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Tesis Doctoral

Study on the presence of *Campylobacter* spp. along the poultry food chain

Estudio sobre la presencia de *Campylobacter* spp. a lo largo de la cadena alimentaria de carne de pollo

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Memoria presentada para optar al grado de Doctor en Ciencia y Tecnología de los Alimentos por la Universidad de Burgos con Mención Internacional

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Que la memoria titulada **"Study on the presence of *Campylobacter* spp. along the poultry food chain/Estudio sobre la presencia de *Campylobacter* spp. a lo largo de la cadena alimentaria de carne de pollo"** presentada por **Dña. Lourdes García Sánchez**, Licenciada en Veterinaria, ha sido realizada en el Área de Tecnología de los Alimentos bajo la dirección del Doctor Jordi Rovira Carballido y la Doctora Beatriz Melero Gil, y en representación de la Comisión Académica del programa de Doctorado, autoriza su presentación para ser defendida como Tesis Doctoral.

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"Nunca se alcanza la verdad total , ni nunca se está totalmente alejado de ella"

Aristóteles

A mi familia

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A todos, MUCHAS GRACIAS

SIN VOSOTROS, NO HUBIERA SIDO POSIBLE

Lourdes

JUSTIFICACIÓN
JUSTIFICATION

JUSTIFICACIÓN

Este estudio se basa en los resultados obtenidos en trabajos anteriores realizados en el área de tecnología de los alimentos de la Universidad de Burgos, por Melero (2012). En estos, se aportaban algunas evidencias de que *C. jejuni* podía subsistir a lo largo de la cadena alimentaria de carne de pollo, sugiriendo la posibilidad de que este patógeno pudiera sobrevivir en el ambiente del matadero y planta de procesado. Así pues, esta tesis recoge el testigo de los trabajos anteriores con la idea de profundizar en la persistencia de *Campylobacter*, no solo a lo largo de la cadena alimentaria, sino también en cada una de las etapas que la constituyen. Asimismo, se ha ampliado el espectro del estudio anterior, a la presencia de *Campylobacter* en casos diagnosticados de campilobacteriosis y su relación con los genotipos encontrados a lo largo de la cadena alimentaria. Por último, se han sentado las bases para la realización de estudios epidemiológicos sobre *Campylobacter*, tanto en la ciudad de Burgos como en toda la Comunidad de Castilla y León.

JUSTIFICATION

This study is based on the results obtained in previous work carried out in the laboratory of food technology of the University of Burgos, by Melero (2012). In that work, some evidence was provided that *C. jejuni* might subsist throughout the chicken food chain, suggesting the possibility that, this foodborne pathogen could survive in the environment of the slaughterhouse and processing plant. Thus, this thesis takes the results of the previous work with the clear idea to get insight in the persistence of *Campylobacter* strains, not only along the food chain, but also in each step of it. Moreover, the scope of the previous study has been extended to the presence of *Campylobacter* in human cases diagnosed with campylobacteriosis, and its relationship with the genotypes found along the food chain. Finally, the foundations for epidemiological studies on *Campylobacter* have been established, both in the city of Burgos and in the Community of Castile and Leon.

RESUMEN
SUMMARY

RESUMEN

Actualmente, la campilobacteriosis está considerada como un problema de Salud Pública tanto en países desarrollados como en países subdesarrollados, siendo la primera causa de gastroenteritis de origen bacteriano en humanos. En 2016, el número de casos declarados en la Unión Europea fue de 246.307, mostrando un incremento del 6,1 % con respecto al año 2015. Aunque se trata de una enfermedad auto-limitante; puede derivar en complicaciones como el síndrome Guillain-Barré, el síndrome Miller Fisher o manifestaciones extra-gastrointestinales, requiriendo el uso de antibióticos.

El consumo de aves, especialmente de carne de pollo, es considerado como la mayor ruta de infección en humanos. Esta tesis tiene como objetivo profundizar en un mayor conocimiento de la presencia de *Campylobacter* spp. a lo largo de la cadena alimentaria de la carne de pollo, desde la granja hasta el consumidor, haciendo especial mención a los mecanismos implicados en la persistencia, virulencia y resistencia a antibióticos. Por ello, en la presente tesis se ha estudiado cada una de las etapas de la cadena alimentaria de la carne de pollo y su posible relación con la incidencia de campilobacteriosis en el mismo período de tiempo.

En primer lugar, se estudió la presencia de *Campylobacter* en granjas avícolas en dos estaciones, otoño y primavera, con el fin de determinar la prevalencia y la variabilidad genotípica existente, así como los factores de virulencia y resistencia a diferentes antibióticos en las cepas aisladas. Este estudio, se planteó para conocer de forma real cuál era la situación en la producción primaria y que variabilidad de genotipos podían existir. Este dato es útil; ya que posteriormente, los pollos son sacrificados en el matadero y pueden comprometer la seguridad alimentaria a lo largo de los siguientes pasos de la cadena alimentaria. La prevalencia de *Campylobacter* fue del 42,9 % y 30,8 % en otoño

y primavera respectivamente, siendo *C. jejuni* la especie más abundante. Además; se comprobó que esta especie, era portadora de los genes de virulencia *cadF, flaA, cdtA, cdtB* y *cdtC* a diferencia de *C. coli*. Sin embargo, *C. coli* presentó mayor resistencia a los antibióticos; siendo algunas cepas de esta especie clasificadas como multi-resistentes.

En segundo lugar, se estudió en profundidad la presencia y variabilidad de *Campylobacter* en un matadero avícola. Los resultados mostraron una prevalencia del 48,2 %. Mediante técnicas de tipificación molecular (PFGE) y secuenciación (MLST) se analizaron dichas cepas, que se clasificaron en siete pulsotipos: A (ST 443; ST 443 CC); B (ST 775; ST 52 CC); C (ST 904; ST 607 CC); D (ST 464; ST 464 CC); E, F (ST 1074;ST 460 CC) y G (ST 3769; ST 21 CC). Entre estos, los pulsotipos G (63,1 %) y C (23 %) fueron los mayoritarios, siendo aislados durante 17 y 21 días respectivamente, e independientemente de la granja correspondiente y tanto antes como después de aplicar los protocolos de limpieza y desinfección. Mediante la técnica wgMLST; se determinó que ambas poblaciones eran altamente clonales, lo que sugiere que ambos pulsotipos pueden permanecer en el ambiente del matadero y planta de procesado, más de lo que en un principio se pensaba.

Los mecanismos por los cuáles *Campylobacter* puede sobrevivir en el ambiente son complejos, ya que se trata de un proceso multifactorial, en el que pueden estar involucrados: la formación de biofilms, la presencia de ciertos genes de virulencia y la resistencia a compuestos antimicrobianos, entre otros. En base a los resultados obtenidos, la secuencia tipo ST 904; ST 607 CC (pulsotipo C) fue la que mostró una capacidad mayor para formar biofilms a diferentes temperaturas, en condiciones de aerobiosis y microaerofilia y en distintas superficies. Además, presentó una mayor resistencia frente a diferentes compuestos antimicrobianos. Este estudio sugiere que la combinación de pequeños cambios en el genoma de la secuencia tipo ST 904 (pulsotipo C), incluidas

mutaciones e inserciones en genes relacionados con la resistencia frente a antimicrobianos, la presencia de genes que codifican el T6SS, así como la presencia de un gran número de genes relacionados con factores de virulencia, podrían explicar su mayor capacidad para formar biofilms. La suma de este conjunto de pequeños cambios, podría explicar la capacidad, para sobrevivir y persistir durante períodos de tiempo más largos, en condiciones ambientales hostiles, como puede ser el ambiente del matadero.

En tercer lugar, se estudió la prevalencia y diversidad de *Campylobacter* en productos de pollo comerciales, tanto envasados (MAP) como sin envasar. En base a los resultados, se obtuvo una prevalencia del 39,4 %; siendo los productos sin envasar los que presentaron mayor porcentaje de *Campylobacter* (45,3 %) frente a los productos envasados (33,6 %). En ambos casos, mediante la técnica de tipificación (PFGE) se obtuvo un gran número de pulsotipos, lo que indica la gran variabilidad genética existente en dichos productos. Aunque la atmósfera modificada puede reducir la prevalencia de microorganismos como *Campylobacter* spp., esta no puede evitar su presencia en productos de pollo envasados. Los resultados sugieren, que algunos pulsotipos podrían persistir en el ambiente de la planta de procesado de la cual proceden o en las carnicerías, o en los productos envasados, durante más tiempo de lo que en un principio se pensaba. Por ello, es necesario instaurar medidas de control más estrictas en los pasos previos de la cadena alimentaria, para evitar la presencia de *Campylobacter* spp., en la carne de pollo, último eslabón de la cadena alimentaria, antes de alcanzar al consumidor y comprometer así la salud pública.

Finalmente, este estudio ha demostrado una relación genotípica entre cepas de la cadena alimentaria de la carne de pollo y cepas aisladas en pacientes con campilobacteriosis. Los resultados muestran que hay 35 clústeres en los que se comparten cepas aisladas en alguna etapa de la cadena alimentaria de pollo y las causantes de la

enfermedad. Así, se ha encontrado, que hay 9 clústeres con cepas compartidas en la granja, 4 en matadero y 22 en productos de pollo. Además, tres clústeres comparten cepas de *Campylobacter* asiladas en dos etapas de la cadena alimentaria y en pacientes. La presente tesis, tiene una especial relevancia epidemiológica ya que el estudio se ha realizado en una misma región geográfica y durante el mismo tiempo relacionando todas las etapas. También, se ha realizado un estudio epidemiológico de la región, determinando que el sexo masculino y pacientes menores de 5 años (pediátricos) son los más afectados. Por último, es de destacar las altas tasas de resistencia a antibióticos encontradas en cepas aisladas durante toda la cadena alimentaria incluidas las de pacientes. *Campylobacter* fue especialmente resistente a ciprofloxacino, ácido nalidíxico y tetraciclinas. Asimismo, se han detectado varias cepas de *C. coli* que se pueden considerar como multi-resistentes.

SUMMARY

Currently, campylobacteriosis is considered a public health problem in both developed and in developing countries, being the first cause of gastroenteritis of bacterial origin in humans. In 2016, the number of reported cases in the European Union was 246,307, showing an increase of 6.1 % over 2015. Although it is a self-limiting disease can lead to complications such as Guillain-Barré syndrome, Miller Fisher syndrome or extra-gastrointestinal clinical manifestations, requiring the use of antibiotics.

The consumption of poultry, especially chicken meat, is considered the greatest route of infection in humans. This thesis aims to deepen in a greater knowledge of the presence of *Campylobacter* spp. along the chicken food chain, ´from farm to fork`, making special mention in the mechanisms involved in persistence, virulence and resistance to antibiotics. Therefore, this thesis has studied each step of the chicken food chain and its possible relationship with the incidence of campylobacteriosis in the same period of time.

Firstly, the presence of *Campylobacter* in poultry farms was studied in two seasons, autumn and spring, in order to determine the prevalence and the existing genotypic variability, as well as the virulence and resistance factors to different antibiotics in the isolated strains. This study was designed to know, in a real way, what the situation was in primary production and what genotype variability could exist. This data is useful; since later, the chickens are slaughtered in the slaughterhouse and can compromise food safety along the next steps of the food chain. The prevalence of *Campylobacter* was 42.9 % and 30.8 % in autumn and spring respectively, with *C. jejuni* being the most abundant species. Further; it was found the presence of the virulence genes *cadF*, *flaA*, *cdtA*, *cdtB* and *cdtC*

in this specie unlike *C. coli*. However, *C. coli* showed greater resistance to antibiotics; being some strains of this species classified as multi-resistant.

Secondly, the presence and variability of *Campylobacter* in a poultry slaughterhouse was studied in depth. The results showed a prevalence of 48.2 %. Using molecular typing (PFGE) and sequencing (MLST) techniques, these strains were analyzed and classified into seven pulsotypes: A (ST 443, ST 443 CC); B (ST 775; ST 52 CC); C (ST 904; ST 607 CC); D (ST 464; ST 464 CC); E, F (ST 1074; ST 460 CC) and G (ST 3769; ST 21 CC). Among these, pulsotypes G (63.1 %) and C (23 %) were the majority, being isolated for 17 and 21 days respectively, and independently of the corresponding farm and both before and after applying the cleaning and disinfection protocols. Using the wgMLST technique; it was determined that both populations were highly clonal, suggesting that both pulsotypes can remain in the slaughterhouse and processing plant environment, more than previously thought.

The mechanisms by which *Campylobacter* could survive in the environment are complex, since it is a multifactorial process, in which; biofilms formation, the presence of virulence genes and resistance to antimicrobial compounds, among others, may be involved. Based on the results obtained, the sequence type ST 904; ST 607 CC was the one that showed a greater capacity to form biofilms at different temperatures, in aerobic and microaerobic conditions and in different surfaces. In addition, it showed greater resistance to different antimicrobial compounds. This study suggests that the combination of small changes in the genome of the sequence type ST 904, including mutations and insertions in genes related to antimicrobial resistance, the presence of genes encoding the T6SS, as well as the presence of a large number of genes related to virulence factors, could explain their greater capacity to form biofilms. The sum of this set of small changes,

could explain the duration, to survive and persist for longer periods of time, in hostile environmental conditions, such as the environment of the slaughterhouse.

Third, the prevalence and diversity of *Campylobacter* in commercial chicken products, both packaged (MAP) and unpacked, was studied. Based on the results, a prevalence of 39.4 % was obtained; unpacked products were those with the highest percentage of *Campylobacte*r (45.3 %) compared to packaged products (33.6 %). In both cases, by typing technique (PFGE) a large number of pulsotypes was obtained, which indicates the great genetic variability existing in these products. Although the modified atmosphere can reduce the prevalence of microorganisms such as *Campylobacter* spp., it could not avoid its presence in packaged chicken products. The results suggest that some pulsotypes could persist in the environment of the processing plant from which they come or in the butcher shops, or in packaged products, for longer than initially thought. Therefore, it is necessary to establish stricter control measures in the previous steps of the food chain, to avoid the presence of *Campylobacter* spp., in chicken meat, as the last step in the food chain, before chicken products reaching the consumer and thus compromise public health.

Finally, this study has shown a genotypic relationship between strains of the chicken food chain and strains isolated in patients with campylobacteriosis. The results show that there were 35 clusters with shared isolated strains from both origins . Thus, it has been found that there are 9 clusters with strains shared on the farm, 4 at the slaughterhouse and 22 in chicken products. In addition, three clusters share isolated *Campylobacter* strains in two stages of the food chain and in patients. This thesis has a special epidemiological relevance since the study has been performed in the same geographical region and during the same time, relating all the chicken food chain. Also, an epidemiological study of the region was carried out, determining that the male sex and

patients under 5 years of age (pediatric) are the most affected. Finally, it is important to highlight the high rates of antibiotic resistances found in isolated strains throughout the chicken food chain, including those of patients. *Campylobacter* was especially resistant to ciprofloxacin, nalidixic acid and tetracyclines. Also, several strains of *C. coli* can be considered as multi-resistant.

ÍNDICE

ÍNDICE

INTRODUCTION

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- **Chapter "*Campylobacter* in the food chain", 2018. Advances in Food and Nutrition Research Book, Elsevier, article nº 8.**

  Lourdes García Sánchez, Beatriz Melero Gil, Jordi Rovira.

- **Artículo Técnico: "*Campylobacter* en pollos: prevención y control en la granja", 2016. Revista Producción Animal, nº 299; páginas 45-51.**

Lourdes García Sánchez, Juan Carlos Marijuán, Olga Vega, Beatriz Melero, Isabel Jaime, Jordi Rovira.

I. INTRODUCTION

1. THE ORGANISM

1.1 History

The history of the genus *Campylobacter* starts with the first description by Theodor Escherich in 1886, of a nonculturable spiral form bacteria associated to children stools suffering diarrhoea. In the following years several publications appeared, describing the occurrence of such "spirilla" in cases of enteric diseases such as "cholera-like" and "dysentery" (Kist, 1986)

In 1913, McFadyean and Stockman isolated a *Vibrio*-like bacterium from aborted ovine fetuses. Six year later, Smith and Taylor, (1919) isolated a spirilla bacterium from aborted bovine fetuses and named it as *Vibrio fetus*. In 1931, *Vibrio jejuni* was named after isolation in cows and calves with intestinal disorders (Jones et al., 1931). *Vibrio coli* was named and detected in 1944 from swine with dysentery (Doley, 1948).

Although *Campylobacter* was assigned as a veterinary disease for more than 40 years, it was not until the 1970s when campylobacters were recognised as human pathogens. This microorganism was first isolated in humans from blood cultures from women aborted (Vinzent et al., 1947). However, the first documented human infection due to this spiral bacterium occurred in 1938 in a milk-outbreak among prisoners in the USA (Levy, 1946). In 1963 this “related vibrios” were renamed by Sebald and Véron as *Campylobacter* due to differences between *Vibrio* and the new genus in DNA base composition, growth requirements and metabolism (Sebald and Véron, 1963).

Elisabeth King was committed to find a methodology to culture this microorganism from faeces, because she believed that the few cases reported until her death were not connected to the reality, and the disease was not as rare (Butzler, 2004). The development of a special filtration technique, used in veterinary medicine in the early

1970s, allowed Butzler to isolate *Campylobacter* from human stools of patients with diarrhoea and high fever (Butzler et al., 1973). However, the main achievement for its cultivation was reached when a selective supplement comprising a mixture of vancomycin, polymyxin B, and trimethoprim was added to a basal medium. This development was a crucial step in the evaluation of the *Campylobacter* epidemiology and their clinical role (Dekeyser et al., 1972; Skirrow 1977).

1.2 Taxonomy

The taxonomy of the genus *Campylobacter* has been extensively revised since 1963; Véron and Chatelain (1973) proposed four species within the genus *Campylobacter* after a taxonomy study: *C. fetus, C. coli, C. jejuni*, and *C. sputorum* with two subspecies, *C. sputorum* subsp. *sputorum*, and *C. sputorum* subsp. *bubulus*. Due to the development of new methods and selective supplement to isolate campylobacters (Butzler et al., 1973; Skirrow, 1977) the number of *Campylobacter*-like organisms (CLO) isolated from different sources increased. However, some of these CLO were classified subsequently as novel genera, species or variants of previously defined species (Vandamme, 2000). Currently the immediate family is that of *Campylobacteriaceae*, which includes three distinct genera: *Arcobacter, Campylobacter*, and *Sulfurospirillum* (Lastovica et al., 2014). The genus *Campylobacter* includes 31 species and 10 subspecies (http://www.bacterio.net/campylobacter.html, accessed 18.03.2018). *C. ornithocola* (Caceres et al., 2017), *C. pinnipediorum* subsp. *caledonicus*, *C. pinnipediorum* subsp. *pinnipediorum* (Gilbert et al. 2017), *C. geochelonis* (Piccirillo et al. 2016), and *C. hepaticus* (Van et al., 2016) are the most recent species identified. Within the genus *Campylobacter, Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli* are most frequently associated with human illness, accounting for up to 90 % of infections (Debruyne et al., 2008). Moreover, *C. jejuni* comprises two subspecies: *C. jejuni* subsp.

jejuni and *C. jejuni* subsp. *doylei.* Although the pathogenic role of *C. jejuni* subsp. *doylei* is unclear, it has been isolated in a low percentage in children and adults with diarrhoea all over the world (Lastovica and Allos, 2008). The two subspecies differ biochemically, and can be identified by multiplex polymerase chain reaction (PCR) based on the nitrate reductase locus (*nap*) (Miller et al., 2007). *C. jejuni* subsp. *jejuni* will hereafter be referred to as *C. jejuni*. Moreover, other species than *C. jejuni* and *C. coli* have been also related with human infections. *C. fetus* subsp. *fetus* is related with diarrhoea and bacteraemia (Lastovica, 1996; Skirrow et al. 1993); *C. concisus* has been isolated from faecal samples and intestinal biopsies from patients with inflammatory bowel disease (IBD) (Mahendran et al. 2011; Zhang et al. 2009); *C. lari* has been also related with diarrhoea, bacteraemia and reactive arthritis (Borczyk et al., 1987; Goudswaar et al., 1995; Morishita et al., 2013) as well as *C. upsaliensis* has been isolated from blood and faecal samples (Lastovica et al., 1989; Lastovica, 2006). On the other hand, other *Campylobacter* species are less frequently isolated from human samples as *C. ureolitycus* (Vandamme et al., 2010); *C. hominis* (Lawson et al., 2001); *C. concisus, C. curvus, C. rectus* and *C. gracilis* isolated from gingival crevices (Ngulukun, 2017). Species isolated from animals are *C. cuniculorum* related with rabbits (Zanoni et al., 2009); *C. insulaenigrae* isolated from marine mammals (Foster et al., 2004); related with birds as *C. avium* (Rossi et al., 2009), *C. canadensis* (Inglis et al., 2007) and *C. volucris* (Debruyne et al., 2010); *C. corcagiensis* from captive lion-tailed macaques (Koziel et al., 2014) and *C. iquaniorom* from reptiles (Gilbert et al., 2015) among others.

1.3 Microbiology

Campylobacter species are Gram-negative spiral, rod-shaped, or curved bacteria and motile due to the presence of a single polar flagellum, bipolar flagella. However, *C. gracilis* and *C. homninis* are nonmotile species. Moreover, at microscope *C. showae*

appears as straight rods. The size of this genus is approximately 0.2 to 0.8 μm wide and 0.5 to 5 μm long. It is non-spore-forming and in old cultures may form coccoid or spherical shapes (Man et al., 2011; Vandamme et al., 2005).

C. jejuni is chemoorganotroph -carbohydrates are not used to obtain energy-; however, they obtain the energy from amino acids or tricarboxylic acid cycle intermediates. *Campylobacter* species reduce fumarate to succinate, are oxidase positive (except for *C. gracilis*), and are indole negative. Except for *C. jejuni* subsp. *doylei*, campylobacters will reduce nitrate. No lecithinase or lipase activity is present, and starch, gelatine, and casein are not hydrolysed by this genus.

All *Campylobacter* species grow under microaerobic conditions (85 % N_2, 3-5 % CO_2 and 5-10 % O_2). However, certain species (*C. concisus, C. curvus, C. rectus*) prefer anaerobic conditions for growth (Kaakoush et al., 2015). Thermophilic campylobacters such as *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* grow optimally at 42º C while the other species grow better at 37º C, but always above 30º C (Rovira et al., 2006; Kärenlampi and Hänninen, 2004). Identification of *Campylobacter* spp. is challenging due to their biochemical inertness and fastidious growth requirements. Biochemical approaches like hippurate hydrolysis test is routinely used in the differentiation between *C. jejuni* and *C. coli*. Although some *C. jejuni* subsp. *jejuni* strains can produce negative reaction, this specie hydrolyses the hippurate while *C. coli* not (Debruyne et al., 2008). Moreover, molecular identification such as multiplex PCR is also required to identify *Campylobacter* species based on diverse genes (Vondrakova et al., 2014; Wang et al., 2002).

1.4 Genome of *Campylobacter* spp.

The whole genome of *C. jejuni* strain NCTC 11168 was firstly sequenced by Prakhill et al. (2000) and further re-annotated by Gundogdu et al. (2007). Afterwards,

other three *C. jejuni* reference strains such as RM1221, 81116 and 81-176 were also sequenced (Person et al., 2007). The size of *C. jejuni* genome is relatively small, between 1.6 and 1.8 Mbp, in comparison with other genomes and indeed among other bacteria (table 1). It has a circular chromosome with 1,641,481 base pairs (bp) in length (1.64 Mbp) with a relatively low G+C content (around 32 %). From the 1,654 predicted coding sequences (CDS), at least 20 probably represent pseudogenes; the average gene length is 948 bp, and 94.3 % of the genome codes for proteins (Parkhill et al., 2000).

Table 1. Bacterial comparative genomes.

