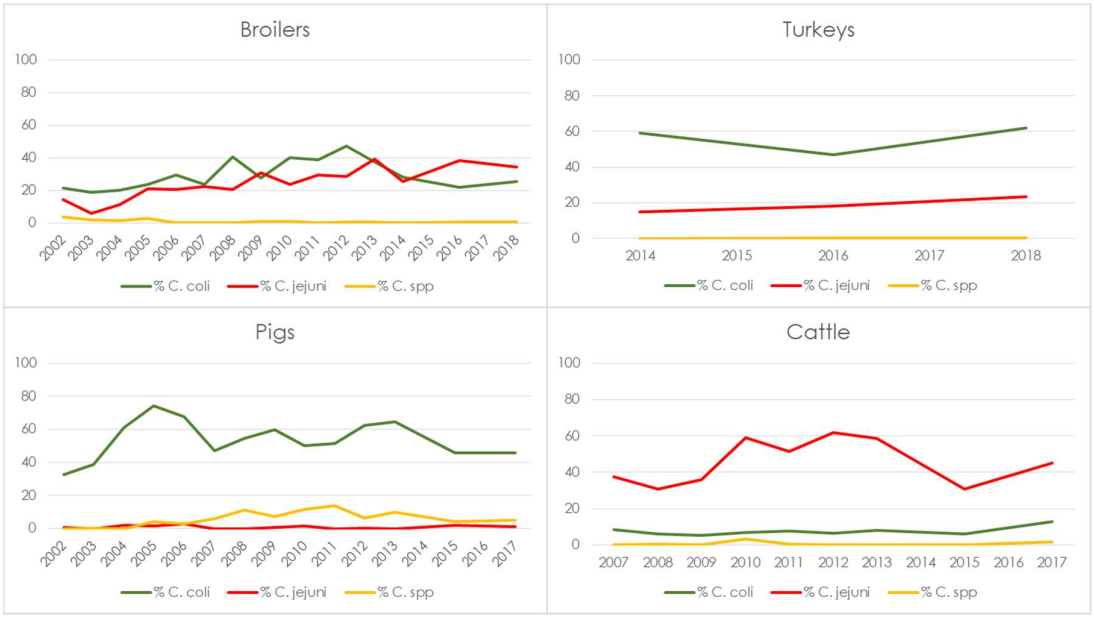


Figure 16. Percentage of samples positive for *Campylobacter* (*C. coli*, *C. jejuni*, and *Campylobacter* spp.) for each host species throughout the study period.

Significant increasing trends were observed in the proportion of positive samples for *C. jejuni* in broilers and turkeys, with annual-biannual rates of increase of 9.7% (95% CI: 6.16 to 13.33%) and 26.2% (95% CI: 15.81 to 37.43%), respectively (Figure 16).

The number of samples analysed, isolates recovered, and isolates subjected to AST varied depending on the year and host species (Table 13). In general, more than 70% of the isolates retrieved every year were subjected to AST for broilers, pigs and cattle (at least 170 *C. jejuni* isolates per host, except in pigs), but in the case of turkeys this percentage was around 50% because the number of samples recovered and positive isolates found were proportionally larger than in the other three hosts (Table 13).

The geographic distribution over the Spanish provinces of *Campylobacter* positive samples (both for *C. coli* and *C. jejuni*) and the proportions of these isolates resistant to gentamicin, streptomycin and erythromycin (the three antimicrobials objective of this thesis) in broilers, turkeys, pigs and cattle are displayed on Figures 17-20.

In broilers, the highest percentages of thermotolerant *Campylobacter* samples were observed in the Northeast (NE) and the provinces of Ourense and Toledo. STR-R isolates were present in most of the country but GEN-R and ERY-R isolates were only present in half of the provinces from which *Campylobacter* positive samples were collected. ERY-R isolates were mainly found in the regions with the highest percentage of isolates and in Andalucía, while GEN-R and STR-R isolates were mainly found in the province of Alicante (Figure 17).

Table 13. Number and proportion of *Campylobacter* spp. samples and isolates, including the ones where AST was completed, in broilers, turkeys, pigs and cattle.

	Broilers			Turkeys			Pigs			Cattle		
Year	Samples	Positive samples (%)	AST on isolates (%)	Samples	Positive samples (%)	AST on isolates (%)	Samples	Positive samples (%)	AST on isolates (%)	Samples	Positive samples (%)	AST on isolates (%)
2002	85	33 (38.8)	29 (87.9)				290	97 (33.4)	94 (96.9)			
2003	160	42 (26.2)	32 (80.0)				319	125 (39.2)	113 (90.4)			
2004	159	52 (32.7)	33 (63.5)				183	116 (63.4)	87 (75.0)			
2005	76	36 (47.4)	31 (86.1)				195	156 (80.0)	140 (89.7)			
2006	98	49 (50.0)	46 (93.9)				196	145 (74.0)	100 (69.0)			
2007	89	41 (46.1)	40 (97.6)				310	165 (53.2)	143 (86.7)	163	75 (46.0)	69 (92.0)
2008	113	69 (61.1)	69 (100)				171	112 (65.5)	93 (54.4)	168	63 (37.5)	61 (96.8)
2009	198	118 (59.6)	115 (97.5)				284	192 (67.6)	167 (87.0)	258	107 (41.5)	105 (98.1)
2010	202	131 (64.8)	127 (96.9)				214	135 (63.1)	105 (77.8)	200	139 (69.5)	130 (93.5)
2011	237	162 (68.3)	156 (96.3)				257	167 (65.0)	129 (77.2)	239	142 (54.4)	132 (93.0)
2012	239	182 (76.1)	86 (47.3)				236	163 (69.1)	73 (44.8)	221	151 (68.3)	75 (49.7)
2013	370	284 (76.7)	140 (49.3)				338	252 (74.5)	108 (42.9)	347	232 (66.8)	115 (49.6)
2014	500	268 (53.6)	170 (63.4)	500	370 (74.0)	170 (46.0)						
2015							373	193 (51.7)	170 (88.1)	367	136 (37.0)	120 (88.2)
2016	427	257 (60.2)	162 (63.0)	488	319 (65.4)	170 (53.3)						
2017							384	200 (52.1)	170 (85.0)	384	228 (59.4)	170 (74.6)
2018	460	276 (60.0)	170 (61.6)	467	401 (85.9)	170 (42.4)						
Total	**3,413**	**2,000 (58.6)**	**1,406 (70.3)**	**1,455**	**1,090 (74.9)**	**510 (46.8)**	**3,750**	**2,218 (59.1)**	**1,692 (76.3)**	**2,347**	**1,273 (54.2)**	**977 (76.7)**

In turkeys, most samples originated from the Northwest (NW), Northeast (NE) and Southwest (SW) with positive samples and GEN-R, STR-R and ERY-R isolates distributed in these same areas. Again, GEN-R and ERY-R isolates were both isolated in the Southeast (SE) (mainly Murcia) and STR-R isolates in the NE (mainly Teruel) (Figure 18).

In pigs, the majority of samples were retrieved from the Northeast (NE), the centre and Murcia, STR-R isolates covered most of the country and yet again both GEN-R and ERY-R isolates were isolated in the same regions of the country. In terms of provinces with higher proportions of resistance, there was a mixed pattern, although high in all (Figure 19).

In cattle, most samples were retrieved in the West (W) and Northeast (NE). STR-R isolates were found in most provinces and in fewer provinces in the case of GEN-R and ERY-R. The highest proportions of resistant isolates to the three antimicrobials were found in the Northeast (NE) and the province of Córdoba (Figure 20).

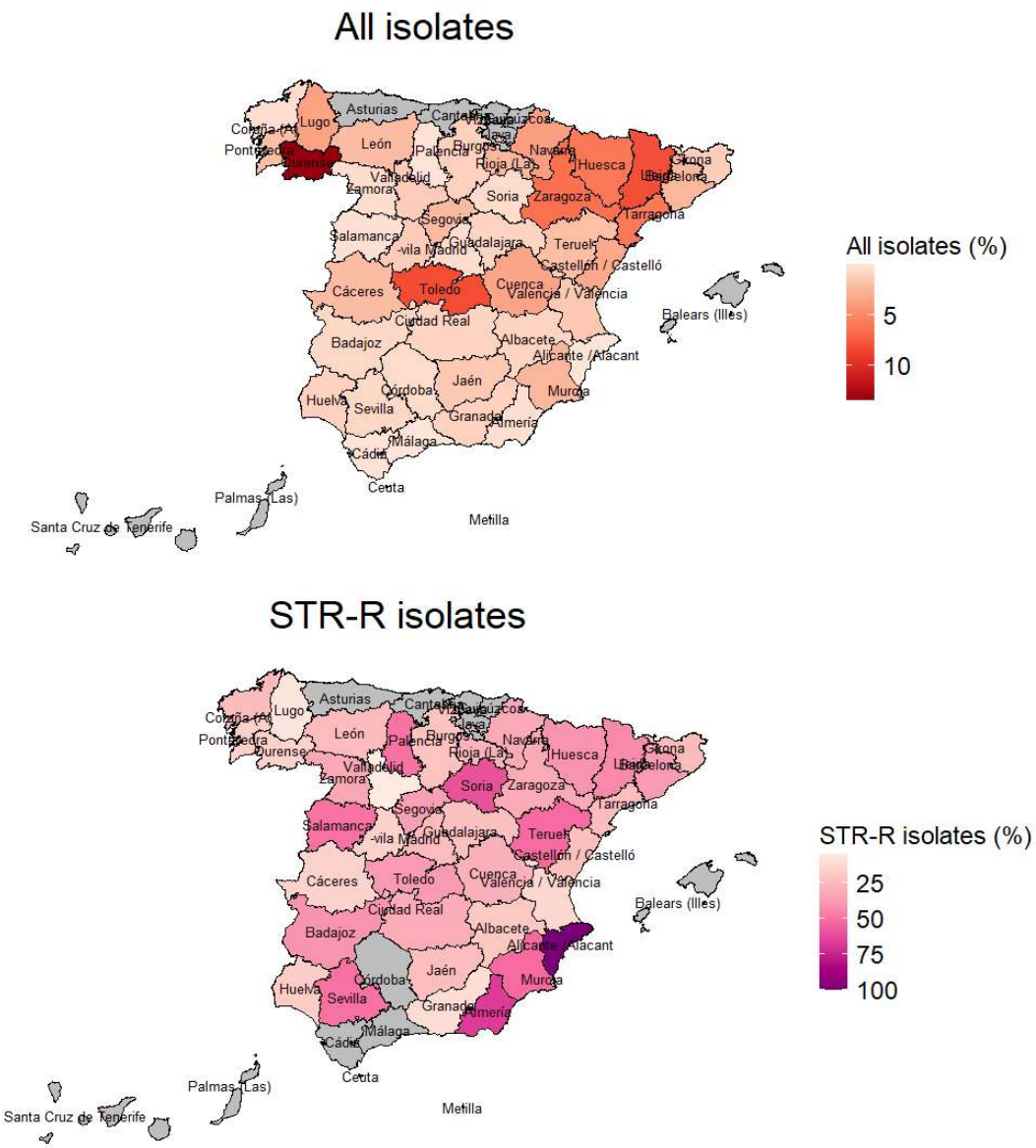


Figure 17. Maps showing the distribution and percentage of all *Campylobacter* positive isolates for broilers and the STR-R isolates among them across the different provinces in Spain (grey areas represent no data).

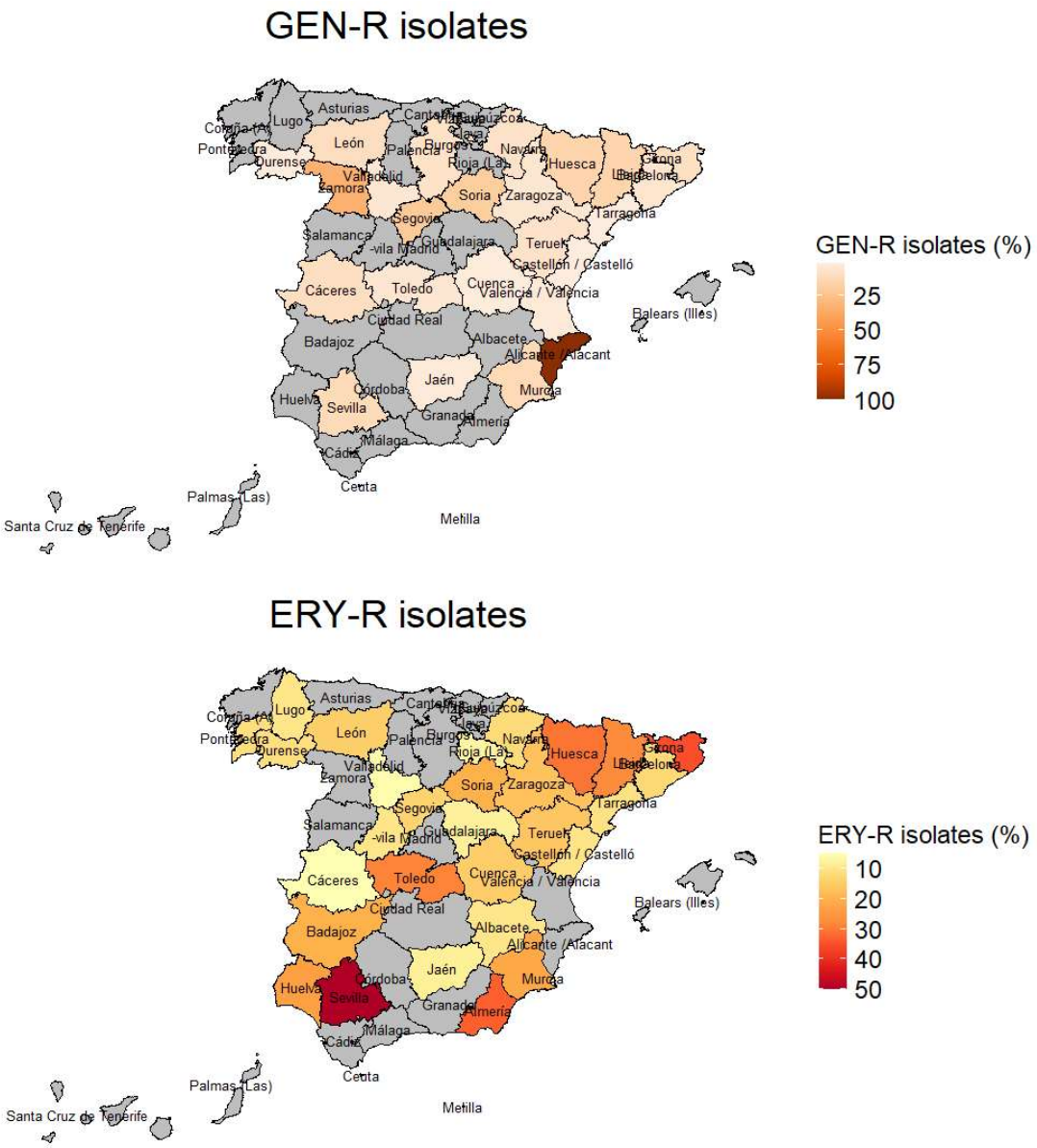


Figure 17 (continued). Maps showing the distribution and percentage of GEN-R and ERY-R isolates among all *Campylobacter* positive isolates for broilers across the different provinces in Spain (grey areas represent no data).

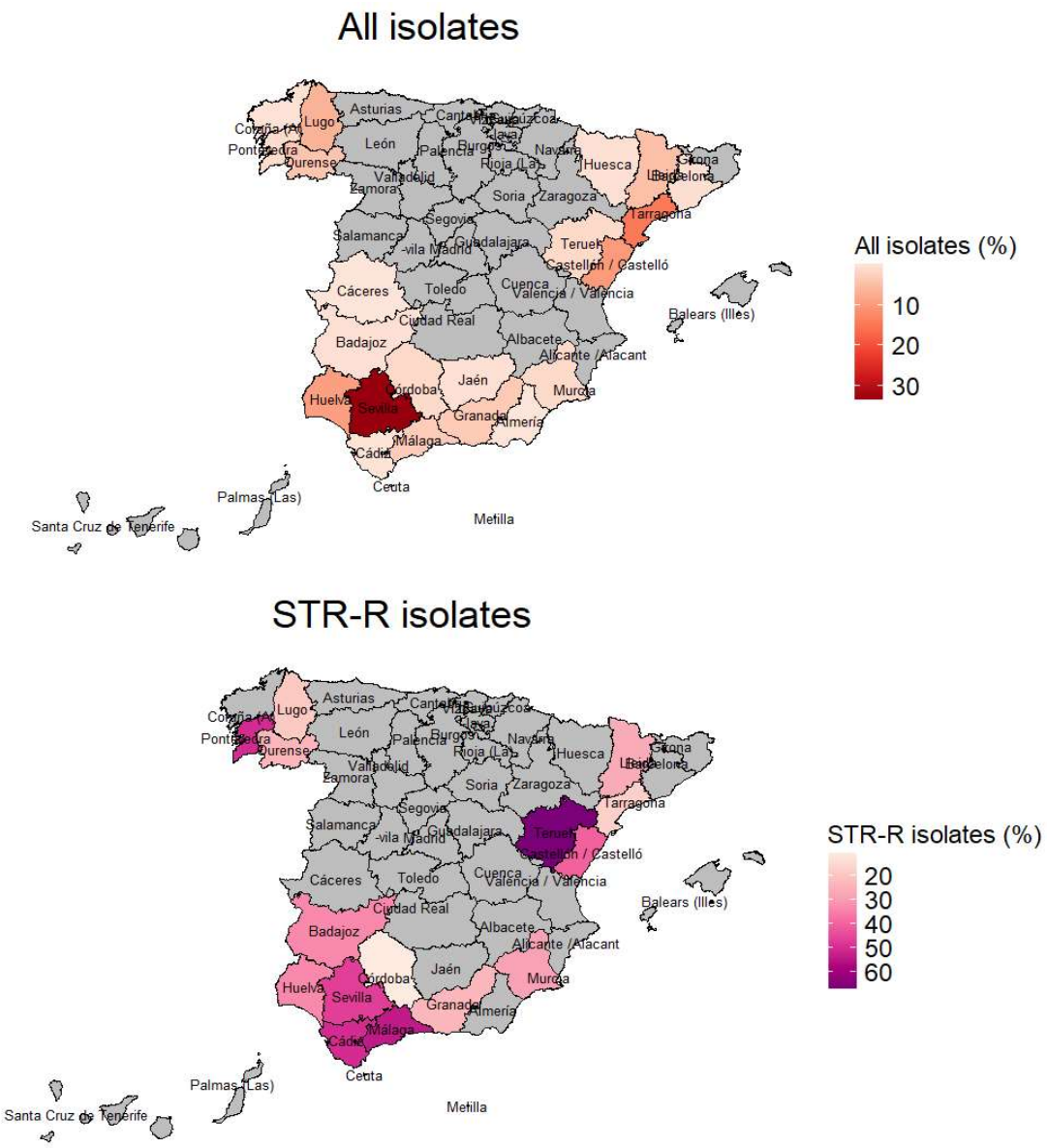


Figure 18. Maps showing the distribution and percentage of all *Campylobacter* positive isolates for turkeys and the STR-R isolates among them across the different provinces in Spain (grey areas represent no data).

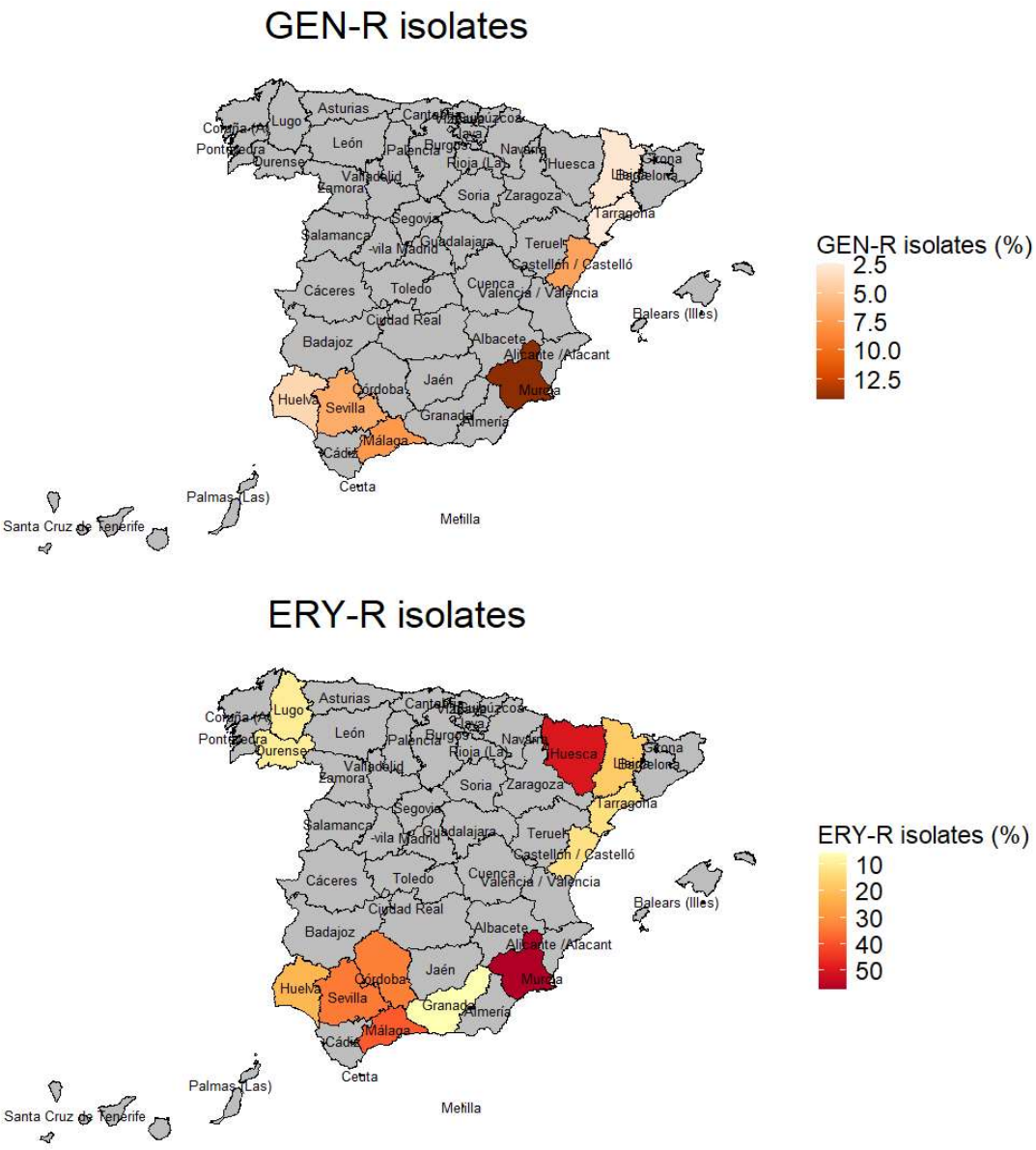


Figure 18 (continued). Maps showing the distribution and percentage of GEN-R and ERY-R isolates among all *Campylobacter* positive isolates for turkeys across the different provinces in Spain (grey areas represent no data).

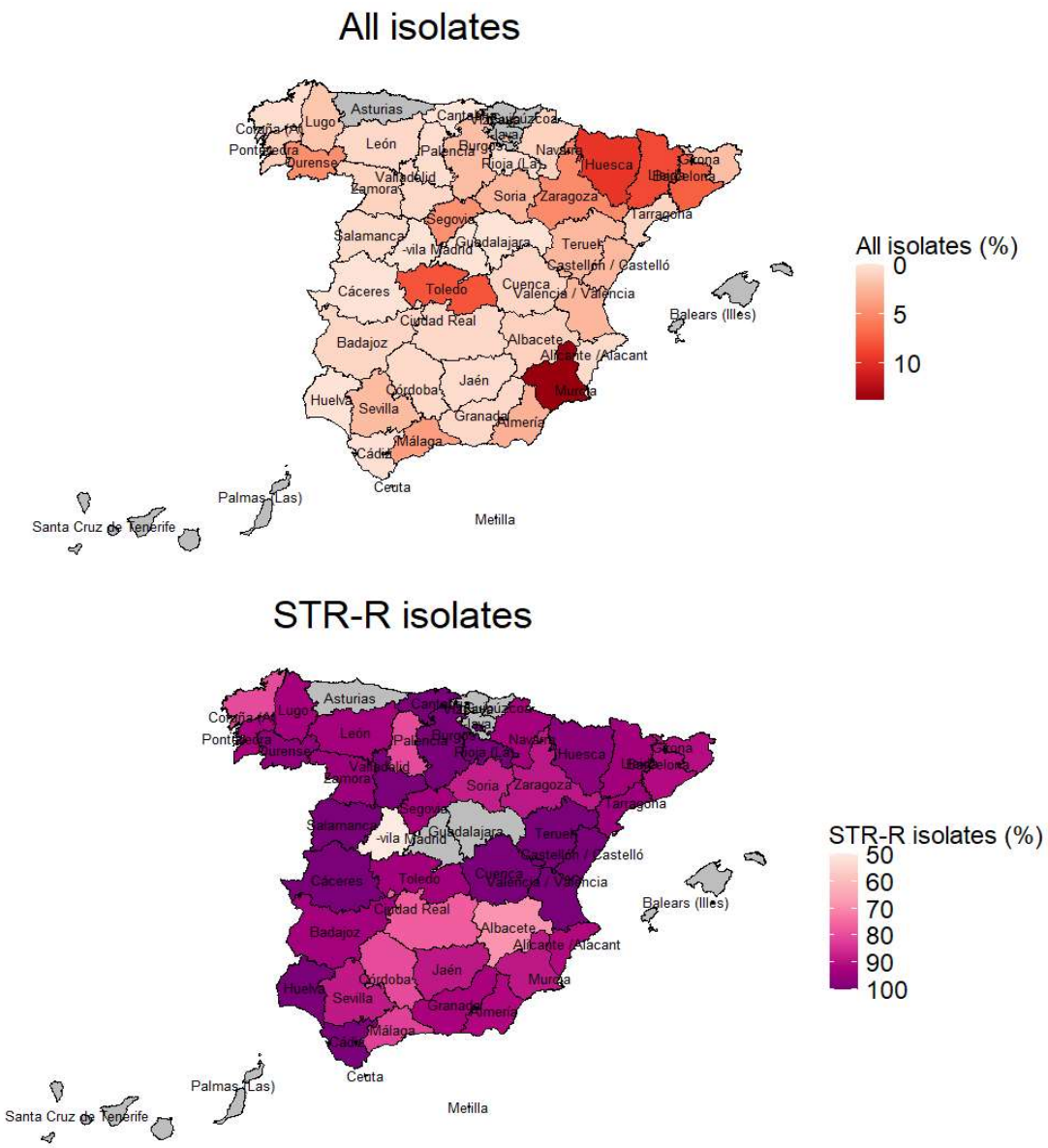


Figure 19. Maps showing the distribution and percentage of all *Campylobacter* positive isolates for pigs and the STR-R isolates among them across the different provinces in Spain (grey areas represent no data).

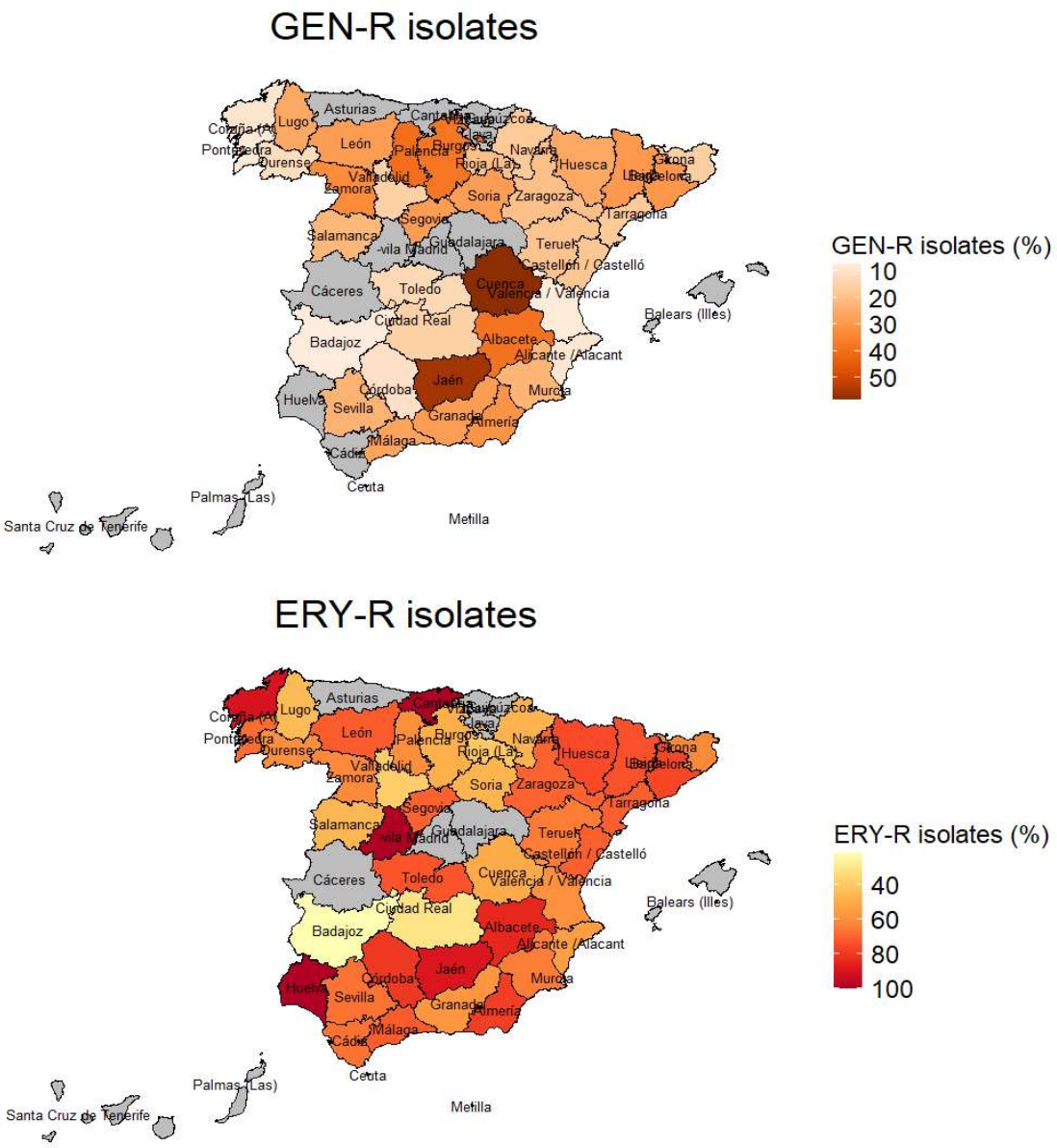


Figure 19 (continued). Maps showing the distribution and percentage of GEN-R and ERY-R isolates among all *Campylobacter* positive isolates for pigs across the different provinces in Spain (grey areas represent no data).

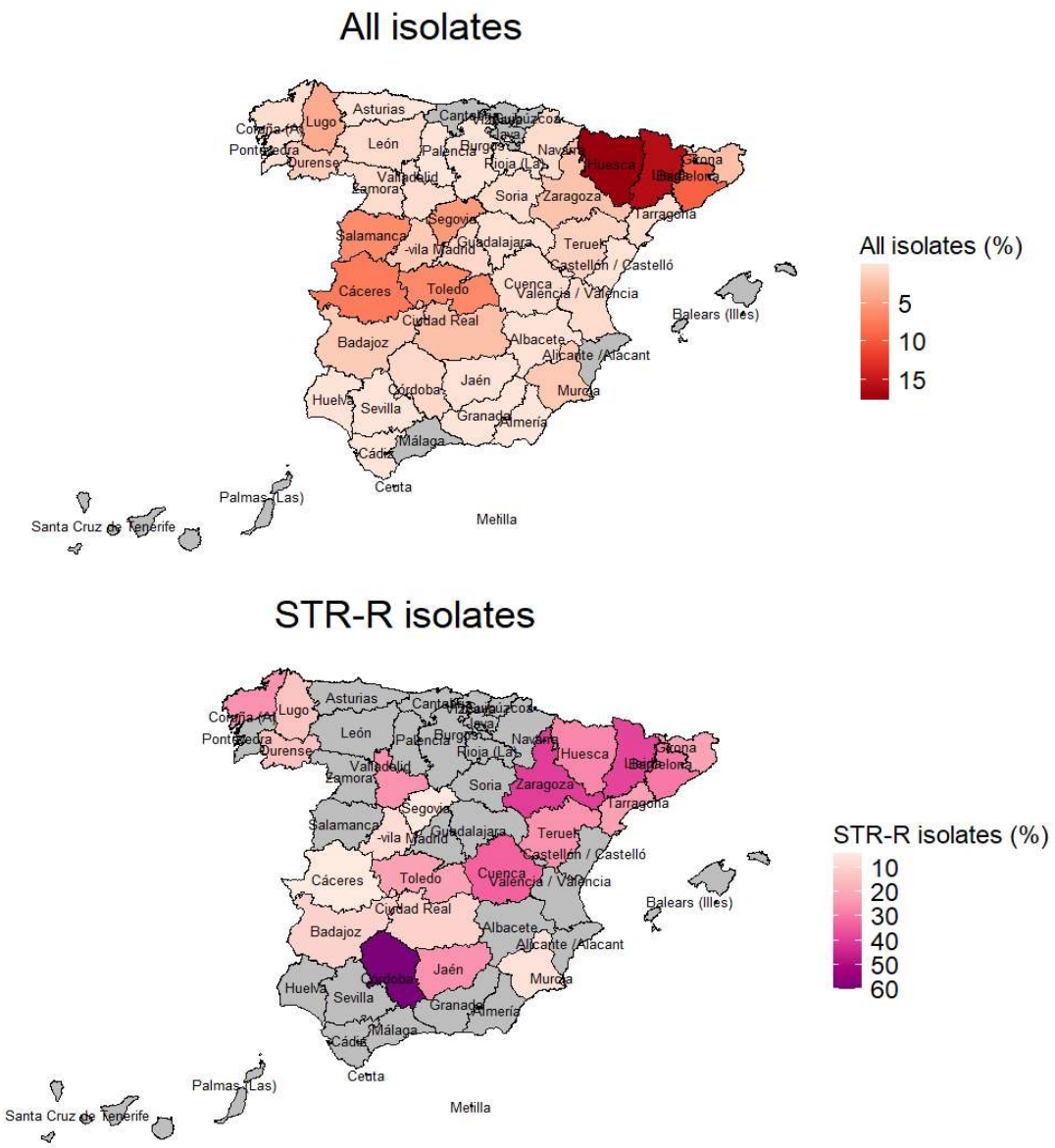


Figure 20. Maps showing the distribution and percentage of all *Campylobacter* positive isolates for cattle and the STR-R isolates among them across the different provinces in Spain (grey areas represent no data).