Genome	Mb	G+C	References
Listeria monocytogenes	2.9	39	Glaser et al. (2001)
Salmonella Typhimurium	4.9	52	Vinuesa et al. (2016)
Escherichia coli	4.6-5.7	50.6	Engelbrecht et al. (2017)
Yersinia enterocolitica	4.6	47.3	(www.sanger.ac.uk)
Campylobacter jejuni	1.6-1.8	32	Parkhill et al. (2000)

C. jejuni species can be described by a pan-genome consisting of a core genome, between 866-1350 genes, shared by all isolates and an accessory genome (2500-4000 genes) composed of partially shared and strain specific genes. While the core genome is thought to be involved in vital functions for the cell, the accessory genome is composed by plasmids, integrated elements, hypervariable regions and single or paired variable genes (Llarena, 2015).

C. jejuni and *C. coli* differ from each other by around 15 % at the nucleotide level, corresponding to an average of nearly 40 amino acids per protein-coding gene. In *Campylobacter*, recombination is a particularly strong evolutionary force, leading to the emergence of new lineages an even large-scale genome-wiede interspecies introgression between *C. jejuni* and *C. coli.* This phenomenon might compensate the small genome size by providing each organism with the ability to import genes that confer adaptations to specific environments (Sheppard et al., 2008; Sheppard et al., 2013).

Moreover, *Campylobacter* is subject to evolutionary changes, which can be mediated via mobile genetic elements, the uptake of DNA via natural transformation, and changes in hypervariable sequences. *Campylobacter* are naturally competent for DNA uptake from environment (Wang and Taylor, 1990); therefore, contributing to its wide phenotypic and genotypic heterogeneity.

The development of new sequencing technologies has made it feasible to carry out much larger and more detailed *Campylobacter* comparative genomics to better identify genes or genomic regions associated with isolates from particular sources (Brownoski et al., 2014).

2. BACTERIA DETECTION METHODS

Traditionally, culture-dependent methods have been used to analyzed microorganism in samples. Regulation EN ISO 10272-1:2017 is the horizontal method used for detection and enumeration of *Campylobacter* spp. This standard shall apply in food for humans and animal feeding stuffs as well as in environmental areas where production and manipulation of food will be performanced.

Since last first January, control and analysis of *Campylobacter* spp. is mandatory in broilers slaughter by the regulation EU 1495/2017 which is a modification of Annex I of Regulation 2073/2005 of 15 November on microbiological criteria for foodstuffs. The regulation lays down that carcass of broilers after chilling conditions will not contain over 1,000 cfu/g according to the sampling procedure. In cases of unsatisfactory results, actions such as; improvements in slaughter hygiene, review of process controls of animals' origin and biosecurity measures in the farms origin should be required.

Culture-dependent methods are not able to recover some forms of microorganism. In that sense, ´viable but non-culturable` state (VBNC) of *Campylobacter* needs independent method as Real-Time Polymerase Chain Reaction (qPCR) or conventional

PCR to allow the rapid detection and quantification of pathogen in foods (Melero et al., 2011).

2.1 Polimerase Chain Reaction (PCR)

In the 80´s, Kary Mullis developed the PCR technique, it is based on the amplification of a single or a few copies of a piece of DNA chain across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Mullis and Faloona, 1987). This technique can be used for a qualitative or a quantitative purpose, either to study the properties of a target molecule or to determine the number of a target molecule.

PCR is performed on a DNA template where several compounds are needed to carry out the reaction; dNTPs, which are the four nucleotide triphosphates, a heat-stable polymerase, magnesium ions in the buffer and two oligonucleotide primers are needed that flank the DNA sequence to be amplified. The reaction is performed by temperature cycling. High temperature is applied to separate (melt) the strands of the double helical DNA, then temperature is lowered to let primers anneal to the template, and finally the temperature is set around 72 °C, which is optimum for the polymerase that extends the primers by incorporating the dNTPs (Kubista et al., 2006). In the present thesis, conventional PCR and real-time PCR were used for *Campylobacter* identification.

Conventional PCR were used according to the methods described by Wang et al. (2002), which simultaneously detect genes from the five major clinically relevant *Campylobacter* spp. Those genes selected were hipO and 23S rRNA from *C. jejuni*; glyA from each of *C. coli*, *C. lari*, and *C. upsaliensis*; and *sapB2* from *C. fetus* subsp. *fetus.* On the other hand, real-time PCR needs a fluorescent reporter that binds to the product formed and reports its presence by fluorescence. A number of probes and dyes are available. In chapter two, real-time PCR was used, together with FAM and VIC

fluorescent dyes in probes, in order to identify two genes; *CeuE* from each *C. coli* and *hipO* from *C. jejuni* (Hong et al., 2007; Schnider et al., 2010).

3. BACTERIA TYPING METHODS

Microbiologists are often asked to determine the relatedness of a group of bacterial isolates in clinical procedures, in this sense, typing techniques are useful to known this possible correlation. *Campylobacter* spp. needs subtyping schemes for a range of different applications such as, prevention, control, monitoring and surveillance of the disease. Therefore, different methods are available for typing; such as; phenotypic and genotypic methods.

Phenotypic typing, including biotyping, serotyping and phage typing, are methods based on the presence or absence of biological or metabolic activities expressed by the organism (Belkum et al., 2007; Duarte et al., 2016). However, although these schemes might be suitable for large-scale screenings, their low discriminatory power limits their usefulness and is evident that a better resolution is needed. Therefore, a wide genotyping method has been used by the high discriminatory power.

Genotyping is used to distinguish between different *C. jejuni* subpopulations and have been proven to be an irreplaceable working tool in a range of different applications, such as molecular epidemiology and identification of pathogenicity and virulence. (Llarena et al., 2015). Some of these techniques are, ribotyping, pulsed-field electrophoresis (PFGE), flagellin typing (*fla* typing), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), DNA microarray, multilocus sequence typing (MLST) and whole genome sequencing (WGS).

In this thesis, PFGE, MLST and WGS (wgMLST) were used in order to understand better the epidemiology and relatedness among different *Campylobacter* spp. strains isolate along chicken food chain and human infections.

3.1 Pulsed Field Electrophoresis (PFGE)

This subtyping method is a powerful tool in epidemiological studies of *Campylobacter.* In this method, genomic DNA is digested by rare cutting restriction enzymes. *Sma*I and *Sac*II/*Kpn*I have been the most commonly enzymes used in this process; which results in large DNA fragments that can be resolved on an agarose gel by applying an electrical field that periodically changes direction. The resulting banding patterns are compared to determine similarity (Allerberger et al., 2003; Hänninen et al., 1998).

To interpret the DNA fragment and transform them into epidemiologically useful information, the microbiologist must understand how to compare PFGE patterns and how random genetic events can alter the patterns. Ideally, the PFGE patterns of isolates representing the outbreak strain would be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains. Categories of genetics and epidemiology relatedness can be classified into; indistinguishable, closely related, possibly related or unrelated (Tenover et al., 1995).

Center for Disease Control and Prevention (CDC) has been standardized and disseminated the PulseNet protocol order to be adopted and used by laboratories in worldwide (www.cdc.gov/pulsenet/pathogens/pfge.html). When clusters of isolates with similar PFGE profiles are detected, laboratories share these data with the epidemiologists, who then perform food history investigations to track the source of the organism. However, difficulties in reproducing the interpretation of the results among different

laboratories make it challenging to use PFGE as a unified typing scheme on a wider scale (Boer et al., 2000; Hänninen et al., 1998; Hänninen et al., 2000).

3.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was proposed in 1998 and it was the first time developed and validated for *C. jejuni* and *C. coli* in 2001 by Dingle et al. (2001). This genotyping technique is consisted in studying seven indexes variations at several chromosomal loci-encoding housekeeping genes, that is, essential genes found in all isolates that are under stabilizing selection for the conservation of metabolic function. (Sheppard and Maiden, 2015). The 86.5 % of nucleotide sequence are identity in *C. jejuni* and *C. coli* housekeeping genes (Sheppard et al., 2008). Among these genes are involved: aspartase A (*aspA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxymethyltranferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*) and adenosinetriphosphate synthase subunit (*uncA*). MLST scheme stablish a combined allele profile of these genes determined the sequence type (ST) or ´allelic profile` of the bacterium. Those ST can be clustered in clonal complexes (CCs) that consist on STs with at least four alleles in common with the founder genotype (Dingle et al., 2001). The primary advantage of this sequence-based method, compared to PFGE, is that the results of different laboratories can be easily compared. MLST data from different study groups are accessible and comparable online (http://pubmlst.org/campylobacter/).

3.3 Next Generating sequencing: wgMLST

Over the last decade, the ´Sanger sequencing` chain-termination methods that were initially used for MLST have been increasingly replaced by highly parallel next-generation sequencing (NGS) techniques (Sheppard and Maiden, 2015). This makes possible the rapid determination of large numbers of complete bacterial genome sequences. The possibility of comparing any number of gene targets among multiple

disparate isolates, allows the assembled data resource to be used to address a wide range of research questions concerning bacterial evolution, ecology and pathogenicity (Jolley and Maiden, 2010). The work flow of NGS involves a DNA extraction step, a library preparation and finally, a sequencing and data recording step.

C. jejuni was among the first bacterial species to be analyzed by NGS technologies, this has led to a proliferation of studies in strains of interest. However, the expertise and tools required for rapid bioinformatic analyses of WGS data remain a challenge (Taboada et al., 2013). Nowadays, WGS is progressively replacing traditional typing methods because it offers the highest possible discriminatory power. It has been applied to study the genomic diversity and changes that can accumulate during human infection and animal colonization. The integration of genome sequencing in surveillance would be expected to facilitate the identification of possible case clusters, which could help to install strategies targeted at the prevention and control of cases of campylobacteriosis (Llarena et al., 2017)

Some studies have already examined the use of genomics in *Campylobacter* surveillance, and all applied a wgMLST methodology as a powerful tool (Llarena et al., 2017). It resolves using gene-by-gene approach the relationship of specially closely related bacterial isolates by indexing allele differences in the shared loci in the whole-genome sequencing (WGS) data (Zhang et al., 2015). The wgMLST approach is applicable to single-clone pathogens with closed genomes or to very closely related variants of more diverse organisms (Maiden et al., 2013). In combination with other epidemiological data, wgMLST enables the recognition of related isolates, providing more accurate information of the potential sources of infection and investigation of bacterial infectious outbreaks (Kovanen et al., 2016).

4. EPIDEMIOLOGY OF CAMPYLOBACTER AND CLINICAL HUMAN ASPECTS

Environmental niches and different animals are considered as important contributors to human infection, however, their particular role in the complex epidemiology of *Campylobacter* infection is still unknown (Whiley et al., 2013). In that sense, important advances in understanding the epidemiology of *Campylobacter* infection were driven by the development of the molecular typing methods as this Thesis has described in the previous paraghrap (3).

4.1 Epidemiology: reservoirs and transmission routes

Reservoir

A reservoir is defined as animal species or a non-animal substance upon which the pathogen depends for its survival (Llarena, 2015) *Campylobacter* spp. are considered normal microflora of the gastrointestinal tract of a variety of birds and mamals, both domesticated and wild, who are mainly considered as asymptomatic carriers (Whiley et al., 2013). Epidemiological studies have reported that poultry is the natural host for thermophilic *Campylobacter* species and they are responsible for an estimated 80 % of human campylobacteriosis. In addition, other animals such as wild birds, cattle, swine and pets have been reported as source of *Campylobacter* contamination (Epps et al., 2013). Nonenteric sources such as non-treated water and milk have been also identified as causative of some cases of campylobacteriosis.

The wide type of hosts and the high genetic variability of *Campylobacter* spp. makes difficult the tracing and attribution of the original source of infection (Gölz et al., 2014; Skarp et al., 2016). In that sense, genotyping techniques make easier this tracing. Moreover, it is known that *C. jejuni* and *C. coli* isolates from a particular genotype cluster are often associated with a given isolation source, especially particular host animals. For

instance, *C. jejuni* and *C. coli* are both found in chicken and cattle at a ratio of 9:1, although this ratio is reversed in pigs, in which most of the isolates are *C. coli* (Sheppard and Maiden, 2015) and appreciable niche overlap among species might be occurred by genetic exchange making difficult characterize the population biology and evolution (Sheppard et al., 2008).

Routes of transmission

Routes of transmission flowing through the environment, farm animals and wild animals through to humans interact in complex ways. These interactions would be driven by factors such as the defecation of wild birds or farm animals, presence of pets, water flow, climatic conditions and other complex ecological parameters (Brownoski et al., 2014). Non-vertebrae vectors, such as flies (Hald et al., 2008), beetles (Skov et al., 2004) and slugs (Sproston et al., 2010) have been also identified as *Campylobacter* carriers and may act as transmission vectors.

Transmission of *Campylobacter* has been a controversial issue; whether *Campylobacter* is transmitted by vertical or horizontal pathways. Some studies described that vertical transmission thought contaminate shell; shell membranes and albumen of fertile eggs might be occurred (Cox et al., 2012). However, an extended literature lead that horizontal transmission is the main route of colonization in farms (McDowell et al., 2008; Schallegger et al., 2016). Once *Campylobacter* is introduced into a chicken flock, it spreads rapidly and results in colonization of the intestinal tracts of the majority of chickens within one week. Once colonized, chickens remain colonized until the slaughter (Ingresa-Capaccioni et al., 2015). During slaughter and subsequently steps, *Campylobacter* can be spread through the plant environment and finally, contaminating the whole poultry food chain reaching final consumers (García-Sánchez et al., 2017; Melero et al., 2012).

Human exposure to risk occurs through direct contact with animals or through contaminated food by either consumption of undercooked meat or due to cross-contamination from raw meat or environmental reservoirs to ready-to-eat food (Humphey et al., 2001; Skarp et al., 2016). Other possible routes of transmission include drinking raw milk and untreated water as well as other practices like swimming in natural waters (Fajo-Pascual et al., 2010; Kapperud et al., 2003). Although it is not common, person-to-person transmission is recognized as another potential source (Kaakoush et al., 2015).

Therefore, all of these stages have a role in the transmission of *Campylobacter* from farm to fork. Moreover, another route as international travel has been considered as one of the most important risk factor for campylobacteriosis. However, in many cases, the direct link between a specific source and the human infection remains unknown (Kaakoush et al., 2015; Domingues, 2012). The lack of knowledge about *Campylobacter* transmission to humans has contributed to the failure of public health system to adequately address this problem.

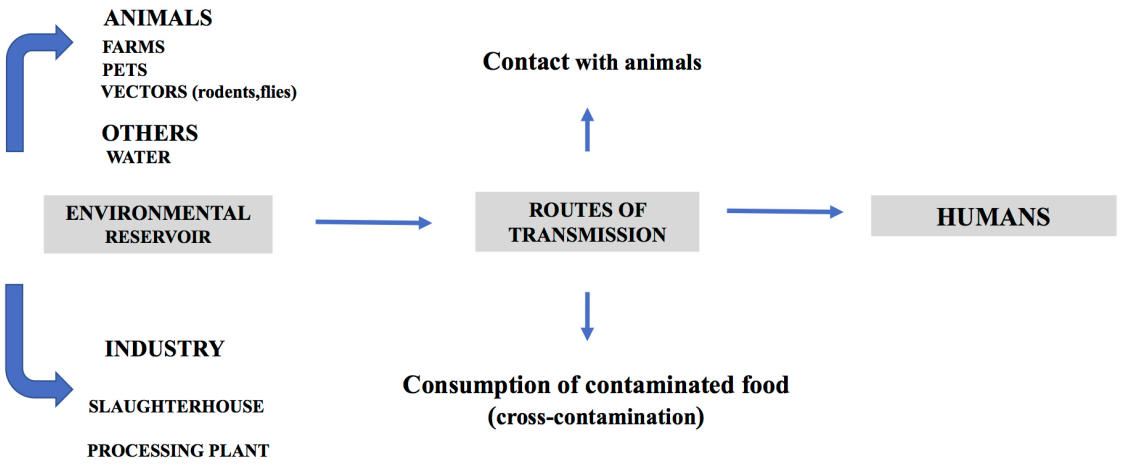


Figure 1. Scheme of possible sources and routes of *Campylobacter* human infections.

4.2 Clinical *Campylobacter* human aspect

Campylobacter jejuni and *Campylobacter coli* have been the most common causes of human bacterial gastroenteritis in worldwide. In European Union, campylobacteriosis has been the first reported cause of zoonoses. In 2016, the number of confirmed cases of human campylobacteriosis was 246,307, which represents 66.3 cases per 100,000 population. This means an increase of 6.1 % compared with 2015, a trend that has been observed over the last 8 years. Although the number of human campylobacteriosis cases remains really high, their severity in terms of reported case fatality is low (0.03 %). Nonetheless, campylobacteriosis is the third most common cause of mortality amongst the pathogens considered (EFSA, 2017). Similar situation occurs in North America and Australia. Despite epidemiological data from Africa, Asia, and the Middle East are still incomplete, these indicate that *Campylobacter* infection is endemic in these regions. However, differences in the incidence and number of cases reported from different countries or regions within the same country may vary substantially (Kaakoush et al., 2015).

The great majority of *Campylobacter* infections are sporadic and self-limiting. It is characterized by watery and sometimes bloody diarrhoea, fever, abdominal cramps and vomiting (Skarp et al., 2016). However, the pathogen has been associated with a wide range of gastrointestinal diseases, such as inflammatory bowel disease, Barrett's esophagus, esophageal and colorectal cancer and can also be responsible for nongastrointestinal problems such Guillian-Barré Syndrome as well as bacteraemia, lung infections, brain abscesses, meningitis, or reactive arthritis among others (Kaakoush et al., 2015).

C. jejuni can be shed in faeces of infected individuals for 2-7 weeks in untreated infections, humans, unlike animals, rarely become chronic or asymptomatic carriers of

the disease; it means that humans are not a primary host for *Campylobacter* (Romich, 2008). In spite of this, several outbreaks have been reported. According to the extended literature, it is well known that most of outbreaks are linked to consumption of undercook chicken, untreated water or milk and contact with animals. However, table 2 shows other less frequent sources of campylobacter's outbreaks.

Table 2. Different human *Campylobacter* outbreaks and the relationship with sources of transmission.

Source	Species	References
Food		
Russian salad, chicken	*Campylobacter* spp.	Calciati et al. (2012)
Commercial catering serving chicken livers	*C. jejuni* and *C. coli*	Lahti et al. (2017a); Moffatt et al. (2016)
Milk	*C. jejuni*	Burakoff et al. (2018)
Cheese	*C. fetus*	Koppenaal et al. (2017)
Raw peas	*C. jejuni*	Kwan et al. (2014)
Sausage (cross-contamination)	*Campylobacter* spp.	Graham et al. (2005)
Water	*C. jejuni*	Revez et al. (2014)
Municipal water supply	*C. jejuni*	Kuusi et al. (2005)
Contact with animals		
Pets	*Campylobacter* spp.	(CDC, 2018)
Raccoon	*C. jejuni*	Saunders et al. (2017)
Pheasant	*C. jejuni*	Heryford et al. (2004)
Social events		
Barbecue party	*C. jejuni*	Allerberger et al. (2003)
School farm visit	*C. jejuni*	Lathi et al. (2017b)
Obstacle adventure race	*C. coli*	Zeigler et al. (2014)
Mountain bike race	*C. jejuni*	Stuart et al. (2010)
Man Sex (homosexual)	*C. coli*	Gaudreau et al. (2013)
	C. jejuni	Gaudreau et al. (2015)

5. PATHOGENESIS AND VIRULENCE FACTORS

Although several putative virulence and survival factors may be important for *Campylobacter* pathogenesis, the relevance of these particular genes and the proteins they encode is generally poorly understood. Host colonization by *Campylobacter* requires motility and chemotaxis, adhesion, invasion and toxin production.

Virulence factors are considered to be important for the induction of gastroenteritis. The clinical and epidemiological characteristics of the disease provide clues to the molecular mechanisms that play a role in *C. jejuni* infection. Some genes have been recognized as responsible for the pathogenicity expression; for instance, *flaA* (flagellin gene), *cadF* (adhesin gene), *racR* and *dnaJ* were selected as pathogenic genes responsible for adherence and colonization, *virB11, ciaB* and *pldA* as pathogenic genes responsible for invasion, and *cdtA*, *cdtB* and *cdtC* as pathogenic genes responsible for the expression of cytotoxin production (Bolton, 2015; Dasti et al., 2010; Datta et al., 2003; Zhang et al., 2016).

5.1 Motility

Motility is essential for escaping from stressful environments and the associated genes are usually up-regulated under such conditions (Guerry, 2007). The motility system in *Campylobacter* requires flagella and a chemosensory system that drives flagellar movement based on environment conditions. The corkscrew shape and the presence of flagella in *campylobacter* are suitable for swimming through the mucus layer that covers the epithelial lining of the intestine, allowing this pathogen to efficiently reach its favoured colonization site, the inner mucus layer of the intestine. The axial part of the flagellum is composed of a hook-basal body and the extracellular filament structural components. The hook-basal body includes (i) a base embedded in the cytoplasm and inner membrane of the cell; (ii) the surface localized hook and (iii) the periplasmic rod and associated ring structures. This rod is connected to the proteins of the motor that provides energy for the movement of the flagellum. This system is composed by a high number of proteins with different roles. Among them, the principal flagellum filament proteins are the major flagellin subunits FlaA, and the minor subunit FlaB, encoded by

flaA and *flaB* genes respectively (Bolton, 2015). Other important protein is CheY, which is a response regulator needed for flagella rotation (Yao et al., 1997).

Bacterial flagellum is one of the most important virulence factors which is also associated with adhesion and invasion as well. In that sense, apart from serving to confer movement, the flagellum of *C. jejuni* have components homologous to the classical Type III secretion systems (T3SS) that serves to transport non-flagellar proteins, playing a central role in *C. jejuni* pathogenesis. T3SS are complex macromolecular structures, which allow Gram-negative bacteria to secrete proteins across the inner and outer membranes, without a periplasmic intermediate, acting as a "molecular syringe" (Cornelis, 2006). Several proteins have been proposed to be exported via the flagellum such as CiaB, CiaC, CiaI, FlaC, and FspA (Neal-McKinney and Konkel, 2012).

In addition to their role in mobility, flagellins are major bacterial proteins that can modulate host responses via Toll-like receptor 5 (TLR5) or other pattern recognition receptors. TLR5 usually initiates a powerful host response that provides crucial signals for maintaining intestinal immune homeostasis. However, *C. jejuni* escapes TLR5 recognition because its flagellin protein has a structure that clearly differs from other bacterial flagellins that do activate this system (Zoete et al., 2010).

5.2 Adhesion

The ability of *C. jejuni* to adhere the host gastrointestinal tract by binding to epithelial or other cells is a prerequisite for colonisation. This action is mediated by several adhesins located on the bacterial surface and it is essential to trigger the disease (Konkel et al., 1997). *Campylobacter* adhesion to fibronectin is mediated by CadF, a 37-kDa fibronectin-binding outer membrane protein. Several studies with mutants have revealed that the lack of this protein avoids colonization by *Campylobacter* (Monteville et al., 2003; Ziprin et al., 1999). Moreover, other proteins have been identified in the

colonisation process, such as the autotransporter CapA, the periplasmic binding protein PEB1 and the surface-exposed lipoprotein JlpA (Bolton, 2015). It has been perceived that a correlation exists between the severity of clinical symptoms in infected individuals and the degree to which *C. jejuni* isolates adhere to cultured cells (Dasti et al., 2010; Fauchere et al., 1986).

5.3 Invasion

Invasion ability of *C. jejuni* is an important pathogenicity-associated factor. The clinical presentation of acute campylobacteriosis is consistent with cellular invasion. The key to host cell invasion and survival are a set of proteins called *Campylobacter* invasion antigens (Cia), which are delivered to the cytosol of host cells via a flagellar T3SS (Barrero-Tobon and Hendrixson, 2012). In that sense, flagellar mutants had significantly reduced its invasion ability (Konkel et al., 1997). There are three Cia proteins: CiaB related with adherence to the target cells, CiaC required for full invasion of INT-407 cells and CiaI that has been reported to play a crucial role in intracellular survival. More recently, a fourth protein, CiaD has been identified as an important factor required for maximal invasion of the host cells (Samuelson et al., 2013). Moreover, CiaB mutans have reduced their invasion ability by decreasing significantly the adherence and the potential invasiveness. Other proteins, such as FlaC, IamA, CeuE, HtrA, VirK and FspA have been suggested to have a role in cell host invasion, although the proper mechanisms are still poor understood (Bolton, 2015).