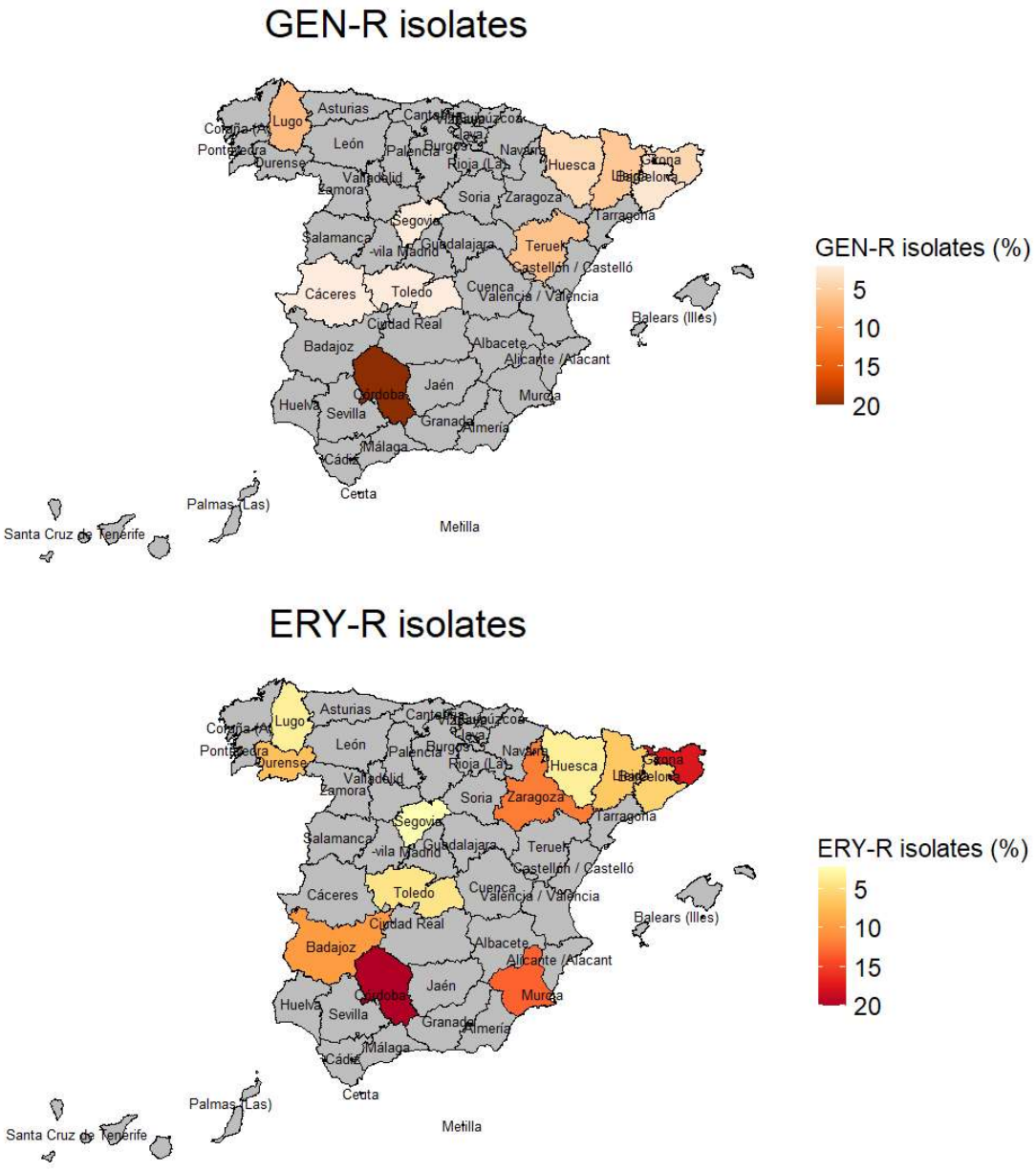


Figure 20 (continued). Maps showing the distribution and percentage of GEN-R and ERY-R isolates among all *Campylobacter* positive isolates for cattle across the different provinces in Spain (grey areas represent no data).

The overall proportion of isolates resistant to ciprofloxacin, nalidixic acid and tetracycline out of those tested was extremely high (>80%) in both *C. coli* (Figure 21) and *C. jejuni* (Figure 21b) from all host species (Table 14), with yearly values exceeding 70% throughout the study period except in *C. jejuni* from cattle (Figure 21b).

Significant differences in the proportion of resistant isolates to these three antimicrobials depending on the host were observed for most years (Table 14). The proportion of ciprofloxacin and nalidixic acid resistant *C. coli* isolates was significantly lower in cattle compared with broilers and turkeys ($p<0.05$) (and in pigs compared with turkeys for ciprofloxacin, $p<0.001$). In the case of tetracycline, *C. coli* isolates from pigs were significantly more resistant than *C. coli* from cattle and broilers ($p<0.001$), but resistance was still above 95% in all species (Table 14). In the case of *C. jejuni*, cattle isolates were significantly less resistant to the three antimicrobials compared with isolates from poultry ($p<0.05$).

The proportion of resistance to the remaining three antimicrobials (streptomycin, erythromycin and gentamicin) was much more variable (Figures 21-21b, Table 15).

In the case of streptomycin, extremely high (80-91%) or high to very high (approximately 55%) levels were found in *C. coli* from cattle and pigs and from broilers and turkeys, respectively (Figure 21), with significant differences between all hosts species except between broilers and turkeys (pigs>cattle>poultry) (Table 15). In contrast, values <11% were observed in *C. jejuni* from broilers, turkeys and cattle (the three host species from which *C. jejuni* was recovered) (Figure 21b). Although levels of resistance in *C. jejuni* were always significantly lower than in *C. coli* for any given host species, no significant differences between host species were observed (Table 15).

Table 14. Percentage of *Campylobacter* isolates resistant (NWT) to tetracycline (TET), nalidixic acid (NAL) and ciprofloxacin (CIP) in the four host species throughout the studied period.

Bacterial species	Host species	TET *C. coli* (MIC>2) *C. jejuni* (MIC>1)	NAL *C. coli*/*C. jejuni* (MIC>16)	CIP *C. coli*/*C. jejuni* (MIC>0.5)
***C. coli* (%)**	Broilers (n=634)	95.9[a]	93.3[a]	94.5[a,b]
	Turkeys (n=279)	97.5[a,b]	95.3[a]	98.2[b]
	Pigs (n=1,692)	99.1[b]	91.7[a,b]	91.7[a,c]
	Cattle (n=149)	95.3[a]	86.7[b]	87.3[c]
***C. jejuni* (%)**	Broilers (n=772)	83.1[a]	88.5[a]	91.1[a]
	Turkeys (n=231)	83.1[a]	86.1[a]	88.7[a]
	Cattle (n=828)	74.1[b]	63.1[b]	63.8[b]

Different superscripts indicate significant differences between hosts for each bacterial species and antimicrobial combination.

*Disk diffusion technique: streptomycin in broilers and pigs (2002-2005) and erythromycin in broilers (2002-2004).

The proportion of gentamicin resistant-isolates was low (<25%) in *C. coli* from all species (Figure 21), although the proportion in pigs was significantly higher than that observed in other host species ($p<0.001$). Resistance levels in *C. jejuni* (Figure 21b) were lower (<2%) and significantly different from those recorded in *C. coli* from the same host but, once again, no significant differences between hosts were observed.

Table 15. Percentage of *Campylobacter* isolates resistant (NWT) to streptomycin (STR), erythromycin (ERY) and gentamicin (GEN) in the four host species throughout the studied period.

Bacterial species	Host species	STR* *C. coli*/*C. jejuni* (MIC>4; IZD<13)	ERY* *C.coli* (MIC>8; IZD<24) *C. jejuni* (MIC>4; IZD<22)	GEN *C. coli*/*C. jejuni* (MIC>2)
***C. coli* (%)**	Broilers (n=634)	54.7[a]	34.8[a]	14.7[a]
	Turkeys (n=279)	55.9[a]	36.6[a]	7.5[b]
	Pigs (n=1,692)	90.6[b]	66.6[b]	22.2[c]
	Cattle (n=149)	82.0[c]	19.3[c]	12.0[a,b]
***C. jejuni* (%)**	Broilers (n=772)	7.7	2.9	1.0
	Turkeys (n=231)	6.1	2.6	0.0
	Cattle (n=828)	10.2	1.7	1.4

Different superscripts indicate significant differences between hosts for each bacterial species and antimicrobial combination.

*Disk diffusion technique: streptomycin in broilers and pigs (2002-2005) and erythromycin in broilers (2002-2004).

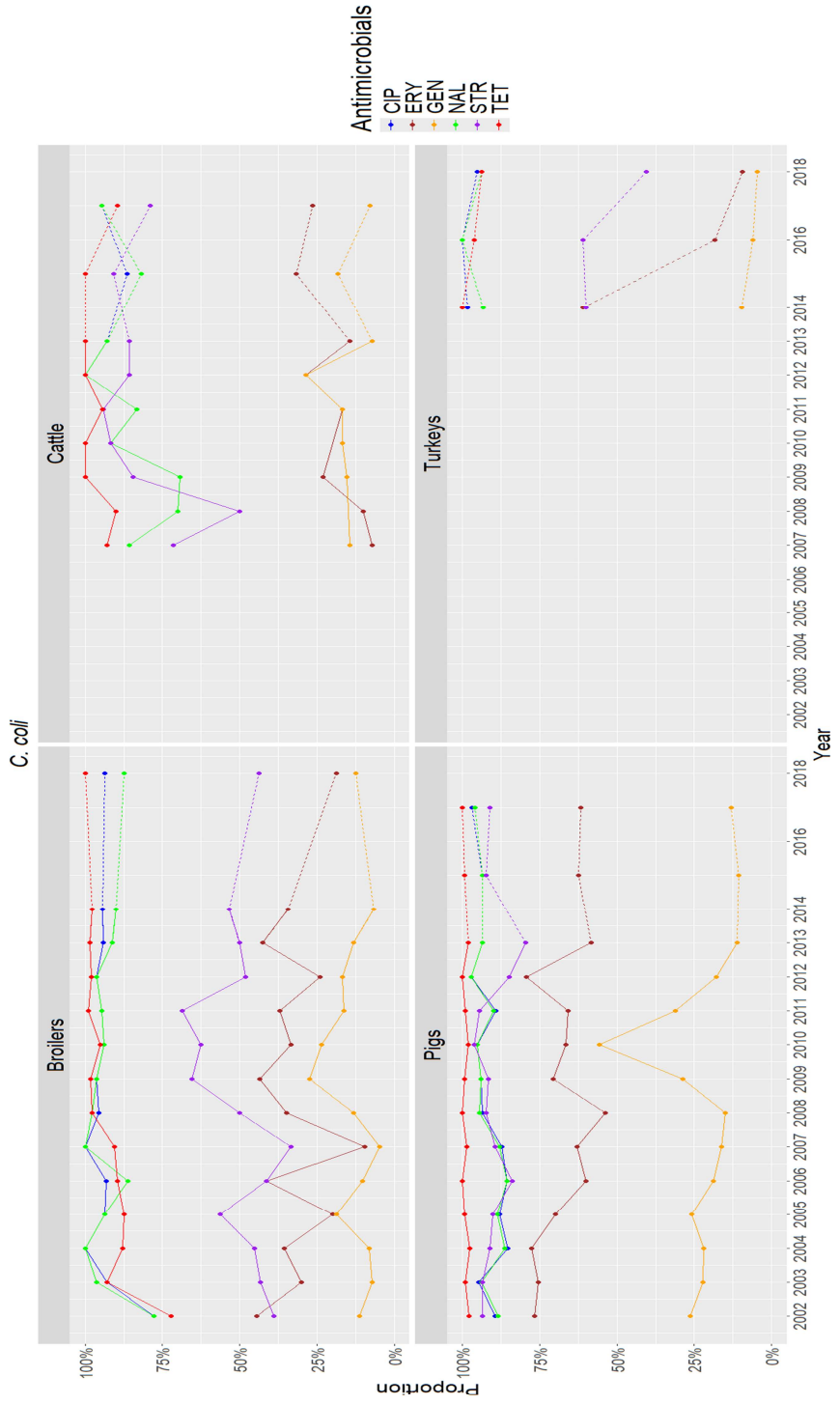


Figure 21. Proportion of *C. coli* isolates retrieved from livestock in 2002-2018 resistant to ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR) and tetracycline (TET); dashed lines indicate periods in which AST was not performed yearly (NB: AST not performed in broilers in 2016).

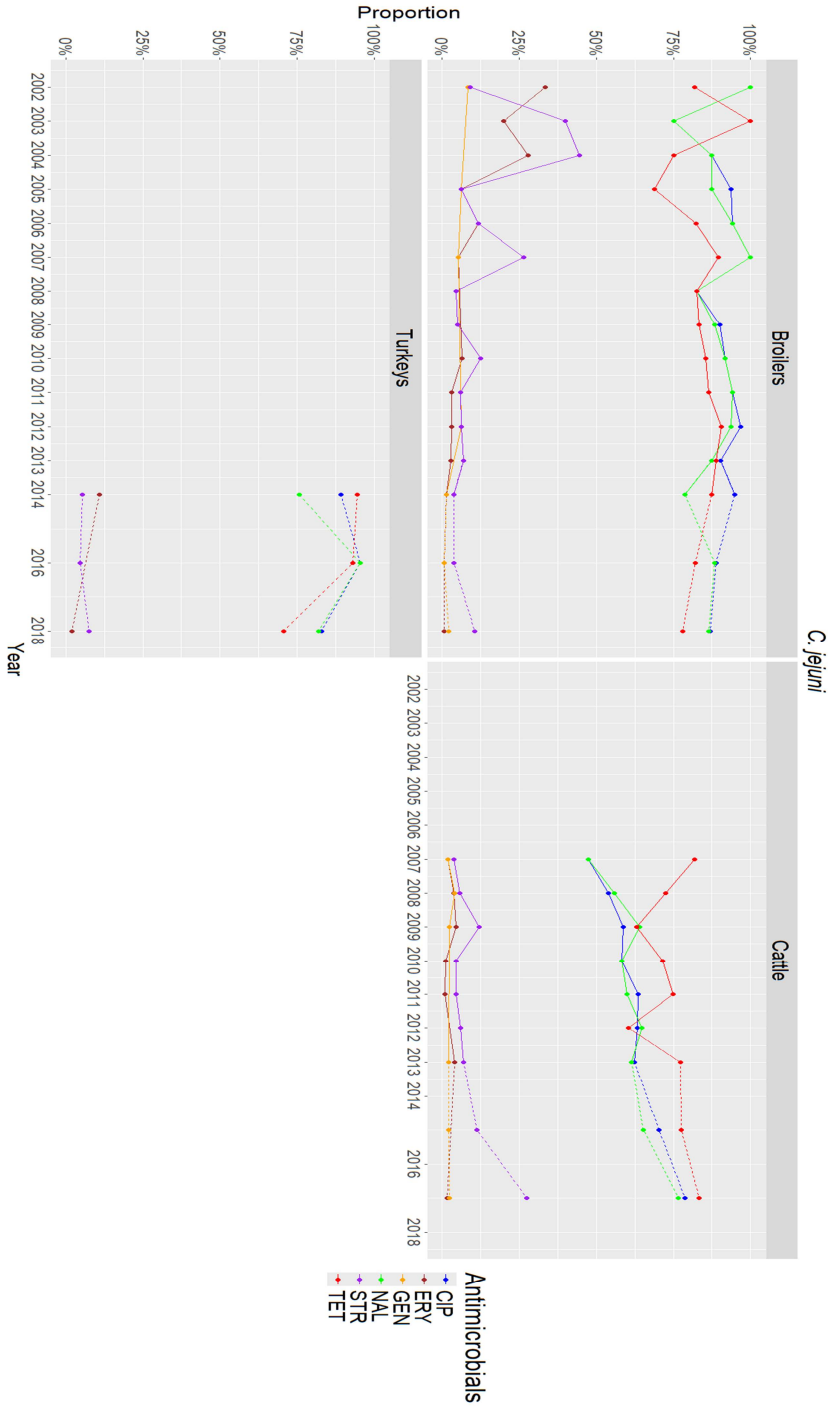


Figure 21b. Proportion of *C. jejuni* isolates retrieved from livestock in 2002-2018 resistant to ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR) and tetracycline (TET); dashed lines indicate periods in which AST was not performed yearly.

The proportion of resistance to erythromycin in *C. coli* was very high (67%) for pigs, high (35%) for broilers and turkeys, and moderate (19%) for cattle (pigs>poultry>cattle) (Figures 21-21b, Table 15). Overall values in *C. jejuni* from all host species were <3% and significantly lower than those in *C. coli*, again with no significant differences across hosts.

Significant ($p<0.05$) temporal trends in the proportion of cattle resistant isolates were observed for erythromycin in *C. coli* (annual rate of increase of 29.9% although with a wide 95% CI: -31.36 to 145.77) (Figure 21) and streptomycin in *C. jejuni* (annual rate of increase of 16.7%, 95% CI: 2.89 to 32.28) (Figure 21b). Significant ($p<0.001$) decreasing trends in the proportion of resistant *C. jejuni* isolates recovered every year from broilers were also observed for two antimicrobials: streptomycin (-9.9% annual decrease rate, 95% CI: -16.13 to -3.14) and erythromycin (-27.5% annual decrease rate, 95% CI: -44.60 to -5.10) (Figure 21b).

Other significant ($p<0.001$) trends found associated with moderate annual rates of increase were observed in *C. jejuni* in cattle for ciprofloxacin (5.22%, 95% CI: 3.91 to 6.54) and nalidixic acid (4.11%, 95% CI: 2.20 to 6.06), in *C. coli* in broilers for tetracycline (1.61%, 95% CI: 0.93 to 2.29) and in *C. coli* in pigs for ciprofloxacin (0.64%, 95% CI: 0.20 to 1.09). For the rest of the antimicrobials, host and bacterial species, no significant trends were found (Figures 21-21b).

A statistical analysis of the squashtograms distributions (Appendices I-IV) for all hosts in *C. coli* and for all hosts in *C. jejuni*, assessing independently susceptible and resistant distributions, revealed significant differences ($p=0.032$) only between ERY-R *C. jejuni* isolates, with higher significantly higher MICs among turkey isolates compared with those from other species.

4.2. Specific Objective 2

4.2.1. Co-resistance and MDR phenotypic profiles

The main resistance profiles observed in all host species for *C. coli* and *C. jejuni* are shown on Table 16 and 17, respectively. More than 85% of the *C. coli* from all host species were resistant to three (CIP-TET-NAL, TET-ERY-STR) or more antimicrobials and over 60% were resistant to three or more antimicrobial classes (MDR). The most common resistance profiles for *C. coli* from each host were CIP-TET-NAL and CIP-TET-NAL-STR in broilers and

turkeys (18-28% of all isolates in each host species for each profile), CIP-TET-NAL-STR in cattle (approximately 50% of all *C. coli* isolates) and CIP-TET-NAL-STR-ERY in pigs (approximately 40% of all *C. coli* isolates). The proportion of pan-susceptible *C. coli* isolates in all host species was low (0-4%), and lower than the proportion of resistant isolates to all six antimicrobials in all host species (0-16%).

The proportion of *C. jejuni* isolates from all hosts resistant to three or more antimicrobials was 54-76% whereas the proportion resistant to three or more antimicrobial classes was 6-9%. The most common resistance profile for *C. jejuni* from all hosts was CIP-TET-NAL, representing from approximately 45% of all cattle to 60-70% of all broiler and turkey isolates. However, in *C. jejuni* the proportion of pan-susceptible isolates (5-15%) was higher than that of resistant isolates to the six antimicrobials in all species (<1%).

Table 16. Antimicrobial phenotypic resistance profiles across animal host species in *C. coli*.

Number of antimicrobials	Antimicrobial profiles	*C. coli* broilers	*C. coli* turkeys	*C. coli* pigs	*C. coli* cattle
0	Pan-susceptible	7 (1.10)	2 (0.72)	1 (0.06)	5 (3.35)
1	CIP | TET | NAL | ERY | STR	8 (1.26)	0 (0)	7 (0.41)	5 (3.35)
2	CIP/NAL	12 (1.89)	4 (1.43)	0 (0)	1 (0.67)
	(GEN | CIP)/TET	28 (4.42)	6 (2.15)	2 (0.12)	0 (0)
	TET/STR	14 (2.21)	1 (0.36)	47 (2.78)	9 (6.04)
	OTHERS	2 (0.31)	0 (0)	7 (0.41)	0 (0)
3	CIP/TET/NAL	**160 (25.2)**	**76 (27.2)**	40 (2.36)	**14 (9.39)**
	TET/ERY/STR	1 (0.16)	1 (0.36)	56 (3.31)	0 (0)
	CIP/TET/(ERY | STR)	39 (6.15)	3 (1.07)	2 (0.12)	1 (0.67)
	OTHERS	10 (1.58)	1 (0.36)	13 (0.77)	1 (0.67)
4	CIP/TET/NAL/STR	**119 (18.8)**	**77 (27.6)**	**371 (22.0)**	**75 (50.3)**
	CIP/TET/NAL/ERY	48 (7.57)	30 (10.8)	84 (4.96)	1 (0.67)
	OTHERS	21 (3.31)	2 (0.72)	18 (1.06)	0 (0)
5	CIP/TET/NAL/ERY/STR	**84 (13.3)**	**56 (20.1)**	**687 (40.6)**	**19 (12.8)**
	GEN/CIP/TET/NAL/STR	28 (4.42)	9 (3.22)	77 (4.55)	9 (0.64)
	OTHERS	7 (1.10)	1 (0.36)	14 (0.83)	0 (0)
6	Pan-resistant	46 (7.25)	10 (3.58)	**266 (15.7)**	9 (0.64)
	TOTAL	634 (100)	279 (100)	1,692 (100)	149 (100)

In bold the top three resistance profiles in each host species; GEN=Gentamicin; CIP=Ciprofloxacin; TET=Tetracycline; NAL=Nalidixic acid; ERY=Erythromycin; STR=Streptomycin.

Table 17. Antimicrobial phenotypic resistance profiles across animal host species in *C. jejuni*.

Number of antimicrobials	Antimicrobial profiles	*C. jejuni* broilers	*C. jejuni* turkeys	*C. jejuni* cattle
0	Pan-susceptible	46 (5.96)	**14 (6.06)**	**129 (15.6)**
1	CIP | TET | NAL | ERY | STR	32 (4.14)	9 (3.90)	**157 (19.0)**
2	CIP/NAL	**73 (9.46)**	**24 (10.4)**	66 (7.97)
	(GEN | CIP)/TET	**70 (9.07)**	7 (3.03)	14 (1.69)
	TET/STR	2 (0.26)	1 (0.43)	7 (0.84)
	OTHERS	2 (0.26)	2 (0.86)	5 (0.60)
3	CIP/TET/NAL	**474 (61.4)**	**159 (68.8)**	**374 (45.2)**
	TET/ERY/STR	0 (0)	0 (0)	0 (0)
	CIP/TET/(ERY | STR)	3 (0.39)	0 (0)	0 (0)
	OTHERS	2 (0.26)	0 (0)	7 (0.84)
4	CIP/TET/NAL/STR	47 (6.09)	11 (4.76)	52 (6.28)
	CIP/TET/NAL/ERY	10 (1.29)	2 (0.86)	1 (0.12)
	OTHERS	3 (0.39)	0 (0)	2 (0.24)
5	CIP/TET/NAL/ERY/STR	4 (0.52)	2 (0.86)	5 (0.60)
	GEN/CIP/TET/NAL/STR	1 (0.13)	0 (0)	5 (0.60)
	OTHERS	1 (0.13)	0 (0)	0 (0)
6	Pan-resistant	2 (0.26)	0 (0)	4 (0.48)
	TOTAL	772 (100)	231 (100)	828 (100)

In bold the top three resistance profiles in each host species; GEN=Gentamicin; CIP=Ciprofloxacin; TET=Tetracycline; NAL=Nalidixic acid; ERY=Erythromycin; STR=Streptomycin.

4.2.2. Association between resistance to gentamicin, streptomycin and erythromycin

In *C. coli*, a strong significant association was observed in the case of broilers and cattle for both streptomycin and gentamicin, while this association was only observed for gentamicin in pigs and for streptomycin in turkeys and the strength of the association was milder (Table 18). Likewise, pigs were slightly (RR=1.18) more likely to be resistant to gentamicin if they were already resistant to erythromycin.

In *C. jejuni*, significant associations were observed in broilers, turkeys and cattle, for gentamicin but especially for streptomycin, with a stronger association across species and antimicrobials when significant (Table 18).

Table 18. Association between phenotypic resistance to gentamicin or streptomycin and erythromycin in *C. coli* and *C. jejuni* isolates from livestock.

Bact.	Hosts	N	ERY-R (%)	Antimicrobial	STR/GEN R (%)	STR/GEN R among ERY-R (%)	*p-value*	RR*	95%CIs
C. coli	Broilers	634	34.5	Streptomycin	54.4	68.9	<0.001	**1.86**	**1.46 - 2.36**
				Gentamicin	14.7	24.7	<0.001	**1.90**	**1.54 - 2.36**
	Turkeys	279	36.6	Streptomycin	56.0	66.7	0.008	**1.58**	**1.13 - 2.21**
				Gentamicin	7.5	10.8	0.156	1.48	0.96 - 2.31
	Pigs	1692	66.7	Streptomycin	90.7	90.7	0.953	1.00	0.89 - 1.13
				Gentamicin	22.0	25.0	<0.001	**1.18**	**1.10 - 1.26**
	Cattle	149	19.5	Streptomycin	82.6	96.6	<0.001	**2.77**	**1.96 - 3.91**
				Gentamicin	12.1	31.0	0.002	**3.27**	**1.77 - 6.05**
C. jejuni	Broilers	772	2.5	Streptomycin	7.6	31.6	<0.001	**5.58**	**2.20 - 14.1**
				Gentamicin	1.0	15.8	<0.001	**17.90**	**6.47 - 49.5**
	Turkeys	231	2.6	Streptomycin	6.0	33.3	0.045	**7.75**	**1.55 - 38.7**
				Gentamicin	0.0	0.0	0.007	N/A	N/A
	Cattle	828	1.7	Streptomycin	10.3	78.6	<0.001	**32.05**	**9.12 - 113**
				Gentamicin	1.4	28.6	<0.001	**27.20**	**9.91 - 74.7**

*RR=Relative risk of any isolate presenting resistance to erythromycin when it is already resistant to streptomycin/gentamicin; in bold = significant RR values in all hosts

4.2.3. Analysis of *flaA* and AMR genes

Over 300 base-pairs (bp) (including the 267 bp-long SVR used in the analysis) of the *flaA* gene sequence were determined in 168 of the 176 chosen isolates (as explained in the Material and methods section). Figure 22 displays the phylogenetic tree constructed using the selected final 168 isolates including two main categories: those with at least simultaneous phenotypic resistance to gentamicin-erythromycin, streptomycin-erythromycin or both (co-resistant group, n=59) and all the rest (non-co-resistant group, n=109), plus the reference strain.

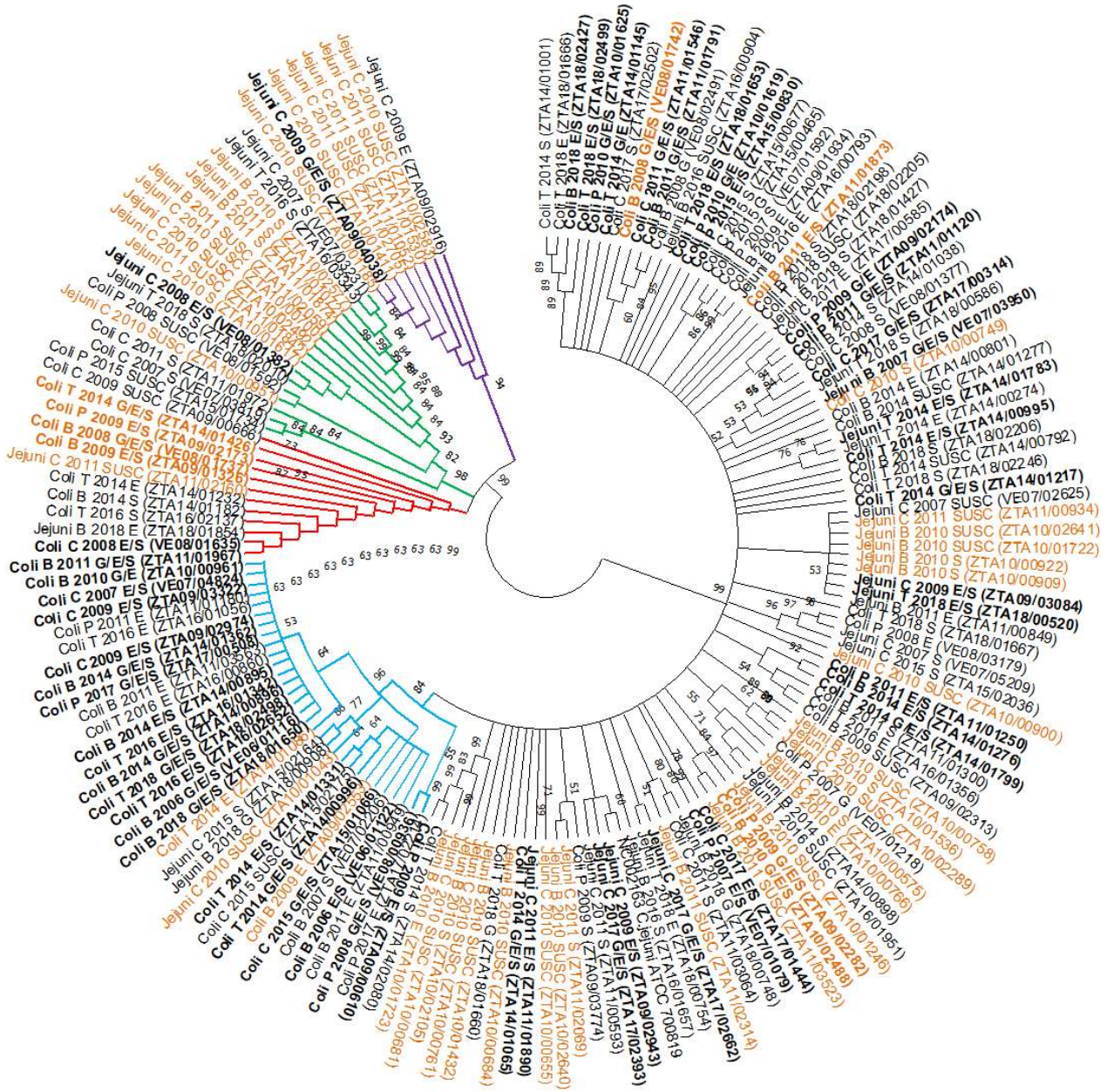


Figure 22. Neighbor-joining phylogenetic tree of the sequences of the short variable region (SVR) of flaA genes from 168 selected *Campylobacter* isolates [Group 1 = black; Group 2 = blue; Group 3 = red; Group 4 = green; Group 5 = violet; NC002163 = Reference strain; B = Broilers; T = Turkeys; P = Pigs; C = Cattle; G = Gentamicin; E = Erythromycin; S = Streptomycin; SUSC = susceptible isolates; aminoglycosides/macrolides co-resistant strains in bold; strains subjected to WGS in orange]. Only branches with bootstrap > 50 are shown.

Altogether, a total of 127 single nucleotide polymorphisms (SNPs) located in 100 polymorphic sites were found, leading to 73 unique *flaA*-SVR gene sequences. The haplotype diversity (Hd – probability that two randomly selected sequences are different) was 0.975, and every two sequences differed on average by 27 SNPs, with an overall mean evolutionary distance (d) between the two sequences of 0.09 substitutions per site.

The 168 isolates were classified into five well supported groups (bootstrapping>60) identified based on the topology of the tree: group 1, including the majority of the sequenced isolates (n=100 isolates), group 2 (n=31 isolates), group 3 (n=12 isolates), group 4 (n=17 isolates) and group 5 (n=8 isolates) (Figure 22), with certain groups containing more isolates from specific hosts (Table 19). Groups 2 and 3, consisting mainly of *C. coli* strains (28/31 and 10/12, respectively) predominantly from poultry (>60%), showed the highest proportions of resistant isolates, including also those resistant to both aminoglycosides and macrolides (Table 19).