5.4 Toxin production

Campylobacter, like other Gram-negative bacteria, produce a cytolethal distending toxin encoded by the *cdtABC* operon. While *cdtA* and *cdtC* are involved with binding and internalization into the host cell, *cdtB* encodes the enzymatically active/toxic subunit. The interaction between the three proteins conforms a tripartite CDT holotoxin,

which is necessary for the delivery of the toxin. In that way, CdtB is translocated into the host cell membrane and causes cell cycle arrest in the G2/M phase resulting in cell death (Dasti et al., 2010; Koolman et al., 2016).

CDT activity can differ somewhat depending on the eukaryotic cell types affected, showing three types of pathways; 1) In epithelial cells, endothelial cells, and keratinocytes undergo G_2 cell cycle arrest, cellular distension, and death; 2) in fibroblasts undergo G_1 and G_2 arrest, cellular distension, and death; and 3) in immune cells undergo G_2 arrest followed by apoptosis. As a result of these actions, CDT contributes to pathogenesis by inhibiting both cellular and humoral immunity, as mentioned, via apoptosis of immune response cells (Smith and Bayles, 2006). Moreover, cytotoxic activity of the *C. jejuni* CDT is dependent on its endocytosis. The toxin could be blocked by changes occurring at the level of the microtubules and actin filaments; therefore, it is unable to reach the nucleus avoiding the DNA damage and the alteration of the cell cycle (Mendez-Olvera et al., 2016).

5.5 Carbohydrate structures

Four different classes of carbohydrate structures such as lipooligosaccharides (LOS), capsular polysaccharides (CPS), O- and N-linked glycans can be found on the surface of the *Campylobacter* cell. The LOS molecule is formed with a core oligosaccharide and lipid A, and has been associated with different activities including immune evasion, host cell adhesion, invasion and protection from complement-mediated killing. Addition of sialyl groups to the LOS molecule increases invasive potential and reduces immunogenicity of *C. jejuni* strains (Bolton, 2015). Sialylated LOS of *C. jejuni* are capable of mimicking human antigens, like those involved in the appearance of Guillian-Barré (GBS) and Miller Fisher syndromes. The majority of patients with GBS

have had an antecedent infection with *C. jejuni* strains with LOS belonging to the locus class A (Revez et al., 2012).

On the other hand, CPS have been described to be associated with many functions; such as, protecting bacteria from adverse environmental conditions like increasing resistance to desiccation, biofilm formation and contribution to virulence in the gastrointestinal tract (Nachamkin et al., 2008).

C. jejuni has shown a remarkable diversity in gene encoding for CPS and LOS. Further characterization of these gene clusters established eleven classes for CPS and 18 for LOS. This structural variation of the CPS and LOS may represent important strategies for evading the immune response of *C. jejuni*. The highly variation in the CPS and LOS gene has been related to the existence of multiple mechanisms, including (i) lateral gene transfer, (ii) gene inactivation, duplication, deletion, and fusion, and (iii) phase variable homopolymeric tracts (Richards et al., 2013).

Additionally, the N-linked glycosylation system of *C. jejuni* is responsible for post-translational modification of over 60 periplasmic proteins, including flagellin. This activity is encoded by the pgl multigene locus present in *C. jejuni*. N-linked glycosylation of surface proteins facilitates immune evasion and protects *C. jejuni* against gut proteases. Contrary to the widespread of N-linked glycosylation, O-linked glycosylation is only limited to the flagellin subunits (Bolton, 2015).

5.6 Iron uptake system

For many microorganisms, iron acquisition is essential for colonization and infection of the host. Iron is a cofactor in many proteins involved in metabolism and basic cellular pathways in both pathogens and their hosts. Under aqueous aerobic conditions at physiological pH, iron is predominantly present as Fe^{3+}. To acquire essential iron, bacteria produce and secrete siderophores with high affinity and selectivity for Fe^{3+} to

mediate its uptake into the cell (Miller et al., 2009). *C. jejuni* is not capable of synthesizing siderophores itself; however, it possesses an uptake system that is able to use siderophores from competing species. This strategy gives a competitive advantage to *C. jejuni* taking into account the large number of genes dedicated to iron uptake, regulation and homeostasis in its relatively small genome (Parkhill et al., 2000). Nevertheless, comparative genomics found variations in iron uptake genes among the sequenced strains.

Several ferric iron uptake systems have been proposed for *Campylobacter* being the enterochelin system one of the most used. This is mediated by CfrA receptor outer membrane protein and the binding-protein-dependent inner membrane ABC transporter system encoded by the *ceuBCDE* genes. *C. jejuni* can use iron to growth taken from host sources such as haem-containing compounds and transferrin proteins. Ferrous iron (Fe^{2+}) diffuses through the outer membrane and only requires a transport protein across the cytoplasm membrane encoded by the gene *feoB* (Miller et al., 2009). Most of the genes involved in iron uptake are primarily regulated by the protein Fur.

6. SURVIVAL STRATEGIES OF *Campylobacter* spp.

As general knowledge *Campylobacter* are fastidious microaerophillic, unable to multiply outside a host and generally unable to grow in atmospheric levels of oxygen. Nonetheless, it can adapt and survive in the environment, exhibiting aerotolerance and resistance to starvation. The survival period of time depends on the species and the environmental conditions, including; temperature, light, biotic interactions, oxygen and nutrient concentrations. For instance, the pathogen can survive up to 9 days in faeces, 3 days in milk and 2-5 days in water (Romich, 2008). To overcome this, many genes and biochemical pathways are involved in these strategies as it has been described in different reviews on this issue (Bolton, 2015; Bronowski et al., 2014; Zhang et al., 2016).

6.1 Biofilms

Biofilm is a polymeric matrix synthesized by aggregates of microbial cells from the same or different species that is attached to different types of surfaces (Teh et al., 2014). This structure allows microorganisms involved to a better survival in hostile environments. *C. jejuni*, as other bacteria, is able to form biofilms both *in vitro* and on processing surfaces made of different materials such as plastic, stainless steel or glass among others among the poultry food chain (Gunther and Chen, 2009; Teh et al., 2014). However, the biofilm structure and its consistency are strain and environmental-dependent due to differences in gene content between strains, different expression of the genes involved in the synthesis of biofilms and those related with survival in the environment (Buswell et al., 1998; Joshua et al., 2006). So far, genes described to be involved in the process include those responsible for cell motility (*flaA, flaB, flaC, flaG, fliA, fliS,* and *flhA),* cell surface modifications (*peb4*, *pgp1*, and *waaF),* quorum sensing (*luxS*), and stress response (*ppk1*, *spoT*, *cj1556*, *csrA*, *cosR*, *cprS*). Studies with mutants have revealed that surface proteins flagella and quorum sensing appear to be required for maximal biofilm formation (Brownoski et al., 2014; Turonova et al., 2015).

Furthermore, biofilms confer upon *C. jejuni* the protection from stressful conditions that has to withstand both in the processing environment and during host infection. Indeed, the resistance to antimicrobial agents such as antibiotics compounds and detergents and disinfectants might be increased during the coexistence in these biofilms (Melo et al., 2017; Teh et al., 2014).

6.2 Stress responses

Campylobacter must overcome a wide range of harsh and stressful conditions from farm to consumer intake, and even the transition through the gastrointestinal tracts of humans. Particularly, this microaerophilic foodborne pathogen must survive in

atmospheric conditions prior to the initiation of infection (Kim et al., 2015) or other stresses in food industry as cleaning and disinfection procedures (Melero et al., 2012; Peyrat et al., 2008).

Reactive Oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical can induce DNA and protein damage in bacteria. In order to avoid the toxicity effect of ROS, *Campylobacter* activates and/or increases the activity of the antioxidant defence system. Among this, superoxide dismutase (SodB), alkyl hydroperoxide reductase (AhpC) and a catalase (KatA) play an important role. Although other regulators like SoxRS and OxyR commonly present in others Gram-negatives bacteria are not present in *C. jejuni,* this bacterium could deal with oxidative stress due to the presence of other regulators such as, the peroxide-sensing regulator PerR, the ferric uptake regulator Fur and the LysR-type transcriptional regulator *Cj1000* (Koolman et al., 2016).

6.3 VBNC and Coccoid forms of *Campylobacter*

Rollins and Colwell (1986) described the viable but non-culturable (VBNC) state as a survival mechanism used by *Campylobacter jejuni*. In this state, the pathogen can growth under stressful conditions such as; lack of nutrients, suboptimal growth temperatures, oxygen tension, pH, osmolartiy changes and high-pressure conditions. *C. jejuni* is able to survive in this state due to the entrance in a minimal metabolic activity (Jackson et al., 2009; Nachamkin et al., 2008).

During this state, the pathogen suffers some morphological changes, reducing in size and change to coccoid form. The capacity of some strains to enter in this dormancy state might explain the reason why some genotypes are more related and persistence in some niches and environmental sources (Brownoski et al., 2014). After a period of resuscitation, the culturability is restored and therefore, campylobacters recover the

infectivity pathways. The importance of this state lies in its implication in public health as a possible reservoir of infection.

7. ANTIMICROBIAL RESISTANCE

Antimicrobial resistance in emerging and re-emerging farm-borne has increased over the last years, for instance in poultry or pig farms it is considered as growing problem. Therefore, prevention zoonoses must be a priority from farm to fork. This prevention might be start at farm level with the concept of "*One Health*" where technical support such as veterinarians, epidemiologists and public health workers must be joined to work in the same direction by reducing antibiotics supply and therefore, possible resistances. Welfare management and using restrictive antimicrobial measures are needed to control the spread of antibiotic resistance elements among food chain (Cantas and Suer, 2014).

Campylobacter is exposed to a widely classes of veterinary antibiotics because it is a commensal of many animal species. Among these classes, quinolones as ciprofloxacin or enrofloxacin has been involved in high resistances in farms and food chain products. Moreover, *Campylobacter* has a natural competence and hypervariable genomic sequences, conferring a considerable genomic plasticity that might confer these resistances (Iovine, 2013). They are likely inherently resistant to cloxacillin, nafcillin, oxacillin, sulfamethoxazole, trimethoprim and vancomycin. However, other types of resistance might be a result occurring during therapeutic antimicrobial drug use in humans and animals (Fouts et al., 2005; Llarena, 2015).

Campylobacter pathogens have evolved multiple mechanism for antimicrobial resistance, including (i) synthesis of antibiotic-inactivating/modifying enzymes (e.g. β-lactamase), (ii) alteration or protection of antibiotic targets (e.g., mutations in *gyr*A or 23S rRNA genes), (iii) active extrusion of drugs out of bacterial cells through drug efflux

transporters (e.g., CmeABC), and (iv) reduced permeability to antibiotics due to unique membrane structures. Some of the resistance-associated traits are endogenous to *Campylobacter*, whereas others are acquired by mutation or genetic transfer (Luangtongkum et al., 2009; Nachamkin et al., 2008).

7.1 Multidrug efflux pump system

Resistance to bile salts, heavy metals and a broad range of other antimicrobial agents is often mediated by the *Campylobacter* multidrug efflux pump (CME). It is encoded by the *cmeABC* operon, consisting of a periplasmic fusion protein (CmeA), an inner membrane efflux transporter belonging to the resistance-nodulation-cell division superfamily (CmeB), and an outer membrane protein (CmeC). The expression is modulated by CmeR, a transcriptional repressor, possibly by inhibiting the *cj0561c* gene, which encodes a putative periplasmic protein. CmeABC contributes to the intrinsic resistance of *C. jejuni* to a broad range of structurally unrelated (Lin et al., 2002).

7.2 Quinolones

According to the European Food Safety Authority (EFSA), very high resistance levels to ciprofloxacin were reported in human *Campylobacter* isolates from all European State Members except Denmark and Norway. Eleven out of 17 reporting countries had levels of ciprofloxacin resistance in *C. coli* between 80-100 % with increasing trends in 2013-2015 in two Member State (MSs) and increasing trends of fluoroquinolone resistance was observed for *C. jejuni* in five MSs. The level of acquired resistance to fluoroquinolones can no longer be considered appropriate for routine empirical treatment of human *Campylobacter* infection (EFSA, 2017). Therefore, other human treatment drugs as macrolides (erythromycin or/and azithromycin) has been required and probably soon, fluoroquinolones might be entered in disuse.

Many studies have shown a clear positive association between the use of fluoroquinolone in poultry production and increases resistance among chicken and human *Campylobacter* isolates (Luangtongkum et al., 2009). Resistance to quinolones in *Campylobacter* is mediated by a single point mutation in the quinolone resistance determining region (QRDR) of the gene *gyrA* and also by the increased activity of the CmeABC efflux pump (Iovine, 2013). There are several different single *gyrA* modifications reported to be associated with fluoroquinolone resistance in *Campylobacter* species: Thr86Ile, Asp90Asn, Thr86Lys, Thr86Ala, Thr86Val, and Asp90Tyr. However, the most frequently observed mutation in quinolone resistant *Campylobacter* is the C257T change in the *gyrA* gene, which leads to the Thr86Ile substitution in the gyrase and confers the high-level resistance to this group of antimicrobials (Wieczorek and Osek, 2013).

7.3 Tetracycline

Tetracycline resistances in *Campylobacter* has mediated by the ribosomal protection protein TetO encoded by the *tet(O)* gene. This protein recognizes an open A site on the bacterial ribosome and binds it in such a manner that it induces a conformational change that results in the release of the bound tetracycline molecule (Luangtogkum et al., 2009).

Exactly how each pathway contributes to tetracycline entry in *Campylobacter* is not completely clear. According to Iovine (2013) two are the mechanisms of tetracycline resistance: (i) alteration of tetracycline's ribosomal target and (ii) multidrug efflux pump. Moreover, in most strains, the *tet(O)* gene is plasmid-encoded, however, some isolates do have a chromosomally encoded copy of the gene (Avrain et al., 2004).

7.4 Aminoglycoside

Antibiotics belonged to this class include: gentamicin, kanamycin, amikacin, neomycin, tobramycin and streptomycin. Mainly, aminoglycosides have two ways to exert antimicrobial activity: (i) interference with the translocation of the nascent peptide chain from the ribosomal A site to the P site leading to premature termination, and (ii) interference with proof-reading, leading to incorporation of incorrect amino acids and dysfunctional protein. However, the main mechanism of amino- glycoside resistance in *C. jejuni* is via aminoglycoside modifying enzymes (AphA, AphD, AadE, aacA, Sat) which are usually plasmid-borne (pCG8245). Additionally, contribution of efflux is not clear (Iovine, 2013; Yao et al., 2017).

7.5 Macrolide

According to EFSA studies, macrolides resistance in *Campylobacter* has increased in the last years and it is frequently detected in many European Union Members at high levels (EFSA, 2017). Historically, the incidence of resistance to macrolides has been low, especially in *C. jejuni*, even though there are several mechanisms by which *Campylobacter* can acquire resistance to these antimicrobial agents. Four are the main ways are involved in *Campylobacter* resistances: (i) target mutations in 23S rRNA genes (ii) target mutations in ribosomal proteins (iii) ribosomal methylation encoded by *erm* (B) and (iv) efflux through CmeABC and possibly others (Bolinger and Kathariou, 2017).

The third way described above, has recently been described in a single isolate of *C. coli* from broilers in Spain. This appear as the first report of *erm(B)* in *Campylobacter* in Europe. The isolate showed high-level erythromycin resistance (MIC ≥ 1,024 mg/L erythromycin) and the *erm(B)* gene was located within a multidrug resistance island containing five antimicrobial resistance genes. Moreover, it was resistant to nalidixic

acid, ciprofloxacin, tetracyclines and streptomycin and susceptible to gentamicin (EFSA, 2017).

Macrolides interrupt protein synthesis in bacterial ribosome by targeting the 50S subunit and inhibit bacterial RNA-dependent protein synthesis. Nucleotides 2058 and 2059 in 23S rRNA can act as key contact sites for macrolide binding. This joining lead to conformational changes in the ribosome and subsequent termination of the elongation of the peptide chain. The chromosome of *Campylobacter* contains three copies of the 23S rRNA gene. In erythromycin-resistant strains, generally all copies carry macrolide resistance-associated mutations, but the co-existence of wild-type alleles does not seem to affect the resistance level (Wieczorek and Osek, 2013).

Resistant in *Campylobacter* strains with the A2074G or A2075G mutation has been observed as well as mutations in the ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98). Additionally, the synergy between the CmeABC efflux pump and mutations has also shown to confer macrolide resistance in *C. jejuni* and *C. coli*. The target mutations and active efflux confer resistance in *Campylobacter* not only to macrolides (erythromycin, clarithromycin, azithromycin and tylosin), but also to ketolides (telithromycin) (Luangtogkum et al., 2009).

8. CONTROL STRATEGIES AND LEGISLATION

Nowadays, *Campylobacter* infections have evolved as one of the major concerns in public health all around the world, affecting equally to develop and developing countries and number of campylobacteriosis cases are still increasing year by year. Moreover, movement of food product, especially from poultry, due to the international trade of goods and the movement of travellers between countries help to expand such concern. Epidemiological studies using molecular typing techniques like MLST found the same *Campylobacter* genotype in different geographical locations, even though they

are at a great distance from each other. Only in the EU, it has been calculated a total annual cost of 2.4 billion € due to the campylobacteriosis and their sequels (EFSA, 2011). The same report states that broiler meat may account for 20 % to 30 % of campylobacteriosis cases in the EU and expand to 50 % to 80 % to the chicken reservoir as a whole (broilers as well as laying hens). Those data demand that some preventive measures and interventions along the poultry/chicken food chain, together with a new legislative framework are needed to achieve a drastically reduction in the presence of *Campylobacter* in those products increasing consumer's protection.

8.1 Control measures in poultry

Due to the high plasticity and adaptation level shown by *Campylobacter* to different environmental conditions, despite its fastidious grow, and its high genetic variability, it seems difficult to rely on only one strategy to reduce its presence in the poultry food chain. On the contrary, it seems than only an approach based on the barrier concept can achieve the necessary control of this pathogen. In that sense, several preventive measures and interventions have been proposed in the different steps of the poultry food chain:

Farm interventions. For instance, the application of strict biosecurity measures can efficiently reduce the risk of *Campylobacter* colonization and infection of the flocks. These measures should include at least: 1) a strict control of established accesses to minimize the entry of unauthorized people 2) hygiene barriers to avoid entering of wild birds, rodents, and insects, especially flies using flying screens and the presence of other farm animals and pets in the proximity of the broiler houses; 3) access to treated water supply (chlorination) and 4) cleaning and disinfection of the whole plant and of all the equipment between flocks, as well as change of footwear and the use of footpaths before entering in the plant. Moreover, other strategies at this level can include as well: the

avoidance or better control of discontinuous thinning, reduce the slaughter age, the use of natural feed additives, the addition of organic acids in drinking water, the use of bacteriocins or vaccination (EFSA, 2011). However, results of the application of those different strategies are still not conclusive.

Slaughter/processing plant interventions. Several strategies have been proposed to reduce the presence of *Campylobacter* in this step. The less costly is to improve the GMP/HACCP during slaughtering and processing, especially by enhancing the cleaning and disinfection procedures. Normally, slaughter productivity is enhanced in comparison with the accessibility to clean the equipment used in the birds' slaughter process. In that sense, slaughter automatic lines are built with very complex equipment, making its proper cleaning difficult. An effort should be done for improving hygienic design of slaughter equipment, in order to avoid the growth or persistence of bacteria. Good manufacturing practices aimed to prevent leakage of intestinal contents during evisceration, detection and re-processing of poultry carcasses highly contaminated with faecal matter or cloacal plugging should be taken into account, especially in colonised flocks. However, those measures only decrease carcasses *Campylobacter* contamination less than 2 logs.

Post slaughter interventions. A risk reduction between 50-90 % in *Campylobacter* presence in broiler carcasses can be obtained if they are freezing for 2-3 days, or by application of hot water or chemical carcass decontamination. This risk reduction can be increased over 90 % if freezing period extends to 2-3 weeks. Currently in the EU, chemical decontamination, using organic acids solutions, chlorine dioxide or trisodium phosphate, is not allowed. Moreover, a 100 % reduction can be achieved by irradiation or cooking broiler carcasses or meat. However, irradiation is not well accepted by consumers, and cooking changes the sensory appearance from fresh chicken meat into

a cooked product. Other interventions such as crust-freezing or application of steam alone or combined with ultrasounds have also been proposed, although they show some practical limitations (EFSA, 2011).

Retail interventions. Cross-contamination between different meats and handling by retailers should be avoided. In that sense, strict cleaning and disinfection procedures, keeping the adequate refrigeration temperature in the counters and proper packaging might limit cross-contamination and therefore reduce the presence of *Campylobacter* in chicken products. Warning labels, with food safety warnings and handling instructions on all fresh poultry packaging may also help for a better consumer handling to avoid cross-contamination. When preparing poultry products at home, consumer must take care to avoid also cross-contamination between chicken meat and other kitchen utensils or with consumers' hands. Moreover, poultry products must be cooked properly. Consumers must take into account that washing chicken prior to cooking is a potential risk of cross-contamination and this practice must be avoided.

8.2 Other measures

Although chicken meat has been designated as the main responsible for the transmission of *Campylobacter* to humans, it is not responsible for 100 % of the cases of campylobacteriosis. This fact suggests that there must be other sources of contamination, a part of the chicken meat. This is true since almost 20 % of the campylobacteriosis cases detected in the EU in 2016, were diagnosed in children under 4 years, which is strange that they eat uncooked poultry meat. In that sense, is important to avoid drinking untreated water or milk or swallowing water while swimming, and washing hands thoroughly, always before handling food or eating, and especially after having contact with animals and pets or after children play in playgrounds or gardens. Nevertheless, further epidemiological studies should be done to get insights in the different sources and

transmission routes to avoid contamination of consumers by *Campylobacter* a part of the well-established poultry route.

AIMS
OBJETIVOS

II. AIMS OF THE STUDY

The general aim of the present PhD Thesis has been the study of the presence of *Campylobacter* spp. along the chicken food chain, form farm to fork, highlighting some persistence mechanisms involved in survival, virulence factors and antimicrobial resistances. To achieve this objective, the study has been divided in the following specific aims:

Aim I. (i) To characterize the genetic diversity of *Campylobacter* spp. in chicken farms during two seasonal periods (spring and autumn), to identify virulence genes and evaluate antimicrobial resistance in the recovered isolates. (ii) To analyze the risk of *Campylobacter* spp. associated in farms and the possible measures involved in the biosecurity to control and prevent this pathogen (Chapter1).

Aim II. To investigate the prevalence and persistence of *Campylobacter* spp. in a chicken processing plant (Chapter 2).