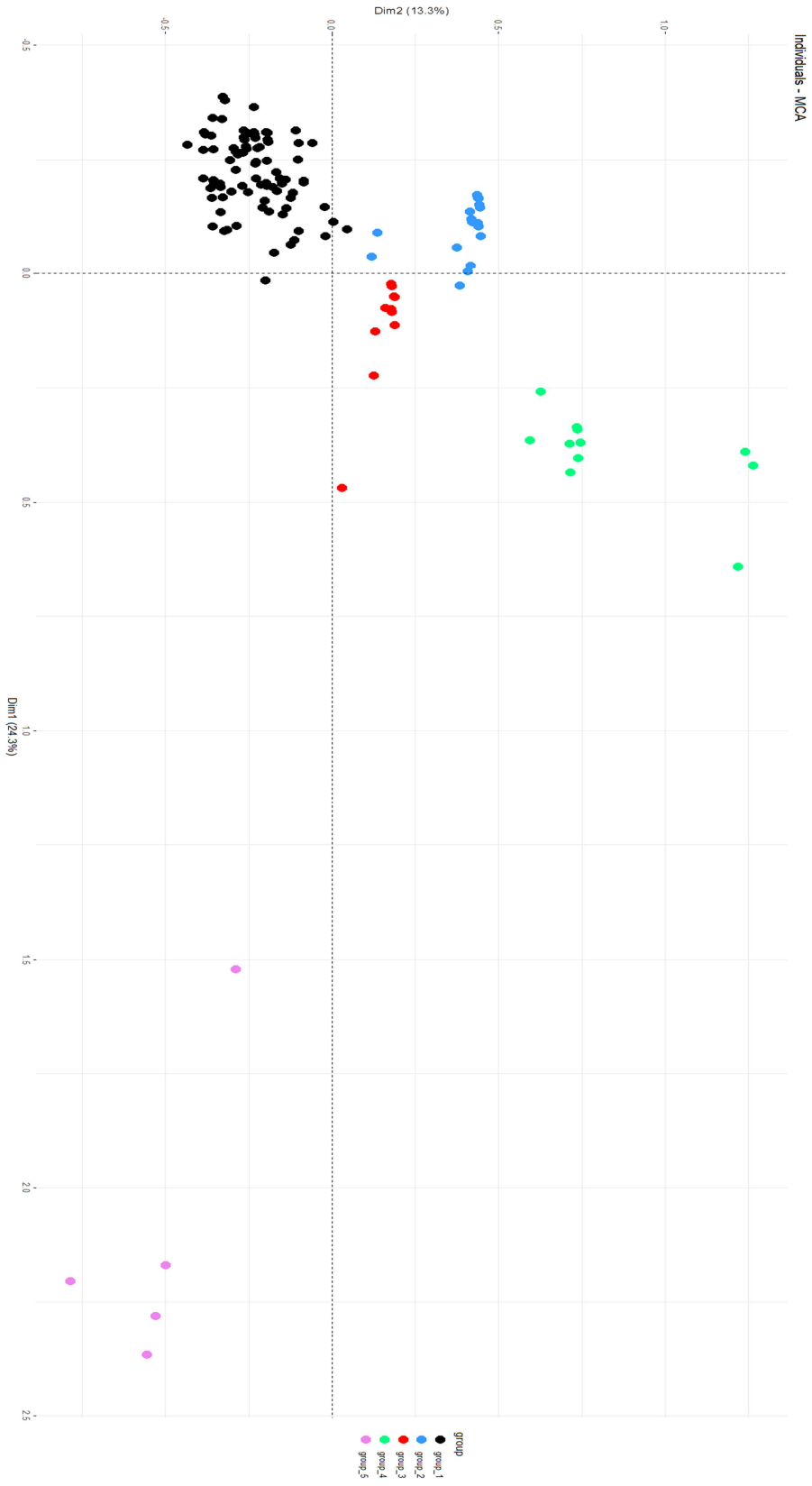
Groups 4 and 5 showed a higher proportion of *C. jejuni* isolates and those from cattle, and the frequency of isolates with simultaneous resistance to both antimicrobial classes in these groups was lower (Table 19).

Only complete RSCU values from the 20 variable codons for each of the 168 isolates were included in the MCA analysis, whose first two dimensions were able to explain 38% of the total variability observed. Isolates included in each of the five groups identified in the *flaA*-SVR phylogenetic tree were also clustered according to the first two dimensions of the MCA further supporting the grouping obtained through the phylogenetic tree, with group 5 and to a lesser extent group 4 isolates (mostly *C. jejuni* from cattle)well separated from other groups (Figure 23).

Table 19. Numbers and proportions of isolates included in each of the groups formed from the *flaA*-SVR gene phylogenetic analysis by phenotypic susceptibility, bacterial species, host species and co-resistant status (in bold =predominant clusters in each category).

Group No.	1	2	3	4	5	Total
N	(n=100)	(n=31)	(n=12)	(n=17)	(n=8)	(n=168)
C. coli	54 (54.0%)	**28 (90.3%)**	**10 (83.3%)**	4 (23.5%)	0 (0.0%)	96 (57.1%)
C. jejuni	46 (46.0%)	3 (9.7%)	2 (16.7%)	**13 (76.5%)**	**8 (100.0%)**	72 (42.9%)
Broilers	37 (37.0%)	**12 (38.7%)**	**5 (41.7%)**	4 (23.5%)	0 (0.0%)	58 (34.5%)
Cattle	27 (27.0%)	7 (22.6%)	3 (25.0%)	**9 (52.9%)**	**8 (100.0%)**	54 (32.2%)
Pigs	**14 (14.0%)**	**4 (12.9%)**	1 (8.3%)	2 (11.8%)	0 (0.0%)	21 (12.5%)
Turkeys	22 (22.0%)	**8 (25.8%)**	**3 (25.0%)**	2 (11.8%)	0 (0.0%)	35 (20.8%)
GEN-R	22 (22.0%)	**11 (35.5%)**	**3 (25.0%)**	0 (0.0%)	1 (12.5%)	37 (22.0%)
ERY-R	45 (45.0%)	**26 (83.9%)**	**8 (66.7%)**	1 (5.9%)	2 (25.0%)	82 (48.8%)
STR-R	58 (58.0%)	**19 (61.3%)**	**8 (66.7%)**	10 (58.8%)	1 (12.5%)	96 (57.1%)
Co-resistant	33 (33.0%)	**18 (58.1%)**	**6 (50.0%)**	1 (5.9%)	1 (12.5%)	59 (35.1%)

Figure 23 (next page). Distribution of the 168 isolates subjected to *flaA*-SVR gene sequencing according to the first two dimensions of a multiple correspondence analysis (MCA) performed considering information on the RSCU of variable codons, bacterial and host species, resistance to gentamicin, erythromycin and streptomycin, and group as determined in the NJ phylogenetic tree (observations are colored according to the group in black, blue, red, green and purple).


Individuals - MCA
Dim2 (13.3%)
Dim1 (24.3%)
group
group_1
group_2
group_3
group_4
group_5

4.3. Specific Objective 3

4.3.1. AMR determinants

A graphical representation of all AMR determinants found in the 194 sequenced *C. coli* and *C. jejuni* isolates, selected based on their susceptible/ resistant phenotype to aminoglycosides (gentamicin, streptomycin) and their host species, is shown in Figure 24. It displays the number of isolates phenotypically resistant to one or two aminoglycosides (gentamicin and/or streptomycin) (AMG-R, n=85; GEN-R, n=25; STR-R, n=81) and macrolides (erythromycin) (ERY-R, n=51), along with the types and number of genetic determinants found. Isolates with phenotypic resistance to one or more of the three antimicrobials (gentamicin, streptomycin and erythromycin) (n=100) included 60 of the 72 *C. coli* (14 broilers, 20 turkeys, 17 pigs and nine cattle) and 40 out of the 122 *C. jejuni* (25 broilers, six turkeys and nine cattle).

AMG-R determinants

All isolates phenotypically resistant to streptomycin and/or gentamicin harbored at least one aminoglycoside resistance determinant, except seven *C. jejuni* isolates (five resistant to streptomycin, one resistant to gentamicin and one resistant to both). Similarly, almost all 109 isolates susceptible to both aminoglycosides did not carry aminoglycoside resistance determinants except three *C. jejuni* (carrying the genes *aph(3')-IIIa*, *aad9* and *ant(6)-Ia*) and four *C. coli* (carrying the genes *aph(3')-IIIa*, *aad9*, *ant(6)-Ia*, *aadE-Cc* and *spw*).

The number of AMG-related AMR determinants present in AMG-R isolates ranged between zero and six (median=2), with a significantly (Mann-Whitney, $p<0.001$) higher number of determinants observed in *C. coli* compared with *C. jejuni* (median 2 *versus* 1, respectively), in line with the higher proportion of AMG-R isolates found in *C. coli* (71%) compared with *C. jejuni* (28%). These medians were maintained across host species in *C. jejuni* but not in *C. coli*, with more AMR determinants in pigs (median=4) and less in cattle (median=1.5), although this difference was not significant (Kruskal-Wallis, $p=0.28$) (Figure 24). These findings were also in line with the phenotypic proportions of resistance observed in the four host species for *C. coli*, at 62%, 64%, 73% and 89%, in broilers, turkeys, cattle and pigs, respectively.

Independently of their phenotype, the GEN-R associated determinants *aac(6')-Im* and *aph(2")-Ib* were found in a single isolate (a *C. coli* of swine origin). The rest of GEN-R related determinants (*aph(2")-Ic*, *aph(2")-If* and *aph(2")-Ih*) were found exclusively in *C. coli* isolates. In contrast, STR-R associated determinants were widely distributed among both *C. coli* and *C. jejuni*, except ribosomal *S12 rpsL-K43R* point mutations, which were found only in one *C. coli* and one *C. jejuni* from cattle (Figure 24). The AMG-R generic determinant *aph(3')-IIIa* (associated to resistance to gentamicin, streptomycin and other aminoglycosides) was more common in *C. coli* (n=23/72; 32%) than in *C. jejuni* (n=14/122; 11%) isolates, whereas other AMG-R genes (*aad9*, *spw* and *apmA*) were found almost exclusively among *C. coli* isolates (with only two *C. jejuni* isolates carrying the *aad9* gene) (Figure 24).

ERY-R resistance determinants

Out of all ERY-R strains (42 *C. coli* and nine *C. jejuni*), 35 *C. coli* and three *C. jejuni* harbored at least one ERY-R associated determinant. Similarly, the majority (n=113/143; 79%) of the erythromycin susceptible isolates in the collection did not carry ERY-R associated determinants (Figure 24).

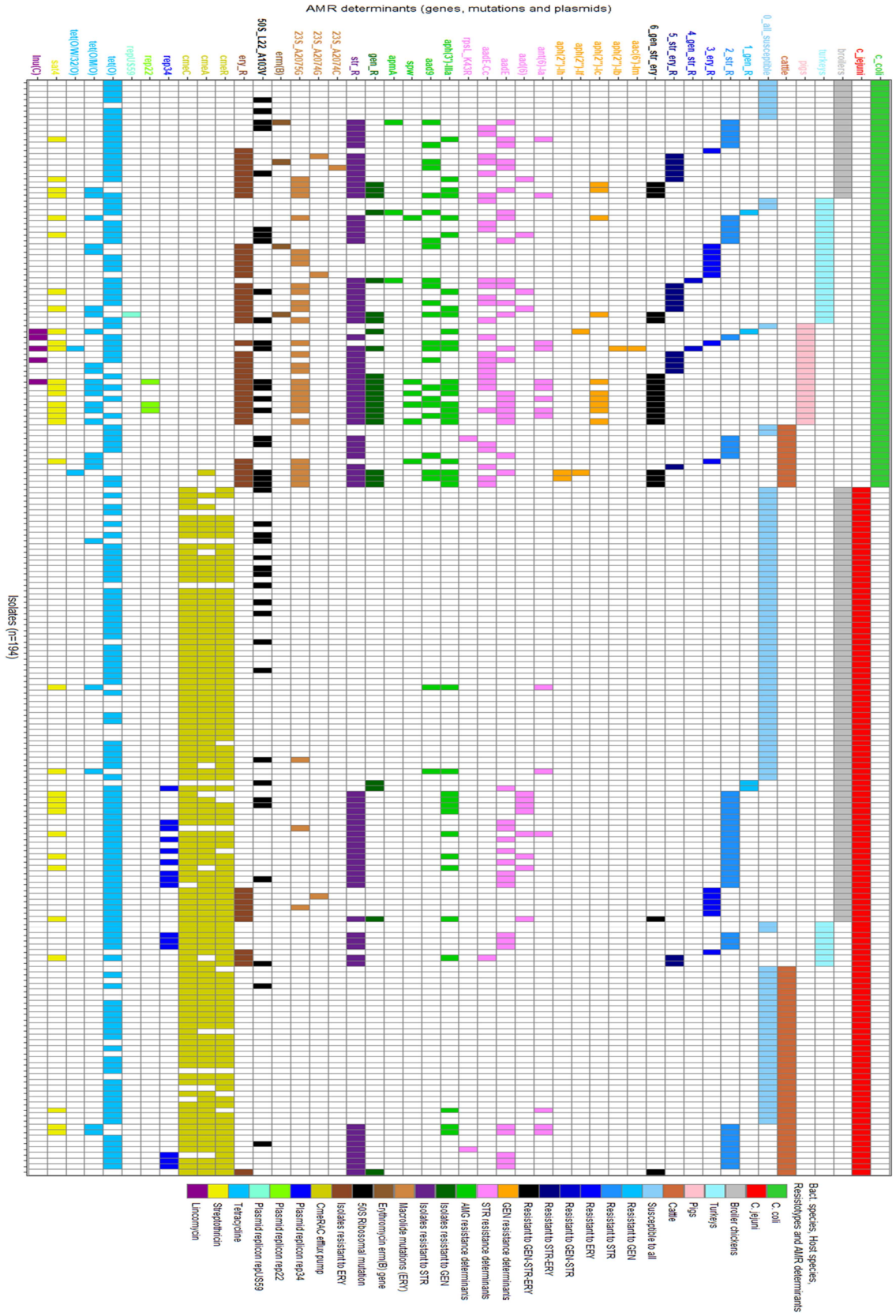
The number of ERY-R-related AMR determinants present in ERY-R isolates ranged between zero and two (median=1), with again significantly (Mann-Whitney, p=0.02) more determinants observed in ERY-R *C. coli* compared with *C. jejuni* (median 1 *versus* 0, respectively), in line with the higher proportion of ERY-R isolates found in *C. coli* (57%) compared with *C. jejuni* (8%). These medians were maintained across bacterial and host species, except for *C. coli* in cattle (median=2) and *C. jejuni* in turkeys (median=0.5), though significant differences were not found (Kruskal-Wallis, p=0.09) (Figure 24).

Independently of their phenotype, the macrolide resistance determinants identified among the isolates in the collection included mutations in the 23S ribosomal subunit (*A2074C* and *A2074G* observed in one and three strains, respectively, and *A2075G* present in 32 strains), the *erm(B)* gene (found in four *C. coli* isolates) and mutations in the 50S ribosomal subunit (*L22-A103V*, present in 41 strains).

AMR determinants for other antimicrobial classes

Regarding other antimicrobial classes, 168 out of 194 (87%) isolates harbored between one and two tetracycline resistance genes, with *tet(O)* being found in a large number of both, *C. coli* (51/72, 71%) and *C. jejuni* (94/122, 77%) isolates whereas *tet(O/M/O)* and *tet(W/N/W)* were more abundant in *C. coli* (21/72, 29%) than in *C. jejuni* (5/122, 4%). One hundred and sixty-six out of 194 (86%) isolates had a chromosomal mutation in the quinolone-resistance determinant region (*gyrA_T86I* (C-257-T)) and 152 out of 194 (78%) isolates harbored one or more class D beta-lactamase encoding *blaOXA* genes (143 and nine isolates presented one and two genes, respectively). The majority of these genes (89%) belonged to the *blaOXA-61-like* family with 12 protein types and the remaining 11% belonged to the *blaOXA-184-like* family with four protein types. The streptothricin resistance encoding AMR gene *sat4* was found in 33 out of 194 (17%) isolates and the lincosamide resistance encoding gene *lnc(C)* was only found in five *C. coli* isolates. For beta-lactamases, streptothricin and lincosamides no phenotypes were available to include in the study as they were not part of official panels. Genes associated with the *CmeRABC* efflux pump were present in most *C. jejuni* (116/122) while missing in all but one of the 72 *C. coli* isolates (Figure 24).

Figure 24 (next page). Distribution of aminoglycoside and erythromycin resistance determinants in *C. coli* and *C. jejuni* among broilers, turkeys, pigs and cattle.


AMR determinants (genes, mutations and plasmids)
Isolates (n=194)
C_coli
C_jejuni
broilers
turkeys
pigs
cattle
0_all_susceptible
1_gen_R
2_str_R
3_ery_R
4_gen_str_R
5_str_ery_R
6_gen_str_ery
aac(6')-Im
aph(2")-Ib
aph(2")-Ic
aph(2")-Id
aph(2")-If
aph(3')-Ih
ant(6)-Ia
aadE
aadE-Cc
aadS
ant(6)-I
aph(3')-IIa
rpsL_K43R
spw
aad9
aphA
gen_R
str_R
23S_A2074C
23S_A2074G
23S_A2075G
emrB
ery_R
50S_L22_A103V
cmeR
cmeA
cmeC
rep34
rep22
repUS59
tet(O)
tet(O/M/O)
tet(O/W/32/O)
sat4
lnu(C)
Bact. species, Host species, Resistotypes and AMR determinants
C. coli
C. jejuni
Broiler chickens
Turkeys
Pigs
Cattle
Suscepible to all
Resistant to GEN
Resistant to STR
Resistant to ERY
Resistant to GEN-STR
Resistant to STR-ERY
Resistant to GEN-STR-ERY
GEN resistance determinants
STR resistance determinants
AMG resistance determinants
Isolates resistant to GEN
Isolates resistant to STR
Isolates resistant to ERY
50S Ribosomal mutation
Erythromycin emrB) gene
Macrolide mutations (ERY)
CmeR-4C efflux pump
Plasmid replicon rep34
Plasmid replicon rep22
Plasmid replicon repUS59
Tetracycline
Streptothricin
Lincomycin

4.3.2. Genetic prediction of AMG-R/ERY-R phenotypes

The concordance between the presence of AMR determinants and resistance phenotypes was evaluated for gentamicin, streptomycin and erythromycin in all *Campylobacter* and stratified by *C. coli* and *C. jejuni*, including and excluding the *aph(3')-IIIa* gene in AMG-R isolates and the *50S_L22_A103V* mutation in ERY-R isolates due to their apparent lack of association with resistance phenotypes (Tables 20-22).

For GEN-R, a moderate agreement between phenotypic resistance and carriage of AMR determinants was found with or without the *aph(3')-IIIa* gene, considering all *Campylobacter* (*kappa*=0.63 *versus* 0.48, respectively) or only *C. coli* isolates (*kappa*=0.66 *versus* 0.62, respectively). In contrast, the agreement was very low in *C. jejuni* GEN-R isolates both with or without the *aph(3')-IIIa* gene though this could be influenced by the very low number of GEN-R *C. jejuni* isolates in the collection (Table 20). For STR-R isolates, the level of agreement was high, both including (*kappa*=0.83) and excluding (*kappa*=0.85) the *aph(3')-IIIa* gene. When stratifying by bacterial species, the level of agreement was slightly lower for *C. coli* than for *C. jejuni* both including or excluding the *aph(3')-IIIa* gene (Table 21).

For ERY-R isolates, the agreement was higher when excluding the *50S_L22_A103V* mutation, both overall (*kappa*=0.72 *versus* 0.47 including it), for *C. coli* (*kappa*=0.75 *versus* 0.51 including it) and for *C. jejuni* (kappa= 0.25 *versus* 0.08 including it) though lower values were observed in this case due to the inability to predict resistant phenotypes (Table 22). The presence of *CmeRAC* efflux pump genes in our collection was not related to phenotypic resistance to either aminoglycosides or macrolides, therefore they were excluded from further analyses (Figure 24).

Table 20. Agreement between the carriage of aminoglycoside resistance genes and phenotypic resistance to gentamicin (GEN) for all *Campylobacter* (ALL) and for *C. coli* and *C. jejuni* separately.

	R isolates number	S isolates number	True Pos. (G+/P+)	False Neg. (G-/P+)	False Pos. (G+/P-)	True Neg. (G-/P-)	Sensitivity	Specificity	PPV[1]	NPV[2]	Kappa	% agreement
GEN ALL [including aph(3')-IIIa]	25	169	18	7	21	148	0.72	0.88	0.46	0.95	0.48	86%
GEN ALL [excluding aph(3')-IIIa]	**25**	**169**	**13**	**12**	**1**	**168**	**0.52**	**0.99**	**0.93**	**0.93**	**0.63**	**93%**
GEN *C. coli* [including aph(3')-IIIa]	21	51	17	4	8	43	0.81	0.84	0.68	0.91	0.62	83%
GEN *C. coli* [excluding aph(3')-IIIa]	**21**	**51**	**13**	**8**	**1**	**50**	**0.62**	**0.98**	**0.93**	**0.86**	**0.66**	**88%**
GEN *C. jejuni* [including aph(3')-IIIa]	4	118	1	3	13	105	0.25	0.89	0.07	0.97	0.06	87%
GEN *C. jejuni* [excluding aph(3')-IIIa]	**4**	**118**	**0**	**4**	**0**	**118**	**0**	**1**	**0**	**0.97**	**-**	**97%**

[1]Positive Predictive Value; [2]Negative Predictive Value; **bold = excluding specific determinants**

Table 21. Agreement between the carriage of aminoglycoside resistance genes and phenotypic resistance to streptomycin (STR) for all *Campylobacter* (ALL) and for *C. coli* and *C. jejuni* separately.

	R isolates number	S isolates number	True Pos. (G+/P+)	False Neg. (G-/P+)	False Pos. (G+/P-)	True Neg. (G-/P-)	Sensitivity	Specificity	PPV[1]	NPV[2]	Kappa	% agreement
STR ALL [including aph(3')-IIIa]	81	113	74	7	9	104	0.91	0.92	0.89	0.94	0.83	92%
STR ALL [excluding aph(3')-IIIa]	**81**	**113**	**74**	**7**	**7**	**106**	**0.91**	**0.94**	**0.91**	**0.94**	**0.85**	**93%**
STR *C. coli* [including aph(3')-IIIa]	49	23	48	1	3	20	0.98	0.87	0.94	0.95	0.87	94%
STR *C. coli* [excluding aph(3')-IIIa]	**49**	**23**	**35**	**14**	**2**	**21**	**0.71**	**0.91**	**0.95**	**0.60**	**0.55**	**78%**
STR *C. jejuni* [including aph(3')-IIIa]	32	90	26	6	4	86	0.81	0.96	0.87	0.93	0.78	92%
STR *C. jejuni* [excluding aph(3')-IIIa]	**32**	**90**	**18**	**14**	**1**	**89**	**0.56**	**0.99**	**0.95**	**0.86**	**0.63**	**88%**

[1]Positive Predictive Value; [2]Negative Predictive Value; **bold = excluding specific determinants**

Table 22. Agreement between the carriage of erythromycin resistance determinants and phenotypic resistance to erythromycin (ERY) for all *Campylobacter* (ALL) and for *C. coli* and *C. jejuni* separately.

	R isolates number	S isolates number	True Pos. (G+/P+)	False Neg. (G-/P+)	False Pos. (G+/P-)	True Neg. (G-/P-)	Sensitivity	Specificity	PPV[1]	NPV[2]	Kappa	% Agrement
ERY ALL [including 50S mutation]	51	143	38	13	31	112	0.75	0.78	0.55	0.90	0.47	77%
ERY ALL [excluding 50S mutation]	**51**	**143**	**36**	**15**	**5**	**138**	**0.71**	**0.97**	**0.88**	**0.90**	**0.72**	**90%**
ERY *C. coli* [including 50S mutation]	41	31	35	6	11	20	0.85	0.65	0.76	0.77	0.51	76%
ERY *C. coli* [excluding 50S mutation]	**41**	**31**	**34**	**7**	**2**	**29**	**0.83**	**0.94**	**0.94**	**0.81**	**0.75**	**88%**
ERY *C. jejuni* [including 50S mutation]	10	112	3	7	20	92	0.30	0.82	0.13	0.93	0.08	78%
ERY *C. jejuni* [excluding 50S mutation]	**10**	**112**	**2**	**8**	**2**	**110**	**0.20**	**0.98**	**0.50**	**0.93**	**0.25**	**92%**

[1]Positive Predictive Value; [2]Negative Predictive Value; **bold = excluding specific determinants.**

4.3.3. Analysis of Minimum Inhibitory Concentrations

A significant association between the number of AMR determinants and the log-transformed MIC values was only found among *C. coli* isolates for gentamicin and erythromycin ($p<0.05$) (Figures 25-25b). In the case of the other bacteria-antimicrobial combinations no significant increase in the MIC values was observed as the number of AMR determinants increased in a given isolate.

The relationship between the MIC and the presence of specific genetic AMR determinants was evaluated separately for each determinant for both gentamicin (Figure 26) and erythromycin (Figure 27). In the case of the relationship between *C. coli* and gentamicin, all isolates but one bearing GEN-R specific determinants displayed high MICs, whereas the AMG-R generic determinant *aph(3')-IIIa* was also distributed along low MICs (Figure 26). When stratifying by host, this significant association was still observed but only for pigs and cattle *C. coli* isolates in the case of gentamicin ($p<0.05$) (Figure 28). No significant association was observed in *C. jejuni* isolates (Figure 28b).

The stratification by determinants in *C. coli* for erythromycin showed that all isolates but one bearing *23S* mutations displayed high MICs, whereas isolates bearing *50S* mutations and *erm(B)* genes were distributed along the whole MIC spectrum (Figure 27).

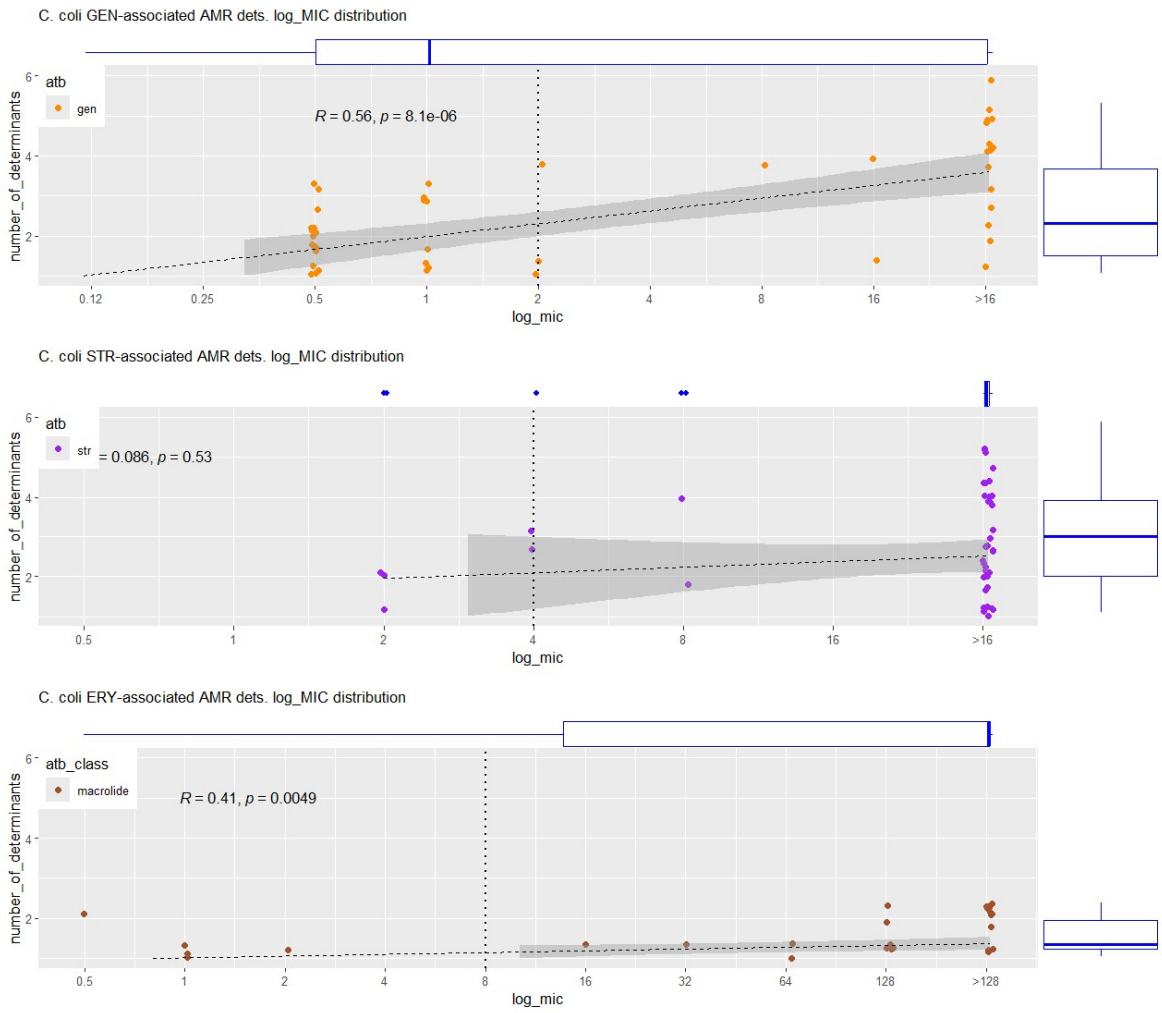


Figure 25. Number of AMR determinants along log-transformed MICs in gentamicin, streptomycin and erythromycin for *C. coli* (vertical dotted lines denote ECOFFs for the different antimicrobials; fitted with Spearman correlation lines (regression lines) for linear trend (best fit of data) and box-and-whisker plots; shadowed areas around the lines represent the confidence intervals of the regression).

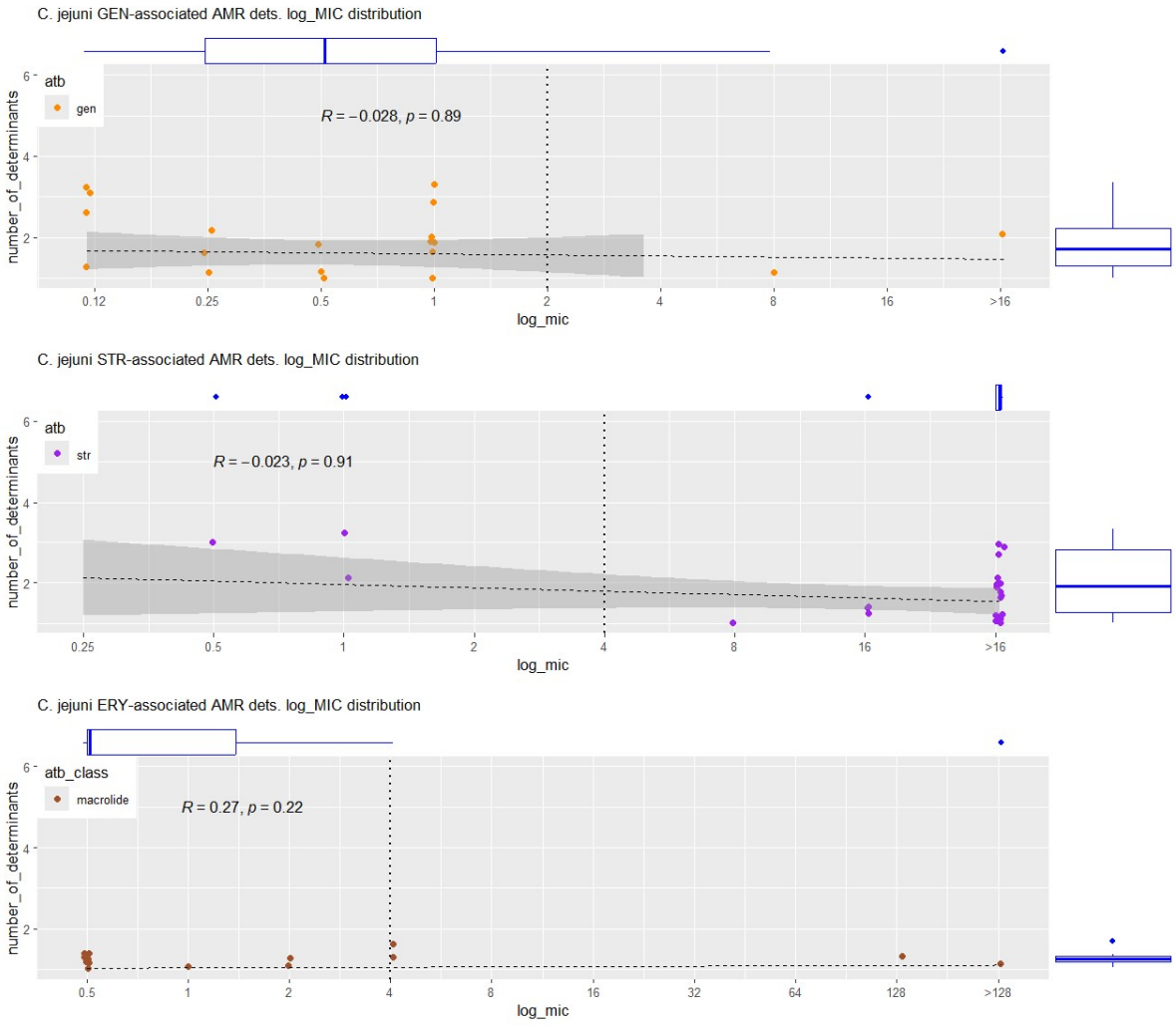


Figure 25b. Number of AMR determinants along log-transformed MICs in gentamicin, streptomycin and erythromycin for *C. jejuni* (vertical dotted lines denote ECOFFs for the different antimicrobials; fitted with Spearman correlation lines (regression lines) for linear trend (best fit of data) and box-and-whisker plots; shadowed areas around the lines represent the confidence intervals of the regression).

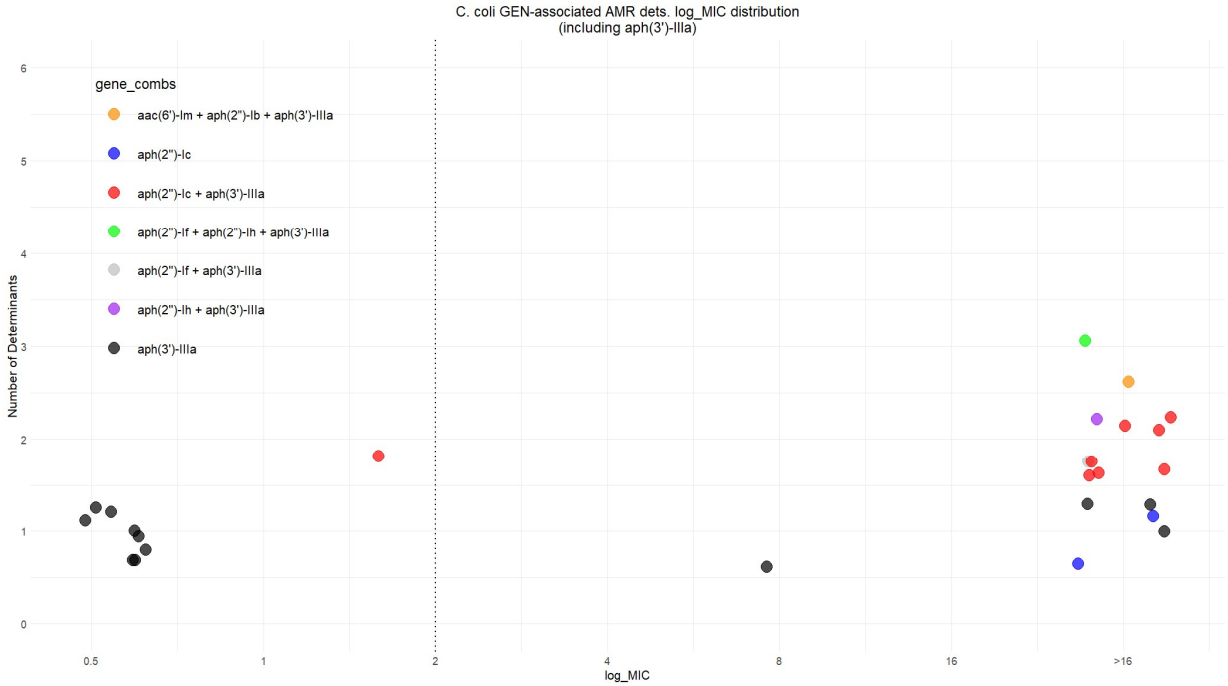


Figure 26. Distribution of *C. coli* GEN-associated determinants (both generic and specific) along the log MIC spectrum.