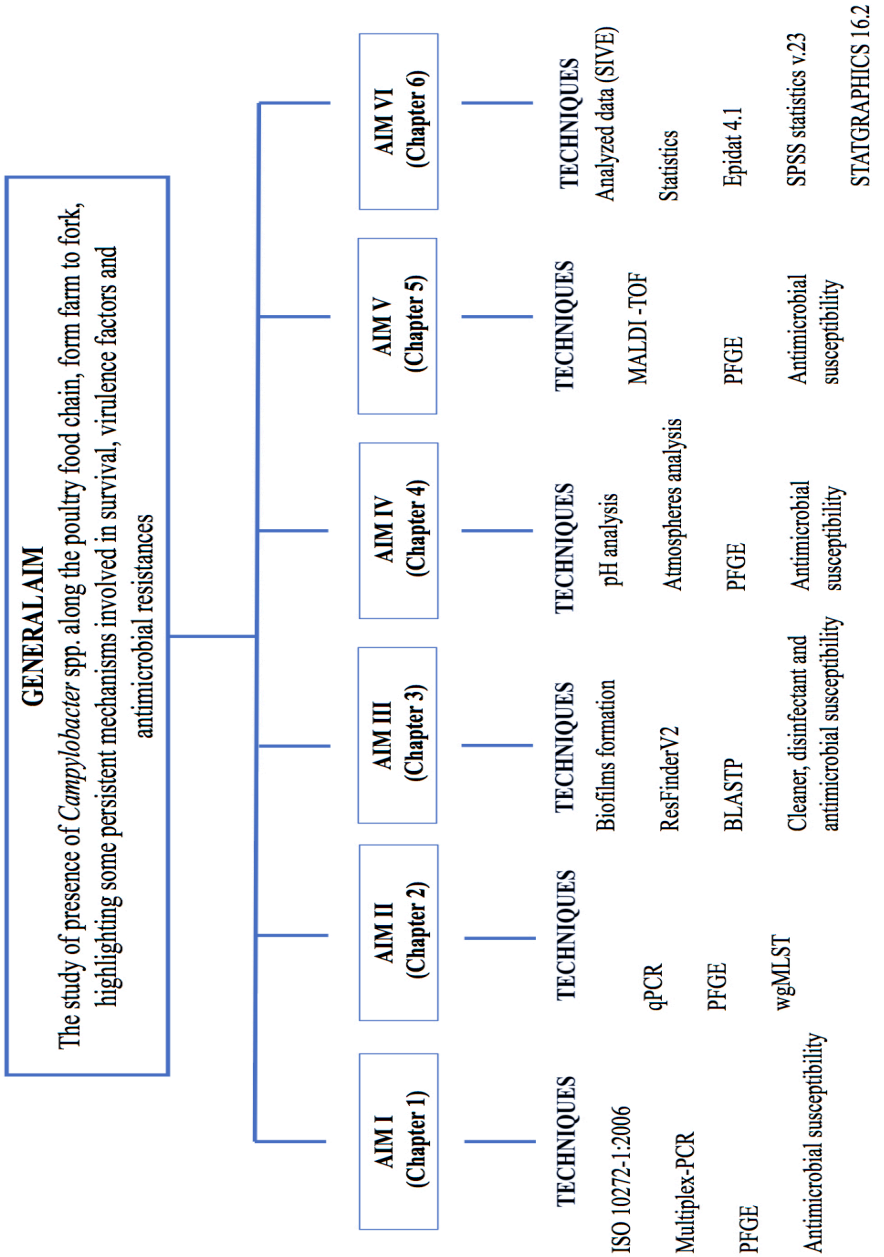
Aim III. To characterize genetically and phenotypically the mechanism and strategies involved in the survival of *Campylobacter* spp. in the poultry plant environment (Chapter 3).

Aim IV. To investigate the prevalence of *Campylobacter* spp. in different commercial chicken meat products in retail shops, both unpacked and packed with modified atmosphere packaging (MAP) and their genetic relation with geographical origins and suppliers (Chapter 4).

Aim V. To investigate the genetic relation between *Campylobacter* spp. isolates through the chicken food chain and isolates from human campylobacteriosis cases, recovered during the same period of time, and their antimicrobial resistance profile (Chapter 5).

Aim VI. To get insights in the epidemiology of campylobacteriosis in Castile and Leon during the period between 2008-2015 (Chapter 6).

The following scheme shows the relation among the different specific aims and Thesis chapters, as well as, the principal laboratory technique used in each case.



III. OBJETIVOS

El objetivo principal de la presente Tesis Doctoral es profundizar en el estudio de la presencia de *Campylobacter* spp. a lo largo de la cadena alimentaria de carne de pollo, desde la granja hasta el consumidor, haciendo especial mención a los mecanismos implicados en la persistencia, virulencia y resistencia a antibióticos.

Para llevar a cabo este objetivo, se han desarrollado los siguientes objetivos parciales:

Objetivo I. En este objetivo se ha desarrollado (i) el estudio genotípico de *Campylobacter* spp. en granjas avícolas durante dos estaciones: primavera y otoño; así como la determinación de genes de virulencia y resistencia a antimicrobianos (ii) el estudio de factores de riesgos asociados en las granjas y su implicación con las medidas de bioseguridad para la prevención y el control de *Campylobacter* spp. (Capítulo 1).

Objetivo II. Estudio de la prevalencia y persistencia de *Campylobacter* spp. en el matadero y planta de procesado avícola (Capítulo 2).

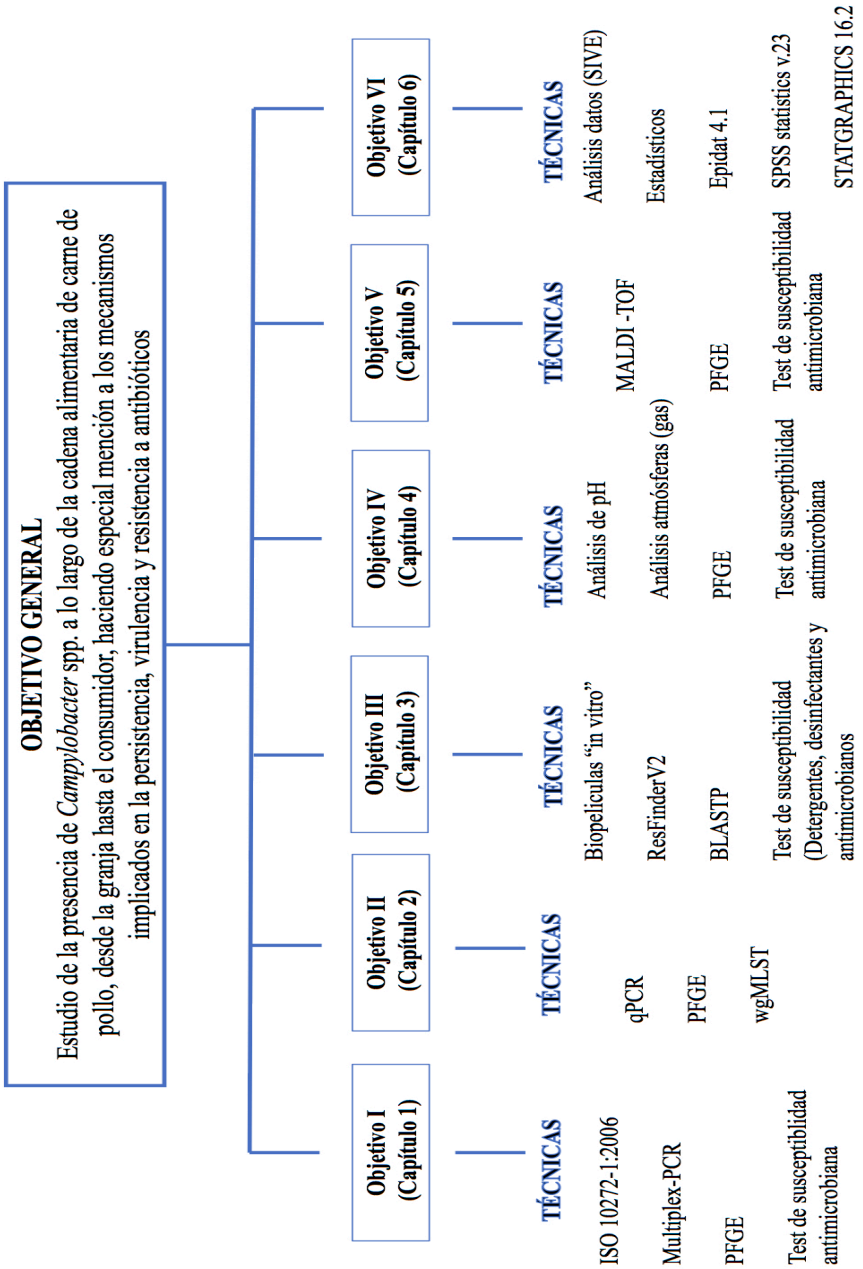
Objetivo III. Determinación genotípica y fenotípica de mecanismos implicados en las estrategias de supervivencia de *Campylobacter* spp. en el ambiente del matadero (Capítulo 3).

Objetivo IV. Determinación de la prevalencia de *Campylobacter* spp. en diferentes tipos de productos de pollo comerciales, tanto envasados en atmósfera modificada (MAP) como sin envasar y su caracterización, prestando especial atención al origen y procedencia de las muestras estudiadas (Capítulo 4).

Objetivo V. Estudio genotípico entre cepas de *Campylobacter* spp. aisladas a lo largo de la cadena alimentaria de carne de pollo y cepas procedentes de casos de campilobacteriosis humana, aisladas durante el mismo período temporal, y establecer su perfil de resistencia antimicrobiana (Capítulo 5).

Objetivo VI. Estudio epidemiológico de la campilobacteriosis en Castilla y León durante el período 2008-2015 (Capítulo 6).

El siguiente esquema muestra la relación entre los objetivos y los capítulos correspondientes de la presente Tesis, así como las técnicas utilizadas.



RESUMEN DE LOS CAPÍTULOS

III. RESUMEN DE LOS CAPÍTULOS

1. RESUMEN CAPÍTULO 1

ESTUDIO GENOTÍPICO DE *CAMPYLOBACTER* EN GRANJAS AVÍCOLAS DURANTE DOS PERÍODOS, OTOÑO Y PRIMAVERA, ASÍ COMO LA DETERMINACIÓN DE GENES DE VIRULENCIA, FACTORES DE RIESGO Y RESISTENCIA A ANTIMICROBIANOS

El principal reservorio de *Campylobacter* spp. es el intestino del pollo, concretamente el ciego, siendo una de las principales fuentes de contaminación de la cadena alimentaria. Los pollos se ven afectados principalmente a partir de las dos semanas de edad y es a partir de ese momento, cuando se produce la colonización de la manada en la granja. Existen numerosos factores, que pueden influir en la colonización y prevalencia del patógeno, como son; la estacionalidad, las medidas de bioseguridad y el manejo.

Por ello, el objetivo de este trabajo ha sido el estudio de la presencia de *Campylobacter* spp. en granjas avícolas con el fin de determinar la prevalencia y la variabilidad genotípica existente en las aves durante dos estaciones (otoño y primavera) antes de ser transportadas al matadero, así como la relación con otras fuentes de contaminación. Para ello, se analizaron un total de 1.188 muestras procedentes de 27 granjas. Los diferentes puntos de muestreo y el número de muestras tomadas fueron: la cloaca de los pollos (n=30), comederos (n=5), bebederos (n=3), bandeja anti-goteo de los bebederos (n=3) y el agua de suministro de la granja (n=3). Las cepas aisladas durante este estudio se han caracterizado mediante la determinación de genes de virulencia (*cadF, flaA, cdtA, cdtB, cdtC*) involucrados en la patogenicidad y la determinación de la resistencia frente a diferentes antibióticos como son: ciprofloxacino, ácido nalidíxico, tetraciclina, eritromicina, azitromicina y gentamicina de importancia tanto en Sanidad Animal como en Salud Pública. Además, durante el estudio, se realizaron encuestas

epidemiológicas sobre factores y prácticas higiénicas implicadas en la transmisión de *Campylobacter* spp.

El estudio ha mostrado una prevalencia en granjas del 42,9 % en otoño, siendo esta menor durante el muestreo de primavera (30,8 %). Dentro de cada granja muestreada también se encontraron diferencias, existiendo rangos de prevalencia entre el 43,1 % y el 88,6 %. *C. jejuni* fue la especie más abundante en todos los muestreos, siendo la única especie aislada en primavera. Mediante el análisis por electroforesis en campo pulsado (PFGE), se observó que las cepas de *Campylobacter* mostraron una alta diversidad genética, estableciéndose 21 pulsotipos (19 correspondientes a *C. jejuni* y 2 a *C. coli*) procedentes de 309 cepas aisladas durante todo el estudio. En cuanto a los genes de virulencia, *C. coli* no presentó *CdtA* ni *CdtC*, por el contrario, *C. jejuni* presentó todos los genes testados involucrados en la patogenicidad.

Este capítulo demuestra que, en general, las granjas analizadas tienen insuficientes medidas de bioseguridad y el ambiente podría tener un importante papel en la transmisión del patógeno. Es de destacar que en algunas granjas analizadas factores como; la ausencia de antesala en la nave, ausencia de un sistema de prevención de entrada de roedores/insectos, el tipo de material de la yacija o el sistema de desinfección del agua podrían favorecer la presencia de *Campylobacter*. Por lo que, la implantación de medidas de bioseguridad podría reducir la contaminación del patógeno, no solo en la granja, si no en los siguientes pasos de la cadena alimentaria. Por otra parte, son alarmantes las resistencias encontradas a los antibióticos analizados, principalmente en (fluoro)quinolonas y tetraciclinas. Por lo que establecer estas medidas en las granjas puede ser crucial para reducir la colonización de *Campylobacter* spp. y disminuir los riesgos para la Salud Pública.

2. RESUMEN CAPÍTULO 2

ESTUDIO DE LA PREVALENCIA Y PERSISTENCIA DE *CAMPYLOBACTER* EN UN MATADERO Y PLANTA DE PROCESADO AVÍCOLA

La campilobacteriosis es la mayor causa de gastroenteritis bacteriana en el mundo. El consumo de aves, especialmente de carne de pollo, es considerado como la mayor ruta de infección en humanos. Durante el faenado de las aves en el matadero, el paquete intestinal puede romperse y este puede contaminar el ambiente.

El presente estudio tiene como objetivo determinar si *Campylobacter* spp. puede persistir en el ambiente del matadero durante un tiempo mayor del que se pensaba. Para ello, se analizaron 494 muestras en diferentes puntos de la línea de procesado: desplumadora, evisceradora, suelo, fregadero, ganchos y canal de pollo antes y después del protocolo de limpieza y desinfección. Los resultados muestran que *Campylobacter* spp. estuvo presente en todos los puntos analizados, aunque con diferencias, presentando la zona del matadero una mayor tasa de contaminación que la planta de procesado. La prevalencia general de *Campylobacter* fue de un 48,2 %. *C. jejuni* fue la especie mayoritaria con un 94,5 % del total de muestras positivas, a diferencia de *C. coli* que estuvo representada por un 5,5 %.

Para determinar la variabilidad genética, se llevó a cabo el genotipado mediante PFGE y tipificación multilocus de secuencias (MLST), determinando siete poblaciones de *C. jejuni* diferentes: pulsotipo A (ST 443; ST 443 CC), pulsotipo B (ST 775; ST 52 CC), pulsotipo C (ST 904; ST 607 CC), pulsotipo D (ST 464; ST 464 CC), pulsotipo E, pulsotipo F (ST 1074; ST 460 CC) y pulsotipo G (ST 3769; ST 21 CC). Entre estas, dos fueron las mayoritarias: el pulsotipo G (63,1 %) y pulsotipo C (23 %), la cuales, se aislaron durante 17 y 21 días respectivamente. El resto de pulsotipos (13, 9 %) se aislaron durante un menor número de días, siendo los pulsotipos A y E aislados durante unos cinco

días. Mediante técnicas de secuenciación, wgMLST, se determinó que las poblaciones más abundantes (ST 3769 y ST 904), eran altamente clonales. El pulsotipo C (ST 904) mostró solamente entre 5 a 14 diferencias alélicas sobre 1836 loci; compartiendo el 98,8 % del total de genes. De la misma forma, el pulsotipo G mostró entre 3 a 17 diferencias alélicas sobre los 1714 loci compartidos (99,1 % del total de genes).

En conclusión, este estudio muestra que algunos genotipos de *C. jejuni* pueden sobrevivir a los procesos de limpieza y desinfección en el ambiente de un matadero y planta de procesado avícola, siendo una posible fuente de contaminación para los lotes que se procesan con posterioridad. Cabe destacar, que hubo dos poblaciones; ST 3769; ST 21 CC y ST 904; ST 607 CC que persistieron durante un período de tiempo más largo de lo que hasta ahora estaba descrito en la bibliografía en estas condiciones.

3. RESUMEN CAPÍTULO 3

ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LAS ESTRATREGIAS DE SUPERVIVENCIA DE *CAMPYLOBACTER JEJUNI* EN EL AMBIENTE DEL MATADERO Y PLANTA DE PROCESADO

Aunque *Campylobacter jejuni* es muy sensible a las condiciones ambientales y tiene unas necesidades de crecimiento fastidiosas, es capaz de sobrevivir en situaciones o circunstancias que no son favorables para su crecimiento, como pH ácido, temperaturas extremas, estrés osmótico y oxidativo, así como a los procesos de limpieza y desinfección.

En el capítulo anterior se mostró que *Campylobacter jejuni* se podía aislar del ambiente del matadero y planta de procesado de pollos, y que algunos genotipos podían persistir en él. Por ello, el presente capítulo plantea el estudio de los posibles mecanismos o estrategias involucrados en dicha persistencia. Para ello, se caracterizaron 45 cepas pertenecientes a los diferentes genotipos aislados en matadero; comprobando su capacidad para formar biofilms, la resistencia frente a diferentes antimicrobianos, entre los que se probaron seis detergentes y desinfectantes y seis antibióticos como; ciprofloxacino, ácido nalidíxico, tetraciclina, azitromicina, eritromicina y gentamicina. Asimismo, se analizó la presencia de 45 genes relacionados con factores de virulencia en todas las cepas estudiadas. Dicha caracterización se realizó tanto a nivel fenotípico como a nivel del genotipo con el fin de encontrar alguna relación entre ellos.

Los resultados han mostrado que tres de las seis secuencias tipo (STs) estudiadas: ST 443 (PFGE A); ST 904 (PFGE C) y ST 3769 (PFGE G), presentaron capacidad de formar biofilms, aunque con ligeras diferencias en las condiciones analizadas, en las cuales se evaluaba: el tipo de material (acero inoxidable, plástico), la temperatura (37 ºC, 30 ºC, 25º C) y la atmósfera (aerobiosis/microaerobiosis). Por otra parte, se ha observado una alta tasa de resistencia frente a antimicrobianos; principalmente a ciprofloxacino,

ácido nalidíxico y tetraciclina; así como a dos de los detergentes evaluados. Además, se ha observado una posible relación entre los resultados genotípicos y fenotípicos de resistencias a antibióticos.

Este estudio sugiere que la combinación de pequeños cambios en el genoma de la secuencia tipo ST 904 (PFGE C), incluidas mutaciones, inserciones, una mayor resistencia frente a antimicrobianos, la presencia de genes que codifican el T6SS, así como la presencia de un gran número de genes relacionados con factores de virulencia, podrían explicar la capacidad de formar biofilms. La suma de este conjunto de pequeños cambios, podría explicar la capacidad, para sobrevivir y persistir durante períodos de tiempo más largos, en condiciones ambientales hostiles, como puede ser el matadero. Sin embargo, la ausencia de algunos genes como *capA*, *pgp1* and *ilvE,* todos ellos relacionados con la capacidad de adhesión, muestra la complejidad de las estrategias adoptadas por algunas cepas de *C. jejuni* para adaptarse a medios hostiles. Esto nos lleva a pensar que, existen múltiples soluciones para que las cepas puedan persistir y sobrevivir a lo largo de la cadena alimentaria de carne de pollo, y, por tanto, ser capaces de causar la enfermedad en humanos. Es necesario la realización de más estudios, para obtener un mejor conocimiento de la ecología y las características genéticas de las distintas cepas de *C. jejuni,* con el fin de prevenir su persistencia a lo largo de la cadena alimentaria de la carne de pollo y así disminuir el riesgo de exposición para los humanos.

4. RESUMEN CAPÍTULO 4

ESTUDIO Y CARACTERIZACIÓN GENOTÍPICA DE LA PREVALENCIA DE *CAMPYLOBACTER* EN DIFFERENTES TIPOS DE PRODUCTOS DE POLLO COMERCIALES, TANTO ENVASADOS EN ATMÓSFERA MODIFICADA (MAP) COMO SIN ENVASAR

Existen diferentes productos alimenticios que han estado involucrados en la transmisión de *Campylobacter* a los humanos, entre estos se encuentran: agua sin tratar, queso, leche, vegetales, etc. Sin embargo, un alto número de infecciones en humanos están relacionadas con la contaminación cruzada por manipulación, preparación y consumo de carne de pollo; siendo este reconocido como uno de los principales vehículos de dicha transmisión.

Por ello, el capítulo 4 tiene como objetivo estudiar la prevalencia y variabilidad genética de *Campylobacter* spp. en el último eslabón de la cadena alimentaria. En este estudio se analizaron un total de 512 muestras de productos comerciales de pollo, comprados en supermercados y/o carnicerías. Los productos muestreados fueron: pechugas, muslos, productos marinados y picados como hamburguesas o salchichas. Se muestrearon 256 productos envasados (MAP) y otros 256 productos sin envasar. Mediante electroforesis de campo pulsado (PFGE) se determinó la variabilidad genética existente en las cepas aisladas y se estudió la relación existente con sus orígenes de procedencia, distribuidor y tiempo de persistencia durante el muestreo. Además, se determinaron las resistencias antimicrobianas de las cepas frente a los siguientes antibióticos: ciprofloxacino, ácido nalidíxico, tetraciclina, azitromicina, eritromicina y gentamicina

Los resultados mostraron una prevalencia del 39,4 % en todos los productos de pollo analizados, siendo los productos sin envasar los que presentaron mayor porcentaje de *Campylobacter* (45,3 %). La prevalencia en productos envasados fue menor (33,6 %).

Aunque la atmósfera modificada puede reducir la prevalencia de microorganismos como puede ser *Campylobacter* spp. esta no puede evitar su presencia como se demuestra en los resultados obtenidos.

Por otra parte, los estudios de genotipado han determinado una alta heterogeneidad. Del total de aislados, se tipificaron 204, que se agruparon en 76 clústeres o genotipos: 55 genotipos de *C. jejuni*, 19 *C. coli* y 2 de *C. lari*. Entre estos, se observaron relaciones entre los diferentes orígenes de procedencia y el producto analizado. *Campylobacter* presentó principalmente resistencias a (fluoro)quinolonas y tetraciclina, siendo los genotipos de *C. coli* los que presentaron un mayor número de resistencias frente a los antibióticos testados.

Como conclusión de este estudio se menciona que algunos pulsotipos podrían persistir en el ambiente de la planta de procesado o en las carnicerías/supermercados durante más tiempo de lo que en un principio se pensaba. Por lo que, es necesario instaurar medidas de control más estrictas en los pasos previos de la cadena alimentaria, para evitar la presencia de *Campylobacter* spp., en la carne de pollo, último eslabón de la cadena alimentaria, antes de alcanzar al consumidor y comprometer así la salud pública. Además, se deberían instaurar campañas de educación sanitaria a la población, para concienciar del riesgo que supone una mala manipulación y cocinado de la carne de pollo, y así extremar las precauciones para evitar contaminaciones cruzadas y por tanto, posibles campilobacteriosis.

5. RESUMEN CAPÍTULO 5

CARACTERIZACIÓN DE CEPAS DE *CAMPYLOBACTER* AISLADAS A LO LARGO DE LA CADENA ALIMENTARIA DE LA CARNE DE POLLO COMO POSIBLE RUTA DE CASOS ESPORÁDICOS DE CAMPILOBACTERIOSIS EN ESPAÑA

La campilobacteriosis es considerada como un problema de salud pública tanto en países desarrollados como en países subdesarrollados, siendo la primera causa de gastroenteritis en humanos. Debido a la importancia de este patógeno como agente zoonótico, en este capítulo se ha estudiado el posible nexo de unión entre las cepas de *Campylobacter* aisladas a lo largo de la cadena alimentaria y cepas de pacientes que han sido diagnosticados con campilobacteriosis.

Para ello, se ha comparado el perfil genético de las cepas de *Campylobacter* aisladas a lo largo de la cadena alimentaria de la carne de pollo (capítulos I, II, IV) con las cepas aisladas en pacientes con campilobacteriosis (aisladas en el principal Hospital de la zona), mediante la técnica de electroforesis en campo pulsante (PFGE). El período de tiempo analizado comprende los años 2014 y 2015. Durante este período se han aislado un total de 1039 cepas: 749 de la cadena alimentaria de la carne de pollo (granjas, matadero, productos de pollo) y 290 en humanos. Con el fin de facilitar la comparación de los diferentes pulsotipos, se han establecido cuatro períodos de estudio: granja-hospital (otoño); granja-hospital (primavera), matadero-hospital y productos de pollo-hospital.

La especie más abundante, responsable de campilobacteriosis humanas fue *C. jejuni* (90,7 %), seguido con gran diferencia por *C. coli* (9,3 %); al igual que ocurre en la cadena alimentaria, donde la incidencia de *C. jejuni* también fue más alta (capítulos I, II, IV). En cuanto a los datos epidemiológicos, parece que el sexo masculino se vio más afectado que el femenino. Con respecto a la edad, los niños (1-5 años) fueron los más afectados, siendo el servicio de pediatría seguido del servicio de urgencias los que más

casos declararon. La hospitalización fue relativamente baja (17,8 %), siendo las edades ≥ 60 las más afectadas.

Los resultados muestran que hay 35 clústeres en los que se comparten cepas aisladas en alguna etapa de la cadena alimentaria de pollo y las causantes de campilobacteriosis. Así, se ha encontrado que hay 9 clústeres con cepas compartidas en la granja, 4 en matadero y 22 en productos de pollo. Además, la caracterización antimicrobiana ha establecido altas resistencias en humanos, siendo el 90,3 % de los aislados resistentes a alguno de los antibióticos testados. Entre estas resistencias, el 96,6 % de los aislados de *Campylobacter* presentó resistencia frente a ciprofloxacino, seguido de un 1,9 % de cepas que presentaron resistencia frente a ciprofloxacino, eritromicina y azitromicina y, en último lugar, un 1,5 % presentaron resistencia a ciprofloxacino y gentamicina.

En conclusión, este estudio ha demostrado una relación genotípica entre cepas de la cadena alimentaria de la carne de pollo y cepas aisladas en pacientes con campilobacteriosis. Este estudio tiene una especial relevancia epidemiológica ya que se ha realizado en una misma región geográfica y durante el mismo período de tiempo. Además, cabe destacar las altas tasas de resistencia a los antibióticos, principalmente a ciprofloxacino, lo que hace, que la eficacia de este antibiótico esté cada vez más en entre dicho para el tratamiento de casos de campilobacteriosis en el área estudiada.

6. RESUMEN CAPÍTULO 6

ESTUDIO EPIDEMIOLÓGICO DE LA CAMPILOBACTERIOSIS EN CASTILLA Y LEÓN DURANTE EL PERÍODO 2008-2015

La campilobacteriosis ha sido la zoonosis más comúnmente declarada, mostrando un incremento de los casos desde el año 2005. En el año 2014, el número de casos confirmados fue de 236,851 con una tasa de notificación de 71 y 82,3 casos por 100.000 habitantes en la Unión Europea y España, respectivamente. Ello supone a nivel europeo un incremento del 9,6 % comparándolo con el año 2013. Los casos de fatalidad fueron relativamente bajos (0,01 %).

El objetivo de este este capítulo fue conocer la incidencia de los casos de campilobacteriosis declarados al Sistema de Vigilancia Epidemiológica (SIVE) de Castilla y León y analizar la tendencia y los patrones de estacionalidad durante el período 2008-2015. Asimismo, describir el tipo de presentación y nivel asistencial de declaración de esta enfermedad, y estudiar la influencia de la edad, sexo y la especie de *Campylobacter* en las personas afectadas.

Los resultados mostraron que al igual que ocurre en la Unión Europea, en Castilla y León la campilobacteriosis sigue una tendencia ascendente. El análisis de la serie temporal evidenció la existencia de dos ciclos diferenciados: un ascenso lineal regular desde 2008 hasta 2012 y un ascenso exponencial en 2013 y 2014 que se limitó en el último año de declaración. Durante este período, hubo un total de 4.330 casos confirmados, de los cuales el 49,4 % correspondieron a edades menores de 5 años (niños) y el ratio hombre/mujer fue de 1.43. Aunque en todos los casos se confirmó el diagnóstico de campilobacteriosis; en el 59,9 % de las encuestas se registró la especie de *Campylobacter*, atribuyéndose el 72,3 % a infecciones por *C. jejuni*. Con respecto a la gravedad de los casos, el 24,7 % precisaron hospitalización. Principalmente, la

hospitalización fue registrada en pacientes mayores de 60 años. Durante el período analizado también se observó la estacionalidad de los casos, observándose dos picos máximos en verano en las cuatrisemanas 6 y 9, siendo la cuatrisemana 9 la que mayor número de casos obtuvo.

En este estudio, se refleja una tendencia ascendente en los casos notificados de campilobacteriosis en Castilla y León durante el período 2008-2015, con una clara estacionalidad en los meses de verano, siendo los sujetos menores de 5 años y el sexo masculino los más implicados. *C*. *jejuni* fue la especie más frecuentemente implicada. La mayoría de los casos fueron esporádicos y la hospitalización representó la cuarta parte de las personas afectadas. La notificación fue más frecuentemente realizada desde atención especializada.

CHAPTER 1

IV. CHAPTER 1. Genotyping, virulence genes and antimicrobial resistance of *Campylobacter* spp. in Spanish poultry farms isolated during two seasonal periods

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Abstract

Campylobacter spp. are the leading causes of bacterial human gastroenteritis worldwide; being poultry farms the main source of infections. To obtain information on prevalence and diversity of *Campylobacter*-infected flocks in the North of Spain, fourteen farms were studied along autumn and spring in 2014 and 2015 years. Moreover, virulence genes involved in pathogenicity and antimicrobial resistance were investigated. A survey about preventive hygiene practices of the farms was performed in order to create a reference for preventing and controlling *Campylobacter* at farm level. The objective was to determine the practices on farms that are risk factors which contribute to the presence of *Campylobacter*. Concerning to the influence of the season, during autumn, 43 % of the farms were positive, whereas only 31 % were positive in spring. Very high within-flock prevalence was observed (43.1 % to 88.6 %); *C. jejuni* being the most prevalent species in both periods. Genotyping by pulsed field gel electrophoresis **(**PFGE) showed a high heterogeneity among farms; being clustered 309 isolates into 21 pulsotypes. Virulence genes were present in all *C. jejuni* isolates. However, two of the five genes studied (*cdtA* and *cdtC)* were absent in *C. coli.* On the contrary, the latter showed higher antimicrobial resistance than *C. jejuni.* This study suggests that environment might be one of the main sources for *Campylobacter* transmission; in a specific farm water supply seen to be a clear cause of the contamination, however elsewhere the source should be other environmental factors, confirming the multifactorial origin of *Campylobacter* colonization in broilers. Then, biosecurity measures in farms are crucial to reduce *Campylobacter* contamination, which may have important implications for human and animal health.

1. Introduction

Campylobacteriosis is the most prevalent zoonotic diseases in the European Union with 246,307 human cases reported in 2016 (EFSA, 2017). *Campylobacter* causes enteric disorders in humans characterized by fever, diarrhea, abdominal pain, nausea and vomiting (Jurado-Tarifa et al., 2016). Although the majority of these cases occur as self-limiting enteritis, more severe and long-lasting cases could require antibiotic treatment, particularly in immune-comprised patients (Ma et al., 2014).

Humans currently consume nearly 60 billion chickens per year, numerically more than other food animal (Colles et al., 2015). It is generally accepted that poultry is a natural reservoir of *Campylobacter* species, constituting the most important source of human infection. The prevalence of *Campylobacter* spp. in broiler chicken flocks ranges from 3 % to 90 % depending on their location (Marotta et al., 2015). *Campylobacter* colonization in chickens takes place at poultry farms, approximately 7 days after hatching (Gibbens et al., 2001). After infection, *Campylobacter* rapidly colonizes the two ceca to a high level and leads to fecal shedding (Marotta et al., 2015). This high-level shedding combined with the coprophagic behavior of chickens means that once the first bird in a broiler flock becomes colonized, the bacterium is able to pass rapidly through the entire flock in just a few days (Chaloner et al., 2014).

Multiple risk factors have been associated with increased proportion of *Campylobacter* positive flocks. Among these factors, season (Jonsson et al., 2012), presence of other animals on the farm or in close proximity (Hansson et al., 2010), presence of rodents (Sommer et al., 2013), vectors such as flies (Borck et al., 2016), flock size (Barrios et al., 2006), several flock houses in the same farm (McDowell et al., 2008), thinning or depopulation practices (Allen et al., 2008; Hansson et al., 2010), drinking

water distribution (Näther et al., 2009) and administration of antibiotics (Allain et al., 2014) have been identified.

Campylobacter infections in animals are usually subclinical. However, recent studies have reported that this infection can lead to diarrhea and inflammatory damage in the birds (Humpherey et al., 2014). A poor welfare in farm environment may result in stress, which reduces chicken immunocompetence making them more susceptible to *Campylobacter* spp. As a result of diarrhea, positives-flocks present litter humidity that, among other things, cause footpad dermatitis. Then, *Campylobacter* is not a merely harmless commensal as it was thought (Alpigiani et al., 2017). The primary colonization site in poultry is the ceca, where the *Campylobacter* population may reach 10^6-10^8 cfu/g and, afterwards, colonization requires motility, adhesion, invasion and toxin production (Bolton, 2015; Epps et al., 2013). However, the exact mechanisms by which *C. jejuni* or *C. coli* cause infection are unknown. According to previous research, some genes have been involved, such as *flaA* (motility), *CadF* (invasion) and cytolethal distending toxin encoded by the cdtABC operon (Bolton, 2015; Dasti et al., 2010; Nguyen et al., 2016).

In veterinary medicine, the use of antibiotics leads to the selection of resistant bacteria in their commensal microbiota (Cerf et al., 2010). Fluoroquinolones and macrolides have been the primary antimicrobials used for the treatment of human *Campylobacter* infections. Studies have shown a clear positive association between the use of fluoroquinolone in poultry production and increases resistance among chicken and human *Campylobacter* isolates (Luangtongkum et al., 2009).

Due to the diversity and variability that *Campylobacter* spp. show as an adaptive mechanism to survive in harsh conditions in the environment, it is necessary to collect epidemiological information from poultry farms and geographical locations, as the primary source of contamination, in order to get insights in these survival mechanism,

and try to develop new strategies to control this pathogen. Therefore, the first aim of the present study was to examine the prevalence and diversity of *Campylobacter* spp. in different broiler flocks, in the same farms, in two seasonal periods to check the diversity of *Campylobacter* species and identify virulence genes and antimicrobial resistance. Moreover, the second aim was to undertake a survey on preventive hygiene practices in the farms, to determine which practices applied in different farms can be considered risk factors that contribute to the occurrence of the colonization of *Campylobacter*.

2. Material and Methods

2.1 Study and sampling procedure

The present study was performed in conventional broiler farms in the North of Spain (Burgos region). All the farms which supply broilers in the slaughterhouse located in Burgos were involved. The same farm was analyzed in two different periods: 14 farms in autumn 2014 and 13 farms in spring 2015, because in the meantime one farm dropped out of the study.

The sampling unit was the flock ranging from 25,000 to 40,000 birds per shed. For the analysis, farms were distributed in four geographical areas of Burgos region according to the proximity between each other: North (Farms VIII, IX, XI), Center (Farms I, II, III, IV, VI, XII, XIV), West (Farms VII and X) and South (Farms V and XIII). Those areas were separated by a minimum distance of 60 km and a maximum of 125 km, whereas the distance between farms of the same area ranged from 4 to 30 km.

Samples were collected in the previous week before birds were sent to the slaughterhouse (28-35 days). Forty-four samples were taken per flock and 1,188 samples were taken in total during the course of the experiment. Samples were taken from cloaca of the birds, feeder, water from nipples, nipple´s tray and farm water supply. The birds were selected at random, 10 from each third of the shed, selecting also one nipple and one

nipple tray from each third of the flock. The feeder samples were taken at random in the four corners and the center of the flock. The sampling procedure is shown in table 1.

Table 1. Sampling procedure performed in each flock studied.

Sampling location	Type of sample	Sampling method	(n)*
Cloacal samples	Feces	Swabs	30
Feeder	Surface (100 cm^2)	Sponge	5
Water from nipples	Water (50 mL)	Tubes	3
Nipple´s tray	Surface	Sponge	3
Water tank	Water (50 mL)	Tubes	3
Total			44

(n)* Samples taken at each sampling location per flock

2.2 Isolation and Identification of *Campylobacter* spp.

Fecal samples were collected using swabs (Copan, Brescia, Italy) and sponges (3M™, Saint Paul, Minnesota, USA). Each swab and each sponge were wet with ringer solution (Oxoid, Basingstoke, England) under sterile conditions before samples were collected in farms. Once in the laboratory, each swab was submerged in 10 mL of Preston broth made with Nutrient broth Nº 2 (Oxoid) supplemented with Preston Campylobacter Selective Supplement (Oxoid) and Campylobacter Growth Supplement Liquid (Oxoid). Likewise, each sponge was submerged in 90 mL of Preston broth and was homogenized for 120 s and incubated microaerobically using a commercial gas-generating system CampyGen (Oxoid) and sealed jar at 41.5 ºC for 48 h. In water samples, 50 mL were individually collected in sterile tubes (Labbox, Barcelona, Spain) and from these, 25 mL were incubated with 90 mL of Preston broth as usual. After enrichment for 48 h, a loop-full from each sample was streaked on a plate of modified charcoal cefoperazone deoxycholate agar (mCCDA) prepared with Campylobacter blood-free selective agar

base (Oxoid) supplemented with CCDA selective supplement (Oxoid). Plates were incubated as described above for enrichment broths. From each plate, two suspected *Campylobacter* spp. colonies were randomly selected for further analysis.

Colonies isolated from mCCDA agar were grown on 5 mL of Brain Heart Infusion broth (Oxoid) for an overnight. Following, DNA was extracted as described Yamada et al. 2015. Briefly, isolates were suspended in 100 µL of Tris-EDTA buffer (pH 8.0) and was incubated at 95 ºC for 10 min before centrifugation at 16,000×*g* for 1 min. The supernatants were subsequently used as templates for PCR. All isolates were analyzed using multiplex PCR to identify *C. jejuni, C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* sub. *fetus* as described Wang et al. (2002). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3 Pulsed field gel electrophoresis (PFGE)

C. jejuni and *C. coli* isolates were cultured on Columbia agar (Oxoid) supplemented with 5 % defibrinated sheep blood (Oxoid) under microaerobic conditions (24 h at 41.5 ºC) for the purpose of PFGE typing. PFGE analyses were performed applying the restriction enzymes *Sma*I and *Kpn*I, according to the PulseNet International standardized protocol (www.cdc.gov/pulsenet/pathogens/pfge.html). *Kpn*I was used to check the diversity of all isolates with similar *Sma*I genotype.

Restricted DNA was electrophoresed for 22.5 h on 1 % (w/v) SeaKem gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories). The electrophoresis conditions consisted in an initial switch time of 5 s and a final switch time of 55 s (gradient of 6 V/cm and an included angle 120º). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories). BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical

analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0 %). The unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns. Isolates with <90 % similarity according to the dendrogram were clustered as separate pulsotypes (Boer et al., 2000).

2.4 Identification of putative *Campylobacter* virulence genes

The presence of five *Campylobacter* virulence genes (*cadF, flaA, cdtA, cdtB, cdtC)* was tested using conventional PCR. The primers and amplification conditions for these genes were described by Bang et al. (2003), Datta et al. (2003) and Konkel et al. (1999). PCR was performed in a Mycycler ™ Thermal Cycler System (Bio-Rad Laboratories). The PCR products were subject to agarose gel electrophoresis. The DNA bands were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories).

2.5 Antimicrobial susceptibility

Campylobacter jejuni and *C. coli* isolates were sub-cultured on Nutrient agar supplemented with 5% sheep blood (Oxoid) and incubated at 41.5 ºC for 24 h in microaerobic conditions. After incubation, bacterial inoculum was performed into 2 mL of 0.9 % NaCl and the turbidity was adjusted to 0.5 McFarland scale to carry out the inoculation. The inoculate plates were incubated in microaerophilic conditions for 48 h at 41.5 ºC. Six antimicrobials belonging to four different classes were tested in different range concentrations: two fluoro(quinolone); ciprofloxacin (0.03-64 mg/L) and nalidixic acid (4-128 mg/L); two macrolide, erythromycin (0.12-16 mg/L) and azithromycin (0.015-1 mg/L); one aminoglycosides, gentamicin (0.12-8 mg/L) and tetracycline (0.12-128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values established by European Committee on Antimicrobial

Susceptibility Testing (www.eucast.org). According to Mäessar et al. (2016) one isolate was considered that presents multidrug resistance (MDR) when it was resistant to three or more no related antimicrobials. Strain *C. jejuni* ATCC 33560™ was used as a control.

The presence of virulence genes and the antimicrobial resistance profile were analyzed by duplicate for each pulsotype.

2.6 Questionnaire

A questionnaire was designed to collect information about farm management practices associated with *Campylobacter* colonization. Important variables involved in biosecurity and risk contributing factors for *Campylobacter* spp. infection were included; as well as, questions about farm facilities, preventive measures and water treatment (Table 2). The questionnaire design was based on previous studies (Torralbo et al., 2014) and was administered on the sampling day by the veterinary technician who took the samples. The data collected were based on a combination of the personal observation of technician at the sampling visit and information obtained from each farmer during a direct interview.

2.7 Statistical study

Statistical analysis was carried out using Statgraphics Centurion XVI. The relation between the variables under study listed in Table 3 and the occurrence of *Campylobacter* colonisation on farms was checked by a univariate analysis by a chi-square test with a 5% level of statistical significance.

Table 2. Data related to farm risk factors obtained from the questionnaire.

Risk factor/Farms	I	II	III	IV	V	VI	VII
Presence of *Campylobacter* (2014/2015)	-/-	-/-	-/-	+/-	+/-	+/+	-/+
Rodent control	Yes	Yes	Yes	No	No	No	No
Vehicle wheel disinfection system	No	No	No	No	No	No	No
Dogs/cats on the farm	No	No	Yes (Dogs)	No	No	Yes (Dogs/Cats)	Yes (Dogs)
Water disinfection	HP*	HP*	HP*	Chlorine	Chlorine	Chlorine	Chlorine
Cleaning footbath	No	No	No	No	No	No	No
Anteroom to poultry house	Yes	Yes	Yes	No	No	No	No
Nº flock/farm	2	4	3	3	2	2	3
Nº broilers/flock	28000	26000	25000	25000	25000	25000	20000
Litter material	Straw from cereal	Straw from cereal	Shaving wood	Straw from cereal	Straw from cereal	Shaving wood	Shaving wood
Thinning	No	No	No	No	No	No	No
Building (old/new)	new	new	new	new	new	new	old

HP* Hydrogen peroxide, pH corrector

Table 2 (continuation). Data related to farm risk factors obtained from the questionnaire

Risk factor/Farms	VIII	IX	X	XI	XII	XIII	XIV
Presence of *Campylobacter* (2014/2015)	-/-	+/+	-/+	-/-	-/-	+/-	+/-
Rodent control	Yes	No	Yes	Yes	Yes	Yes	Yes
Vehicle wheel disinfection system	No	No	No	No	No	No	No
Dogs/cats on the farm	Yes (Dogs/Cats)	Yes (Dogs)	No	Yes (Dogs)	Yes (Dogs)	No	No (Dogs)
Water disinfection	Chlorine	Chlorine	Chlorine	HP*	Chlorine	HP*	HP*
Cleaning footbath	No	No	No	No	No	No	No
Anteroom to poultry house	Yes	Yes	No	Yes	Yes	No	No
Nº flock/farm	4	3	1	2	2	4	4
Nº broilers/flock	28000	20000	34000	25000	40000	39000	26000
Litter material	Shaving wood	Shaving wood	Straw from cereal	Shaving wood	Shaving wood	Straw from cereal	Straw from cereal
Thinning	No	No	No	No	No	No	No
Building (old/new)	new	old	new	new	new	new	new

HP* Hydrogen peroxide, pH corrector

3. Results

A total of 1,188 samples were analyzed from 14/13 farms in two different seasons. The percentage of positive *Campylobacter* spp. farms during autumn 2014 was 43 %, higher than in spring 2015 where the percentage was 31 %. Only farms VI and IX were positive in both periods (table 4). Farm VI was *C. jejuni* positive in the same percentage in both periods (79.5 %), whereas farm IX showed higher *Campylobacter* spp. percentage in autumn (86.4 %) than in spring (52.3 %). Six farms were positive only in one season (farms IV, V, VII, X, XIII and XIV) and the rest of the farms were negative in both periods. Farm XIV exited the study after the first sampling period. The prevalence within all farms investigated in this study ranged from 43.2 % in farm IV to 88.6 % in farm XIII. Geographical location was also associated with the prevalence of *Campylobacter* spp. having the southern farms the highest odds ratio values (table 3).

In autumn, 193 samples out of 616 were *Campylobacter* spp. positive representing 31.3 % (table 4). In this season, 92.2 % of the isolates corresponded to *C. jejuni* and 7.8 % to *C. coli* that was detected in two farms. However, in spring, only 116 samples out of 572 were positive (20.3 %) being *C. jejuni* the only species isolated in all positive sampled farms. Considering the *Campylobacter* positive samples, the prevalence was higher in autumn as it shows the statistical analysis (Table 3).

Cloacal samples were the ones showing the highest contamination level ranging from 63.3 % to 100 %, followed by feeder and nipple`s tray with 40 % and 33 % to 100 % respectively (table 5). The lowest percentage was found in farm water supply, where only farm XIII showed a positive result.

Table 3. Effect on *Campylobacter* contamination of different factors related to broiler farms analyzed by chi-squared test.

Variable	Level	C+[a]	C+ (%)	Odds ratio	95 CI	p-value
Season	Spring	116	10	1		
	Autum	196	16	1.79	1.38-2.34	<0.0001
Location	Center	114	10	1		
	South	76	6	3.05	2.12-4.39	
	West	58	5	1.97	1.36-2.87	
	North	61	5	1.21	0.85	<0.0001
Prevention of rodents	Yes	89	7.5	1		
	No	220	18.5	7.40	5.54-9.89	<0.0001
Dog/cats on the farm	Yes	164	14	1.06		
	No	145	12	1	0.72-1.21	0.617
Entrance room	Yes	61	5	1		<0.0001
	No	284	24	8.97	6.57-12.25	
Water treatment	Chlorine	245	21	5.26		
	Hydrogen peroxide	64	5	1	0.14-0.26	<0.0001
Nº flock per farm	1	25	2	1		
	2	168	16.3	1-18	0.71-1.94	
	3	52	4	0.62	0.36-1.08	
	4	64	5	0.66	0.39-1.80	0.002
Litter material	Straw	206	17	1.87		
	Shaving wood	103	9	1	0.4	<0.0001
Nº broilers	20,000-25,000	220	18	5.56		
	26,000-30,000	25	2	1	0.12-0.28	
	>30,000	64	5	1.53	0.48-0.91	<0.0001

[a] *Campylobacter* positive samples

Table 4. *C. jejuni* and *C. coli* prevalence in each farm and in each season.

		Autumn 2014			Spring 2015	
Farm	**Location**	***Campylobater* spp. (%)**	***C. jejuni* (%)**	***C. coli* (%)**	***Campylobacter* spp. (%)**	***C. jejuni* (%)**
I	Center	Negative			Negative	
II	Center	Negative			Negative	
III	Center	Negative			Negative	
IV	Center	19/44 (43.2)	10/19 (52.6)	9/19 (47.4)	Negative	
V	South	37/44 (84.1)	37/37 (100)		Negative	
VI	Center	35/44 (79.5)	35/35 (100)		35/44 (79.5)	35/35 (100)
VII	West	Negative			33/44 (75)	33/33 (100)
VIII	North	Negative			Negative	
IX	North	38/44 (86.4)	32/38 (84.2)	6/38 (15.8)	23/44 (52.3)	23/23 (100)
X	West	Negative			25/44 (56.8)	25/25 (100)
XI	North	Negative			Negative	
XII	Center	Negative			Negative	
XIII	South	39/44 (88.6)	39/39 (100)		Negative	
XIV	Center	25/44 (56.8)	25/25 (100)		ND*	
Total		193/616 (31.3)	178/193 (92.2)	15/193 (7.8)	116/572 (20.3)	116/166 (100)

ND*: Not determined, the farm dropped out of the study in spring period.