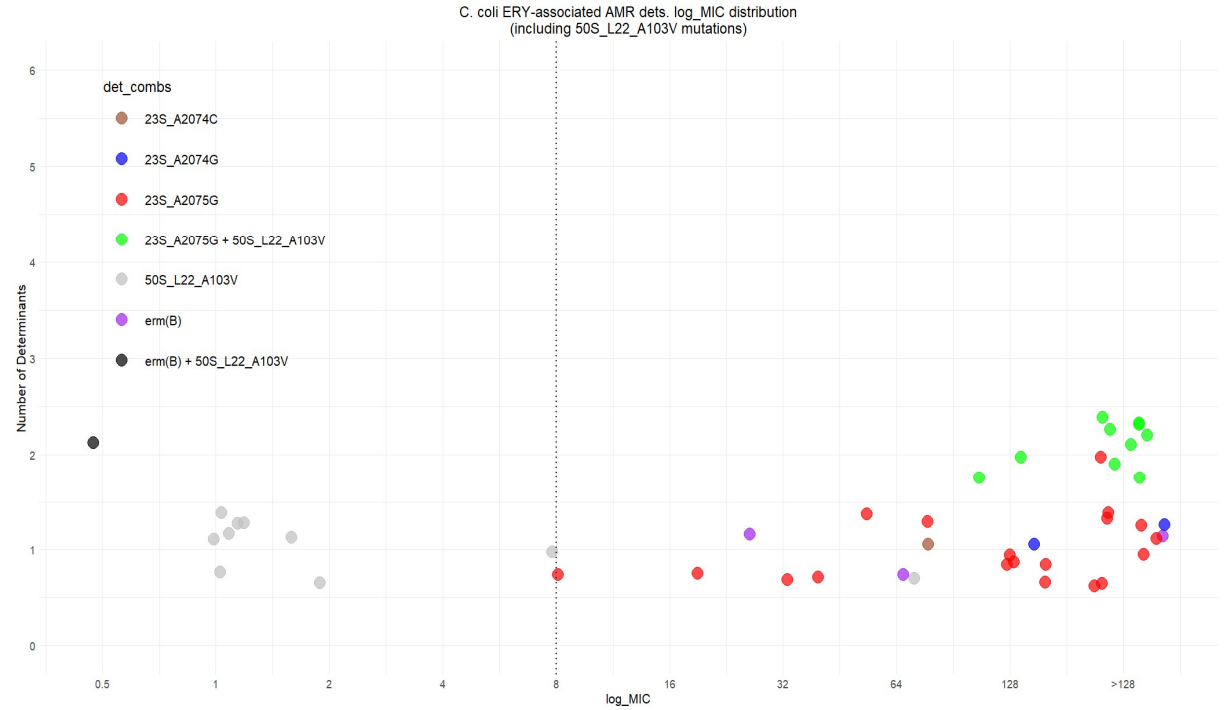


Figure 27. Distribution of *C. coli* erythromycin determinants along the log MIC spectrum.

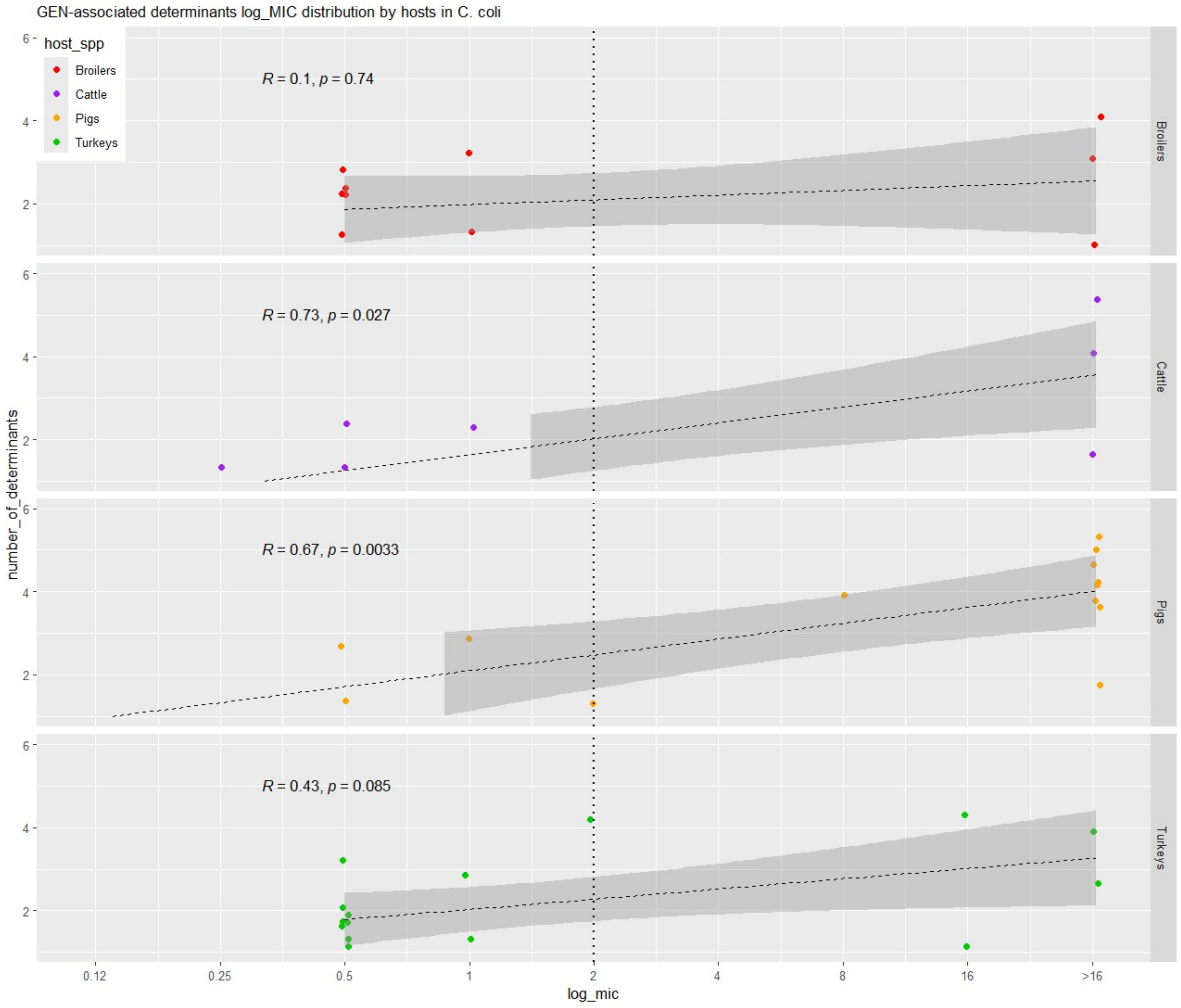


Figure 28. Distribution of AMR determinants associated with resistance to GEN along log-transformed MICs stratified by hosts in *C. coli* (vertical dotted line denotes ECOFF for gentamicin in *C. coli;* fitted with Spearman correlation lines (regression lines) for linear trend (best fit of data); shadowed areas around the lines represent the confidence intervals of the regression).

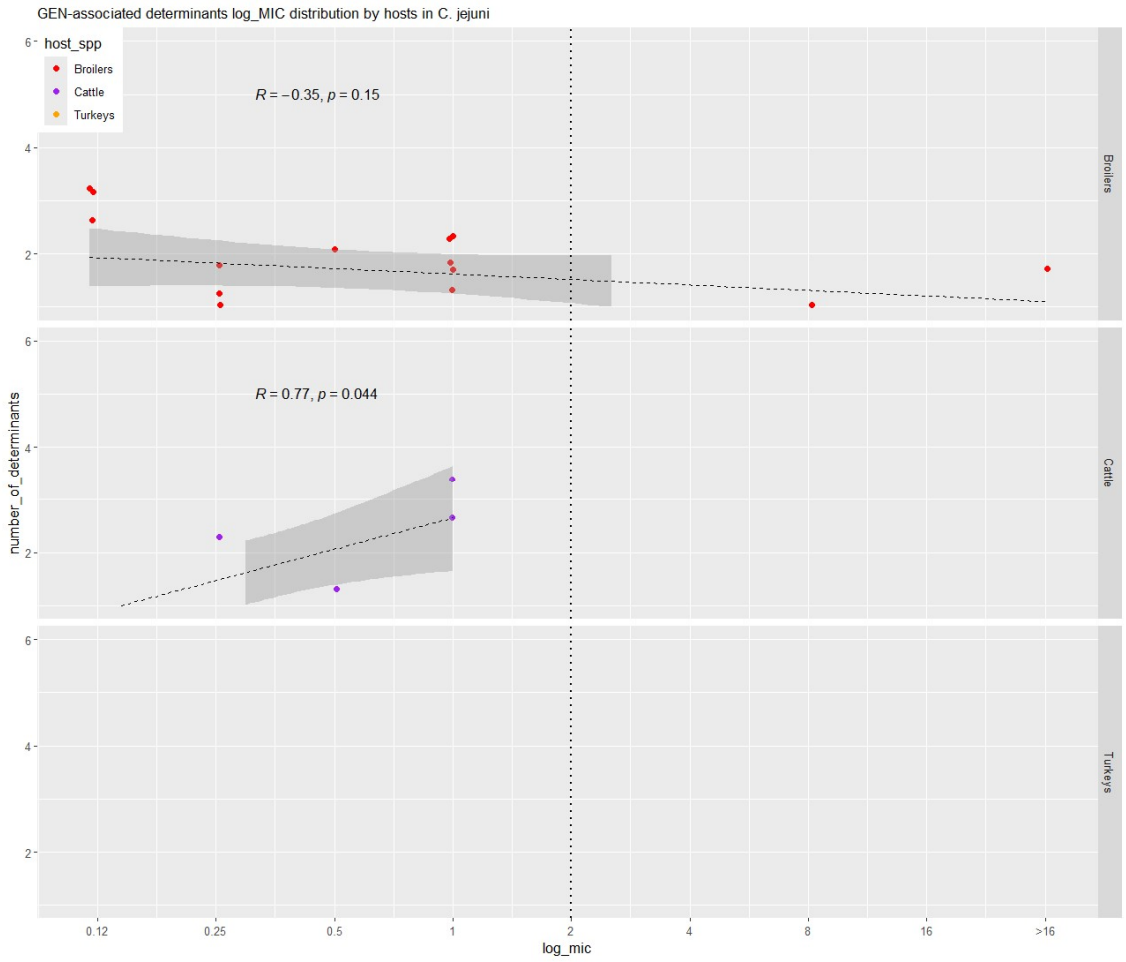


Figure 28b. Distribution of AMR determinants associated with resistance to GEN along log-transformed MICs stratified by hosts in *C. jejuni* (vertical dotted line denotes ECOFF for gentamicin in *C. jejuni*; fitted with Spearman correlation lines (regression lines) for linear trend (best fit of data); shadowed areas around the lines represent the confidence intervals of the regression).

The association between the simultaneous presence of different pairs of AMR determinants is shown as a matrix on Figure 29. Several genes associated to STR-R (*aadE*, *aad9, aadE-Cc* and *ant(6)-Ia*) and GEN-R (*aph(2'')-Ic*) were positively associated. In addition, a positive association between some of these genes and certain MGEs (*IS30(ISHahy9), IS1595(ISCco2)* and *rep22*) was also observed (Figure 29).

Networks displayed in Figures 30-30b show the number of STR-R (n=74) and GEN-R (n=17) isolates, respectively, in which each pair of AMR genes/MGE determinants was found together, with genes *aph(3')-IIIa* and *aadE/aadE-Cc* often found along other determinants among STR-R isolates, while a lower connectivity was observed for GEN-R, with the pair *aph(3')-IIIa* and *aph(2'')-Ic* being the most frequent. The *aph(3')-IIIa* gene was thus found in high proportion in most isolates, both STR-R and GEN-R isolates.

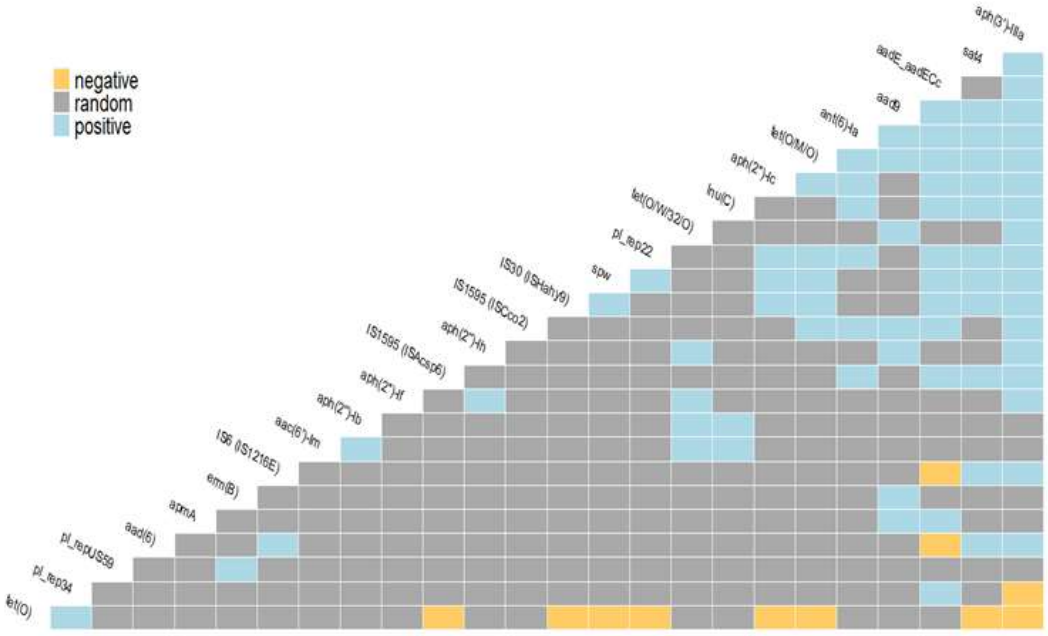


Figure 29. Co-occurrence matrix between gentamicin, streptomycin and erythromycin associated resistance determinants in all *Campylobacter* (yellow colour=lack of co-occurrence (negative result); grey colour=undetermined (random result); blue colour=co-occurrence (positive result)).

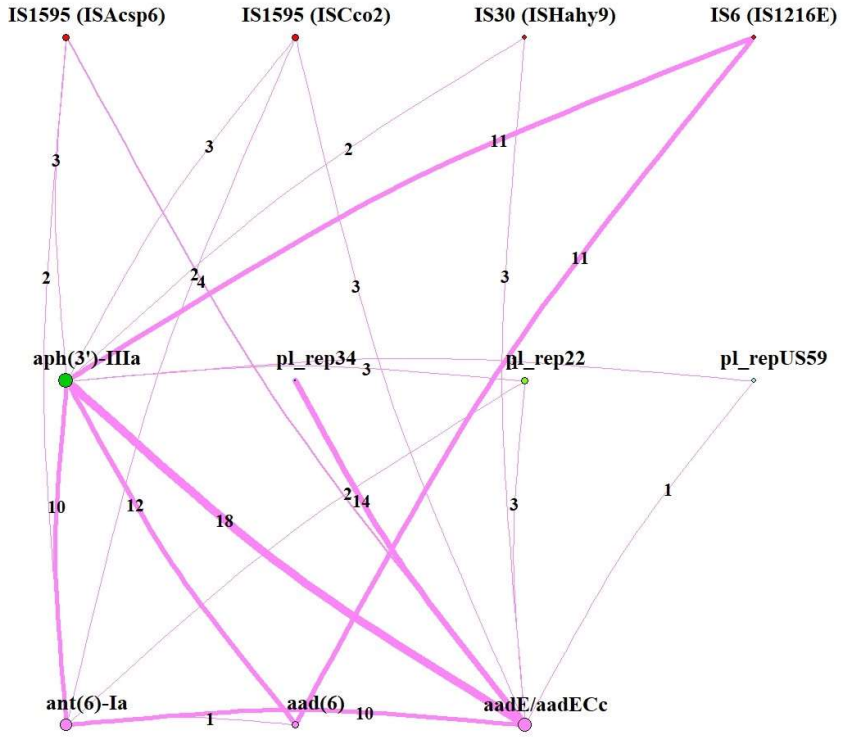


Figure 30. Network analysis of determinant and MGE associations in STR-R isolates (n=74).

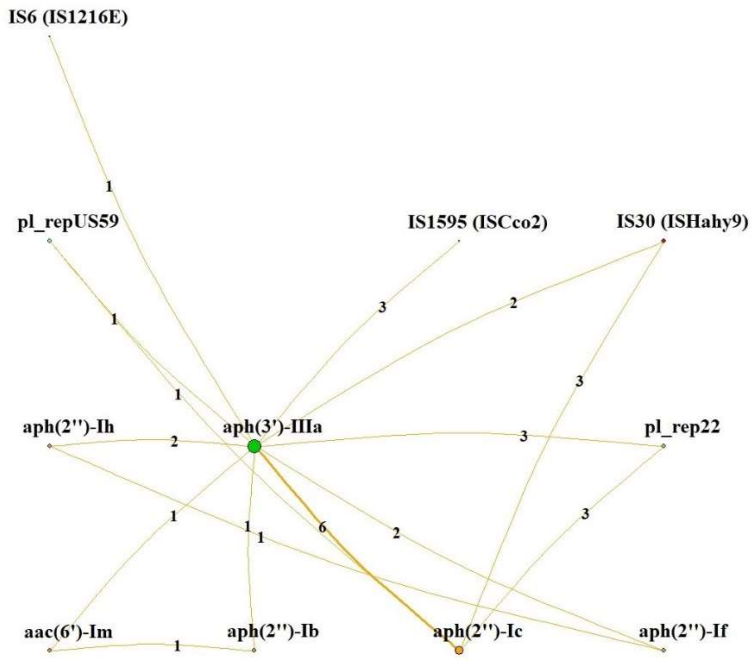


Figure 30b. Network analysis of determinant and MGE associations in GEN-R isolates (n=17).

4.3.4. Genetic contexts and MGEs

A total of 110 isolates harbored either aminoglycoside resistance determinants, macrolide resistance determinants or both. The ARGs for aminoglycosides and macrolides (excluding point mutations) were never located in the same contig with the exception of the *erm(B)* gene, found together with several AMG-R genes in the same contig in three out of four isolates that harbored this gene.

Forty-nine out of 83 (59%) isolates with AMG-R determinants harbored two (n=23/49) or more (n=26/49) determinants, and in 88% (n=43/49) and 94% (n=46/49) of them these determinants were located on the same contig and/or on genomic islands, respectively. In contrast, only one of the 36 isolates with a single aminoglycoside determinant carried it on a genomic island.

Fifty-six out of 69 (81%) isolates with ERY-R determinants harbored only one determinant, and the remaining 13 (19%) isolates presented a combination of *23S A2075G* and *50S_L22_A103V* mutations (n=11), the *erm(B)* gene and the *50S_L22_A103V* mutation (n=1) or two copies of the *23S A2075G* mutation (n=1; *C. coli* from cattle, on the same contig).

The AMG-R and ERY-R genes were found in the bacterial chromosome in 63% (70/110) of the isolates carrying them, including both *Campylobacter* species and all four hosts, with the remaining isolates carrying them in contigs belonging to plasmids according to MOB-Suite (23 non-mobilizable, 16 conjugative and one mobilizable plasmid).

As mentioned above, in this study 49 isolates harbored two or more AMG-R determinants, representing 36% (n=18/50), 48% (n=12/25), 71% (n=12/17) and 39% (n=7/18), of broiler, turkey, pig and cattle isolates, respectively. Sixty-eight isolates bore at least one ERY-R determinant, representing 62% (n=31/50), 64% (n=16/25), 71% (n=12/17) and 50% (n=9/18), of broilers, turkeys, pigs and cattle, respectively.

The classification of isolates per host species according to the likely location (plasmid or chromosomal) of AMR determinants conferring resistance to any antimicrobial class and to the presence of MDRGIs in their genome is shown in Table 23. Pigs (n=12/17; 71%) and turkeys (n=11/20; n=55%) were the host species with the highest frequency of isolates carrying MDRGIs. Likewise, the location of AMR determinants in plasmids (including conjugative, mobilizable and non-mobilizable) was more common in pigs (Table 23).

Table 23. Classification of bacterial species per host according to presence of MDRGI among isolates carrying at least one AMR determinant and likely location of the AMR determinant.

				Location of AMR determinants		
Bacterial species	**Hosts**	**N**	**MDRGIs**	**Chromosome**	**NC/NM plasmids**	**C/M plasmids**
C. coli	Broilers	**16**	**7 (44%*)**	**9 (56%)**	5 (31%)	2 (13%)
	Turkeys	**20**	**10 (50%)**	**14 (70%)**	4 (20%)	2 (10%)
	Pigs	**17**	**12 (71%)**	6 (35%)	**8 (47%)**	3 (18%)
	Cattle	9	3 (33%)	**5 (56%)**	2 (22%)	2 (22%)
C. jejuni	Broilers	**34**	10 (29%)	**27 (79%)**	4 (12%)	3 (9%)
	Turkeys	5	1 (20%)	**4 (80%)**	0 (0%)	1 (20%)
	Cattle	**9**	**4 (44%)**	**5 (56%)**	0 (0%)	4 (44%)

**Percentage of trait among total of traits per Bacteria-Host combination for both categories.*

In bold: higher values per trait and category; NC/NM=Non-conjugative or non-mobilizable plasmids; C/M=conjugative or mobilizable plasmids

The number of AMR determinants found in *C. coli* and *C. jejuni* isolates over time did not experience variation throughout the period for any of the four host species.

An MCA analysis using the presence/absence of AMR determinants conferring resistance to gentamicin, streptomycin and erythromycin and of MGEs along with resistance phenotype to these three antimicrobials and bacterial and host species was performed in a new attempt to identify subgroups among *C. coli* and *C. jejuni* depending on these characteristics. The first three components identified in this second MCA explained 33% of the variance present in the data set (Figure 31). Variables contributing to each of the first two dimensions are shown in Figure 32.

Five distinct clusters were identified in the HCPC based on the results from the MCA (Figure 33, Table 24). The variability observed between clusters 1, 3, 4 and 5 was mostly explained along Dimension 1 of the MCA whereas cluster 2 was mostly explained by Dimension 2.

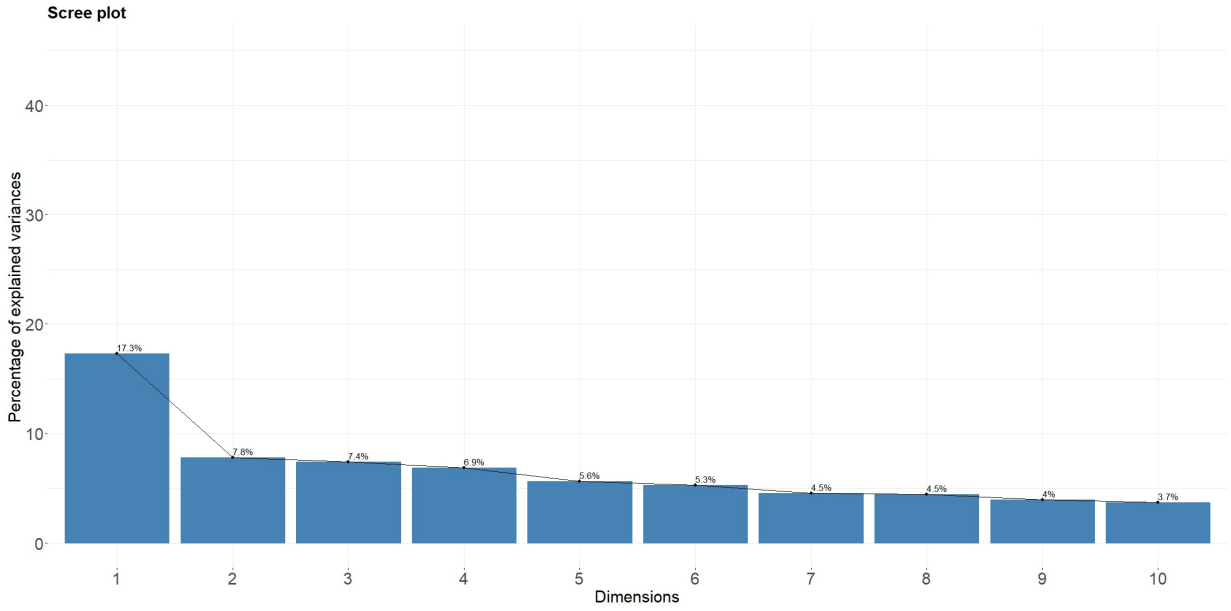
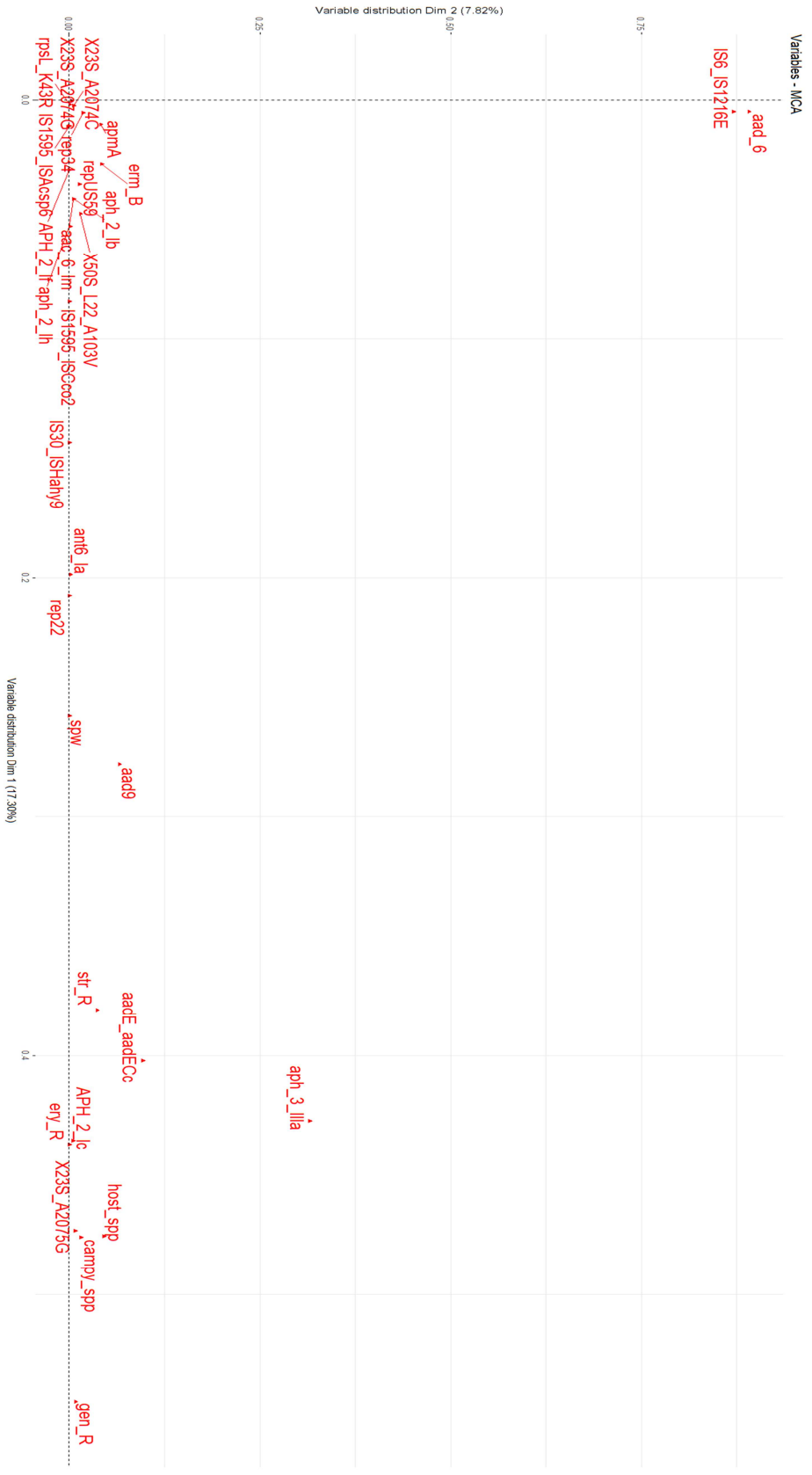


Figure 31. Proportion of variability explained by the different variables across the first 10 dimensions of the MCA (scree plot).

Figure 32 (next page). Distribution of the different variables (components) over Dimensions 1 and 2 of the MCA.


Variables - MCA
Variable distribution Dim 2 (7.82%)
Variable distribution Dim 1 (17.30%)
0.00
0.25
0.50
0.75
0.0
0.2
0.4
aad_6
IS6_IS1216E
ermA
ermB
aph_2_Ib
repUS59
X23S_A2074C
X23S_A2074G
rep34
aac_6_Im
X50S_L22_A103V
IS1595_ISCco2
rpsL_K43R
IS1595_ISAcsp6
APH_2_If
aph_2_Ih
IS30_ISHahy9
ant6_Ia
rep22
spw
aad9
aadE_aadECc
str_R
aph_3_IIIa
APH_2_Ic
ery_R
host_spp
X23S_A2075G
campy_spp
gen_R

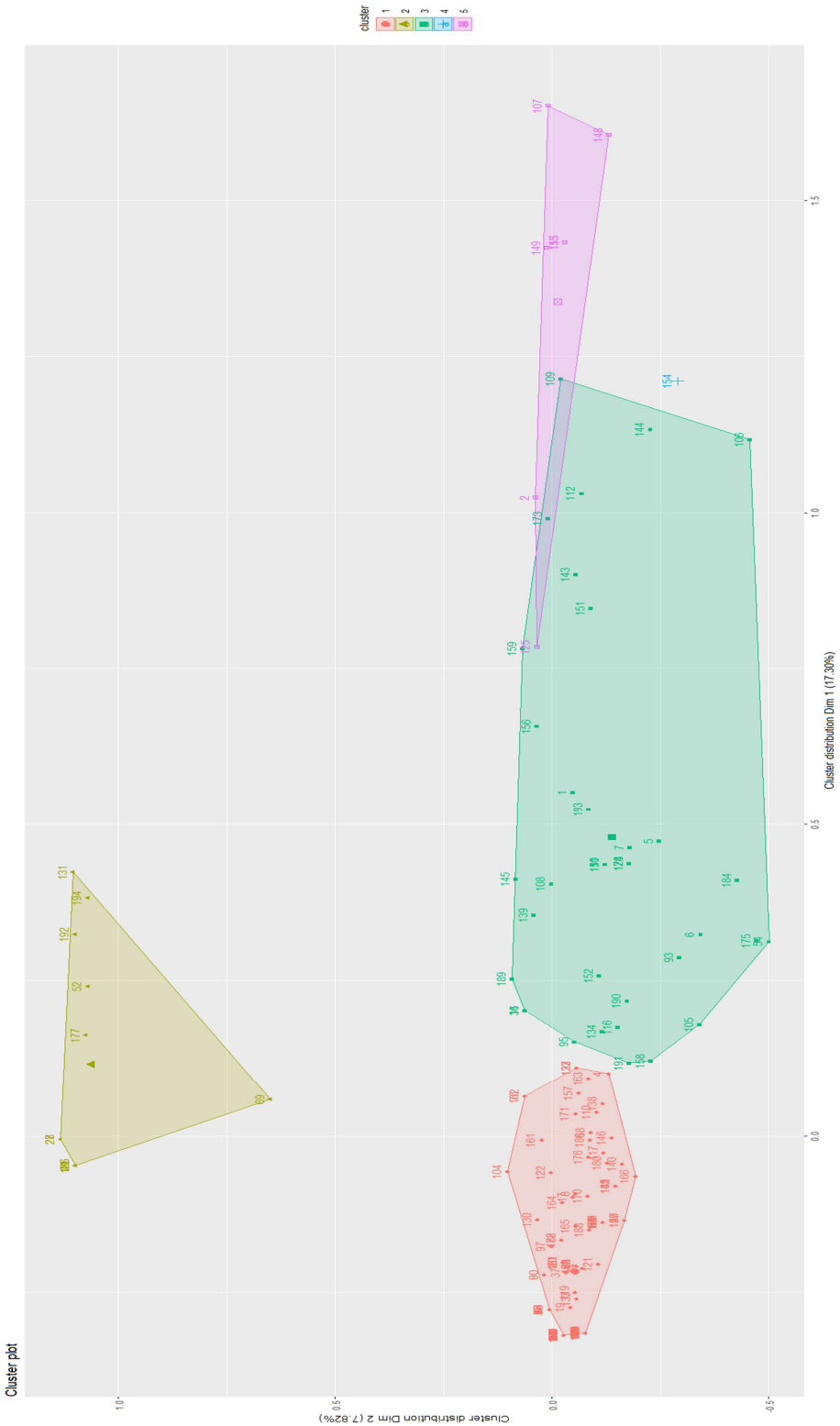


Figure 33. No. and distribution of clusters identified over dimensions 1 and 2 in the MCA analysis of AMG determinants and MGEs from 194 *Campylobacter* isolates.

Table 24. Characteristics of the clusters according to host species, bacterial AMR determinants and MGEs.

Clusters	1		2		3		4		5	
Isolates	137		12		37		1		7	
Bacterial species	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*
Bacterial isolares	25	112	4	8	35	2	1		7	
Host groups*:										
Broilers (98)	11	69	1	8	8				1	
Turkeys (30)	9	7	3		10	1			1	
Pigs (18)	1				11		1		5	
Cattle (48)	4	36			6	2				
GEN-R (25)		3		1	14		1		6	
STR-R (81)	8	21	4	8	30	2	1		7	
ERY-R (51)	7	8	3	1	26				6	
MDRGIs (47)		4	8	4	20	3	1		7	
Conjugative and mobilizable plasmids (17)		4		1	7	3			2	
Non-conjugative non-mobilizable plasmids (20)		1	1	2	12				5	
Chromosome located ARGs (157)	15		3	5	12	4	1			
Transposon Tn5405 (20)		2	4	7	4	2			1	

* Different colours assigned to different hosts

CLUSTER 1 (n=137, 70% of all the collection) included the majority (86%) of *C. jejuni* isolates from broilers that were sequenced and a majority (56-100% from each host species) of AMG-S *C. coli* sequenced isolates from the other three hosts (Table 24). In 96% of the isolates in this cluster the AMR determinants were located in the chromosome All 15 isolates harboring the *rep34* replicon were located in this cluster, with a tetracycline resistance ribosomal protection gene at the 5' end and a tetracycline resistance gene at the 3' end (*tet(O)-sat4-topB-rep34-aadE-tetO*) (Appendix VI) where the *aadE* gene confers resistance to streptomycin.

CLUSTER 2 (n=12, 6%) included 9/12 AMG-R *C. jejuni* isolates from broilers that were sequenced and all three ERY-R *C. coli* isolates from turkeys (Table 24). All AMG-R genes in isolates in this cluster were located in MDRGIs, either in the bacterial chromosome (n=8/12) or less frequently in non-conjugative non-mobilizable (NC-NM) plasmids (n=4/12). All isolates in this cluster included the genes *aad(6)-sat4-aph(3')-IIIa* and 11 out of 12 carried insertion sequence *IS6(IS1216E)* and the *Tn5405* type transposon (Appendix VII).

CLUSTER 3 (n=37, 19%) included *C. coli* isolates mostly resistant to gentamicin, streptomycin and erythromycin from all hosts (75-100% of all *C. coli* isolates from each species), and 62% (n=23/37) of the isolates in this cluster harbored AMG-R genes in MDRGIs (Table 24). This cluster included three of the four isolates carrying the *erm(B)* gene present in the collection. The main mobile genetic elements (MGEs) in this group linked to AMR determinants were insertion sequence *IS1595(ISCco2)*, carrying *aph(2")-If* as 'passenger' genes (which confer high resistance levels to gentamicin), insertion sequence *IS1595(ISAcsp6)* and plasmid replicon *repUS59* (Appendix VIII).

CLUSTER 4 was formed by a single pig GEN-R and STR-R *C. coli* isolate, bearing the genes *aph(2")-Ib-aac(6')-Im* in a MDRGI and the STR-R *aad9* gene (Table 24).

CLUSTER 5 (n=7, 3.6%) included only AMG-R *C. coli* isolates mainly from pigs (n=5/7) with the *aph(2")-Ic* preceded by either plasmid replicon *rep22* (Appendix IX) or insertion sequence *IS30 (ISHahy9)*. Spectinomycin resistance ARGs in this cluster were *spw* genes (complete or truncated) (Table 24).

The ML phylogenetic tree built from the consensus sequences of the core genome of all 194 isolates revealed a limited genetic variability among isolates. *C. coli* and *C. jejuni* isolates were separated in two BAPS clades each (1 and 2 for *C. coli* and 3 and 4 for *C. jejuni*) (Figures 34-35).

BAPS 1 contained 18 *C. coli* isolates resistant to gentamicin and streptomycin (harboring insertion sequences *IS1595(ISCco2)*, *IS30(ISHahy9)* and plasmid replicon *rep22)* and BAPS 2 consisted of 41 isolates that were mostly STR-R but GEN-S (Figure 34). Isolates from all four host species considered, as well as the three MCA clusters including *C. coli* isolates, were found in both clades (Figure 34).

Regarding the *C. jejuni* isolates, BAPS 3 was closest to the *C. coli* group and harbored mostly plasmid replicon *rep34* as MGE, linked to STR-R (Figure 35).

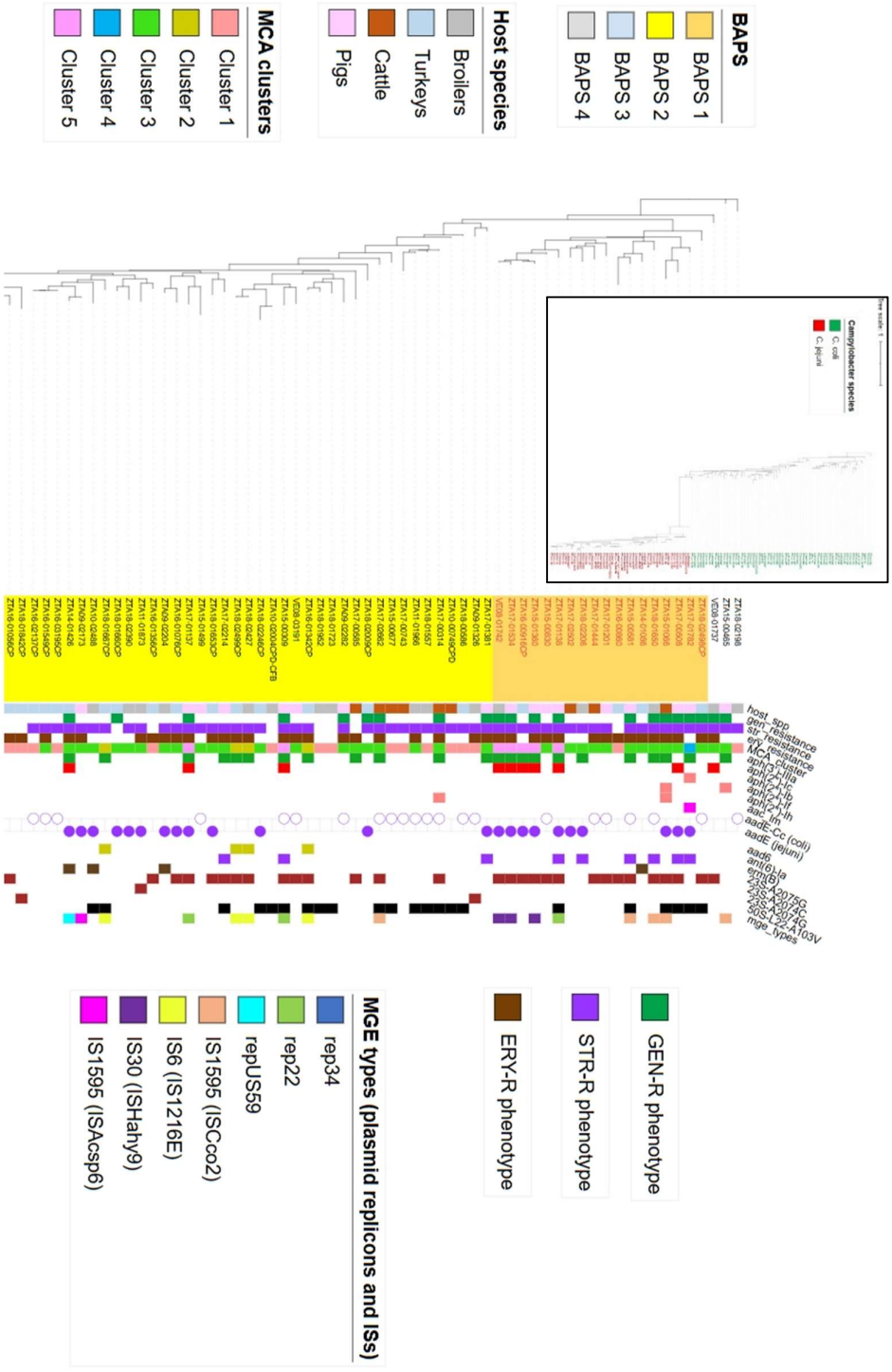


Figure 34. Phylogenetic tree of *C.coli* showing hosts, phenotypes, MCA clusters, MGEs and AMR determinants (Inset=ML phylogenetic tree of all *Campylobacter* showing differentiation *C. coli* / *C. jejuni*).

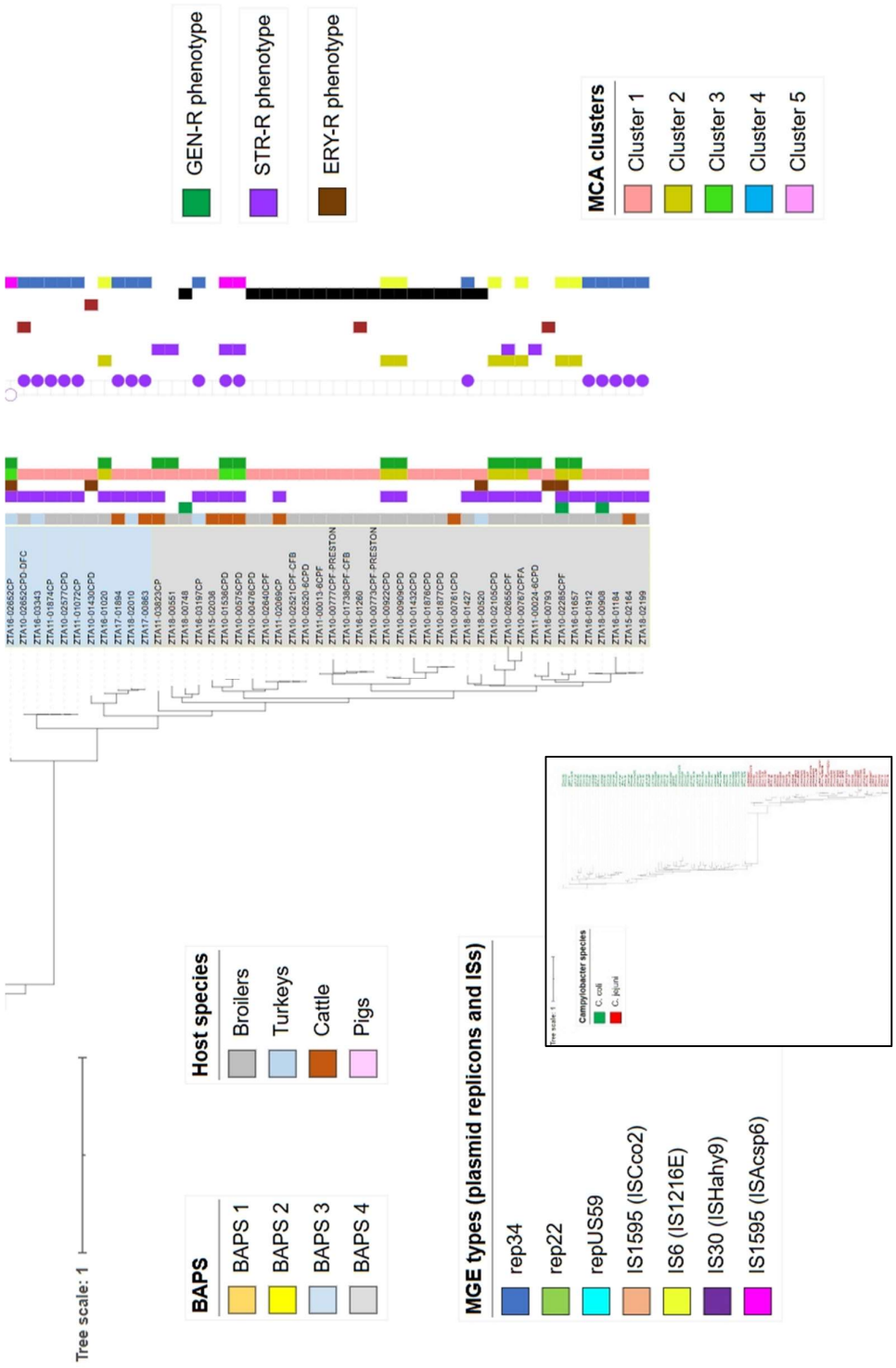
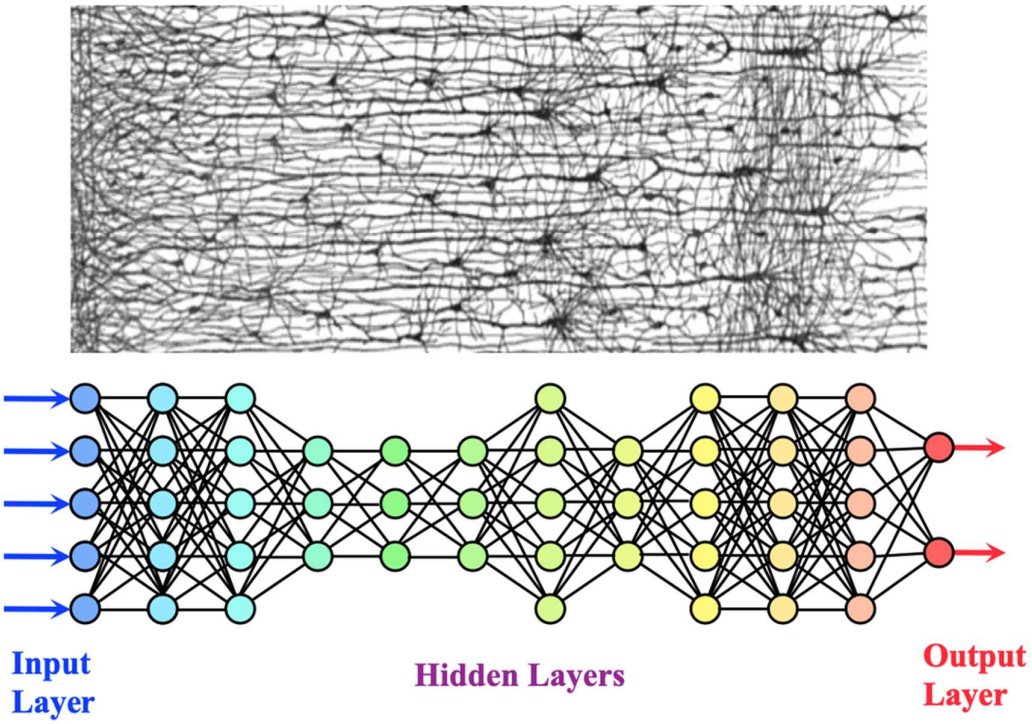


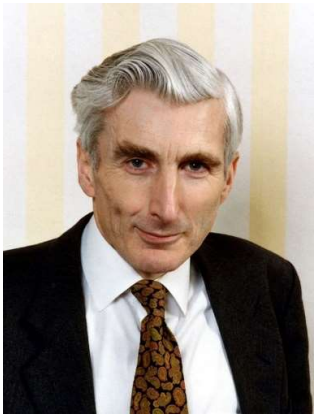
Figure 35. Phylogenetic tree of *C. jejuni* showing hosts, phenotypes, MCA clusters, MGEs and AMR determinants (Inset=ML phylogenetic tree of all *Campylobacter* showing differentiation *C. coli* / *C. jejuni*).

DISCUSSION



"In this century, not only has science changed the world faster than ever, but in new and different ways. Targeted drugs, genetic modification, artificial intelligence, perhaps even implants into our brains – may change human beings themselves."

Martin John Rees OM
Astronomer and Member of the British House of Lords



5. DISCUSSION

Antimicrobial resistance is becoming a major problem for the treatment of diseases caused by zoonotic bacteria such as *Campylobacter*. The mechanisms by which AMR can spread in bacterial populations (vertically or horizontally via HGT) may have enormous consequences because they can determine the speed at which AMR phenotypes disseminate through food animal hosts, humans and the environment. Of particular concern are genetic traits conferring resistance to three or more antimicrobial classes (MDR phenotype), particularly when transmitted together.

In the case of *Campylobacter* infections, aminoglycosides and macrolides are the two antimicrobial classes most commonly used to treat clinical cases when necessary [237]. The treatment of infections associated with *Campylobacter* resistant to these two antimicrobial classes can be seriously compromised. Hence, agencies such as EFSA have included in their recommendations the evaluation of the distribution and spread dynamics of these genetic mechanisms in *C. coli* and *C. jejuni* isolated from food animals [470].

The use of phenotyping methods for microbial characterization is widespread and well developed, but the extent to which these techniques allow making inferences on the specific genetic mechanisms behind the antimicrobial resistance phenotypes observed is limited. As opposed to 'bottom-up' approaches based on the identification of a gene and the evaluation of its function, this thesis followed a 'top-down' approach in which isolates were first grouped by phenotype to then identify the genetic elements associated with a particular phenotype. Earlier applications of this approach have identified host-associated genes and alleles [471], and there is considerable potential for understanding other more complex phenotypes associated with isolates from the food chain, such as its survival and virulence.

Starting from the individual isolate – thermotolerant *Campylobacter* in this case – several epidemiological analyses were carried out to characterize the occurrence of different resistance phenotypic traits and how these were matched with the genetic background of the isolates. This contributed to understanding the possible mechanisms of AMR spread to aminoglycosides and macrolides in *Campylobacter* isolates from food animals in Spain. This study of individual isolate-based *versus* aggregated phenotypic data has

proven a reliable means of gaining insights into mechanisms of antimicrobial resistance as described elsewhere [1, 415], and for this reason this approach was adopted here.

Distribution of thermotolerant *Campylobacter* and resistance phenotypes in livestock in Spain (Specific Objective 1)

The annual proportion of broiler samples collected over the 17 years of the study period included in this thesis that were positive for *Campylobacter* varied widely from 26% to 77%. From 2004 to 2008, positive isolation levels were lower than 50%, while in the 2009-2017 period this figure was over 50%, with an average of 60% and peaks of 75%.

Average figures reported by EFSA for the same periods and animal species (2004-2008 and 2009-2017) for EU Member States were similar to that in Spain [472, 473]. Sampling and culture methods can influence recovery rates [474], thus from 2004 to 2018 it may be that *Campylobacter* sampling and recovery rates have improved.

While the more limited available timeframe for turkey makes comparisons difficult, percentages of *Campylobacter* positive samples found in turkeys in this thesis were somewhat higher (75% in 2014, 65% in 2016 and 85% in 2018) than those found on average in five EU countries with available data (Denmark, Germany, United Kingdom, Poland and the Netherlands), which were below 70% for all these three years [473, 475, 476]. Both the small sample size and the study period make it difficult to extrapolate possible reasons for these variations.

The percentage of pig samples from which *Campylobacter* isolates were retrieved over this first part of the study period (2004-2007) was variable (33-80%) with average reporting levels of 50%, as in the rest of EU countries for the same period [477]. Positive reporting figures were still frequently around 50% at the end of the period in Spain [386] whereas average EU figures were higher (70%) at the end of the period.

The proportion of *Campylobacter* positive samples in cattle from 2007 to 2017 also varied widely (37% to 70%) with the proportion being highest in 2010-2013, while the average for the period was 50-60%. This level contrasts with values as low as 5% (or less) reported by EFSA in EU countries [386, 473].

Altogether, the prevalence of thermotolerant *Campylobacter* in all livestock species investigated here has remained relatively stable over the studied years (or experienced

a slight increase in broilers as in all EU countries). It is also noticeable the higher *Campylobacter* levels in cattle in Spain compared to other EU countries.

The impact of surveillance programmes in the different countries could have an influence on these findings in all four host species, as well as other factors such as environmental (relative humidity or temperature) [478] or even farm management practices, as reported elsewhere [479], or maybe due to seasonal changes in production having an effect in all EU countries, or only in some of them. Another factor that could be contributing to increases of *Campylobacter* levels being reported may be improvements in detection techniques, which would be translated into undersampling/underdetection in previous years but no evidence has been found about this. Finally, the role played by wild birds should also be taken into consideration [480, 481], in Spain and elsewhere.

As expected, host species was strongly associated with the specific *Campylobacter* species retrieved in positive samples, although proportions varied between the different hosts.

In this thesis a near 50/50 distribution for *C. coli*/*C. jejuni* was found in broilers. Although according to EFSA reports *C. jejuni* is the predominant *Campylobacter* species found in positive broiler samples in EU Member States (>80%) [473], *C. coli* is becoming more common in broilers since the beginning of the century [263] (EFSA baseline survey), and this trend keeps continuing [482]. The trend of *C. coli* in broilers in the EU has fluctuated like in Spain, but occasional increases of *C. coli* have been reported in the last years of the study period in all the EU [386, 473].

In our collection, *C. coli* was the predominant species in turkeys. However, in EFSA reports of turkey samples from the same period (2014-2018), *C. jejuni* represented the majority with an average of 75% *versus* 25% of *C. coli* [473, 475]. Some studies have reported lower levels of *C. jejuni* in turkeys [483]. These variations may be due to different sample protocols being able to recover differing numbers of isolates, as the protocols used can influence recovery rates [484].

In pigs, *C. coli* is highly predominant [485-487], making up nearly all isolates in studies including porcine samples such as those in the present thesis. According to EFSA's reports covering the study period of this thesis, *C. coli* accounts for over 90% of *Campylobacter* isolates from pigs, with only a minimal proportion attributed to *C. jejuni* [487]. The main database on which this work is based only had 33 samples positive to *C. jejuni* in pigs in all the studied period (2004-2018), thus agreeing with the reviewed literature.

In this thesis, proportions of *Campylobacter* in cattle were variable but with a vast predominance of *C. jejuni* (80%) over *C. coli* (15%). In the EU, levels of *Campylobacter* in cattle for 2014 to 2018 were the same as the ones found in this work [473, 475] typically around 80% of *C. jejuni*.

As presumed, the level of resistance to the antimicrobials used in our study was closely linked with the *Campylobacter* bacterial species found, with higher levels of resistance in *C. coli* than in *C. jejuni* in agreement with previous research in animal samples [488]. In humans, *C. coli* has traditionally shown higher levels of AMR to most antimicrobials compared with *C. jejuni* [296, 383, 489, 490]. Hence, the problem of AMR may intensify if the importance of *C. coli* as a human pathogen keeps increasing, because an increase of the pathogen will bring about an increase of the resistances associated with *C. coli*.

Regarding AMR, as shown in Figures 17-20 (Results section), the provinces with higher levels of *Campylobacter* positive isolates did not always match with the provinces with higher AMR levels. In addition, no clear pattern in the geographic distribution of AMR in Spain at the province level was observed, although this could be related to the limited sample size achieved in certain provinces of the country. The only similarities observed rather frequently were the presence of resistance to gentamicin and streptomycin in the same provinces, an expected finding because these two antimicrobials belong to the same antimicrobial class and thus share similar genetic resistance mechanisms [491].

Trends in the proportion of resistant isolates to the three antimicrobials which were the focus of this thesis in Spain and other EU countries are shown in Figure 36 (broilers, *C. coli* and *C. jejuni*), Figure 37 (turkeys, *C. coli* and *C. jejuni*) and Figure 38 (*C. coli* pigs and *C. jejuni* cattle) (EFSA-ECDC reports 2004 to 2018), though these should be interpreted carefully since not all countries contributed data and not for all years within the thesis studied period. The countries and reporting periods in these three figures were all data that were possible to extract from EFSA reports. Streptomycin data are not shown since only data from two years was available for other EU countries.

Spain systematically recorded higher values compared with most/all other EU countries with data available for gentamicin and erythromycin (Figure 36). For *C. coli* from poultry (broilers and turkeys) and pigs, Spain recorded the highest values among EU countries, whereas for *C. jejuni* the values in Spain for poultry and cattle were similar or lower than other EU countries.

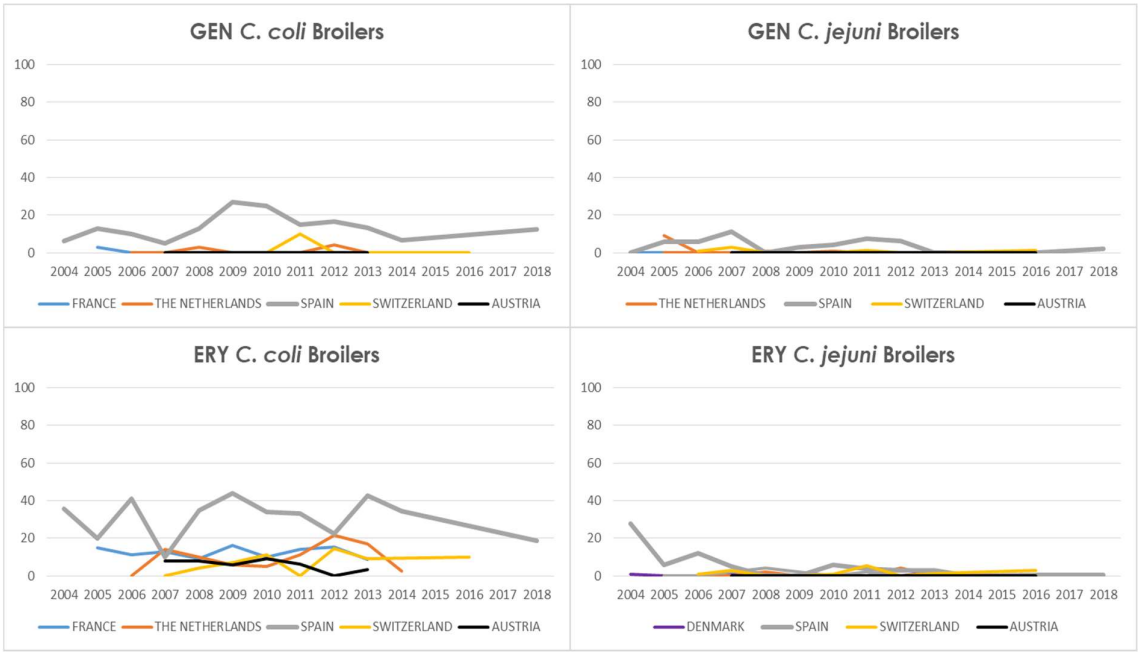


Figure 36. AMR levels in EU countries over 2004-2018 in *C. coli* broilers (left) and *C. jejuni* broilers (right). All comparable data between countries available from EFSA reports have been used to create these maps.

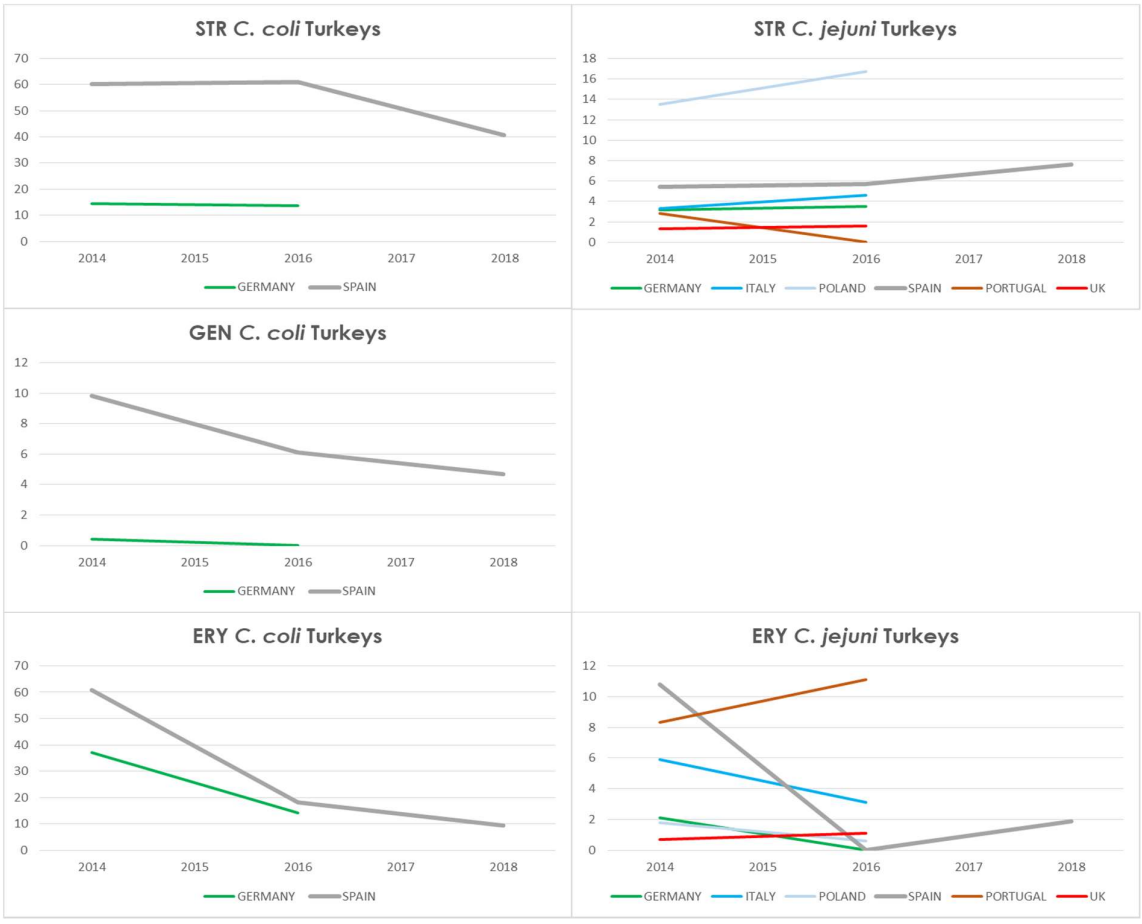


Figure 37. AMR levels in EU countries over 2014-2018 in *C. coli* turkeys (left) and *C. jejuni* turkeys (right).

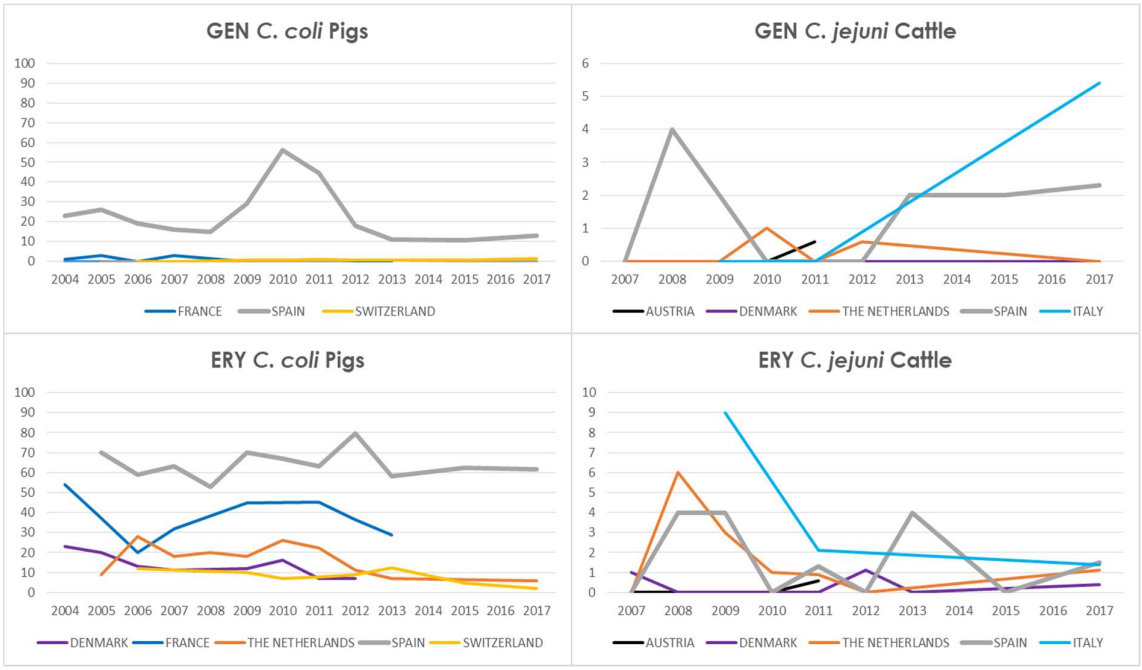


Figure 38. AMR levels in EU countries over 2004-2017 in *C. coli* pigs (left) and *C. jejuni* cattle (right).

The reason for the higher level of resistances found in livestock *C. coli* isolates from Spain compared with other EU countries could be related to the generally higher level of AMU for livestock in Spain compared with at least some other EU Member States, according to data from the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (EMA, 2024), although this hypothesis should be further evaluated, especially given the difficulties interpreting data on sales of antimicrobials as opposed to quantities administered on actual antimicrobial therapies.

Presence of simultaneous resistance to multiple antimicrobials in thermotolerant *Campylobacter* from livestock in Spain

Resistance profiles in *C. coli* from pigs involved a higher number of antimicrobials (among the six considered here) compared with other hosts (Table 5, Results section). This increased resistance in pig isolates compared with those from cattle/poultry is in agreement with what has been reported by other authors [285, 356, 492].

Overall, the simultaneous occurrence of phenotypic resistance to erythromycin (macrolide) and streptomycin and/or gentamicin (aminoglycosides) was observed in both *C. coli* and *C. jejuni* isolates from all livestock species, though the strength of the association was particularly high in *C. jejuni*, also from all livestock species, in which the overall resistance levels were lower (Table 7, Results section).

In addition and unsurprisingly, streptomycin-resistant isolates had a significantly higher probability of being also resistant to gentamicin, which was expected given that they belong to the same antimicrobial class (aminoglycosides) and therefore share resistance mechanisms, mostly based on natural transformation, homologous recombination and sharing of MGEs [237, 287, 344]. The identification of the same trend for erythromycin was however more intriguing. The only case of MDR in relation to macrolides described in the literature involves the *erm(B)* gene when located on MDRGIs containing genes conferring resistance to other antimicrobial classes, including aminoglycosides [291, 363]. However, according to the WGS analysis carried out in the subset of isolates from this thesis, *erm(B)* genes were rare in our collection (found in only four isolates out of 194). Based on our sequencing data, the significant association between the phenotypic resistance to both antimicrobial classes was due to the simultaneous presence of other erythromycin determinants (mainly *23S* mutations) along with a variety of AMG-R genes (Table 7, Results section).