Table 5. *Campylobacter* spp. prevalence in each sampling location per farm (positives/total samples).

Season	Autumn						Spring			
Sampling location	**Farm IV**	**Farm V**	**Farm VI**	**Farm IX**	**Farm XIII**	**Farm XIV**	**Farm VI**	**Farm VII**	**Farm IX**	**Farm X**
Cloacal samples	19/30 (63.3%)	30/30 (100%)	28/30 (93.3%)	30/30 (100%)	27/30 (90%)	25/30 (83.3%)	27/30 (90%)	27/30 (90%)	19/30 (63.3%)	22/30 (73.3%)
Feeder	0	4/5 (80%)	5/5 (100%)	5/5 (100%)	5/5 (100%)	0	5/5 (100%)	3/5 (60%)	3/5 (60%)	2/5 (40%)
Water from nipples	0	0	0	0	1/3 (33.3%)	0	0	0	0	0
Nipple´s tray	0	3/3 (100%)	2/3 (66.7%)	3/3 (100%)	3/3 (100%)	0	3/3 (100%)	3/3 (100%)	1/3 (33.3%)	1/3 (33.3%)
Water Supply	0	0	0	0	3/3 (100%)	0	0	0	0	0
Total	19/44 (43.2%)	37/44 (84.1%)	35/44 (79.5%)	38/44 (86.4%)	39/44 (88.6%)	25/44 (56.8%)	35/44 (79.5%)	33/44 (75%)	23/44 (52.3%)	25/44 (56.8%)

From the original 315 isolated *Campylobacter* strains, 292 out of 297 *C. jejuni* and 17 out of 18 *C. coli* strains were further genotyped by PFGE, while the remaining six strains were lost during processing. These strains were clustered into 21 PFGE types (Figure 1A). Among the 19 *C. jejuni* pulsotypes identified, 8 were isolated in autumn (A, C, K, S, I, E, G and R) and 11 in spring (D, H, J, L, M, N, P, F, B, Q and O). Only two *C. coli* pulsotypes appeared; pulsotype T in farm IV and pulsotype U in farm IX, both in autumn. Table 6 shows the number and distribution of the different pulsotypes found along the two sampling seasons. From the total of positives farms (10), the majority of them (9) harbored between 1 and 3 pulsotypes and the other farm (VI) showed 7 different pulsotypes in spring. Only in two farms pulsotypes from both species, *C. jejuni* and *C. coli*, were isolated (table 6). When *Kpn*I enzyme was used, *C. jejuni* strains were clustered in 16 pulsotypes being H, I and J clustered together (cluster 1) as well as pulsotypes L and M (cluster 5) (Figure 1B).

No relationship between pulsotypes corresponding to the two seasons analyzed was found, showing different patterns in autumn and in spring (table 6). However, during autumn, pulsotypes C and E appeared in several farms. Pulsotype C was identified in farm V (in cloacal samples, feeder and nipple´s tray) and in farm XIV (cloacal samples). This farm also shared pulsotype E with farm IX in cloacal samples as well. Moreover, this pulsotype was identified as the single one in farm IX in all sampling location except water from nipple's tray and tank. All different *C. jejuni* and *C. coli* pulsotypes found in the 10 positive farms in both seasons were isolated in cloacal swabs samples, with the exception of pulsotype P that was only isolated from nipple´s tray in farm VI in spring. In eight of these positive farms the pulsotypes isolated from the cloaca of birds were also isolated from environmental samples, mainly from feeder and nipple's trays. Only pulsotype R was isolated also from farm main water source.

All *C. jejuni* pulsotypes were positive for the genes *flaA*, *cadF*, *cdtA*, *cdtB* and *cdtC*. However, both *C. coli* pulsotypes were negative for *cdtA* and *cdtC* genes. Additionally, *C. jejuni* pulsotypes were resistant to ciprofloxacin, nalidixic acid and tetracycline, with the exception of pulsotype F that was sensitive to all antibiotics except tetracycline. The two pulsotypes of *C. coli* (T and U) showed higher resistance than *C. jejuni*. They have been classified as multi-resistant because at least, they were resistant to four different classes of antibiotics. Pulsotype U was resistant to ciprofloxacin, nalidixic acid, tetracycline and erythromycin, whereas pulsotype T was resistant to ciprofloxacin, nalidixic, acid tetracycline and azithromycin.

Table 4. *Campylobacter* spp. PFGE types and (number) in positive farms in each sampling location (obtained with *Sma*I restriction enzyme)

Season	**Autumn 2014**						**Spring 2015**			
Sampling location	**Farm IV**	**Farm V**	**Farm VI**	**Farm IX**	**Farm XIII**	**Farm XIV**	**Farm VI**	**Farm VII**	**Farm IX**	**Farm X**
Cloacal samples	A (11) *T (10)*	C (12) K (10) S (8)	I (28)	E (28) *U (7)*	R (27)	E (8) C (15) G (2)	D (2); H (18) J (1); L (3) M (1); N (1)	F (26)	B (14) Q (5)	O (21)
Feeder		C (1) K (2) S (1)	I (5)	E (3) *U (1)*	R (5)		H (4) J (1)	F (3)	B (2) Q (1)	O (2)
Water from nipples					R (1)					
Nipple´s tray		C (2) K (1)	I (1)	E (2) *U (1)*	R (3)		J (2) P (1)	F (3)	B (1)	O (1)
Water Tank					R (1)					
Total PFGE types	**2**	**3**	**1**	**2**	**1**	**3**	**7**	**1**	**2**	**1**

In italics *C. coli* pulsotypes

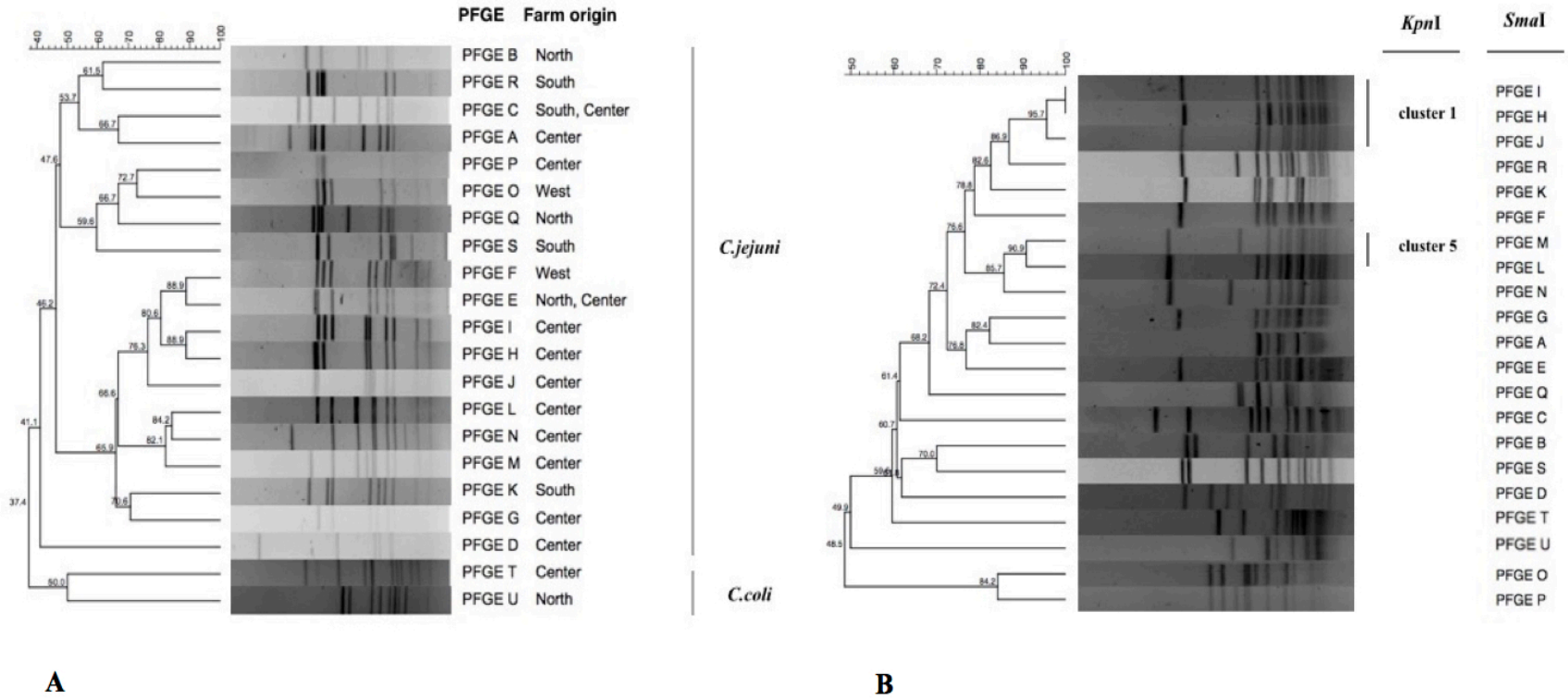

Figure 1. Dendogram of different *C. jejuni* and *C. coli* PFGE profile with *Sma*I and *Kpn*I enzyme. Figure 1A shows the different PFGE profile with *Sma*I enzyme restriction and their relation with farms origins. Figure 1B shows that PFGE I, PFGE H and PFGE J (*Sma*I) were gathered into cluster 1 as well as PFGE M and PFGE L (*Sma*I) were gathered into cluster with *Kpn*I restriction.

Data obtained from the questionnaire completed at the farms that have participated in this study are shown in table 2. In general, few or even a lack of biosecurity measures in the farms has been observed. None of the 14 farms had cleaning footbath neither disinfection systems for vehicle wheels. Half of them had dogs or/and cats wandering around the farm and all farms disinfected water supply with chlorine (8 farms) or hydrogen peroxide plus pH corrector (6 farms). Although most of the farms had new buildings, only 9 farms out of 14 had a well-established system to prevent the entrance of rodents, and half of them a hygienic ante-room to access the poultry houses and around 50 % of them used wood shavings and the other straw as litter material. On average, farms participating in the study have more than 2 poultry houses and produced between 20,000 and 40,000 birds per flock. None of the farms practiced thinning during birds growing cycle. More details related to any particular farm are shown in table 2. Interestingly, several biosecurity measures have shown effective to reduce *Campylobacter* contamination such as control of rodents and anteroom before entering to the poultry shed, as it is evident by the high odds ratios obtained in the statistical analysis when they were absent (Table 3). On the other hand, disinfection of water supply by chlorine implies a higher probability of contamination by *Campylobacter*, as well as the litter material, favoring the straw the presence of *Campylobacter*. However, the presence of animals as dogs and cats in the farm has not influence. Moreover, the numbers of flocks and broilers per farm have not a clear effect (Table 3).

4. Discussion

Recently, EU has amended Regulation 2073/2005 by including a maximum contamination level of *Campylobacter* spp. in broiler carcasses of 1,000 cfu/g at slaughter (European Regulation 1495/2017). EFSA estimates that a public health risk reduction from the consumption of broiler meat of more than 50 % could be achieved, if carcasses

complied with this limit. However, the same regulation states that in case of unsatisfactory results, one of the actions to take is the improvement in biosecurity measures in the farms of origin. This is why, it is still important to collect data about the prevalence and diversity of *Campylobacter* spp. in different geographical and seasons at farm level, in order to obtain a wider scope of the contamination risk factors. These studies are relevant because reducing the prevalence and concentration of this microorganism in the intestinal tract would contribute greater to public health benefits than later interventions in the broiler food chain (Battersby et al., 2016; Marotta et al., 2015).

Campylobacter positive farms in EU remains still really high: 74.21 % in Hungary, 69.35 % in Slovenia, 66.67 % in Luxembourg and 62.17 % in Spain (EFSA, 2015; Torralbo et al., 2014). Although the proportion of positive farms in this study was slightly lower than 40 %, prevalence within-flocks varies from 43.2 % to 88.6 %. In Europe, this prevalence varies from 18 to 90 %, with the north European countries having substantially lower prevalence than southern countries (EFSA, 2014). In this work, the bird's slaughter age (28-32 days) might be related with the high prevalence found within-flocks, since is the period when the highest *Campylobacter* colonization rate occurred at farms according to Zhang et al. (2016). *C. jejuni* was the species more frequently found, appearing in all positive sampled farms, whereas *C. coli* only appeared in two farms in autumn; this fact has been corroborated by other authors (Hald et al., 2001; Näther et al., 2009; Sasaki et al., 2011; Torralbo et al., 2014).

Although, many studies have been conducted regarding identification of the risk factors, for flock colonization, by *Campylobacter* species, no clear conclusions have been reached. Adkin et al. (2006) made a classification, based on an extensive literature, in 14 sources of on-farm contamination and 37 contributing factors. Among the risk

contributing factors seasonal variation has been extensively studied due to higher incidence in human campylobacteriosis during summer, when a higher incidence in poultry colonization happened (McDowell et al., 2008; Meunier et al., 2016). In this study, a significant difference in positive percentage and prevalence within-flock has been observed between the autumn and spring, being higher in autumn. The number of positive farms was higher in autumn (6) than in spring (4) and only two farms were positive in both seasons. Regarding the number of positive samples within-flock was also higher in autumn (31.3 %) than in spring (20.3 %) showing a clear influence of the season in the prevalence of *Campylobacter* spp. at farm level (Table 3). These results agree with previous studies, which have also found a clear correlation between season and *Campylobacter* infection in broiler flocks. The majority of these studies found a peak in the prevalence during summer and autumn and a lower prevalence during spring and winter (Ellis-Iversen et al., 2012; Jorgensen et al., 2011).

Geographical location also has shown a significant effect in the prevalence of *Campylobacter* spp. being the farms located in the South part of the region, the ones with higher prevalence; probably this difference is due to the temperature, which is higher in the South than in the rest of the region. Similar results were observed in a large study conducted in Great Britain (Jorgensen et al., 2011). These authors found that temperature explained about half of the *Campylobacter* prevalence; the rest can likely be explained by other factors, related with farm management.

All positive farms in the study (10) have shown a higher percentage of *Campylobacter* spp. in cloacal than in environmental samples (8). Moreover, in farms IV and XIV (autumn period) environmental samples were negative although cloacal samples were positive. Similar situations have also been described by other authors (Bull et al., 2008; Newell et al., 2011; Thakur et al., 2013). Results of farm XIII suggest that water

supply from farm wells can act as a primary source of campylobacter contamination. In that case, water seems to have infected the nipples and birds might be infected by drinking water from nipples, since a single pulsotype was found in all sampling locations and also in birds.

The epidemiology of *Campylobacter* spp. is complicated by its genetic variability or diversity. Although *C. jejuni* has a relatively small genome, it carries significant levels of variation, potentially indicative of evolution leading to niche specialization (Bronowski et al., 2014). Our study revealed a high diversity of genotypes (21 pulsotypes) among 14 farms sampled, being the heterogeneity quite similar in both seasons, with 11 pulsotypes in spring compared to 8 pulsotypes in autumn; however, the pulsotypes found in spring and autumn were different. Jonsson et al. (2012) reported a protective effect among farms surrounding distance with neighboring farms of more than 4 km and to other livestock in more than 2 km. However, in this study, farm XIV share pulsotype C with farm V and pulsotype E with farm IX, although these farms belong to different geographical area with a distance between them around 60 km.

The diversity of pulsotypes among the farms contrasts with the little diversity of pulsotypes that can be found in the same flock. Mainly, in each farm one to three different profiles were determined, showing flock specific genotypes. Several authors have described a similar situation in different studies (Jorgensen et al., 2011; Schallegger et al., 2016). Only two farms (VI and IX) were *Campylobacter* positive in both seasons. However, farm VI showed a single pulsotype in autumn (I), whereas in spring seven different pulsotypes were found. Some of these pulsotypes (H and J) are located side by side to pulsotype I in the cluster analysis with the enzyme *Sma*I and classified all together with the enzyme *Kpn*I (cluster I). More data are needed to clarify the genetic proximity of those pulsotypes.

In the majority of farms, genotyping pattern identified from cloacal samples were also recovered from environmental samples; except pulsotype P in farm VI (spring period) which only was recovered from nipple´s tray. Those data support the main role of environment in the horizontal transmission of *Campylobacter* within the flock (Smith et al., 2016). Moreover, Farm XIII was the only in which the same *C. jejuni* pulsotype was isolated in water supply and in the rest of analyzed samples, as mentioned above. These data suggest that water supply can contaminate the flock. Similar results were found by Sasaki et al. (2011) in Japan and supported by other authors that described the survival of *Campylobacter* in water (Buswell et al., 1998; Nicholson el al., 2005; Zhang et al., 2016).

Factors which reflect the level of biosecurity (e.g. adopting rodent control around the broiler house, change of footwear and clothes before entering the houses or improvement of the hygiene barriers, adoption of the thinning practice and the slaughter age), have been identified frequently as contributing risk factors for campylobacter colonization in broilers (Allain et al., 2014). It is well known that horizontal transmission occurs, but how the microorganism enters to farms or how long *Campylobacter* spp. could survived in the farm environment is still unknown. In order to obtain a better idea about the influence of farm characteristics on the presence of *Campylobacter*, a questionnaire was filled in the farms during each visit. Interestingly, the six *Campylobacter* negative farms in both seasons have a well-established rodent control, an anteroom before entrance to the shed and new broiler houses. On the contrary, all *Campylobacter* positive farms showed the lacking of the same biosecurity measures; such as, no existence of anteroom to access in the shed, no disinfection footpath at the entrance, no system to prevent the entrance of rodents or insects, having pet animals (dogs/cats) and more than one shed in the farm. Ellis-Iversen et al. (2012) found that these factors, specially shed entrance and

the presence of others animals increased the likelihood of *Campylobacter* positive flocks. Moreover, the presence of dogs and cats on the farm has been described as possible source of *Campylobacter* species, especially *C. jejuni* (Carbonero et al., 2012). Although the relatively small number of farms should be considered, in this work the existence of anteroom to access in the shed and system to prevent the entrance of rodents has been proved as effective measures for the reduction of *Campylobacter* spp. , however the presence of dogs and cats on the farm has not influenced on *Campylobacter* prevalence.

The analysis of the virulence genes and antimicrobial resistance combined with PFGE clusters was applied for a better characterization of the isolates. This study has shown that *C. jejuni* PFGE types have more virulence-associated genes than *C. coli,* as Zhang et al. (2016b) have previously described. Our results showed that the flagella gene (*flaA*) and fibronectin-binding protein encoded by *cadF* were detected in all isolates tested of both species, in agreement with other authors (Sen et al., 2018; Wieczorek et al., 2015). Moreover, all *C. jejuni* PFGE types tested presented *cdtA*, *cdtB* and *cdtC* genes, whereas in *C. coli* PFGE types (T and U) the *cdtA* and *cdtC* genes were absent. Presence of the three *Cdt* genes in *C. jejuni* species has been reported (Mendez-Olvera et al., 2016). In several works, a higher prevalence of *cdt* genes has been found in *C. jejuni* than in *C. coli* (Bardon et al., 2017; Di Giannatale et al., 2014;), being its presence associated with the severity of the disease (Ghorbanalizadgan et al., 2014; Zhang et al., 2016).

High antibiotic resistance rates were observed in this study. *C. jejuni* pulsotypes were resistant to three antibiotics; quinolones (CIP, NAL) and tetracycline (TET). It was also reported by other authors, although the percentages of resistant strains varied from 4.1 % in Turkey, 36.9 % in Poland or 100 % in USA (Abay et al., 2014; Wieczorek et al., 2015; Zhao et al., 2010). *C. coli* showed higher antibiotic resistance; especially pulsotype

T that was multi-resistant. The ability of *C. coli* to exhibit higher antimicrobial resistance than *C. jejuni* observed in the current study was also reported previously (EFSA, 2015).

5. Conclusion

Although there are many studies about *Campylobacter* colonization in farms, the pathways by which poultry flocks acquire *Campylobacter* are not fully understood yet. This study has shown that season and geographical location have an influence on prevalence and diversity of *Campylobacter,* showing a high diversity of pulsotypes among farms. The consistency and the genetic relation among these patterns within-flocks suggest that environment might be one of the main contributing factors of *Campylobacter* transmission; and water supply a possible primary source. The fact, that very close pulsotypes appear in the same farm in different seasons, suggests that survival of *Campylobacter* in the environment could be related to its genetic variability and adaptability. Further studies are required to gather data that support this hypothesis. Virulence analysis has shown that some genes (*cdtA* and *cdtC*) encoding the cytolethal distending toxin were absented in *C. coli*. This fact might explain why campylobacteriosis caused by *C. jejuni* is higher than *C. coli* in human infections. Moreover, C. *coli* exhibited multiresistance to four antimicrobial classes. Poultry farms play a key role in the epidemiology of human *Campylobacter* infection. This work has highlighted that, in general, the broilers farms studied have insufficient biosecurity measures. Additionally, it has shown that some of the analyzed factors, such as the absence of entrance room, systems to prevent the entrance of rodents or insects, type of litter material, system of water disinfection might favor the presence of *Campylobacter*. Therefore, the implementation of farm biosecurity measures might reduce the contamination in subsequent production steps; thereby, decreasing the presence of this pathogen in the chicken food chain, avoiding human infections.

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CHAPTER 2

V. CHAPTER 2. *Campylobacter jejuni* survival in a poultry processing plant

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Abstract

Campylobacteriosis is the most common cause of bacterial gastroenteritis worldwide. Consumption of poultry, especially chicken's meat is considered the most common route for human infection. The aim of this study was to determine if *Campylobacter* spp. might persist in the poultry plant environment before and after cleaning and disinfection procedures and the distribution and their genetic relatedness. During one month from a poultry plant were analyzed a total of 494 samples -defeathering machine, evisceration machine, floor, sink, conveyor belt, shackles and broiler meat- in order to isolate *C. jejuni* and *C. coli*. Results showed that *C. jejuni* and *C. coli* prevalence was 94.5 % and 5.5 % respectively. Different typing techniques as PFGE, MLST established seven C. jejuni genotypes. Whole genome MLST strongly suggest that highly clonal populations of *C. jejuni* can survive in adverse environmental conditions, even after cleaning and disinfection, and persist for longer periods than previous thought (at least 21 days) in the poultry plant environment. Even so, it might act as a source of contamination independently of the contamination level of the flock entering the slaughter line.

1. Introduction

Campylobacteriosis is the most common cause of bacterial gastroenteritis in developed countries with EU-wide notification rate in 2014 of 60 cases every 100,000 persons and an estimated cost to public health systems of ~ EUR 2.4 billion a year (EFSA, 2014). Human campylobacteriosis is a self-limiting gastroenteritis that lasts for approximately 5–7 days and it is characterized by watery and sometimes bloody diarrhea, fever, abdominal cramps and vomiting (Skarp et al., 2016). Severe post-infection complications such as; reactive arthritis, bacteremia and Guillian-Barré syndrome might occur (Theoret et al., 2012).

Campylobacter jejuni and *Campylobacter coli* are responsible around 90 % of all campylobacteriosis diagnosed in humans in EU (Bolton, 2015; EFSA, 2014) and they are commonly found in the gastrointestinal tract of several animal species (Epps et al., 2013). Epidemiological studies have reported chickens are the main reservoir of human campylobacteriosis. The consumption of broiler meat is considered the highest common route of human infection (Oh et al., 2015; Prachantasena et al., 2016; Young et al., 2007). *C. jejuni* and *C. coli* are fastidious microorganisms being unable to grow at temperatures below 30 °C, neither tolerating desiccation nor atmospheric levels of oxygen. They are susceptible to various environmental and food processing-induced stressors such as osmotic stress (Bronowski et al., 2014), cooking temperatures (Wanyenya et al., 2004) and different disinfectants (Gutiérrez-Martín et al., 2011). Nevertheless, both species are widely spread in the environment and can be readily isolated from contaminated food, water and other sources (Teh et al., 2014; Torralbo et al., 2014).