Moreover, phylogenetic studies based on *flaA-SVR* gene sequencing have been used in the past to study the epidemiology of *Campylobacter* spp. from different sources [420]. Previously, studies based on the *flaA*-SVR gene sequence did not find a relationship between AMR and specific genotypes [493]. However, in the strain collection used in this thesis to study the *flaA*-SVR variability and its relationship with antimicrobial resistance, five distinct groups were identified, two of which (predominantly formed by *C. coli* isolates from broilers and turkeys) were associated with an increased proportion of simultaneous resistance to aminoglycosides and macrolides (Table 10, Results section). In contrast, isolates in groups 4 and 5 were primarily *C. jejuni* of cattle origin (exclusively cattle *C. jejuni* in group 5) and not phenotypically co-resistant.

The MCA analysis of synonymous codon usage further confirmed the differentiation between groups, with those in group 5 being well separated, what could indicate they are in fact a separate entity among *C. jejuni*, perhaps part of the 'cattle specialist *C. jejuni* isolates', as described in the literature [494]. The existence of 'cattle specialist *C. jejuni* lineages' has been previously speculated, implying that adaptation of *C. jejuni* to

cattle could be associated with the presence of genetic elements favouring its survival in the intestine of cattle [1]. In this thesis, only eight cattle *C. jejuni* isolates clustered together in that way. More data points would be needed to confirm this.

So, the *flaA*-SVR gene study carried out in this thesis was able to partially characterize the variability present in the dataset in relation to antimicrobial resistance, but the results obtained were not discriminative enough to infer clear associations between certain groups of isolates and specific antimicrobial resistance determinants.

Carriage of AMR determinants in thermotolerant *Campylobacter* from livestock in Spain

In this thesis, *C. coli* and *C. jejuni* differed substantially in the number of AMR determinants they carry, in agreement with other studies, especially in relation to AMG-R determinants [495]. Interestingly, when this analysis was conducted specifically for isolates resistant to AMG or erythromycin, a significantly higher number of resistance determinants was observed among phenotypically resistant *C. coli* isolates compared with *C. jejuni* resistant isolates, as well as in resistant isolates from pigs compared with those originating from other host species.

The proportion of phenotypically resistant isolates without known genetic mechanisms was higher for erythromycin (23%) (Table 12) than for AMGs, which highlights the knowledge gaps on mechanisms responsible for conferring erythromycin resistance in food animals. Overall, if these results are considered excluding *aph(3')-IIIa* genes and *50S_L22_A103V* mutations, the agreement between genotype and phenotype is in line with those described for AMGs and MACs in *Campylobacter* by other authors [350, 417, 418, 496]. The imperfect genotypic prediction of the observed phenotypes could be due to errors in laboratory techniques (typing, sequencing).

All isolates harboring four or more ARGs on the same contig in our collection (n=16) were *C. coli*, and the majority originated from pigs (n=7/16). The presence of multiple ARGs in the same location of the bacterial chromosome in the *Campylobacter* species with the highest phenotypic resistance compared with *C. jejuni* further highlights the therapeutic challenges this pathogen can pose [138, 497]. In addition, the underlying genetic mechanisms present in AMG-R isolates were differently located depending on the *Campylobacter* species: while among *C. coli* isolates harboring AMG resistance determinants, half (n=32/61) had these on genomic islands, this was observed in only one

third (n=15/49) of the *C. jejuni* isolates. This may be due to the higher plasticity of *C. coli* and the need to harbour genes and mobile genetic elements on MDRGIs to move them between other bacteria by HGT.

Moreover, even though the majority of GEN-R and STR-R isolates harbored resistance determinants for these antimicrobials (Table 11, Results section), 14% and 8%, respectively, did not carry known genetic mechanisms that could explain their phenotypes, suggesting there may be additional mechanisms involved in conferring resistance to AMGs. Among these, resistance mechanisms involving other aminoglycosides such as spectinomycin (*aad9* and *spw* genes) and apramycin (*apmA* gene) may provide an answer, so they should be further explored.

Regarding gentamicin resistance, the gene most frequently present in the GEN-R isolates analysed in this thesis was *aph(3')-IIIa* (present in 16 of the 25 GEN-R isolates sequenced) followed by *aph(2'')-Ic* (9 GEN-R isolates), while the remaining GEN-R genes were present in only four isolates (Figure 24, Results section). Another Spanish study on cattle found the *aph(2'')-Ic* gene in six *C. coli* isolates highly resistant to gentamicin (MIC>16mg/L), and five of them were accompanied by the *aph(3')-IIIa* gene with no other AMG-R genes associated [496]. The *aph(2'')-Ic* gene is frequently located in the bacterial genome associated to MGEs on plasmids or transposons, facilitating HGT among bacteria. This configuration enhances the mobility options for the gene, rendering the isolates bearing it potential spreaders or disseminators of AMG-R across other bacterial species and hosts [498]. The *aph(2'')-Ic* gene, which was found in nine isolates in our collection with a GEN-R and STR-R phenotype, was associated to certain mobile genetic elements previously described in the literature such as plasmid replicons *rep22* [266, 499] and *repUS59* (also known as plasmid *PSSG1*) [500], and insertion sequence *IS30(ISHahy9)*. This gene was always found in plasmids (non-conjugative/ non-mobilizable plasmids, n=8; conjugative/ mobilizable plasmids, n=2) in agreement with previous studies linking its presence to plasmids [501].

We found four insertion sequence types within the *IS1595* family, all at low frequencies: *IS6(IS1216E)* (n=11), *IS1595(ISCco2)* (n=5), *IS1595(ISAcsp6)* (n=4) and *IS1595(ISSag10)* (n=1). Two out of the five isolates bearing insertion sequence *IS1595(ISCco2)* in our collection carried the *aph(2'')-If* gene (conferring gentamicin resistance with MICs > 16 mg/L) and both were located in plasmids. Insertion sequence *IS1595(ISCco2)* is very relevant for *Campylobacter* because is a way the bacteria has to transfer important genes by HGT.

In our collection, the only 'passenger gene' found was the *aph(2'')-If* gene which was ocated on plasmids. Some studies have found this same passenger gene [346].

Regarding STR-R genes, the one most frequently present in the 81 STR-R isolates sequenced in this thesis was *aadE* (in 47% of isolates), followed by *aph(3')-IIIa* (38%), *aadE-Cc* (35%) *aad(6)* and *ant(6)-Ia* (14%). Nevertheless, the genes *aadE, aad(6)* (both present in *C. coli* and *C. jejuni*) and *aadE-Cc* (specific of *C. coli*) were never found together in the same isolate (Figure 24, Results section). The *aadE* gene was present in 40 out of the 194 isolates in the collection, and 38 of them were STR-R. Moreover, 24 of the 40 (60%) isolates with this gene carried it in plasmids (more if only considering *C. coli* isolates, with 20/24 with the gene harboring it in plasmids compared with 4/24 specifically for *C. jejuni*).

The frequence of isolation of this gene in our isolates was higher than that described elsewhere [139, 496], and the most logical explanation is the higher STR-R frequency in our isolates (Figures 34-35, Results section).

The *aadE* gene is generally found associated to MGEs in both Gram-negative and Gram-positive bacteria, hence, *C. coli* may have acquired *aadE* genes from *C. coli*, *C. jejuni* or bacteria from other genera, mainly via HGT [502].

Conversely, only one *C. jejuni* isolate out of the 122 in the dataset harbored the *aadE-Cc*, which was located on a CM plasmid. The presence of the *aadE-Cc* gene in *C. jejuni* is very rare since this gene is very specific to *C. coli*, although it has been already reported elsewhere [503]. The *aadE-Cc* gene is generally associated to MGEs in *C. coli*. Since this *C. jejuni* isolate was the phylogenetically closest *C. jejuni* isolate to the *C. coli* clade, this finding suggests that this isolate could belong to an under-sampled lineage that may act as an interspecies *C.coli*/ *C.jejuni* (Figure 35, Results section) [1].

Plasmid replicon *rep34* (belonging to the class B of replicons) [504] was found in 15 *C. jejuni* isolates associated to the *aadE* gene and other genes not related to antimicrobial resistance. The location of *rep34* in this genetic context suggests this replicon may play, or have played (as it had mostly a chromosomic location), a role in conferring streptomycin resistance.

Additionally, regarding streptomycin resistance, the *aad(6)* gene is normally found in transposons or plasmids in Gram-positive bacteria [139], and in this study in 11/12 of isolates carrying this gene it was associated to transposons in the chromosome. This suggests that the primary function of this gene in this genetic context in our dataset may

be to complement the resistance mechanism against streptrothricin conferred by the *sat4* gene, as they both were found together along with *aph(3')-IIIa* in these transposons, as also described elsewhere [505], all in MDRGIs.

Finally, although the *ant(6)-Ia* gene [349] was not found in many isolates in the thesis dataset (14/194) it was frequently associated to transposons (9/14) conferring STR-R, primarily in pigs in the thesis collection.

A considerable proportion of *C. coli* isolates in our collection (46/72, 63.9%) harbored ERY-R determinants, similar to what has been reported elsewhere in isolates from food producing animals and humans [506-509]. When looking into the ERY-R subset of *C. coli* isolates this proportion increased to 78%, with the main one being *23S* mutations (36/51; 70%). However, other studies have reported frequencies of detection of *23S* mutations close to 90-100% in *C. coli* ERY-R isolates [416, 510], higher than our findings. The most common point mutation conferring ERY-R in *Campylobacter* in this study was the *A2075G* mutation in domain V of the 23S rRNA. Three isolates in our collection harbored the 23S_A2074G mutation and only one harbored the 23S_A2074C mutation, findings which are in agreement with what has been published by other authors [370].

Isolates bearing *23S* mutations displayed higher MIC values than those carrying *erm(B)* genes, although other authors have reported otherwise [35, 511]. Still, the low number of isolates with *erm(B)* in our collection precludes extracting definitive conclusions.

The higher frequency of *23S* mutations in ERY-R *C. coli* (n=29/61) *versus C. jejuni* (n=3/39) could be related to different selective pressures to which *C. coli* and *C. jejuni* are exposed to. The higher the exchange of genetic material between bacteria such as *C. coli*, the stronger the pressure, thus the higher the possibilities of developing mutations and accumulating AMR determinants, as has been described in species other than *Campylobacter* [512].

Only 12 out of 41 isolates with the *50S_L22_A103V* ribosomal point mutation in our study presented an ERY-R phenotype. Moreover, we found the *50S_L22_A103V* ribosomal point mutation in twice as many *C. coli* (n=19/60; 32%) than *C. jejuni* isolates (n=6/40; 15%), which could be also due to the higher frequency of mutation in *C. coli*. In contrast, other studies have found more *50S* mutations in *C. jejuni* than in *C. coli* [495]. Hence, it seems that the presence of the *50S_L22_A103V* ribosomal point mutation on its own was not enough to confer erythromycin resistance in our collection because, as shown on Table 11 (Results section), the agreement between phenotype and presence of this

determinant increased when the mutation was removed. Besides, its role is unclear and some authors argue that it may be secondary in the occurrence of such resistance as it changes the structure of the ribosome, obstructing the action of the antimicrobials [357, 495]. Our results show this same finding, as the isolates with higher MICs had a combination of *23S* and *50S* mutations (Figure 27, Results section), not only 50S mutations. There are however other reports of associated erythromycin resistance to this *50S* mutation [93].

During the realization of this thesis a *C. coli* isolate from a broiler carrying the *erm(B)* gene retrieved in 2009 was detected, demonstrating the circulation of this gene several years before it was first reported in Asia as possibly originating from Gram-negative bacteria [308]. This gene was first reported in Spain in one *C. coli* from a broiler in 2015 [363] and in two *C. coli* from turkeys in 2017 [364].

The three genes are included in the collection of isolates used in this thesis. The isolate from 2015 constituted the first European report of this gene, and along with the two genes from 2017, the three were associated with other genes in MDRGIs conferring resistance to erythromycin, ciprofloxacin, tetracycline and nalidixic acid (two of them in plasmids). The new *erm(B)* gene characterized in this thesis, although not part of a MDRGI, was found also in a plasmid, highlighting their potential for HGT.

However, the isolates carrying the four *erm(B)* genes were not closely related according to the phylogenetic tree (with genetic distances between them ranging from 993 to 1972 SNPs), suggesting separate acquisition events. The inclusion of *erm(B)* genes in plasmids containing genes conferring resistance to other antibiotics in *C. coli* from food animals could pave the way to rapid dissemination of macrolide resistance [383, 470]. Besides, reported resistance levels to erythromycin in humans have been consistently higher for *C. coli* than for *C. jejuni* [296], and similar reports have been made in poultry [488] in agreement with our findings. Since macrolides are one of the three Critically Important Antimicrobial classes used for the treatment of human campylobacteriosis (along with fluoroquinolones and aminoglycosides) [240], a more in-depth knowledge into their resistance mechanisms is warranted. The low but still higher frequency of detection of this gene in *C. coli* isolates, more resistant and associated to more AMR mechanisms, deserve further research.

The *CmeABC* efflux pump has been reported as an important mechanism for antimicrobial resistance in *Campylobacter* conferring resistance to multiple drugs [376].

Our data showed that the *CmeABC* efflux pump and all its units were absent from *C. coli* isolates and present in most *C. jejuni* isolates, but this presence was not associated to ERY-R in *C. jejuni* isolates from multiple hosts over the 17-year period of study, even though all units were present. This same finding has been reported elsewhere, describing possible regulatory mechanisms involving the different pump units, but not yet characterized [513]. The unexplained erythromycin susceptibility in *C. jejuni* isolates bearing the pump (or some of its subunits) could be due to the presence of insertions and deletions in the regulatory region of the *CmeABC* promoter [511] (though this option was not explored in this thesis). It could also be due to the presence of phosphotransferases and macrolide esterases or additional efflux systems [514-516].

Certain mechanisms may be involved in the particularly increased MICs observed in certain ERY-R isolates, such as mutational resistance affecting the expression of the *CmeABC* efflux pump in *C. jejuni*, as described in other bacteria [517]. The high proportion of ERY-R isolates found in *C. coli* from turkeys in other European countries [470, 489], as they have also been found in this thesis, further highlights the need to clarify the resistance mechanisms present in ERY-R isolates, as suggested by others [350, 518].

In the study of MICs, the significantly higher MIC values observed in this thesis among ERY-R isolates from turkeys compared with other species (Appendices I-IV, Material and Methods section) was perhaps not linked to the presence of the *erm(B)* gene based on the phenotypes of the only four isolates in which this gene was detected: only one of the four isolates carrying the *erm(B)* gene presented high MIC levels (>128 mg/L) and another one was present in an ERY-S isolate, indicating that the gene may not be functional or not expressed. The lack of association between high MICs and the presence of the *erm(B)* gene has also been described by other authors [290].

In our *Campylobacter* dataset, erythromycin resistance conferred by 23S mutations was more associated to high MIC levels (MIC > 128 mg/L) than the sole presence of the *erm(B)* gene. However, the presence of this gene associated to aminoglycoside resistance determinants represents a public health threat in cases where these two antimicrobial classes are the only treatment option left in clinical settings. EFSA recommends investigating the molecular mechanisms of macrolide resistance, especially in isolates resistant to high concentrations of erythromycin, in order to detect chromosomal mutations or the presence of the transferable *erm(B)* gene [383]. Furthermore, these same guidelines recommend searching for erythromycin resistant genes, not only in

resistant strains presenting concomitant resistance to aminoglycosides or an MDR phenotype, but also in susceptible isolates.

As final remarks, in the isolate collection used in this thesis, *C. coli* was the species with the largest number of determinants forming part of MDRGIs, especially in pigs. In addition, pigs were the hosts with more ARGs located in plasmids, thus highlighting their possible role as reservoirs of resistance that could be spread horizontally.

The co-resistance patterns described on Table 18 (Results section) between aminoglycosides and macrolides in *C. jejuni* from poultry and cattle, showing strong association between both antimicrobial classes with high RR were the product of resistance mechanisms located in different regions of the bacterial chromosome. It was a co-located resistance rather than true co-resistance. Here, WGS was essential to show that the phenotypic co-resistance was not due to resistance mechanisms linked at the genetic level.

The present thesis has helped to gain a thorough understanding of the AMR situation of Spanish livestock, focusing on the associated AMR genetic determinants circulating in the country at host and *Campylobacter* species level. Furthermore, WGS has helped to determine the origin of certain resistances in some hosts and not in others, sometimes in *C. coli* and others in *C. jejuni*. Similarly, some phenotypic resistances did not have any explainable mechanism when looking at genetic context level, and the opposite was also true, some determinants present in the isolates from the collection did not express as a resistance phenotype. Thus, the phenotypic analysis of antimicrobial resistance data from thermotolerant *Campylobacter* (*Campylobacter coli* and *Campylobacter jejuni*) from the national monitoring programme alone was insufficient to identify differences in the nature and distribution of the resistance mechanisms conferring such resistance, often linked with specific bacteria and hosts in Spain.

Returning to the overall hypothesis of this PhD thesis, it can be concluded that although the phenotypic analysis of antimicrobial resistance data from thermotolerant *Campylobacter* (*Campylobacter coli* and *Campylobacter jejuni*) obtained through national monitoring programmes on livestock is a good starting point for characterizing the situation, this analysis alone does not allow the detection and characterization of predominant and/or emergent strains carrying antimicrobial resistance determinants to aminoglycosides and macrolides. WGS methods should be used if a thorough understanding of the resistance situation is necessary.

The main challenges being faced when the option forward is the use of WGS are the high cost of sequencing and the need of a very specific technical expertise to carry out the bioinformatic analyses.

CONCLUSIONS

6. CONCLUSIONS

1 – The proportion of *C. coli* isolates retrieved through the antimicrobial resistance surveillance programme from poultry, pig and cattle during the 2004-2018 period in Spain which were resistant to streptomycin, gentamicin and erythromycin was higher than those observed in other EU countries, while this was not observed in *C. jejuni*.

2 – A significant association between the simultaneous occurrence of phenotypic resistance to erythromycin (macrolide) and streptomycin and/or gentamicin (aminoglycosides) was observed in both *C. coli* and *C. jejuni* isolates from all livestock species considered, although stronger for *C. jejuni*.

3 – When considering only isolates phenotypically resistant to aminoglycosides and/or macrolides, a significantly higher number of resistance determinants was found in *C. coli* compared with *C. jejuni*. Similarly, resistant *C. coli* isolates from pigs harbored significantly more resistant determinants than those retrieved from other livestock species.

4 – The proportion of resistant isolates without the presence of known antimicrobial resistance determinants was low, but this proportion was higher for macrolides than for aminoglycosides.

5 – The presence of the *50S_L22_A103V* ribosomal point mutation on its own was not enough to confer phenotypic resistance to erythromycin in a proportion of *C. coli* and *C. jejuni* isolates with this genotype.

6 – The detection of a *C. coli* broiler isolate carrying the *erm(B)* gene in 2009 demonstrates the circulation of this gene at least five years before it was first reported in Asia in 2014.

7 – Antimicrobial resistance genes were more commonly located in multidrug resistance genomic islands in *C. coli* compared to *C. jejuni*, especially in those retrieved from pigs. In addition, *C. coli* carrying resistance genes from pigs harbored this more commonly in plasmids, thus highlighting their possible role as reservoirs of resistance that could be spread horizontally.

8 – The phenotypic analysis of antimicrobial resistance data from thermotolerant *Campylobacter* (*Campylobacter coli* and *Campylobacter jejuni*) from livestock obtained through national monitoring programmes alone does not allow the detection and characterization of predominant and/or emergent strains carrying antimicrobial resistance determinants; hence, WGS methods should be used to gain a thorough understanding of the resistance situation.

CONCLUSIONES

7. CONCLUSIONES

1 - La proporción de aislados de *C. coli*, obtenidos a través del programa de vigilancia de resistencias a los antimicrobianos en broilers, cerdos, pavos y vacas durante el período 2002-2018 en España, que fueron resistentes a estreptomicina, gentamicina y eritromicina, fue mayor que la observada en otros países de la UE, mientras que esto no se observó en aislados de *C. jejuni*.

2 - Se observó una asociación significativa entre la ocurrencia simultánea de resistencia fenotípica a eritromicina (macrólido) y estreptomicina y/o gentamicina (aminoglucósidos) en aislados de *C. coli* y *C. jejuni* de todos los animales de abasto considerados, aunque esta asociación fue más fuerte en *C. jejuni*.

3 - Al considerar solo los aislados fenotípicamente resistentes a aminoglucósidos y/o macrólidos, se encontró un número significativamente mayor de determinantes de resistencia en *C. coli* que en *C. jejuni*. Igualmente, los aislados resistentes de *C. coli* de cerdos portaban significativamente más determinantes de resistencia que los aislados de otras especies hospedadoras.

4 - La proporción de aislados resistentes sin presencia aparente de determinantes de resistencia antimicrobiana conocidos fue baja, pero esta proporción fue mayor para los macrólidos que para los aminoglucósidos.

5 - La presencia de la mutación puntual ribosomal *50S_L22_A103V* por sí sola no fue suficiente para conferir resistencia fenotípica a eritromicina en una proporción de aislados de *C. coli* y *C. jejuni* con este genotipo.

6 - La detección de un aislado de *C. coli* en broilers portador del gen *erm(B)* en 2009 demuestra la circulación de este gen al menos cinco años antes de que se publicara por primera vez en Asia en 2014.

7 - Los genes de resistencia antimicrobiana se localizaron más comúnmente en islas genómicas de resistencia a múltiples fármacos en *C. coli* en comparación con *C. jejuni*, especialmente en los aislados de cerdo. Además, los aislados de *C. coli* con genes de resistencia procedentes de cerdos los portaban más frecuentemente en plásmidos, lo que destaca su posible papel como reservorios de resistencia que podrían propagarse horizontalmente.

8 - El análisis fenotípico de los datos de resistencia a los antimicrobianos de *Campylobacter* termotolerantes (*Campylobacter coli* y *Campylobacter jejuni*) obtenidos a través de programas nacionales de monitoreo en animales de abasto, por sí solo, no permite la detección y caracterización de cepas predominantes y/o emergentes portadoras de determinantes de resistencia a los antimicrobianos, por lo tanto, se deberían utilizar métodos WGS para conocer en profundidad la situación de la resistencia.

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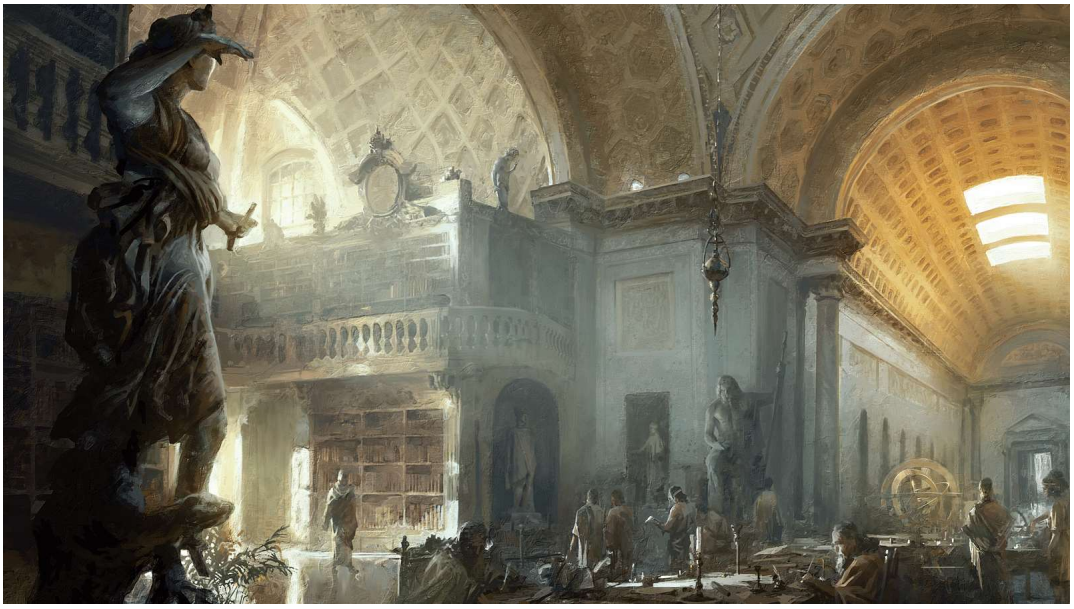
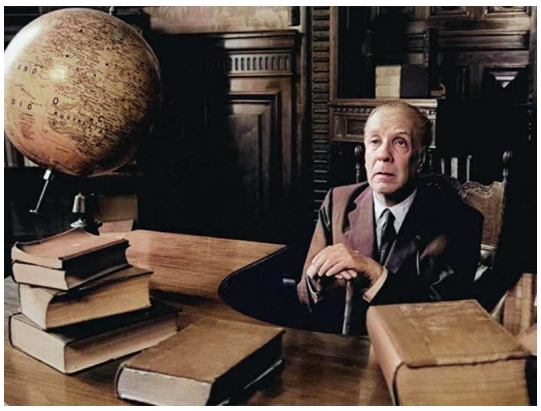


"Siempre he imaginado que el Paraíso será una especie de biblioteca. Quizás, tal como lo imagino, ese paraíso sea la Biblioteca de Alejandría, donde se conservaba todo el conocimiento, donde coexistían todos los pensamientos de la humanidad."

Jorge Luis Borges
Writer and librarian



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APPENDICES

Appendix I. Minimum Inhibitory Concentration distributions for broiler isolates and *Campylobacter* spp. categories.

Campylobacter coli

S														R
MICs	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
GEN		10	69	138	195	152	15	2	6	43	1	10		
CIP	6	20	6	4	2	2	34	281	152	24	8			
TET			8	16	5		2	7	10	297	64	105	12	2
NAL							12	23	8	37	253	258	7	
ERY			6	116	186	59	5		3	7	87	43	20	
STR				14	94	95	52	19	5	157	55	9		

| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Campylobacter jejuni

S														R
MICs	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
GEN		92	270	206	136	20	1	1	3	2		2		
CIP	13	18	6	5	5	10	90	355	123	17	5			
TET		6	25	25	16	7	11	35	53	197	127	26	7	1
NAL						16	35	22	16	45	153	209	18	
ERY			75	247	107	12	4			1	9	1		
STR			29	161	403	72	15	2	3	20	5	3		

| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Appendix II. Minimum Inhibitory Concentration distributions for turkey isolates and *Campylobacter* spp. categories.

Campylobacter coli

S												R
MICs	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
GEN	11	84	129	29	4		1	11	7			
CIP	1		1			29	85	100	19			
TET				1				5	6	43	107	
NAL						3	3	7	16	137	43	
ERY				49	32	3	5	3	12	8	17	52
STR		2	16	51	39	15	2	9	74			

| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Campylobacter jejuni

S												R
MICs	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
GEN	34	198	193	29	7		1	11	7			
CIP	4		6	1	6	56	194	135	19			
TET			2	4	3	2	5	11	29	105	116	
NAL					4	17	9	13	31	185	45	
ERY				82	37	6	6	3	12	9	19	52
STR		18	86	162	51	17	3	10	76			

| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Appendix III. Minimum Inhibitory Concentration distributions for pig isolates and *Campylobacter coli*.

Campylobacter coli

S														R
MICs	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
GEN		14	80	292	329	633	143	11	9	77	12	90		
CIP	25	76	32	8	7	6	118	687	517	79	8	1		
TET			4	**7**	4		7	17	56	573	280	386	97	1
NAL				2	1	3	33	75	30	44	397	855	58	
ERY			9	80	235	109	16	10	6	12	468	154	76	
STR				1	7	45	57	38	16	412	198	103		

= ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Appendix IV. Minimum Inhibitory Concentration distributions for cattle isolates and *Campylobacter* spp. categories.

Campylobacter coli

S													R
MICs	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
GEN		95	217	91	28	35 |	2			11		2	
CIP	31	67	9	2 |	6	7	80	133	47	5			
TET			50	23	3	3 |		12	17	94	76	41	
NAL						28	78	30	8 |	40	154	55	1
ERY				153	176	26	10 |	1			**7**	4	6
STR			32	105	184	30	7	8 |	10	59	9	6	

\| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

Campylobacter jejuni

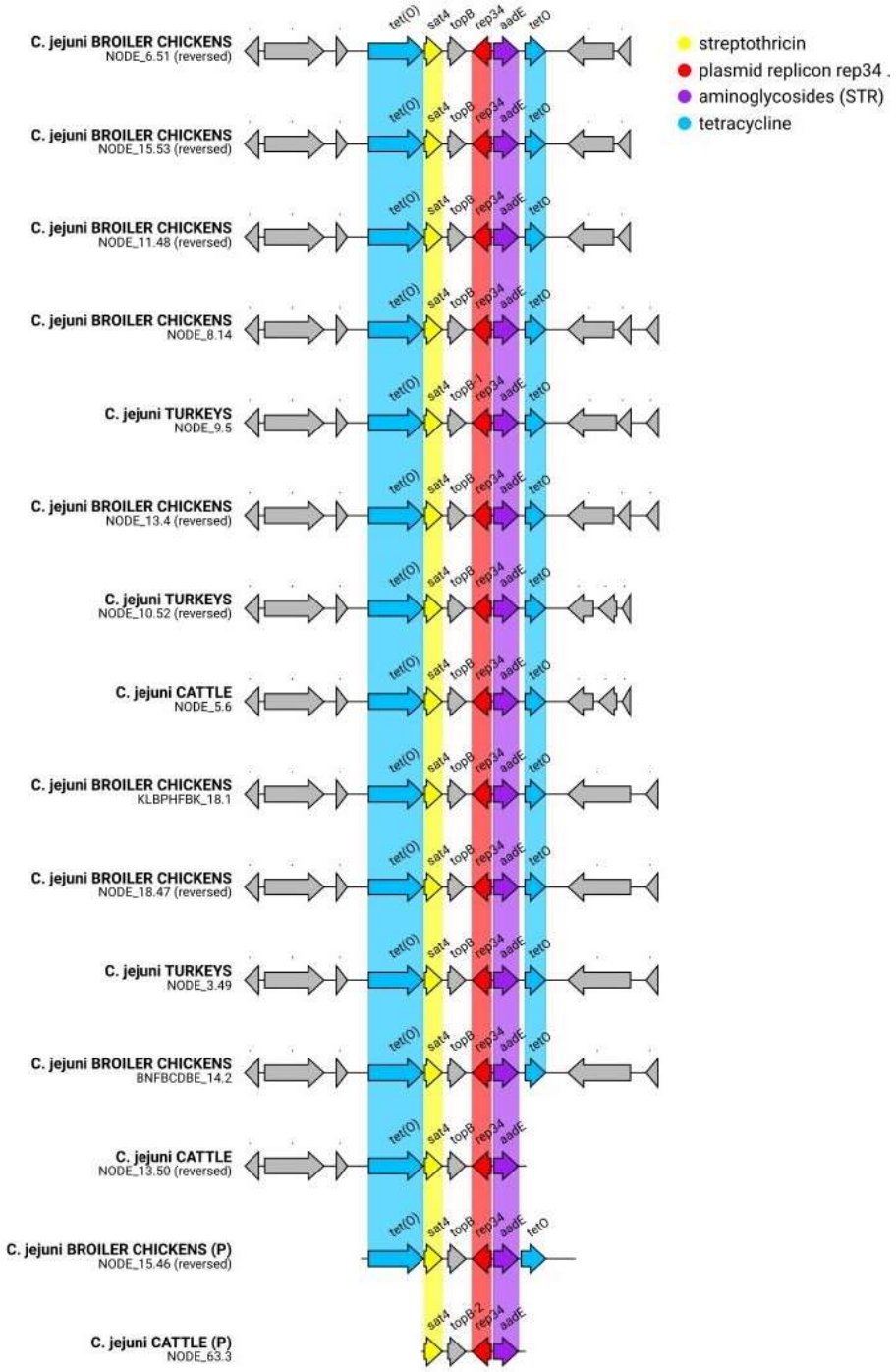
S													R
MICs	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
GEN		100	236	188	217	21 |	2		1	4			
CIP	74	161	36	1 |	5	6	90	279	46	1	1		
TET		15	140	35	4 |	3		18	39	295	76	22	6
NAL						28	142	105	27 |	47	140	204	4
ERY			75	472	131	7	1 |	1		1	5	2	
STR			32	124	405	134	40 |	4	11	24	4		

\| = ECOFF value, separating susceptible 'wild-type' strains (left) from resistant 'non wild-type' strains (right)

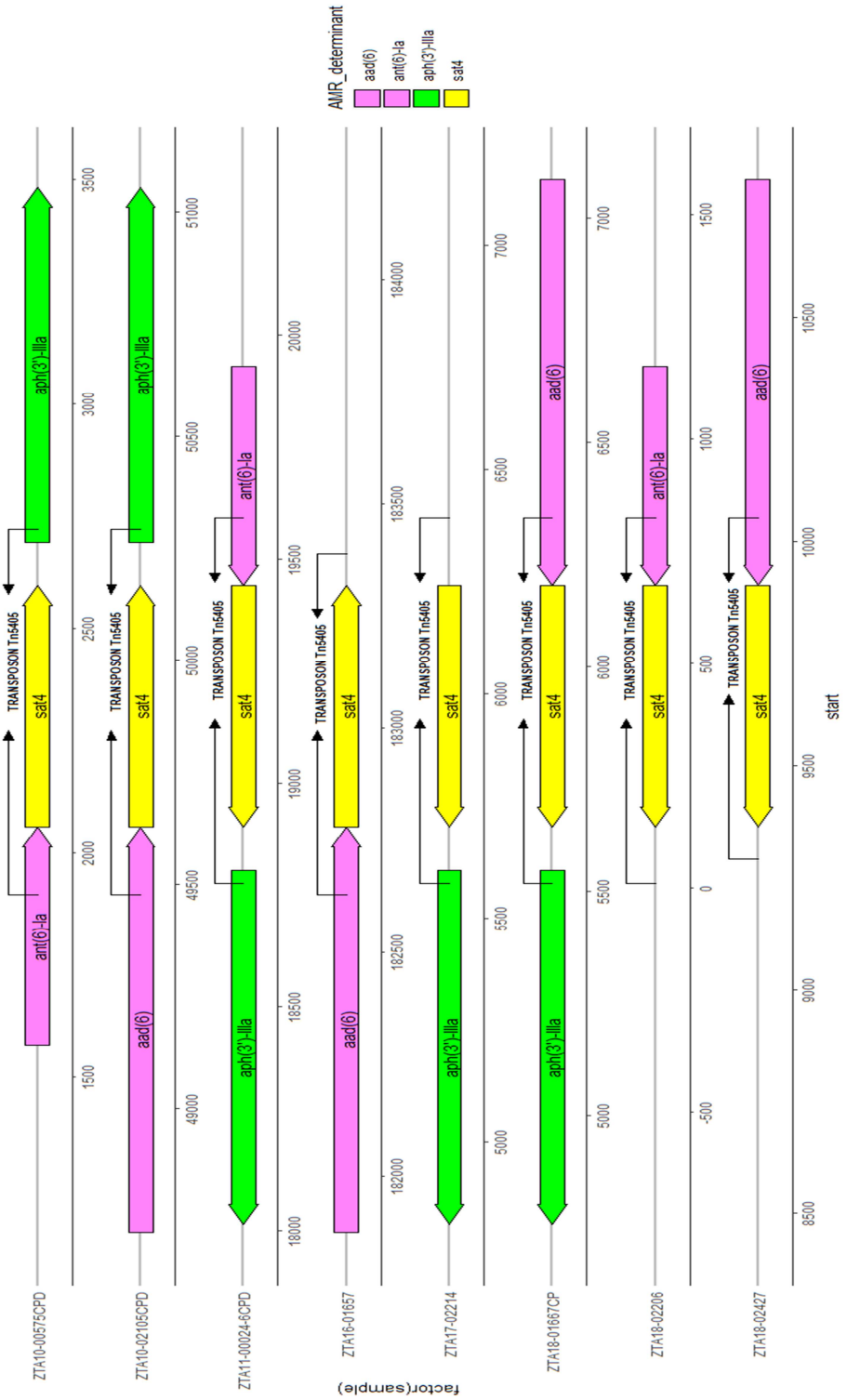
Appendix V. Basic quality parameters of the genomes used in the thesis.

Parameter	Raw reads WGS data (Specific Objective 2) 107 isolates	New WGS data (Specific Objectives 2 + 3) 88 isolates
Number of contigs	49-585	22-3,794
Total length	1.56-1.75 Mbp	1.60-8.12 Mbp
Contig min length	128	9,714
Contig max length	283,000	657,948
N50	4,392–120,859 bp	1,478-384,841 bp

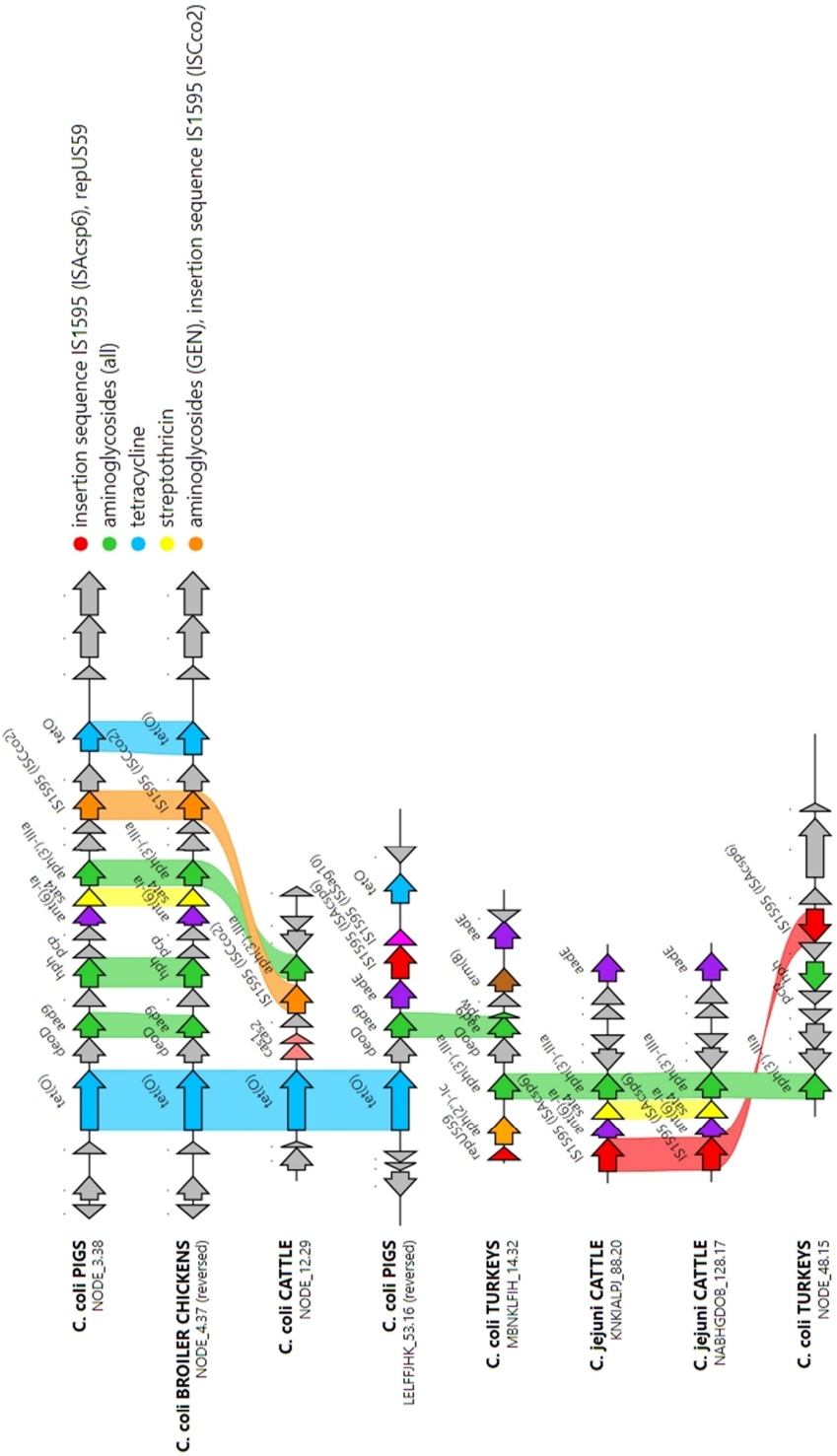
Appendix VI. Genetic contexts of isolates bearing plasmid replicon *rep34*.



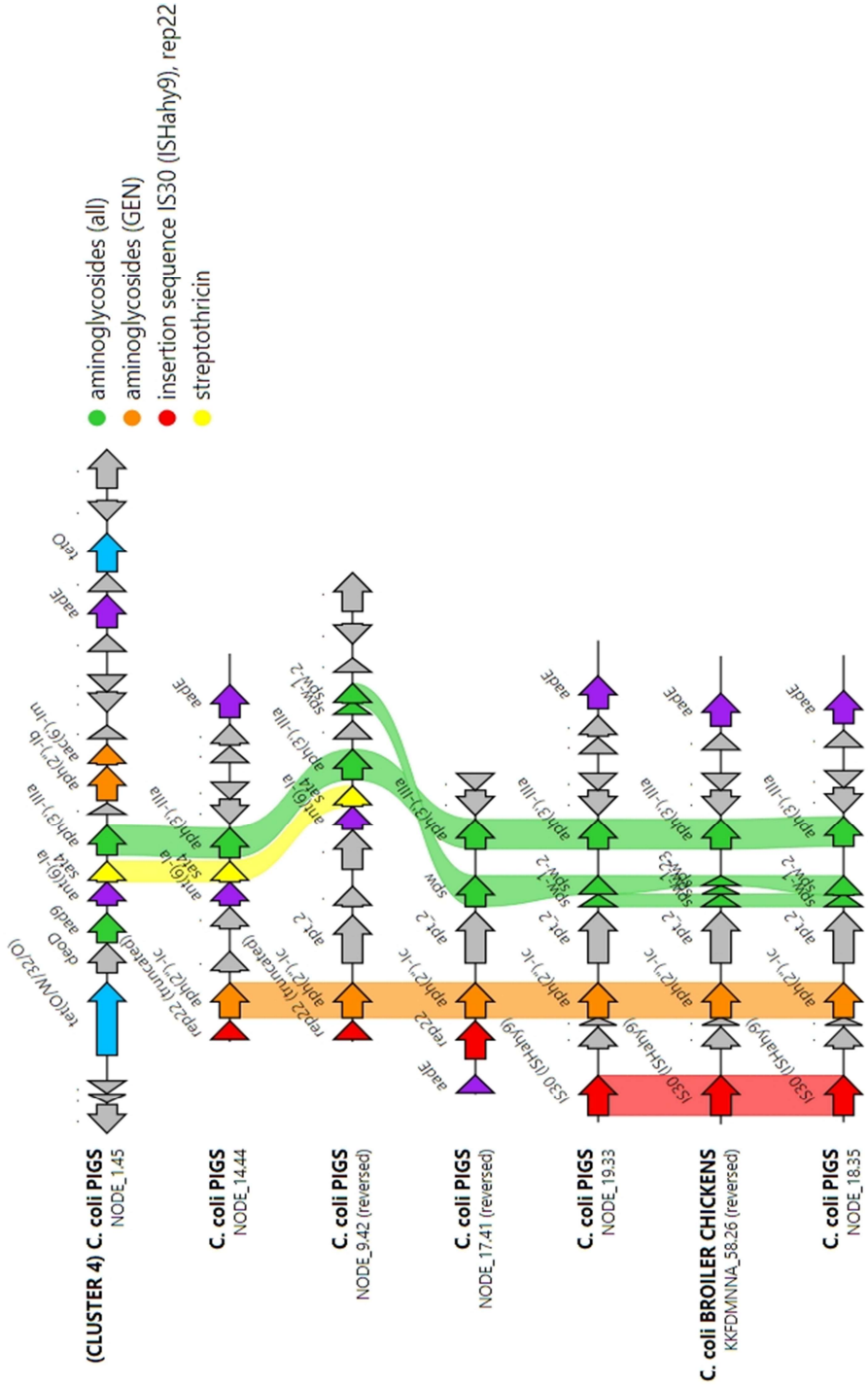
Appendix VII. Precise location and extension of transposon *Tn5405* on contigs harbouring it.



Appendix VIII. Genetic context of isolate bearing plasmid replicon *repUS59*.



Appendix IX. Genetic contexts of isolates bearing plasmid replicon *rep22*.



Appendix X. Thesis peer reviewed publications.

https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.689262/full

frontiers in Microbiology

ORIGINAL RESEARCH
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Monitoring of Antimicrobial Resistance to Aminoglycosides and Macrolides in *Campylobacter coli* and *Campylobacter jejuni* From Healthy Livestock in Spain (2002–2018)

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Antimicrobial resistance (AMR) in *Campylobacter* spp. (*Campylobacter coli* and *Campylobacter jejuni*) is a concern due to its importance in public health, particularly when it involves aminoglycosides and macrolides, drugs of choice for treatment of human cases. Co-resistance to these two antimicrobial classes involves transfer of genetic elements and/or acquisition of mutations in different genetic loci, which can in turn spread through vertical or horizontal gene transfer (HGT) phenomena, with each route having different potential implications. This study aimed at evaluating the association between the presence of phenotypic resistance to these two antimicrobial classes in *C. coli* and *C. jejuni* recovered from livestock at slaughterhouses in Spain (as part of the AMR surveillance program), and at assessing the genetic heterogeneity between resistant and susceptible isolates by analysing the "short variable region" (SVR) of the *flaA* gene. Over the 2002–2018 period, antimicrobial susceptibility test results from 10,965 *Campylobacter* isolates retrieved from fecal samples of broilers, turkeys, pigs and cattle were collected to compare the proportion of resistant isolates and the Minimum Inhibitory Concentrations (MICs) against six antimicrobials including gentamicin (GEN), streptomycin (STR), and erythromycin (ERY). AMR-associated genes were determined for a group of 51 isolates subjected to whole genome sequencing, and the *flaA* SVR of a subset of 168 isolates from all hosts with different resistotypes was used to build a Neighbor-Joining-based phylogenetic tree and assess the existence of groups by means of "relative synonymous codon usage" (RSCU) analysis. The proportion of antimicrobial resistant isolates to both, aminoglycosides and macrolides,

varied widely for *C. coli* (7–91%) and less for *C. jejuni* (all hosts 0–11%). Across hosts, these proportions were 7–56% in poultry, 12–82% in cattle, and 22–91% in pigs for *C. coli* and 0–8% in poultry and 1–11% in cattle for *C. jejuni*. Comparison of the MIC distributions revealed significant host-specific differences only for ERY in *C. jejuni* ($p = 0.032$). A significant association in the simultaneous presentation of AMR to both antimicrobial classes was observed across hosts/bacterial species. The *flaA* gene analysis showed clustering of isolates sharing resistotype and to a lesser degree bacterial species and host. Several resistance markers associated with resistance to aminoglycosides and macrolides were found among the sequenced isolates. The consistent association between the simultaneous presentation of AMR to aminoglycosides and macrolides in all hosts could be due to the persistence of strains and/or resistance mechanisms in *Campylobacter* populations in livestock over time. Further studies based on whole genome sequencing are needed to assess the epidemiological links between hosts and bacterial strains.

Keywords: *Campylobacter*, antibiotics, antimicrobial resistance, aminoglycosides, macrolides, flagellin, genes

INTRODUCTION

Campylobacter coli and *Campylobacter jejuni*, thermophilic bacteria of the genus *Campylobacter* spp., are the most frequently notified human gastrointestinal zoonotic pathogens in the European Union (EU) since 2005 (EFSA, 2005, 2006, 2007). Traditionally, *C. jejuni* was more frequently isolated from human cases than *C. coli*, but in 2017, 24.1% of *Campylobacter* confirmed human infections in the EU were caused by *C. coli* versus 22.2% due to *C. jejuni*, thus suggesting this pattern may vary (EFSA-ECDC, 2019b). Poultry and poultry products are considered the main source of human campylobacteriosis, followed by ruminants (beef, dairy cattle, and their manure) and environmental sources (Ravel et al., 2017; Rosner et al., 2017; An et al., 2018; Thepault et al., 2018). Consumption of pig meat has been linked to human cases caused by *C. coli* (Rosner et al., 2017). Although the prevalence of *Campylobacter* spp. in pork is high, previous studies found it was only associated with 2% of all human cases (Kittl et al., 2013), while beef has been linked to 19% of human cases (Boysen et al., 2014).

Treatment of human cases, when necessary, may be hampered by the increasing threat of antimicrobial resistance (AMR) observed in recent years (Friedrich, 2019). Macrolides [erythromycin (ERY) and azithromycin] and fluoroquinolones [ciprofloxacin (CIP)] are the drugs of choice in human patients requiring antibiotic treatment, but the latter class is not recommended for children. When these drugs are ineffective, systemic administration of aminoglycosides is the only option left (Bolinger and Kathariou, 2017; World Health Organization (WHO), 2017). Levels of AMR to aminoglycosides and macrolides in clinical thermophilic *Campylobacter* from humans are increasing (Aarestrup and Wegener, 1999; Bolinger and Kathariou, 2017; EFSA-ECDC, 2019b), and a similar trend for macrolides has been observed in isolates from pigs and broilers (Moore et al., 2006; Wang et al., 2016). In Europe, the proportion of resistant *Campylobacter* spp. isolates from food producing animals and humans vary depending on the country. Isolates originating from Spain showed higher levels of resistance to aminoglycosides [gentamicin (GEN) and streptomycin (STR)], macrolides (ERY), quinolones [CIP and nalidixic acid (NAL)], and tetracycline (TET) in *C. coli* from broilers, turkeys and pigs, and *C. jejuni* from broilers (EFSA, 2005, 2006, 2007; EFSA-ECDC, 2016, 2017, 2018a, 2019b). *C. coli* has traditionally shown higher levels of AMR to most antimicrobials compared with *C. jejuni* from both humans and animals (EFSA-ECDC, 2017, 2018a, 2019b). Therefore, the AMR problem may intensify if the importance of *C. coli* as a human pathogen keeps increasing. *C. coli* is now as prevalent as *C. jejuni* in broilers in some countries (Wieczorek and Osek, 2013), thus, monitoring of *C. coli* and *C. jejuni* AMR levels, particularly to macrolides and aminoglycosides, is equally important (EFSA-ECDC, 2018a, 2019b).

Resistance to aminoglycosides and macrolides can be mediated by multiple mechanisms, including chromosomal mutations and horizontal gene transferable elements (Davies and Wright, 1997; Saenz et al., 2000; Moore et al., 2006; Luangtongkum et al., 2009; Wieczorek and Osek, 2013; Bolinger and Kathariou, 2017). Antibiotic modifying enzymes (AMEs) are commonly involved in resistance to aminoglycosides (Saenz et al., 2000; Wieczorek and Osek, 2013; Garneau-Tsodikova and Labby, 2016), whereas ribosome methyltransferases (RMTs) are frequently related to resistance to macrolides (Saenz et al., 2000; Aarestrup, 2005). Co-resistance to both aminoglycosides and macrolides, as well as multidrug resistance (MDR) to additional antimicrobial classes, can be acquired through several mechanisms such as 16S rRNA RMTs (RmtB, ArmA) encoded in multi-drug resistance genomic islands (MDRGIs) carrying *erm* genes in *C. coli* (Aarestrup, 2005; Garneau-Tsodikova and Labby, 2016; Bolinger and Kathariou, 2017) and 23S rRNA RMTs (Saenz et al., 2000). Additional mechanisms can involve transferable genomic islands carrying multiple aminoglycoside resistance genes encoding AMEs in *C. coli* (Davies and Wright, 1997; Luangtongkum et al., 2009; Qin et al., 2012; Wieczorek and Osek, 2013) and multidrug macrolide efflux pumps (including

the resistance enhancing *CmeABC* in *C. jejuni*), alone or in combination with target gene mutations (Aarestrup, 2005; Wieczorek and Osek, 2013; Garneau-Tsodikova and Labby, 2016; Bolinger and Kathariou, 2017). Although several of these mechanisms were first discovered in *C. coli*, evidence of transfer to *C. jejuni* was shown thereafter (EFSA-ECDC, 2018a).

Epidemiological studies complemented with genetic analyses are essential to help understand the mechanisms by which co-resistance and MDR to aminoglycosides and macrolides may be emerging in thermophilic *Campylobacter* from animals and humans (Luangtongkum et al., 2009; Wieczorek and Osek, 2013). Here, data from the national surveillance program on AMR in *Campylobacter* spp. from broilers, turkeys, pigs and cattle in Spain were analyzed to assess the prevalence of *Campylobacter* species in different animal hosts and the patterns of phenotypic AMR co-resistance to macrolides and aminoglycosides over the years. The results of this research can contribute to better explain AMR co-selection/MDR phenomena between these two antimicrobial classes in *Campylobacter* from livestock.

MATERIALS AND METHODS

Study Population

The data analyzed here is based on sample collection, culture and antimicrobial susceptibility testing (AST) work carried out during 2002–2018 on isolates retrieved through the Spanish national veterinary AMR monitoring program for *Campylobacter* spp. in poultry (broilers and turkeys), pigs and cattle, according to EU legislation (EC, 2013). Samples for each animal species, originating from multiple farms, were collected at slaughterhouses covering 60% of the national throughput (**Supplementary Figures 1**, **2**). Broiler samples were retrieved every year from 2002 to 2014 and every 2 years thereafter, turkey samples every 2 years from 2014 to 2018, pig samples every year from 2002 to 2013 and every 2 years thereafter, and cattle samples every year from 2007 to 2013 and every 2 years thereafter. Pooled samples collected every year for each host species ranged between 76 and 500 (mean = 228) for broilers, 467 and 500 (mean = 485) for turkeys, 171 and 384 (mean = 268) for pigs, and 163 and 384 (mean = 261) for cattle (**Supplementary Table 1**).

In relation to sampling and culture, pools made of samples from animals belonging to the same farm (from 10 caeca in poultry or from the caecum content of 2 animals in pigs and cattle) were collected at the slaughterhouse and transported refrigerated to the laboratory, where they were processed within 24 h after collection according to ISO 10272-2006-1. Single colonies with morphology compatible with *Campylobacter* spp. were identified as "*C. coli*," "*C. jejuni*," or "*C.* spp." using API strips (up to 2010) and a multiplex PCR (2010 onward) (Ugarte-Ruiz et al., 2012).

Regarding AST, the AMR phenotype of *Campylobacter* spp. isolates was determined using the two-fold broth micro-dilution reference method (calculating Minimum Inhibitory Concentrations – MICs, according to ISO Norm 20776-1:2006) or diffusion technique (calculating Inhibition Zone Diameters – IZDs) (Ugarte-Ruiz et al., 2015).

Antimicrobial susceptibility testing results for the six antimicrobials listed on the AMR surveillance programs for *Campylobacter* spp. in the EU (EC, 2013) were available for isolates from all species and years: CIP, TET, NAL, STR, ERY, and GEN. For ERY in broilers, IZDs were used up to 2004 (included) and MICs were used thereafter. For STR in broilers and pigs, IZDs were used up to 2005 (included) and MICs were used thereafter.

Samples in which information on culture result, molecular identification and/or AMR typing was missing were excluded from the analysis. The following information was available for all samples in the study: host species, *Campylobacter* growth result, *Campylobacter* species, year and AST result. Isolates were classified as "susceptible" (wild-type strains) or "not susceptible" (resistant strains) according to epidemiological cut-off points (ECOFFs) provided by the "European Committee on Antimicrobial Susceptibility Testing" (EUCAST)[1] (**Table 1**). Proportions of resistant isolates for each bacterial and host species and period were defined as very low (<1%), low (1.1–10%), moderate (10.1–20%), high (20.1–50%), very high (50.1–70%), and extremely high (70.1–100%), as recommended by EFSA (EFSA-ECDC, 2019b).

Statistical Analyses

Proportions of resistant *C. coli* and *C. jejuni* isolates to each antimicrobial from the different hosts were compared using Z-tests, adjusted for multiple comparisons by the Holm-method. Cochrane-Armitage logistic regressions were used to test for trends of AMR phenotypic resistance in *C. coli* and *C. jejuni* per antimicrobial and host species, and the relative change in the proportion of resistant isolates per year was computed along with its 95% confidence interval. The association in the simultaneous presentation of phenotypic resistance to STR/ERY, GEN/ERY, and GEN/STR over the whole study period and in different time periods (2002–2006, 2007–2012, and 2013–2018) was further

[1]EUCAST-European Society of Clinical Microbiology and Infectious Diseases, MIC and zone distributions and ECOFFs http://www.eucast.org/mic_distributions_and_ecoffs/ (accessed 14/02/2020).

TABLE 1 | Epidemiological cut-offs (ECOFFs) used for interpretation of MICs in *Campylobacter* spp. (Source EUCAST).

Antimicrobial	*C. coli* MIC (>) Micro-dilution	*C. coli* DIAM (<) Difusion	*C. jejuni* MIC (>) Micro-dilution	*C. jejuni* DIAM (<) Difusion
Gentamicin (GEN)	2	NA	2	20
Streptomycin (STR)	4	13	4	13
Erythromycin (ERY)	8	24	4	22
Ciprofloxacin (CIP)	0,5	26	0,5	26
Nalidixic acid (NAL)	16	NA	16	NA
Tetracycline (TET)	2	30	1	30

evaluated for each bacterial and host species using relative risks and chi-squared and Fisher's exact tests.

In order to evaluate differences in the distribution of MICs values in *C. coli* and *C. jejuni* from the four host species, available data were represented as "squashtograms." The existence of statistical differences in MICs distributions in susceptible and not-susceptible (here referred to as "resistant") isolates depending on bacterial species (for a given host) or on host (for a given bacterial species) was evaluated using Mann–Whitney *U* or Kruskal–Wallis tests followed by Dunn's *post hoc* tests, correcting for multiple comparisons by the Benjamini-Hochberg method.

Molecular Characterization Based on *flaA* Sequencing

A subset of 125 isolates including all combinations of hosts, bacterial species, year of recovery and AMR phenotype, randomly chosen within each category, was used to assess their genetic relatedness by comparing the flagellin *flaA* short variable region (SVR) gene sequence as described by other authors (Ugarte-Ruiz et al., 2013; Zhang et al., 2018). Selected isolates were classified into two categories: isolates with simultaneous phenotypic resistance to aminoglycosides and ERY (n = 53, "cases") and isolates not presenting this simultaneous resistance (n = 72, "controls"). Amplification of the *flaA* SVR gene sequence by PCR was performed as previously described (Ugarte-Ruiz et al., 2013), and the obtained amplicons were sequenced. Additionally, the *flaA* sequence of fifty-one isolates (8 "cases" and 43 "controls") previously subjected to whole genome sequencing (GenBank accession codes SRX5575129 to SRX5587545) was extracted (along with information on the presence of resistance genes) using a homemade Python script. The resulting 176 sequences were then aligned using MUSCLE (Edgar, 2004) and a Neighbour-Joining (NJ) phylogenetic tree with 1,000 bootstraps was built to evaluate the phylogenetic relationship between isolates. The *flaA* gene of the NCTC 1168 *C. jejuni* strain (1719 nucleotides-long, bacterial chromosome positions 1269232 to 1270950) was used as an external reference. A multiple correspondence analysis (MCA) of the relative synonymous codon usage (RSCU) values categorized as > 1 (positive bias) or < 1 (negative bias) was performed as described before (Meinersmann et al., 2005). The MCA included, along with the RSCU of variable codons, other available covariates (bacterial species, host species, resistance to GEN, ERY, and STR, and clade as determined in the NJ phylogenetic tree).

Microsoft Access was used for data handling and database initial analyses. Data were further handled with Microsoft Excel and imported into "R" version 3.6.3 (R Core Team, 2020). The R packages "FSA" (Ogle et al., 2020), "plyr" (Wickham, 2011), and "ggplot2," "dplyr," "reshape2," and "tidyr" (Wickham et al., 2019) were used for the analysis and visual representation of the data. Information on resistance-associated markers from the sequenced strains was extracted using ResFinder (Bortolaia et al., 2020). MEGA-X (Kumar et al., 2018) and DnaSP6 (Rozas et al., 2017) were used on imported DNA sequences for the preparation and analysis of sequence alignments. R packages "BiocManager" ("coRdon") (Morgan and Ramos, 2019) and "seqinr" (Charif and Lobry, 2007) were used for the calculation of RSCUs. "Corrplot" (Wei et al., 2017), "FactoMineR" (Lê et al., 2008), "factoextra" (Kassambara and Mundt, 2020), and "ggtheme" were used for the MCA analysis. All Figures were generated using R except **Figure 1** (Excel) and **Figure 6** (MEGA-X).

RESULTS

In total 3,413 independent samples from broilers, 1,455 from turkeys, 3,750 from pigs and 2,347 from cattle were included in the analysis, of which 2,000 (58.6%), 1,090 (74.9%), 2,218 (59.2%), and 1,273 (54.3%) resulted in the isolation of *Campylobacter* spp., respectively (**Table 2**). The number of samples analyzed, isolates recovered, and isolates subjected to AST varied depending on year and host species (**Supplementary Table 2**).

Over the entire study period, *C. coli* was the most frequently isolated species in pig (88.7%; 1,968/2,218) and turkey (74.8%; 815/1,090) samples, while *C. jejuni* was the most frequent species in cattle (84.4%; 1,074/1,273). In broilers, the proportion of *C. coli* and *C. jejuni* was very similar (51.2%; 1,023/2,000 and 47.8%; 957/2,000, respectively) (**Table 2**).

Although there were differences depending on the year, the proportion of positive samples to *C. coli* and *C. jejuni* remained relatively constant over the whole study period in pigs and cattle, with one bacterial species being more prevalent than the other one, while the situation was more variable in broilers (**Figure 1**). Significant increasing trends were observed in the proportion of positive samples for *C. jejuni* in broilers and turkeys, with annual-biannual rates of increase of 9.7% (95%CI: 6.16–13.33%) and 26.2% (95%CI: 15.81–37.43%), respectively (**Figure 1**).

The overall proportion of isolates resistant to CIP, NAL, and TET was extremely high (>80%) in both *C. coli* and *C. jejuni* from all host species (**Table 3** and **Figures 2**–**5**), with yearly values exceeding 70% throughout the study period except in *C. jejuni* from cattle (**Figure 5**). Still, there were significant differences in the proportion of resistant isolates to these three antimicrobials depending on the host (**Table 3**). The proportion of CIP and NAL-resistant *C. coli* isolates was significantly lower in cattle compared with broilers and turkeys ($p < 0.05$) (and in pigs compared with turkeys for CIP, $p < 0.001$). In the case of TET, *C. coli* isolates from pigs were significantly more resistant than *C. coli* from cattle and broilers ($p < 0.001$), although resistance was still above 95% in all species (**Table 3**). In the case of *C. jejuni*, cattle isolates were significantly less resistant to the three antimicrobials compared with isolates from poultry ($p < 0.05$).

The proportion of resistance to the remaining three antimicrobials (STR, ERY, and GEN) was much more variable (**Table 3** and **Figures 2**–**5**). In the case of STR, extremely high (80–91%) or high to very high (∼55%) levels were found in *C. coli* from cattle and pigs and from broilers and turkeys, respectively, with significant differences between all hosts species except between broilers and turkeys (pigs > cattle > poultry). In contrast, values <11% were observed in *C. jejuni* from all three host species

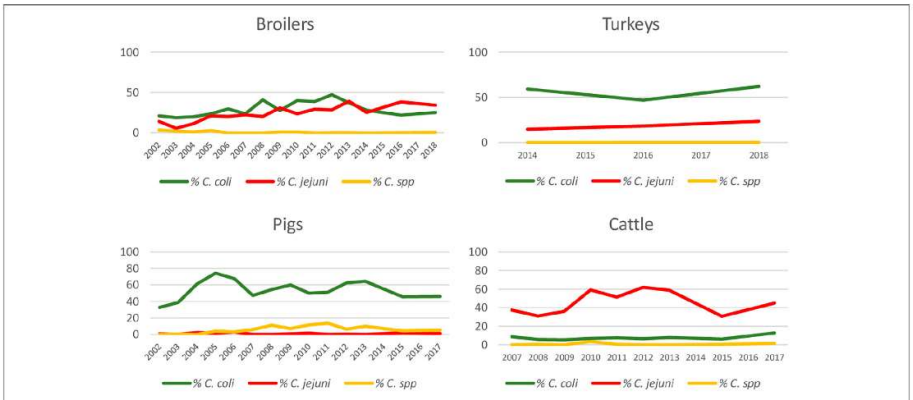

FIGURE 1 | Percentage of *Campylobacter* isolates over total sampled, identified as *C. coli*, *C. jejuni*, and *C.* spp. for each host species throughout study period.

from which this bacterial species was recovered (broilers, turkeys, and cattle). Although levels of resistance in *C. jejuni* were always significantly lower than in *C. coli* for any given host species, no significant differences between host species were observed.

The proportion of resistance to ERY in *C. coli* was very high (67%) for pigs, high (35%) for broilers and turkeys, and moderate (19%) for cattle (pigs > poultry > cattle) (**Table 3** and **Figures 2–5**). Overall values in *C. jejuni* from all host species were < 3% and significantly lower than those from *C. coli*, again with no significant differences across hosts.

Finally, the proportion of GEN resistant-isolates was low (<25%) in *C. coli* from all species (**Table 3** and **Figures 2–5**), although the proportion in pigs was again significantly higher than that observed in other host species ($p < 0.001$). Resistance levels in *C. jejuni* were lower (<2%) and significantly different from those observed in *C. coli* from the same host and, once more, no significant differences between hosts were observed.

Significant ($p < 0.05$) trends in the proportion of cattle resistant isolates were observed associated to increasing annual rates for ERY and *C. coli* (29.9% although with a wide 95%CI: −31.36 to 145.77) and STR and *C. jejuni* (16.7%, 95%CI: 2.89–32.28) (**Figure 5**). Significant ($p < 0.001$) trends in the proportion of resistant *C. jejuni* isolates recovered yearly from broilers were also observed for two antimicrobials, in both cases associated to decreasing annual rates: STR (−9.9%, 95%CI: −16.13 to −3.14) and ERY (−27.5%, 95%CI: −44.60 to −5.10) (**Figure 2**).

Other significant ($p < 0.001$) trends were found associated with modest annual rates of increase in *C. jejuni* in cattle for CIP (5.22%, 95%CI: 3.91–6.54) and NAL (4.11%, 95%CI: 2.20–6.06), in *C. coli* in broilers for TET (1.61%, 95%CI: 0.93–2.29), and in *C. coli* in pigs for CIP (0.64%, 95%CI: 0.20–1.09). For the rest of antimicrobials, host and bacterial species no significant trends were detected (**Figures 2–5**).

For any given bacterial species, an analysis of the quantitative AST results across hosts species revealed significant differences ($p = 0.032$) in the distribution of MIC values between "susceptible" and "not susceptible" isolates only for *C. jejuni* strains from turkeys "not susceptible" to ERY (MIC ≥ 256 mg/L) (**Supplementary Excel File 1**). In contrast, for any given host species, no significant differences were observed between MIC distributions of "susceptible" and "not susceptible" *C. coli* vs. *C. jejuni* isolates.

Co-resistance and MDR Phenotypic Profiles

The main resistance profiles observed in each bacterial and host species are shown on **Supplementary Table 3**. Of all *C. coli* isolates from all host species, >85% were resistant to three (CIP-TET-NAL, TET-ERY-STR) or more antimicrobials and >60% were resistant to three or more antimicrobial classes (MDR). The most common resistance profiles for *C. coli* from each host were CIP-TET-NAL and CIP-TET-NAL-STR in broilers and turkeys (18–28% of all isolates in each host species for each profile), CIP-TET-NAL-STR in cattle (~50% of all *C. coli* isolates) and CIP-TET-NAL-STR-ERY in pigs (~40% of all *C. coli* isolates). The proportion of pan-susceptible isolates for *C. coli* in all host species

TABLE 2 | Collection period, number of fecal samples and positive samples for *Campylobacter* isolation from each host species included in the study.

Host species	Broilers	Turkeys	Pigs	Cattle
Years	2002–2018	2014–2018	2002–2017	2007–2017
Sample size	3,413	1,455	3,750	2,347
C. coli	1,023 (30.0%)	815 (56.0%)	1,968 (52.5%)	183 (7.8%)
C. jejuni	957 (28.0%)	273 (18.8%)	33 (0.9%)	1,074 (45.8%)
C. spp.	20 (0.6%)	2 (0.1%)	217 (5.8%)	16 (0.7%)

TABLE 3 | Percentage of *Campylobacter* isolates not susceptible (resistant) to each antimicrobial in the four host species throughout the studied period.