Once *Campylobacter* is introduced in the slaughter line, it can spread to the poultry meat, especially in the defeathering and evisceration steps (Berrang et al., 2001; Melero et al., 2012). *Campylobacter* spp. could be recovered during slaughter, in

processing equipment and in the plant environment (Berndtson et al., 1996; Berrang et al., 2000; Cason et al., 2007; Ellerbroek et al., 2010). Therefore, *Campylobacter* genotypes found in the farm flock can efficiently survive and persist throughout the poultry food chain till the retail shops (Damjanova et al., 2011; Gruntar et al., 2015; Melero et al., 2012). Possible persistence of *Campylobacter* populations in the plant environment and their role as a continuous source of contamination is still largely unknown. Environmental persistence of *Campylobacter* in the slaughterhouse appears to be ephemeral. Poultry meat from broiler flocks otherwise negative may be contaminated if the previously slaughtered flock was positive (Allen et al., 2007; Miwa et al., 2003). However, it has been shown that negative flocks were contaminated by strains that did not generally originate from the predominating strains recovered from the ceca of the previous positive flocks (Elvers et al., 2011). Moreover, survival of *C. jejuni* overnight on food processing equipment surfaces, after cleaning and disinfection procedures, has been reported (Melero et al., 2012; Peyrat et al., 2008b). These data suggest that some strains of *Campylobacter* might survive longer in the slaughterhouse environment, and they can contaminate carcasses in several batches during the slaughter process.

Pulsed field gel electrophoresis (PFGE) has been widely used for *Campylobacter* typing (Hänninen et al., 2000; Melero et al., 2012). However, PFGE profile cannot conclusively establish the clonal relationship between isolates (Revez et al., 2014a). On the contrary, whole-genome MLST (wgMLST) has revealed as a powerful tool to resolve the relationship of especially closely related bacterial isolates by indexing allele differences in the shared loci (Kovanen et al., 2014; Revez et al., 2014a; Zhang et al., 2015), and it has been successfully applied in analyzing point-source and diffuse *Campylobacter* outbreaks (Kovanen et al., 2016; Revez et al., 2014a, Revez et al., 2014b).

The aims of this study were: (i) investigating the persistence of *Campylobacter jejuni* in slaughter and poultry processing environment, including food contact surfaces (FCS), non-contact surfaces (NFCS) before and after routine cleaning and disinfection procedures as well as in broiler's meat; and (ii) defining the population structure of the *Campylobacter jejuni* population isolated from different slaughterhouse and processing sampling locations and times.

2. Material and Methods

2.1. Sampling procedure

A poultry slaughterhouse and processing plant in the North of Spain was studied for 4 weeks, from 2nd June to 27th June 2014. During this period, seven different farms sent the broilers to the slaughterhouse. The distance among farms varies from 4 to 125 km. Broilers were transported to slaughterhouse by crates, the previous night that they were slaughtered. Each sampling day, an average of 25,000 broilers was slaughtered from two different farms.

The slaughterhouse was visited alternately on 11 occasions during the studied period. Visits were conducted at the time of slaughter during the last part of the slaughter shift. In each visit, samples from the same sampling locations were taken before and after cleaning and disinfection. Equipment was cleaned with sodium hydroxide and potassium hydroxide and disinfected with a quaternary ammonium compound using doses and concentrations according to the supplier recommendations.

The sampling procedure was divided into two areas, taking account the ongoing activity: (1) slaughterhouse and (2) processing. Samples were classified as: food contact surfaces (FCS), non-food contact surfaces (NFCS) and broiler meat (F). During the visits, samples from the different sampling locations were collected every sampling day (Table 1). Half of those samples were taken afterslaughtering, when surfaces were dirty,

the another half in the following 30 min after cleaning and disinfection. As broiler meat samples, one broiler thigh was taken in the conveyor belt at the end of processing line before their distribution into the market. Carcasses of the processing area belong to flocks slaughtered the day before.

Table 1. Sampling locations and sampling procedure used in this study.

Sampling Location	Sanitation status (n)*	Type of sample	Sampling method
Slaughterhouse			
Defeathering	Dirty (n=5)	Surface (Fingers)	Sponge
*(FCS)***	Clean (n=5)	Surface (Fingers)	Sponge
Evisceration	Dirty (n=5)	Inner Surface	Sponge
(FCS)	Clean (n=5)	Inner Surface	Sponge
Sink	Dirty (n=2)	Surface (100 cm^2)	Sponge
*(NFCS)****	Clean(n=2)	Surface (100 cm^2)	Sponge
Floor	Dirty(n=2)	Surface (1000 cm^2)	Wiping towel
(NFCS)	Clean (n=2)	Surface (1000 cm^2)	Wiping towel
Processing			
Shackles	Dirty (n=5)	One shackle	Sponge
(FCS)	Clean (n=5)	One shacke	Sponge
Conveyor belt	Dirty (n=3)	Surface (900 cm^2)	Sponge
(FCS)	Clean (n=3)	Surface (900 cm^2)	Sponge
Sink	Dirty (n=2)	Surface (100 cm^2)	Sponge
(NFCS)	Clean (n=2)	Surface (100 cm^2)	Sponge
Floor	Dirty (n=1)	Surface (1000 cm^2)	Wiping towel
(NFCS)	Clean (n=1)	Surface (1000 cm^2)	Wiping towel
Broiler meat			
Thigh	(n=1)	Meat and Skin (10g)	Sterile bag

n* number of samples taken in each sampling location.
FCS**:Food Contact Surfaces; NFCS ***: Non Food Contact Surfaces.

Samples from FCS and NFCS on dirty surfaces were taken using sterile sponges (3M™, Saint Paul, Minnesota, USA) except floor samples that were collected with sterile wiping towel, both were soaked with ringer solution sterile (Oxoid). Additionally, samples from clean surfaces were taken using commercial sterile pre-moistened sponges with neutralizing buffer (water and buffer salts) (3M™). Broiler thighs were taken entire and placed in a sterile bag. All samples were kept at 4 °C for less than 3 h before further analysis in the lab.

2.2. Isolation and identification of *Campylobacter* spp.

Samples were analyzed using the standard procedure (ISO 10272-1:2006), but using Preston broth instead of Bolton broth in the enrichment step (Melero et al., 2012). Each sponge and wiping towel were submerged individually in 90 mL of Preston broth made with Nutrient Broth Nº 2 (Oxoid, Basingstoke, England) supplemented with Preston *Campylobacter* Selective Supplement (Oxoid) and Campylobacter Growth Supplement Liquid (Oxoid). In the case of food samples (chicken thighs), 10 g were taken aseptically and placed in sterile stomacher bags and homogenize with 90 mL of sterile Preston broth supplemented as described above. All samples were homogenized for 120 s and incubated microaerobically using a commercial gas-generating systems-CampyGen (Oxoid) and sealed jar at 41.5 °C for 48 h. After enrichment for 48 h, a loop-full from each sample was streaked on a plate of modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) prepared with *Campylobacter* blood-free selective agar base (Oxoid) supplemented with CCDA selective supplement (Oxoid). Plates were incubated as described above for enrichment broths. From each plate, two typical isolated *Campylobacter* spp colonies were randomly selected for further analysis.

Isolated colonies from the mCCDA agar were grown on 5 mL of Brain Heart Infusion broth (Oxoid) for an overnight. DNA was extracted as previously described by

Yamada et al. (2015). Briefly, strains were suspended in 100 µL of Tris-EDTA buffer (pH 8.0) and incubated at 95 °C for 10 min, and centrifuged at 16,000×*g* for 1 min. The supernatants were subsequently used as templates for PCR. All isolates were identified by Real-Time PCR following the procedure described by Hong et al. (2007) and modified by Schnider et al. (2010) to identify two target genes: *hipO* and *ceuE*. Briefly, denaturation step was conducted at 95 °C for 10 min and annealing step consisted in 40 cycles at 95 °C for 15 s and 60 °C 1 min. Primers and probes are shown in Table 2. The reactions were performed in StepOneTM (Applied Biosystems, Foster City, California, USA), and the data were analyzed by StepOneTM 2.0 Software (Applied Biosystems). As control, CECT 7572 *C. jejuni* and CECT 7571 *C. coli* were used. Confirmed *C. jejuni* and *C. coli* strains were stored for further typing at -80 °C in Brain Heart Infusion broth containing 20 % of glycerol.

Table 2. Sequence of primers and probes used to differentiate *C. jejuni* and *C. coli.*

Target species	Primer & probe	Sequence
C. jejuni	*hipO-F*	5' CTGCTTCTTACTTGTTGTGGCTTT-3'
	hipO-R	5' GCTCCTATGCTTACAACTGCTGAAT-3'
	hipO-P	5'-FAM-CATTGCGAGATACTACTATGCTTT-MGBNFQ-3'
C. coli	*ceuE-F*	5'-GATAAAGTTGCAGGAGTTCCAGCTA-3'
	ceuE-R	5'-AACTCCACCTATACTAGGCTTGTCT-3'
	ceuE-P	5'-VIC-CTGTAAGTATTTTGGCAAGTTT-MGBNFQ-3'

According to Hong et al. (2007)

2.3. Pulsed field gel electrophoresis (PFGE)

C. jejuni isolates were cultured on Columbia agar (Oxoid) supplemented with 5% defibrinated sheep blood (Oxoid) below microaerobic conditions (24 h at 41.5 °C) for the

purpose of PFGE genotyping. PFGE analysis, applying the restriction enzymes *Sma*I and *Kpn*I was performed according to the PulseNet International standardized protocol (www.cdc.gov/pulsenet/pathogens/pfge.html). *Kpn*I was used to check the diversity of all isolates with similar *Sma*I genotype.

Restricted DNA was electrophoresed for 22.5 h on 1 % (w/v) SeaKem gold agarose in 0.5 x TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.). The electrophoresis conditions used to consist of an initial switch time of 5 s and a final switch time of 55 s (gradient of 6 V/cm and an included angle 120°). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories). BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity, analysis was carried out using the Dice coefficient (position tolerance, 1.0 %). The unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns. Isolates with <90 % similarity according to the dendrogram were clustered as separate genotypes (Boer et al., 2000).

2.4. Whole genome MLST (wgMLST)

DNA was extracted from *C. jejuni* isolates using PureLink Genomic DNA kit (Invitrogen, Carlsbad, California, USA). Quality assurance of the DNA was performed with Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, U.S.A.) concentration was measure with Qubit fluorometer (Life Techologogies; Invitrogen). Library preparation and sequencing was performed by FIMM (Institute for Molecular Medicine. Helsinki, Finland) using Nextera XT library preparation kits and HiSeq2000 platform in HT mode (indexed paired-end 2 × 101bp), respectively (Illumina Inc., San Diego, US). The reads were filtered and assembled using SPAdes 3.2.0 with default settings using the MismatchCorrector function (Bankevich et al., 2012). Assembled

contings were automatically annotated using RAST (http://rast.nmpdr.org/) (Aziz et al., 2008).

MLST database (pubmlst.org/campylobacter) was used to assigned sequence type (STs) and Clonal Complex (CCs) to the different genomes. Ad hoc wgMLST analysis was performed using Genome profile (GeP; Zhang et al., 2015) using default setting.

2.5. Statistical analysis

An analysis for categorical data was applied. After cross-tabulation of categorical data (sampling location: environmental surface and food versus pulsotypes) to obtain the frequency table, a test of independence was done. Chi-square test (P-value <0.05) determines if the different parameters are independent at the 95.0 % confidence level.

3. Results

A total of 238 *Campylobacter* isolates were obtained from 494 samples (48.2 %): 94.5 % were identified as *C. jejuni* whereas 5.5 % were identified as *C. coli* (Table 3). The statistical analysis shows that there is a statistically significant association ($P < 0.05$) between a particular area of the slaughterhouse and processing plant and the frequency of *C. jejuni* isolates. As it was expected the slaughterhouse was the most contaminated area with 153 isolates out of 238 (64.3 %) in comparison with the processing area where only 75 isolates (31.5 %) were obtained. In addition, 10 of the eleven broiler's thighs sampled were also *C. jejuni* positives representing 4.2 % of the total isolates. FCS of both, slaughterhouse and processing, were the most contaminated areas, together with the broiler's thighs (Table 3). It was in the defeathering machine where more isolates from *C. coli* were found 9 out of 13 (69.2 %).

Table 3. *Campylobacter* positives samples in each sampling location before and after cleaning and disinfection along the studied period (11 visits).

Sampling Location	Sanitation status	Campy*/Total (%)	*C jejuni*/Total positives (%)	*C coli*/Total positives (%)
Slaugtherhouse				
Defeathering	Dirty (n=5)	30/50 (60)	24/30 (80)	6/30 (20)
*(FCS)***	Clean (n=5)	30/55 (54.5)	27/30 (90)	3/30 (10)
Evisceration	Dirty (n=5)	39/50 (78)	39/39 (100)	0/39 (0)
(FCS)	Clean (n=5)	31/55 (56.4)	31/31 (100)	0/31 (0)
Sink	Dirty (n=2)	10/10 (100)	10/10 (100)	0/10 (0)
*(NFCS)****	Clean (n=2)	2/10 (20)	1/2 (50)	1/2 (50)
Floor	Dirty (n=2)	5/22 (22.7)	5/5 (100)	0/5(0)
(NFCS)	Clean (n=2)	6/20 (30)	5/6 (83.3)	1/6 (16.7)
Processing				
Clamps	Dirty (n=5)	23/55 (41.8)	23/23 (100)	0/23 (0)
(FCS)	Clean (n=5)	19/50 (38)	18/19 (94.7)	1/19 (5.3)
Conveyor belt	Dirty (n=3)	29/33 (87.9)	28/29 (96.6)	1/29 (3.4)
(FCS)	Clean (n=3)	1/30 (3.3)	1/1 (100)	0/1 (0)
Sink	Dirty (n=2)	0/12 (0)	0/0 (0)	0/0 (0)
(NFCS)	Clean (n=2)	0/12 (0)	0/0(0)	0/0 (0)
Floor	Dirty (n=1)	2/11 (9.1)	2/2 (100)	0/2 (0)
(NFCS)	Clean (n=1)	1/10 (10)	1/1 (100)	0/1 (0)
Broiler meat				
Thigh	(n=1)	10/11(90.9)	10/10 (100)	0/10 (0)
TOTAL (%)		238/494 (48.2)	225/238 (94.5)	13/238 (5.5)

Campy*: Total *Campylobacter* spp.
FCS**: Food Contact Surfaces; NFCS***: Non Food Contact Surfaces.

In the processing area, conveyor belt was the most contaminated step with 29 positives out of 33 samples taken during the working shift. In all NFCS sampling locations, the

number of *C. jejuni* positive samples was lower, with the exception of the sink located in the slaughterhouse, which was contaminated every day during the studied period.

A total of 90 *Campylobacter* isolates (37.8 %) were obtained after cleaning and disinfection (C&D) (Table 3). Again slaughterhouse was the more contaminated area with 69 isolates out of 90 and from those, 61 were isolated in the defeathering and evisceration machines. The slaughterhouse floor also remains contaminated after C&D, even with higher number of positive samples than during the working shift. In the processing area, shackles were the most contaminate sampling location after C&D with 19 positive out of 50 samples taken.

Table 4 shows the distribution of the different *Campylobacter* spp. isolates day per day along the studied period, for each sampling location before and after C&D. In all sampled days C*ampylobacter* spp. were found in multiple sampling locations showing a high expanded contamination all around the poultry plant. The majority of the sampling locations a high number of isolates were also obtained after C&D, with the exception of the conveyor belt, although it has showed a high contamination rate during the working shift, only one isolate was recovered after C&D (on day Thursday 19th). The number of *Campylobacter* isolated per day was very high and the prevalence, in the both areas, ranged from 23.4 % on Wednesday 11th to 70.2 % on Wednesday 18th.

From 238 isolated strains of *Campylobacter* spp., only 222 out of 225 corresponding to *C .jejuni* were further typified by PFGE. These were clustered into 7 different PFGE types with both restriction enzymes (Fig. 1). Among the pulsotypes identified, 140 isolates belong to pulsotype G (63.1 %) followed by 51 isolates from pulsotype C (23 %) and 18, 7, 3, 2 and 1 isolates from pulsotypes A, E, F, B and D

Tabla 4. *Campylobacter* positive samples in each sampling location along the studied period (*C. jejuni*/*C. coli*).

			Week 1		*Week 2*			*Week 3*		*Week 4*		
Sample	**Status**	**2M**	**5TH**	**6F**	**9M**	**10TU**	**11W**	**18W**	**19TH**	**23M**	**25W**	**27F**
Defeathering	Dirty (n=5)	4/-[a]	4/-	4/-	4/-	(nd)	-/3	3/-	2/-	2/-	-/3	1/-
(FCS)	Clean (n=5)	4/-	2/-	2/-	3/-	3/-	1/1	5/-	3/-	2/-	1/2	1/-
Evisceration	Dirty (n=5)	4/-	3/-	4/-	5/-	(nd)	3/-	5/-	5/-	2/-	5/-	3/-
(FCS)	Clean (n=5)	5/-	1/-	2/-	2/-	2/-	-/-	4/-	3/-	5/-	3/-	4/-
Sink	Dirty (n=2)	(nd)	(nd)	(nd)	(nd)	(nd)	(nd)	1/1	2/-	2/-	2/-	2/-
(NFCS)	Clean (n=2)	(nd)	(nd)	(nd)	(nd)	(nd)	(nd)	2/-	-/-	-/-	-/-	-/-
Floor	Dirty (n=2)	1/-	-/-	1/-	1/-	2/-	-/-	-/-	-/-	-/-	-/-	-/-
(NFCS)	Clean (n=2)	1/-	-/-	(nd)	-/-	2/-	-/1	1/-	1/-	-/-	-/-	-/-
Clamps	Dirty (n=5)	-/-	1/-	-/-	-/-	1/-	-/-	3/-	5/-	4/-	4/-	5/-
(FCS)	Clean (n=5)	-/-	2/-	(nd)	5/-	2/-	-/-	3/-	4/-	-/-	1/1	1/-
Conveyor belt	Dirty (n=3)	-/-	2/1	2/-	3/-	3/-	1/-	3/-	3/-	3/-	3/-	3/-
(FCS)	Clean (n=3)	-/-	-/-	(nd)	-/-	-/-	-/-	-/-	1/-	-/-	-/-	-/-
Sink	Dirty (n=2)	-/-	-/-	-/-	-/-	-/-	-/-	(nd)	(nd)	(nd)	(nd)	(nd)
(NFCS)	Clean (n=2)	-/-	-/-	(nd)	-/-	-/-	-/-	(nd)	(nd)	(nd)	(nd)	(nd)
Floor	Dirty (n=1)	-/-	-/-	-/-	-/-	-/-	-/-	1/-	-/-	-/-	-/-	-/-
(NFCS)	Clean (n=1)	-/-	-/-	(nd)	1/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Thigh	(n=1)	1/-	1/-	-/-	1/-	1/-	1/-	1/-	1/-	1/-	1/-	1/-
TOTAL[b]		22/ 47	17/ 47	15/ 34	25/ 47	16/ 37	11/ 47	33/ 47	30/ 47	22/ 47	26/ 47	21/ 47
TOTAL (%)		46.8	36.2	44.1	53.2	43.2	23.4	70.2	63.8	46.8	51.3	44.7

Slaughterhouse | **Processing**

M: Monday, TU: Tuesday, W:Wednesday, TH: Thursday, F: Friday. Numbers before day letter corresponded to the calendar day 2=2nd; 5=5th, etc. (nd): not determined. FCS: Food Contact Surfaces; NFC: Non Food Contact Surfaces.
[a] Number of positives *C. jejuni*/Number of positive *C. coli* (-): No *C. jejuni* or *C.coli* detected in this sampling location.
[b] *Campylobacter* positive/number of samples.

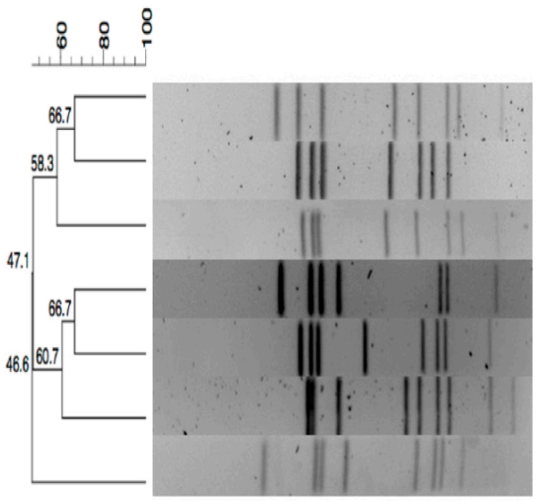
respectively. Table 5 shows the number and distribution of the different pulsotypes found along the sampling period and the slaughter order of the different flocks coming from the different farms. Four pulsotypes G, C, A, and E were isolated at least during more than one day. PFGE type G was the most abundant; it was recovered in almost all sampling locations in the slaughterhouse and processing plant, including broiler's thighs, at least along 17 days. A similar situation happens with PFGE type C that was isolated at least during 21 days. Other minority PFGE types were also present in the last nine sampling days (Table 4).

A total of 45 strains, 33 from pulsotype G, 8 from pulsotype C and 1 from each of the other pulsotypes, were whole sequenced for further analysis by MLST and wgMLST. Each PFGE type corresponded to a single ST and CC type (Fig.1). wgMLST analysis was performed on PFGE type C (ST 904; ST 607 CC) and PFGE type G (ST 3769; ST 21 CC) separately, using as reference genome one represent of each type (Table 6). Isolates of ST 904 (pulsotype C) showed 5 to 14 total allele differences over 1836 shared loci (98.8 % of the total genes). Similarly, isolates of ST 3769 (pulsotype G) showed 3 to 17 allele differences over 1714 shared loci (99.1 % of the total genes).

Table 5. Distribution and number of *C. jejuni* pulsotypes in each sampling location along the studied period and flock slaugther order.

Sample	Status	2M	5TH	6F	9M	10TU	11W	18W	19TH	23M	25W	27F
Defeathering	Dirty	**4G**	**4G**	**4G**	**4G**			**3G**	**1G**	**2C**		**1A**
(FCS)	Clean	**4G**	**2G**	**2G**	**3G**	**3G**	**1G**	**5G**	**3G**	**1C/1E**	**1C**	**1A**
Evisceration	Dirty	**4G**	**3G**	**3G/1C**	**5G**		**1G/2C**	**5G**	**4G/1C**	**1C/1A**	**1C/2A**	**3C**
(FCS)	Clean	**5G**	**1G**	**2G**	**2G**	**1G/1C**		**4G**	**3G**	**4C/1A**	**3C**	**3C/1B**
Sink	Dirty							**1G**	**2C**	**2C**	**1C/1A**	**2C**
(NFCS)	Clean							**2G**				
Floor	Dirty	**1G**		**1G**	**1G**	**2G**						
(NFCS)	Clean	**1G**				**1G/1C**		**1G**	**1C**			
Clamps	Dirty		**1G**			**1C**		**1G/2C**	**4G/1E**	**2C/2E**	**4A**	**1C/1A/2F/1B**
(FCS)	Clean		**2G**		**5G**	**2G**		**2G/1C**	**1G/1C/2E**		**1A**	**1C**
Conveyor belt	Dirty	**2G**	**3G**	**2G**	**3G**	**3G**	**1C**	**3G**	**3G**	**2C/1D**	**3A**	**2C/**
(FCS)	Clean								**1G**			**1F**
Sink	Dirty											
(NFCS)	Clean											
Floor	Dirty							**1C**		**1C**		
(NFCS)	Clean				**1G**							
Thigh		**1G**	**1G**		**1G**	**1C**	**1G**	**1E**	**1G**	**1C**	**1A**	**1A**
TOTAL (222)		**22**	**16**	**15**	**25**	**16**	**6**	**32**	**29**	**22**	**18**	**21**
Flock slaughter order		**FA**	**FB**	**FD**	**FD**	**FD**	**FD**	**FE**	**FE**	**FF**	**FF**	**FG**
		FB	**FC**	**FB**	**FB**	**FC**	**FC**	**FA**	**FD**	**FD**	**FD**	**FD**
Farm distance (km)		**112**	**5.3**	**54.4**	**54.4**	**60**	**60**	**63**	**3**	**18**	**18**	**125**

M: Monday, TU: Tuesday, W: Wednesday, etc.Numbers before day letter correspond to the calenday day.FA-FG: Flocks from different farms



PFGE	MLST	
	Sequence Type (ST)	Clonal Complex (CC)
C (n=51)	904	ST-607 Complex
A (n=18)	443	ST-443 Complex
B (n= 2)	775	ST-52 Complex
E (n= 7)	ND	ND
F (n=3)	1074	ST-460 Complex
G (n=140)	3769	ST-21 Complex
D (n= 1)	464	ST-464 Complex

Figure 1. Dendogram of different PFGE profile and the corresponding MLST Sequence Type (ST) and Clonal Complex (CC) of *Campylobacter jejuni*. A-G: Cluster of strains showing the same PFGE profile; N: number of samples with the same PFGE profile. ND: Not Determined.