Antimicrobials		Tetracycline	Nalidixic acid	Ciprofloxacin	Streptomycin*	Erythromycin*	Gentamicin
		Coli (MIC > 2) *Jejuni* (MIC > 1)	*Coli/Jejuni* (MIC > 16)	*Coli/Jejuni* (MIC > 0.5)	*Coli/Jejuni* (MIC > 4; DIAM < 13)	*Coli* (MIC > 8; DIAM < 24) *Jejuni* (MIC > 4; DIAM < 22)	*Coli/Jejuni* (MIC > 2)
C. coli (%)	Broilers (n = 634)	95.9^{a}	93.3^{a}	$94.5^{a,b}$	54.7^{a}	34.8^{a}	14.7^{a}
	Turkeys (n = 279)	$97.5^{a,b}$	95.3^{a}	98.2^{c}	55.9^{a}	36.6^{a}	7.5^{b}
	Pigs (n = 1,692)	99.1^{b}	$91.7^{a,b}$	$91.7^{a,c}$	90.6^{b}	66.6^{b}	22.2^{c}
	Cattle (n = 149)	95.3^{a}	86.7^{b}	87.3^{c}	82.0^{c}	19.3^{c}	$12.0^{a,b}$
C. jejuni (%)	Broilers (n = 772)	83.1^{a}	88.5^{a}	91.1^{a}	7.7^{a}	2.9^{a}	1.0^{a}
	Turkeys (n = 231)	83.1^{a}	86.1^{a}	88.7^{a}	6.1^{a}	2.6^{a}	0.0^{a}
	Cattle (n = 828)	74.1^{b}	63.1^{b}	63.8^{c}	10.2^{a}	1.7^{a}	1.4^{a}

Different superscripts indicate significant differences between hosts for each bacterial species
**Diffusion technique: streptomycin in broilers and pigs (2002–2005) and erythromycin in broilers (2002–2004).*

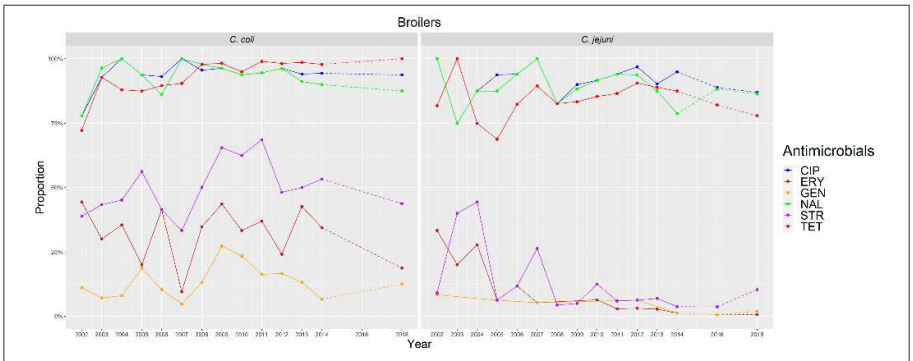

FIGURE 2 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from broilers for 2002–2018. Years in which AST was performed are indicated in the X axis; dashed lines indicate periods in which AST was not performed every year (no AST performed in *C. coli* in 2016).

was low (0–4%), and lower than the proportion of resistant isolates to all six antimicrobials (0–16%).

In comparison, the proportion of *C. jejuni* isolates from all hosts resistant to three or more antimicrobials was 54–76% whereas the MDR proportion was 6–9%. The most common resistance profile for *C. jejuni* from all hosts was CIP-TET-NAL, amounting to between ∼45% of all cattle and 60–70% of all broiler and turkey isolates. In this case, the proportion of pan-susceptible isolates (5–15%) was higher than that of resistant isolates to the six antimicrobials in all species (<1%).

Association Between Resistance to GEN, STR, and ERY

Overall, a significant association between the occurrence of phenotypic resistance to aminoglycosides and macrolides was observed, so that *C. coli* and *C. jejuni* from all host species (except *C. coli* in pigs and turkeys and *C. jejuni* in turkeys) resistant to one of the two aminoglycosides (or to both) were more likely to be also resistant to ERY (**Table 4**). This association was stronger in *C. jejuni* and/or cattle isolates.

Analysis of *flaA* and AMR Genes

Over 300 bp (including the 267 bp-long SVR used in the analysis) of the *flaA* gene sequence were correctly determined in 168 of the 176 chosen isolates (all except 2 "cases" and 6 "controls"). **Figure 6** displays the phylogenetic tree constructed using the selected final 168 isolates (59 "cases" and 109 "controls") plus the reference strain. Overall, a total of 127 single nucleotide polymorphisms (SNPs) located in 100 polymorphic sites were found, leading to 73 unique *flaA* SVR gene sequences. The haplotype diversity (Hd – probability that two randomly selected sequences are different) was 0.975, and every two sequences differed on average by 27 SNPs with an overall mean evolutionary distance (d) between the two sequences of 0.09.

The 168 isolates were classified into five groups based on the topology of the tree: group 1, including the majority of the

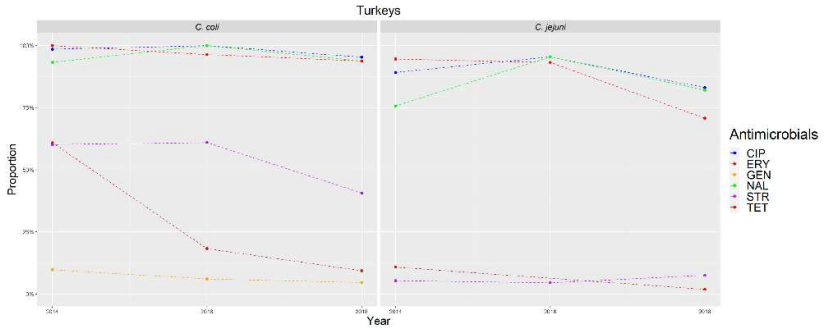

FIGURE 3 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from turkeys for 2014–2018. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

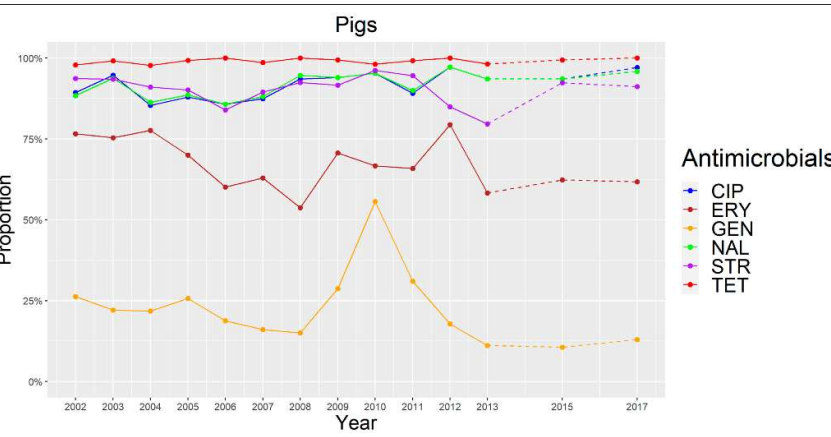

FIGURE 4 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* from pigs for 2002–2017. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

sequenced isolates (n = 100 isolates), group 2 (n = 31 isolates), group 3 (n = 12 isolates), group 4 (n = 17 isolates), and group 5 (n = 8 isolates) (**Figure 6**). Groups 2–5 formed separate clades from group 1 (bootstrap > 60). Groups 2 and 3, consisting mainly of *C. coli* strains (28/31 and 10/12, respectively) predominantly from poultry (>60%), showed similar proportions of isolates resistant to aminoglycosides and macrolides ("cases") and of "controls" (18/31 and 6/12, respectively) (**Table 5**). Groups 4 and 5 showed a higher proportion of *C. jejuni* isolates (13/17 in group 4 and 8/8 in group 5) from cattle (9/17 and 8/8), and the frequency of isolates with simultaneous resistance to both antimicrobial classes ("cases") in these groups was much lower (1/17 and 1/8, respectively) (**Table 5**).

Only complete RSCU values from the 20 variable codons among the 168 isolates were included in the MCA analysis. The first two dimensions identified in the MCA explained 38% of the total variability observed. Isolates included in each of the five groups identified in the phylogenetic tree were also clustered according to the first two dimensions of the MCA (**Figure 7**).

Different resistance markers involved in AMR against macrolides and aminoglycosides were found in the isolates subjected to WGS. Several genes involved in the CmeABC

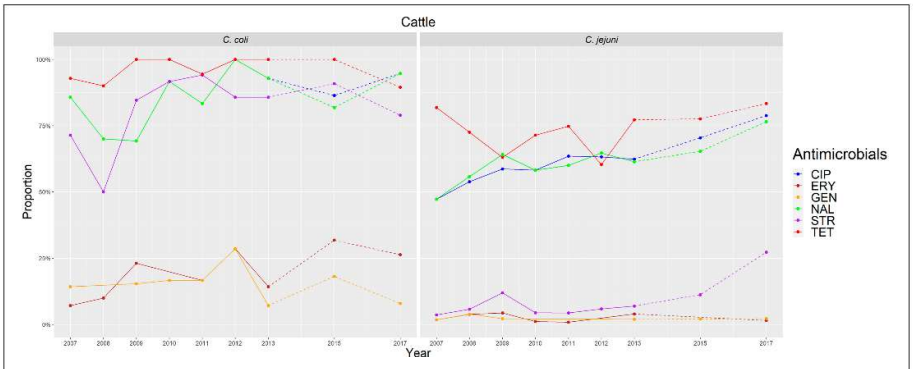

FIGURE 5 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from cattle for 2007–2017. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

TABLE 4 | Association between phenotypic resistance to gentamicin, streptomycin, and erythromycin in *C. coli* and *C. jejuni* isolates from livestock.

Host	Bacterial species	*N*	Erythromycin-R (%)	Antimicrobial	Resistance (%)	Resistance among EryR (%)	*p*-value	RR*
Broilers	*C. coli*	634	34.5	Streptomycin	54.4	68.9	<0.001	1.86
				Gentamicin	14.7	24.7	<0.001	1.90
	C. jejuni	772	2.5	Streptomycin	7.6	31.6	<0.001	5.58
				Gentamicin	1.0	15.8	<0.001	17.90
Pigs	*C. coli*	1692	66.7	Streptomycin	90.7	90.7	0.953	1.00
				Gentamicin	22.0	25.0	<0.001	1.18
Turkeys	*C. coli*	279	36.6	Streptomycin	56.0	66.7	0.008	1.58
				Gentamicin	7.5	10.8	0.156	1.48
	C. jejuni	231	2.6	Streptomycin	6.0	33.3	0.045	7.75
				Gentamicin	0.0	0.0	1	17.85
Cattle	*C. coli*	149	19.5	Streptomycin	82.6	96.6	0.028	5.92
				Gentamicin	12.1	31.0	0.002	3.27
	C. jejuni	828	1.7	Streptomycin	10.3	78.6	<0.001	32.05
				Gentamicin	1.4	28.6	<0.001	27.20

*Relative risk of presenting resistance to erythromycin in isolates resistant to streptomycin/gentamicin.

efflux pump (*cmeB*, *cmeC*, and *cmeR*) were present in 39–40/40 sequenced *C. jejuni* isolates and missing in 10–11/11 *C. coli* strains, while the *cmeA* gene was present in 34/40 and 4/11 of the *C. jejuni* and *C. coli* strains, respectively, but their presence was not associated with ERY resistance (**Supplementary Excel File 2**). Among the 12 ERY-resistant isolates, three presented mutations associated with macrolide-resistance in the 23S rRNA encoding gene and other three carried the *erm*(B) gene, while no resistance marker was found in the remaining six isolates. Regarding aminoglycoside-resistance associated genes, seven different genes were found in one or more strains (between 0 and 3 per strain), and their presence was associated with resistance to STR and/or GEN except in one susceptible *C. jejuni* strain from cattle (**Supplementary Excel File 2**). No apparent association between a specific *flaA* gene group and the presence of any of the resistance markers was observed.

DISCUSSION

Antimicrobial resistance is becoming a major problem for the treatment of diseases caused by zoonotic bacteria such as thermophilic *Campylobacter*. The mechanisms by which AMR can spread in a bacterial population (vertically or horizontally) has enormous implications, since it can determine the speed at which AMR phenotypes disseminate. Of particular concern are genetic traits conferring MDR (Magiorakos et al., 2012), particularly when transmitted together. Hence, it is of paramount importance to explore the genetic mechanisms implicated in AMR in *C. coli* and *C. jejuni* from the different hosts involved in the epidemiology of infection in humans (EFSA-ECDC, 2020). As described elsewhere, this study of isolate-based phenotypic data versus aggregated data has also proven to be a reliable means of gaining insight into such mechanisms (Alvarez et al., 2020),

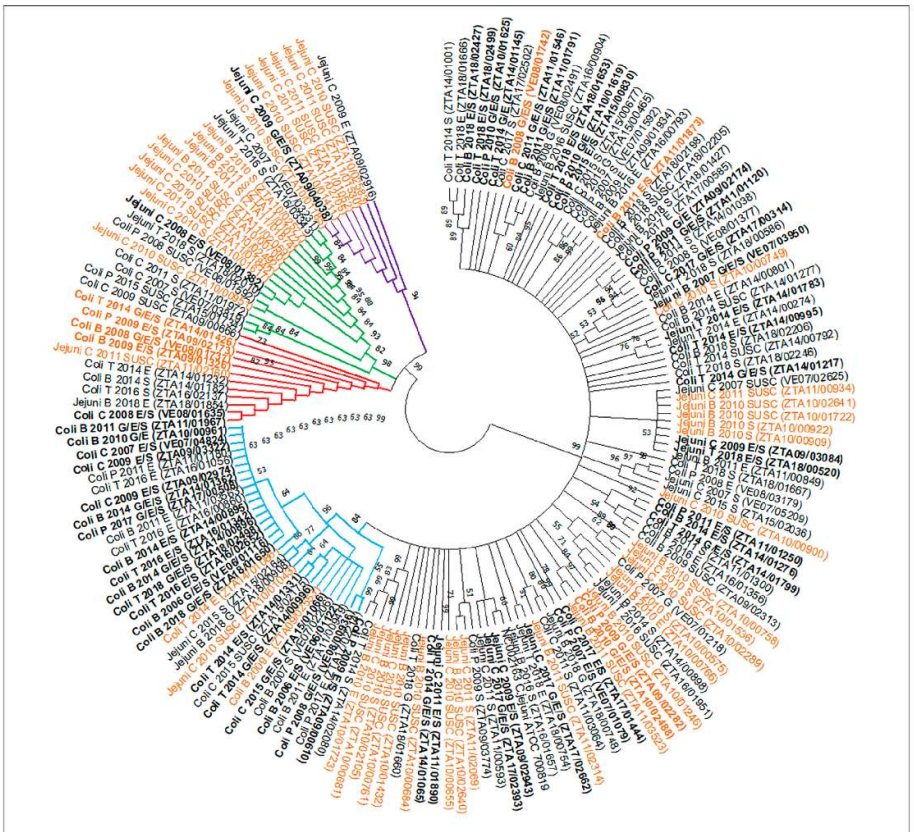

FIGURE 6 | Phylogenetic tree of the short variable region (SVR) of *flaA* genes from 168 selected *Campylobacter* isolates, combining bacterial species, host, year of recovery and AMR phenotype, obtained by the Neighbour-Joining method [Group 1 = black; Group 2 = blue; Group 3 = red; Group 4 = green; Group 5 = violet; NC002163 = Reference strain; B = Broilers; T = Turkeys; P = Pigs; C = Cattle; G = Gentamicin; E = Erythromycin; S = Streptomycin; SUSC = susceptible isolates; aminoglycosides/macrolides co-resistant strains ("cases") in bold; strains subjected to WGS in orange].

and the assessment of phenotypic susceptibility patterns found here can guide the genetic analysis in a "top-down" approach (Sheppard and Maiden, 2015).

The annual proportion of *Campylobacter* positive samples found in our samples from broilers collected over a 17-year period (ranging from 26.2 to 76.7%) was higher than values reported by EFSA from EU member states (26%) (EFSA-ECDC, 2019a) and mostly higher than values reported in other regions of the world such as China (30.2%) (Tang et al., 2020). The proportion of *Campylobacter* positive samples in cattle from 2007 to 2017 (37–69.5%) was also much higher than values reported by EFSA for 10 EU countries (1.5–3.5%) (EFSA-ECDC, 2018b, 2019a), although country-specific studies in Finland (Hakkinen et al., 2007), and Lithuania (Ramonaite et al., 2013) reported values more similar to the ones found here (39.6 and 80%, respectively). Similarly, the percentage of pig samples from which *Campylobacter* isolates were retrieved in our study (33.4–80%) was in the range of results reported for Greece (49.1%) (Papadopoulos et al., 2020), much higher than previously reported by EFSA for 8 EU countries (2–7%) (EFSA-ECDC, 2018b, 2019a) and lower than reported in a Danish study (92%) (Boes et al., 2005). However, the percentages we found in turkeys

TABLE 5 | Numbers and proportions of isolates included in each of the groups formed from the phylogenetic analysis based on phenotypical AMR susceptibility, bacterial species, host species, and case/control categories.

Group #	1	2	3	4	5	Total
N	(*n* = 100)	(*n* = 31)	(*n* = 12)	(*n* = 17)	(*n* = 8)	(*n* = 168)
GEN-R	22 (22.0%)	**11 (35.5%)**	**3 (25.0%)**	0 (0.0%)	1 (12.5%)	37 (22.0%)
ERY-R	45 (45.0%)	**26 (83.9%)**	**8 (66.7%)**	1 (5.9%)	2 (25.0%)	82 (48.8%)
STR-R	58 (58.0%)	**19 (61.3%)**	**8 (66.7%)**	10 (58.8%)	1 (12.5%)	96 (57.1%)
Coli	54 (54.0%)	**28 (90.3%)**	**10 (83.3%)**	4 (23.5%)	0 (0.0%)	96 (57.1%)
Jejuni	46 (46.0%)	3 (9.7%)	2 (16.7%)	**13 (76.5%)**	**8 (100.0%)**	72 (42.9%)
Broilers	37 (37.0%)	**12 (38.7%)**	**5 (41.7%)**	4 (23.5%)	0 (0.0%)	58 (34.6%)
Cattle	27 (27.0%)	7 (22.6%)	3 (25.0%)	**9 (52.9%)**	**8 (100.0%)**	54 (32.1%)
Pigs	**14 (14.0%)**	**4 (12.9%)**	1 (8.3%)	2 (11.8%)	0 (0.0%)	21 (12.5%)
Turkeys	22 (22.0%)	**8 (25.8%)**	**3 (25.0%)**	2 (11.8%)	0 (0.0%)	35 (20.8%)
Cases	33 (33.0%)	**18 (58.1%)**	**6 (50.0%)**	1 (5.9%)	1 (12.5%)	59 (35.1%)
Controls	67 (67.0%)	13 (41.9%)	6 (50.0%)	**16 (94.1%)**	**7 (87.5%)**	109 (64.9%)

In bold = predominant proportions within each group and category.

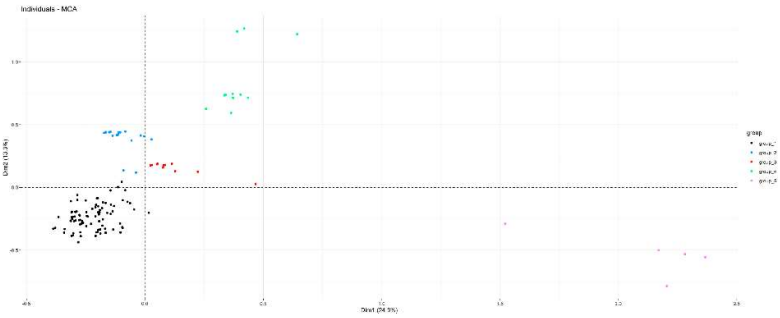

FIGURE 7 | Distribution of the 168 isolates subjected to *flaA* gen sequencing according to the first two dimensions of a multiple correspondence analysis (MCA) performed considering information on the RSCU of variable codons, bacterial and host species, resistance to gentamicin, erythromycin, and streptomycin, and clade as determined in the NJ phylogenetic tree (observations are colored according to the clade).

(65.4–85.9%) were similar to an EU report comprising 5 countries (71.6%) (EFSA-ECDC, 2019a) but lower than found in a German study (90–100%) (Ahmed et al., 2016).

As expected, the host species were strongly associated with the *Campylobacter* species retrieved in positive samples, although proportions found for each bacterial species may vary depending on isolation protocols used. The predominance of *C. coli* in pig samples found in our study is in agreement with previous studies from Denmark (Boes et al., 2005). However, and even though this bacterial species has been traditionally associated with pigs, it is becoming more common in poultry (Miller et al., 2006). In our collection, *C. coli* was in fact the predominant species in turkey, while a more balanced distribution between *C. coli* and *C. jejuni* was reported in turkey samples from Germany (Ahmed et al., 2016). In broilers, a close to 50/50 distribution for *C. coli*/*C. jejuni*, as the one found here, was also observed in samples from China (Tang et al., 2020). However, EFSA reported a predominance of *C. jejuni* with 2,452 *Campylobacter* positive samples from 16 countries (EFSA-ECDC, 2019a). In cattle, a study from Denmark (Nielsen et al., 1997) found similar proportions for each bacterial species (6.8% *C. coli*, 90.9% *C. jejuni*, and 2.3% *C.* spp.) than our study (14.4% *C. coli*, 84.4% *C. jejuni*, and 1.2% *C.* spp.).

As presumed, the level of resistance to the antimicrobials used in our study was closely linked with the *Campylobacter* bacterial species found, with higher levels of resistance in *C. coli* than in *C. jejuni* in agreement with previous research (Pergola et al., 2017; Alvarez et al., 2020). The lack of barriers to horizontal gene transfer (HGT) in *C. coli* may explain the higher levels of MDR observed in this bacterial species compared to *C. jejuni* (Pearson et al., 2015).

Out of the six antimicrobials assessed here, high to extremely high levels of resistance were found for three of them (CIP, NAL, and TET) in *C. coli*, while in *C. jejuni* they ranged between medium to very high. For CIP and NAL these levels were consistently higher than those described for isolates from food animals in other European countries with the exception of

C. jejuni in turkeys (equal levels to Italy, Poland and Portugal at 70%) and cattle (equal levels to Italy at 80%) (EFSA-ECDC, 2020). For TET in *C. jejuni* in cattle, levels in Spain (85%) were between levels reported in Austria, Denmark and the Netherlands (60%) and levels reported in Italy (95%) (EFSA-ECDC, 2020).

Resistance levels to the remaining three antimicrobials analyzed in this study were much more variable, yet again consistently higher than in other European countries across hosts and bacterial species. The exceptions were *C. jejuni* from turkeys (15% ERY in Portugal vs. 2.6% in Spain; 20% STR in Poland vs. 6.1% in Spain) and cattle (10% ERY in Italy vs. 1.7% in Spain) (EFSA-ECDC, 2020).

Overall, a significant association between the presentation of phenotypic resistance to ERY (macrolide) and STR and GEN (aminoglycosides) was consistently found for both *C. coli* and *C. jejuni* from most host species (**Table 3**). When the association between antimicrobial pairs was analyzed stratifying by time-periods (2002–2006, 2007–2012, and 2013–2018) certain categories were not significantly associated, probably due to being smaller sample sizes (data not shown). Unsurprisingly, STR-resistant isolates had a significantly higher probability of being also resistant to GEN, which was expected given that they belong to the same antimicrobial class (aminoglycosides) and therefore share resistance mechanisms, mostly based on natural transformation, homologous recombination and sharing of MGEs (Davies and Wright, 1997; Luangtongkum et al., 2009; Qin et al., 2012; Wieczorek and Osek, 2013).

Campylobacter is considered a high-risk pathogen in terms of AMR due to the high levels of HGT and the association of AMR genes in MDRGIs. Some authors argue that the transfer of MDRGIs is likely to lead to co-selection phenomena after their genetic mobilization. This could explain why *Campylobacter* adapts so quickly in its interaction with the host, constantly obtaining improved phenotypes (Sheppard and Maiden, 2015).

The *erm*(B) gene, previously described only in Asia (Qin et al., 2014) and possibly originating from Gram-positive bacteria, was found in Spain in one *C. coli* from broiler in 2015 (Florez-Cuadrado et al., 2016) and two *C. coli* from turkeys in 2017 (Florez-Cuadrado et al., 2017). This was the first European report of this gene, associated with other genes in MDRGIs bearing resistance to ERY, CIP, TET, and NAL, and involved in AMR to STR (and present in isolates that may be susceptible to GEN). The three *erm*(B)-carrying strains, included in this study, were found in isolates showing simultaneous resistance to aminoglycosides and were clustered in different clades (1, 2, and 3). However, given the very limited number of ERY-resistant sequenced strains no conclusions can be drawn regarding their association with specific genetic populations. The inclusion of *erm*(B) genes in plasmids encoding additional resistance genes to other antibiotics in *C. coli* from food animals could pave the way to rapid dissemination of macrolide resistance (EFSA-ECDC, 2018a, 2019b). Besides, reported resistance levels to ERY in humans have been consistently higher for *C. coli* than for *C. jejuni* (EFSA-ECDC, 2018a), and similar reports have been made in poultry (Pergola et al., 2017) in agreement with our findings. Since macrolides are one of the three "Critically Important Antimicrobial" classes used for the treatment of human campylobacteriosis (along with fluoroquinolones and aminoglycosides) (World Health Organization (WHO), 2017), a more in-depth knowledge into their resistance mechanisms is warranted.

The increasing rates of resistance to ERY in *C. coli* and to STR in *C. jejuni* of cattle origin described here suggest this host species could play an increasingly important role in the epidemiology of AMR in *Campylobacter*. A nationwide case-control study carried out in Luxembourg identified beef consumption as an important source of infection for *C. coli* (Mossong et al., 2016), thus suggesting that cattle may be a relevant reservoir for this foodborne pathogen.

MICs values in isolates classified as "susceptible" or "not susceptible" may indicate the presence or absence of different AMR determinants in the bacterial genome. The significantly higher MICs values observed in this study for ERY in turkey resistant isolates (**Supplementary Excel File 1**) could indicate the presence of the transferable *erm*(B) gene. However, out of the 12 ERY-resistant isolates subjected to WGS, only three carried the *erm*(B) gene (and had MICs ranging between 32 and 256 ug/ml), and additionally, mutations in the 23S rRNA encoding gene were found in just three isolates (**Supplementary Excel File 2**). This suggests that other mechanisms may be involved in the observed increased MICs in certain isolates, such as mutational resistance affecting the expression of the CmeABC efflux pump in *C. jejuni* (Zhang et al., 2017). This, linked with the high proportion of ERY-resistant isolates found in *C. coli* from turkeys in other European countries (EFSA-ECDC, 2017, 2020) further highlights the need of clarifying the resistance mechanisms present in resistant isolates from this host. In fact, EFSA recommends investigating the molecular mechanisms of macrolide resistance, especially in isolates resistant to high concentrations of ERY, in order to detect chromosomal mutations or the presence of the transferable *erm*(B) gene (EFSA-ECDC, 2019b). Furthermore, these same guidelines recommend searching for ERY resistant genes, not only in resistant strains presenting concomitant resistance to aminoglycosides or a MDR phenotype, but also in susceptible isolates. Thus, an in-depth characterization of resistant isolates would be needed to confirm this hypothesis. The integration of phenotypic and genomic analyses may allow predicting differences in resistance levels beyond resistance thresholds (Bolinger and Kathariou, 2017; EFSA-ECDC, 2019b).

Phylogenetic studies based on *flaA* SVR gene sequencing have been used in the past to study the epidemiology of *Campylobacter* spp. from different sources (Zhang et al., 2018). Previously, studies based on the *flaA* gene sequence had not found a relationship between AMR and specific genotypes (Corcoran et al., 2006). However, in our strain collection five distinct groups were identified, two of which were associated with an increased proportion of simultaneous resistance to aminoglycosides and macrolides (groups 2 and 3), predominantly formed by *C. coli* isolates from broilers and turkeys (**Table 5**). In contrast, isolates in groups 4 and 5 were primarily *C. jejuni* of cattle origin. The existence of "cattle specialist *C. jejuni* lineages" has been previously speculated, implying that adaptation of *C. jejuni* to cattle could be associated with the presence of genetic

elements favoring its survival in the intestine of cattle (Sheppard and Maiden, 2015) and with a significant gene gain and loss (Mourkas et al., 2020). Interestingly, cattle *C. jejuni* showed the strongest association between resistance to aminoglycosides and macrolides (with RR > 25), but only 2 co-resistant isolates (out of 7 co-resistant *C. jejuni* cattle isolates sequenced) were classified into groups 4 and 5.

Among its limitations, the sample size used in the first part of this long study period (2002–2008) was relatively small. Furthermore, AST in the first years (2002–2005) was based on determining IZDs and MICs depending on the antimicrobial considered. Therefore, conclusions based on data from that period must be interpreted carefully. In addition, only 1.5% (168) of the total number of isolates were subjected to the *flaA* gene analysis, and only 51 of them were analyzed by WGS.

Nevertheless, our findings confirm that high resistance levels in *Campylobacter* spp. from food producing animals were consistently observed, and that resistance to macrolides and aminoglycosides was strongly associated across hosts and bacterial species. Further studies based on WGS would be needed in order to determine the genetic determinants behind this resistance and the possible existence of more prevalent lineages.

In this study, *C. coli* isolates, more prevalent in pigs and poultry (especially turkeys), showed significantly higher resistance levels than *C. jejuni* strains. The significant association in the simultaneous presentation of phenotypic resistance to aminoglycosides and macrolides, confirmed in *C. coli* isolates independently from host species of origin, suggests the possible circulation of resistance genes against both antimicrobial classes. Such resistance mechanisms could have been transmitted together, or else, have disseminated via resistant clones in the studied livestock species. The genetic analysis revealed the presence of some isolates more genetically related with resistant phenotypes in poultry and others with susceptible phenotypes in cattle. In order to test these hypotheses it would be necessary to characterize the resistance mechanisms present in isolates from the different species by means of a thorough molecular analysis of their whole genome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required because animals included in this study were sampled in the slaughterhouse during routine processing of livestock and were not subjected to any additional handling of any kind. Samples were collected in the frame of official monitoring programs according to EU and national legislation.

AUTHOR CONTRIBUTIONS

VL-C, LD, and JA: conceptualization. VL-C and JA: investigation, writing – original draft preparation, methodology, software, formal analysis, and validation. JA: Funding acquisition and project administration and supervision. JS, CF, TS, IP, MU-R, CB, and MG: data supply. MU-R, CB, and MG: data curation. VL-C and AO: laboratory work. VL-C, MU-R, CB, AO, MG, JS, IP, MM, LD, and JA: writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.689262/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix XI. Forthcoming publications related to the thesis (first author) in preparation.

1 – "Genetic characterization of aminoglycoside and macrolide resistance determinants and associated mobile genetic elements in thermophilic *Campylobacter* from livestock in Spain"

Authors: Lopez-Chavarrias V, Torre Fuentes L, Dieguez Roda B, Ugarte-Ruiz M, Saez JL, Moreno MA, Dominguez L, Alvarez J

2 – "Epidemiological comparison of *Campylobacter jejuni* isolates from Poland and Spain combining mlst and whole genome analyses"

3 – "Multi-locus sequence types and antimicrobial resistance markers in *Campylobacter jejuni* and *Campylobacter coli* isolated from humans, animals, food and the environment across Europe"

Appendix XII. Publications not related to the thesis topic.

PUBLISHED

1 - Saka HK, García-Soto S, Dabo NT, Lopez-Chavarrias V, Muhammad B, Ugarte-Ruiz M, et al. (2020) Molecular detection of extended spectrum β-lactamase genes in Escherichia coli clinical isolates from diarrhoeic children in Kano, Nigeria. PLoS ONE 15(12): e0243130.
https://doi.org/10.1371/journal.pone.0243130

2 - Perestrelo, S.; Amaro, A.; Brouwer, M.S.M.; Clemente, L.; Ribeiro Duarte, A.S.; Kaesbohrer, A.; Karpíšková, R.; Lopez-Chavarrias, V.; Morris, D.; Prendergast, D.; *et al.* Building an International One Health Strain Level Database to Characterise the Epidemiology of AMR Threats: ESBL—AmpC Producing *E. coli* as An Example—Challenges and Perspectives. Antibiotics 2023, 12, 552.
https://doi.org/10.3390/antibiotics12030552

IN PROOF-READING OR SUBMITTED

1 – "Double Deletion of EP402R and EP153R in the Attenuated Lv17/WB/Rie1 African swine fever virus (ASFV) Enhances Safety, Provides DIVA Compatibility, and Confers Complete Protection Against Genotype II Virulent Strain"

(In proof-reading in journal *Vaccines*)

2 – "Source attribution of human *Campylobacter* infection: A multi-country model in the European Union"

(Submitted to journal *Frontiers in Microbiology*)

Appendix XIII. Contribution to conferences, congresses and symposia.

1 - Conference One Health - EJP Annual Scientific Meeting 2019

(Dublin, Republic of Ireland, 21-24 May 2019)

Oral communication:

"Trends in antimicrobial resistance in *Campylobacter coli* from broilers and pigs in Spain from 2002 to 2015"

2 - Conference One Health - EJP Annual Scientific Meeting 2020

(Online due to pandemic, 27-29 May 2020)

Poster communication:

"Characterization of simultaneous antimicrobial resistance to aminoglycosides and macrolides in thermophilic *Campylobacter* in Spanish livestock"

3 – Conference One Health - EJP Annual Scientific Meeting 2022

(Orvieto, Italy, 11-13 April 2022)

Poster communication:

"Epidemiological comparison of *Campylobacter jejuni* isolates from Poland and Spain combining mlst and antimicrobial resistance whole genome analyses"

4 – 16th International Symposium on Veterinary Epidemiology and Economics (ISVEE 2022)

(Halifax, Nova Scotia, Canada, 8-12 August 2022)

Oral communication:

"Mapping antimicrobial resistance markers for aminoglycosides and macrolides in *Campylobacter* in Spanish livestock"