Table 6. Overview of the wgMLST results for *Campylobacter jejuni* pulsotypes C (n=8) and pulsotypes G (n=33).

	Pulsotype C	Pulsotype G
Number of:	**ST 904; ST 607 CC**	**ST 3769; ST 21 CC**
Reference genes	1859	1729
Shared loci	1836	1714
Identical shared loci	1813	1675
Polymorphic shared loci	23	39
Excluded loci (gene duplication)	5	3
Excluded loci (incomplete information)	18	12

4. Discussion

This study was focused on the persistence of *C. jejuni* in the slaughterhouse and processing plant environment where the harsh conditions are present to get survive. As, it happens in previous studies, *C. jejuni* predominated in the poultry plant in comparison with the lower presence of *C. coli* (Pepe et al., 2009; Peyrat et al., 2008a; Wieczorek et al., 2015). This fact is usually associated with flock age, where *C. jejuni* is more common at the end of rearing period just before birds are sent to slaughter, whereas *C. coli* is more common in free-ranged chickens, laying hens or chicken breeders (Prachantasena et al., 2016). Additionally, some authors suggest that *C. coli* is less robust and might be more sensitive to the stress conditions found in the poultry processing plant (Peyrat et al., 2008a). However, in this study 46.2 % of *C. coli* strains were isolated after cleaning and disinfection, the majority of them in the slaughterhouse indicating certain resistance to these stress conditions.

Around 50 % of all samples taken were *C. jejuni* positive, showing the high prevalence of this bacterium in the poultry plant studied. Prachantasena et al. (2016) found a similar prevalence of 43.52 %, as an average of five slaughterhouses in Thailand. As it was expected, the FCS were more contaminated than NCFS, and especially in the slaughterhouse area. In this study, four sampling locations out of nine (defeathering, evisceration, slaughter sink and conveyor belt) were always contaminated along the 11 sampling days, especially the defeathering and evisceration steps. These data confirm that the main entrance of *C. jejuni* in the poultry processing plant is through the external contamination of broilers and via intestinal content. Feathers and legs are in contact with droppings, also with crates during transport, both, have been described as possible source of external contamination of birds (Melero et al., 2012; Slader et al., 2002). Other authors have described defeathering and evisceration as critical contamination steps in poultry

processing (Gruntar et al., 2015; Johnsen et al., 2006; Sasaki et al., 2013). Moreover, the sink located near the evisceration equipment, has showed a high contamination level despite there are not physical contact between the sink surface and birds' carcasses or offals, only serves as personnel hand washing station. This sink might be contaminated by means of airborne microbial dispersal such as; aerosols, water droplets or particles during processing as it was suggested by Allen et al. (2007).

Additionally, Kudirkienè et al. (2011) corroborated that *Campylobacter* will be able to survive in the slaughterhouse air after 8 h. As it was expected, in the processing area less contamination by *C. jejuni* was found in comparison with the slaughterhouse. Shackles and conveyor belt showed 41.8 % and 87.9 % of contaminated samples respectively. This is in agreement with the high contamination rates of the thighs, which was around 90% of the samples taken, suggesting an important cross-contamination rate between poultry meat and processing equipment.

C.jejuni strains have been isolated in all sampling locations after C&D, especially in slaughterhouse area and in shackles with the exception of the sink located in the processing area. This fact might be due to the specific design of the equipment, more focused in reaching high production rates than in facilitate the cleaning process. Particularly, the rubber fingers of the defeathering machine are really difficult to clean properly and organic matter and rests of feathers were visually detected during sampling after C&D. Some authors found that the slaughterhouse environment does not harbor campylobacters during a specific time or after C&D (Cools et al., 2005; Gruntar et al., 2015). However, others authors found some residual contamination making processing equipment a potential source of cross-contamination (Johnsen et al., 2006; Melero et al., 2012; Peyrat et al., 2008a; Prachantasena et al., 2016). Those differences are mainly due to the different equipment used in the different poultry processing lines as well as the

different C&D procedures used in each case. It is necessary that equipment designers take in consideration hygienic principles to minimize the possible cross-contamination between equipment and poultry carcasses.

The persistence of *C. jejuni* strains from farm to the end product have been well established by different authors (Damjanova et al., 2011; Melero et al., 2012). However, the persistence of *C. jejuni* in the poultry processing environment has received little attention; mainly due to the general believe that campylobacters are extremely sensitive to the extra-intestinal environment, harsh conditions present in these facilities. It is a well-known fact, that *Campylobacter* is introduced into the slaughterhouse by *Campylobacter* positive broilers flocks. Frequently, *Campylobacter* contaminating slaughterhouse equipment and meat products were genetically identical to those found in broiler flocks ceca from where they come from Elvers et al. (2011), Gruntar et al. (2015) and Prachantasena et al. (2016). Those strains can also contaminate, via cross-contamination, the following slaughtered flocks, especially if they are negative (Allen et al., 2007; Sasaki et al., 2013). However, Elvers et al. (2011) found that only the first carcasses (about 500 birds) of a negative flock processed after a *Campylobacter* positive flock, showed strains with the same genotype that the ones detected in this previous flock, describing a sort of diluting effect during the time. Moreover, they detected in these negative flocks other genotypes different from the ones found in the ceca of the previous positive flock. As it was mentioned above, another possible source of cross-contamination could be the existence of different *C. jejuni* strains that persist after C&D. However, it is not clear in the literature, how long could those strains persist in the poultry processing environment. Only Melero et al. (2012) suggested that some strains in this environment can persists longer and become a source of cross-contamination along the time. In the present study, genotypes C and G appeared in different sampling locations for at least 21 and 17 days

respectively. Those strains were isolated in all steps of poultry processing from defeathering to the end product despite the slaughtered flocks came from different farms, the slaughter order and the C&D cycles. Moreover, many studies show a high diversity between Campylobacter genotypes from flocks coming from different farms, even from farms that were located adjacent each other (Prachantasena et al., 2016). Taking into account this fact, it was expected a high diversity of pulsotypes in the poultry plant. However, the genetic diversity found in this study was really low. Pulsotype G strains were isolated in 8 sampling days out of 11 that covers 17 days. Along this period, slaughtered flocks came from 6 different farms. Similar situation happens with pulsotype C strains, which was isolated along 9 sampling days (at least 21 days) from different sampling locations all around the poultry plant. In this case, flocks came from 7 different farms. It seems improbable that these different farms showed the same pulsotype.

Several authors suggested in previous works that *C.jejuni* might survive in the slaughter and processing environment (Elvers et al., 2011; Melero et al., 2012; Peyrat et al., 2008b; Prachantasena et al., 2016). However, this is the first time, as far as we know, that *C. jejuni* strains from different pulsotypes C and G were isolated along different days and after several cycles of C&D. Probably, changing C&D routines or the appearance of more competitive strains in the environment produces a sort of succession along the time among different *C. jejuni* pulsotypes. Further studies to check this possibility should be done in the future.

MLST analysis showed that the 7 PFGE types correspond with 7 different sequences types and clonal complexes. In the case of pulsotype G, all 33 strains belong to the ST 3769; ST 21 complex. Only 5 records have been found in the database *Campylobacter jeuni/coli* PubMLST (www.pubmlst.org). Those records were associated mainly to human stool and chicken in UK, Portugal and Luxembourg. As far as we know

this is the first time that this ST-CC is isolated in Spain. All strains from pulsotype C (8) were assigned to the ST 904; ST 607 complex. In that case, 57 records are registered in the database and mainly isolated from human stool and chicken in different countries of Europe, including Spain. Whole genome MLST revealed that all strains from those later pulsotypes were clonal, showing similar diversity previously observed in human outbreaks (Revez et al., 2014a, 2014b; Zhang et al., 2015). The reasons, explaining the long persistence of these clonal populations in the slaughterhouse, are still unclear. It has been showed the presence of successful *C. jejuni* clones (monomorphic genotypes) possibly spread around the globe by rapid animal (migrating birds), food or human movement (Llarena et al., 2015). Therefore, these two clonal populations of ST 904 and ST 3769 might have been continuously introduced by animals, as the consequence of persistence of clonal genotypes in farms. Alternatively, the slaughterhouse environment could have selected those specific clones from a larger population. Indeed, some strains of *C. jejuni* are able to survive in the adverse condition of the slaughterhouse due to their ability of to form a monospecies biofillm (Brown et al., 2014) or to integrate in preexisting ones. Culotti and Packman (2015) showed that some bacteria as *Pseudomonas aeruginosa* not only prolongs the survival of *C. jejuni* under aerobic conditions, but also enables the growth of *C.jejuni* on the surface of *P.aeruginosa* biofilms. Other authors suggested that *C. jejuni* from monoculture biofilms might enter in a "viable but not culturable state" (Ica et al., 2012). The appearance of biofilms normally are related with the accumulation of organic matter on the surface of the equipment that protects bacteria for an efficient cleaning and disinfection, situation that happens in this case, as it was mentioned before.

5. Conclusion

This study has corroborated that some *C. jejuni* genotypes might survive C&D procedures. The difficulty to clean and disinfect properly the processing equipment makes that organic matter residues increase the ability of *C. jejuni* strains to survive. Results strongly suggest that highly clonal populations of *C. jejuni* can stay, for a certain period of time, in the environment and act like a source of contamination independently of the *Campylobacter* positive flock entering the slaughter line. Improvements in the hygienic design of the poultry processing equipment and in the C&D procedures are needed to reduce the possibility of *Campylobacter* strains to form biofilms alone or together with other bacteria, in the slaughter/processing environment, thus reducing the risk of *C. jejuni* in the end product. However, further studies extending the sampling period will be necessary to find out how this microorganism will be able to survive in such adverse conditions and persist in the processing environment for longer periods than previous thought.

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CHAPTER 3

VI. CHAPTER 3. Biofilm formation, virulence and antimicrobial resistance of different *Campylobacter jejuni* isolates from a poultry slaughterhouse

Abstract

The fastidious requirement of the zoonotic pathogen *Campylobacter jejuni* contrasts with its ability to overcome harsh conditions. Different strategies might be involved in the survival and persistence through the poultry food chain. Therefore, the aims of this study were to get insights in these survival strategies in the environment of a chicken slaughterhouse by (i) characterizing factors such as biofilm formation, virulence and antimicrobial resistance in the *C. jejuni* strains isolated and (ii) understanding the possible link between the phenotypic and genetic characterization using whole genome sequencing (WGS). Results has shown that three STs: ST 443 (PFGE A); ST 904 (PFGE C) and ST 3769 (PFGE G), out of the six studied, formed biofilms with variable intensity according to different conditions (temperatures; 37 ºC, 30 ºC, 25 ºC and materials; stainless steel and plastic). High antimicrobial resistances were found to ciprofloxacin, nalidixic acid and tetracycline as well as in two common cleaners used at slaughterhouse. A combination of several changes in the genome of ST 904 (PFGE C) including mutations, insertions in antimicrobial resistance genes, the presence of T6SS and a set of genes related with virulence factors, might explain their ability to form biofilm and its high resistance. Those traits can explain its persistence for a longer period time in the harsh environmental conditions of the chicken processing plant. However, the lack of some genes such as *capA*, *pgp1* and *ilvE* show the complexity of the survival strategies adopted by the different strains of *C. jejuni,* suggesting that there are multiple solutions to persist and survive along the chicken food chain and therefore causing human diseases. Further studies will be needed in order to better understand the relation between the ecological and genetic characteristics of the strains of *C. jejuni* to prevent the persistence along the food chain, decreasing antimicrobial resistance and human risk exposure.

1. Introduction

Campylobacter jejuni, a gram-negative and spiral-shaped microaerophilic bacterium is a leading cause of human food-borne infections in developed and developing countries (Gölz et al., 2014; Lertpiriyapong et al., 2012). Although most human infections seem to be sporadic and the gastrointestinal symptoms are usually self-limiting, extra intestinal complications may develop in some cases, such as; reactive arthritis, Guillain-Barré and Miller-Fisher syndromes (Skarp et al., 2016). These complications are suggested to be a result of an autoimmune response induced by ganglioside-like lipooligosacharides (LOS) expressed by *C. jejuni* (Revez et al., 2011).

Poultry is the most important source of human infection and colonization level in broiler ceca reaching counts as high as 10^9 CFU/g (Ma et al., 2014; Marotta et al., 2015). These high levels allow the bacteria to be easily spread throughout the processing plant (Whiley et al., 2013) and carcass could be contaminated by spilled gut content during slaughter. Although *Campylobacter* have uniquely fastidious growth requirements and it is very sensitive to environmental conditions, the bacteria are able to overcome harsh conditions; such as, cleaning and disinfection procedures (Peyrat et al., 2008), persist longer periods in slaughterhouse environment (García-Sánchez et al., 2017) or survive throughout the poultry food chain (Melero et al., 2012).

Biofilm formation is considered as one of the most important bacteria strategies to survival in suboptimal conditions in the environment. However, molecular mechanisms involved in regulating biofilm formation of *C. jejuni* are still poorly understood. So far, some genes described to be involved in the virulence process have been associated as well to the development of biofilms. For instance, genes responsible for cell motility (*flaA, flaB, flaC, flaG, fliA, fliS,* and *flhA*), cell surface modifications (*peb4*, *pgp1*, and *waaF*), quorum sensing (*luxS*), and stress response (*ppk1*, *spoT*, *cj1556*,

csrA, *cosR*, *cprS, nuok*). CosR is likely to be a key protein in the maturation of *C. jejuni* biofilm and it has also been demonstrated that is involve in the expression of the antimicrobial efflux pump CmeABC in *C. jejuni* (Turonova et al., 2015). This is the predominant efflux pump in this pathogen and plays a key role in bacterial physiology, conferring intrinsic and acquired resistance to diverse toxic compounds; such as bile salts, antibiotics or various detergents (Lin et al., 2002; Lin et al., 2003).

Despite the importance of *C. jejuni* as enteric pathogen and the progress in recent years in comprehending the complicated and multifactorial pathogenesis; there is a gap in understanding the combination of phenotypic and genotypic characteristics. The challenge now is to make the link between the genotypic and phenotypic data to understand better the mechanisms influencing *C. jejuni* persistence in the environment, and the role that this might play in transmission of this important pathogen (Bronowski et al., 2014). Therefore, the first aim of the present study was (i) the phenotypic characterization of different strains isolated from a poultry slaughterhouse to get insights into biofilm formation, virulence profile and antimicrobial resistance and (ii) understanding the possible relation between this phenotypic characterization and their genetic profile using whole genome sequencing (WGS).

2. Material and Methods

2.1. Bacterial isolates

In this study, forty five *Campylobacter jejuni* strains were used. Those strains were isolated in a previous study from a slaughterhouse environment, clustered in six PFGE profile and assigned to six MLST sequence type (ST) and clonal complexes (CC) after whole genome sequencing (García-Sánchez et al., 2017). Sequenced *C. jejuni* isolates were distributed, proportionally to the number of isolates obtained in the previous study as follows: 33 correspond to pulsotype G (ST 3769; ST 21 CC), 8 to pulsotype C

(ST 904; ST 607 CC) and 1 from the rest of pulsotypes PFGE A (ST 443; ST 443 CC), PFGE B (ST 775; ST 52 CC), PFGE D (ST 464; ST 464 CC) and PFGE F (ST 1074; ST 460 CC). Table 1 shows the epidemiological data related with those STs obtained from PubMLST (https://pubmlst.org/ campylobacter/).

Table 1. Epidemiological data of the different pulsotypes used in this study.

ST/CC	Records PubMLST	Origin	Distribution	Presence in Spain	Year*
443/443	58	Human, Chicken	World	Madrid	2010
775/52	83	Human, Chicken	Europe	Yes, Not specified	2002
904/607	101	Human, Chicken	Europe	Vitoria, Madrid	2003/2010
464/464	583	Human, Chicken	World	Castile and Leon	2008
1074/460	6	Human, Chicken	UK/Spain	Vitoria	2002/2004
3769/21	11	Human, Chicken	UK, Luxembourg, Portugal	No	

(*): Year that the Sequence Type appeared in Spain according to PubMLST (https://pubmlst.org/ campylobacter/) May 2018.

2.2. Biofilms formation

The ability of all *C jejuni* strains to form biofilm was determined in two different material: in stainless steel and in 96-well polystyrene microtiter plates (Nunclon Delta Surface, Thermo Scientific). Moreover, three different temperatures 37 ºC, 30 ºC and 25°C were tested under aerobic and microaerobic conditions. Sterile stainless steel coupons (stainless steel type 1.4301 according to European Standard EN 10088-1, with a type 2B finish according to European Standard EN 10088-2) were placed in a six-well polystyrene tissue culture plate and incubated with 3 mL of Nutrient broth (Oxoid, Basingstoke, England). Likewise, 96 well-polystyrene microtiter plates were incubated with 300 microliter of Nutrient broth (Oxoid). *C. jejuni* inoculum was performed with cultures adjusted to A_{600} of 0.05 in Nutrient broth. After 48 h of incubation, in the different conditions, surfaces were washed with an equal volume of phosphate-buffered saline and

dried at 60°C for 30 min. Next, 1 mL of 1% (wt/vol) crystal violet solution was added, and further incubated on a rocker at room temperature for 30 min. The unbound dye was removed by thorough washing in water, followed by drying at 37°C. Bound crystal violet was dissolved by adding 20% acetone - 80% ethanol, followed by incubation on a rocking platform for 15 min at room temperature (Brown et al., 2014). The resulting dissolved dye was measured at a wavelength of 590 nm by using a Gen5 2.00 software and Epoch spectrophotometer (BioTek, Winooski, USA). *C. jejuni* reference strains NCTC 11168 was used as positive control (Kalmokoff et al., 2006; Reuter et al., 2010; Turonova et al., 2015). All assays were performed in triplicate.

2.3. Antimicrobial, cleaner and disinfectant susceptibility testing

Minimal inhibitory concentration (MIC) for antimicrobial compounds: antibiotics, industrial cleaners and disinfectants against all C. *jejuni* strains was determined by agar spotting test and performed by triplicate. Bacterial inoculum was diluted in 2 mL of 0.9 % NaCl and the turbidity was adjusted of 0.5 McFarland units. The different strains were spotted into Nutrient agar (Oxoid) supplemented with 5 % sheep blood agar (Oxoid) containing the respective antimicrobial.

Six antibiotics belonging to four different classes were tested: two fluoro(quinolone); ciprofloxacin (0.03-64 mg/L) and nalidixic acid (4-128 mg/L); two macrolides, erythromycin (0.12-16 mg/L) and azithromycin (0.015-1 mg/L); one aminoglycoside, gentamicin (0.12-8 mg/L) and tetracycline (0.12-128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values according to European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). Multidrug resistance (MDR) was defined as simultaneous resistance to three or more unrelated antimicrobials (Mäessar et al., 2016). *C. jejuni* ATCC 33560™ was used as a positive control strains as it is described by Cantero et al. (2017).

In addition, six commercial sanitizers, commonly used in the cleaning and disinfection of chicken processing plants, were tested. From these, four were cleaners; Desenfort (Betelgeux, Gandia, Spain), Freefoam (Diversey, Barcelona, Spain), Deltafoam (Diversey, Barcelona, Spain), Diverflow (Diversey, Barcelona, Spain), named C_1, C_2, C_3, C_4 respectively in order to simplicity of the terms. The chemical composition of these was: a mix of sodium and potassium hydroxide (C_1), mix of organic acids (C_2), alkaline cleaner with silicates (C_3) and sulfamic acid (C_4). The remaining two compounds were quaternary ammonium disinfectants commercially available: Dexacide (Betelgeux, Gandia, Spain) and Divosan (Diversey, Barcelona, Spain) named D_1 and D_2 respectively. The tested concentrations in the study included, at least 10 dilutions, decreasing or increasing 0.25 % (v/v), depending on the first results obtained in the test performed in the laboratory and starting in the lower concentration recommended by suppliers per each sanitizer.

2.4. Analysis of genes associated to antibiotic resistance and virulence

All isolates were subjected to analysis for the presence of antimicrobial resistance genes using ResFinder v2.1 (Zankari et al. 2012) available at the CGE (https://cge.cbs.dtu.dk/services/ResFinder) (table 2).

Table 2. Antibiotic resistance mechanisms found in *Campylobacter jejuni.*

Antibiotic classes	Encoding gene(s)	Resistance mechanism	References
Quinolones, fluoroquinolones	*gryA*	Modification of the DNAgyrase target (Thr-86-Ile; Asp-90-Asn, Ala-70-Thr). Efflux through CmeABC	Hakanen et al. (2002) Cagliero et al. (2006a) Iovine (2013)
Tetracycline	*tet(O)*	Modifications of the target ribosomal A site by TetO binding. Efflux through CmeABC and possibly others	Iovine (2013) Lin et al. (2002)
Macrolide	23rRNA	Mutations in 23rRNA.Mutations in ribosomal proteins L4/L22 is likely minor. Efflux though CmeABC and possibly others. Decreased membrane permeability due to MOMP.	Cagliero et al. (2006b) Lin et al. (2007) Iovine (2013)
Aminoglycoside	*AphA*, *AadE*	Modification of the antibiotic by modifying enzymes. Contribution of efflux is not clear	Iovine (2013)
Beta-Lactam	*bla*OX-*61* *bla*OX-*184*	Inactivation of the antibiotic by enzyme B-lactamase. Efflux through CmeABC and possibly others. Decreased membrane permeability due to MOMP.	Griggs et al. (2009) Iovine (2013)

Additionally, the genome of all *C. jejuni* were analyzed for the presence of 44 virulence associated genes, selected according to previous published works by other authors (Chen et al., 2005; Dasti et al, 2010; Bronowski et al., 2014; Bolton et al, 2015). Among the selected genes are those involved in stress response (*ppk1*, *ppk2*, *cstA*, *spoT*, *hspR*, *htrA*, *htrB*, *sodB*, *katA*, *perR*, *aphC*, *dnaJ, cprs, cosR, nuok*), motility and chemotaxis (*flaA*, *flaB*, *flaC, fliA*, *rpoN*, *luxS, cheA*, *cheY*), adhesion (*cadF*, *capA*, *peb4*, *pgp1*,*waaF*, *ivle),* invasion (*ciaB*, *ciaC*, *ceuE*, *iam*), capsule (*kspE*, *kspM*, *pgld*), iron uptake system (*fur*, *cfrA*, *cfrB*), toxins (*cdtA*, *cdtB*, *cdtC*, *cgtB*) and the gene related to Guillain-Barré syndrome (*wlaN*). Additionally, Type IV (T4SS) and type VI (T6SS) secretion systems involved in pathogenesis and symbiotic relationship/adaptation to

environmental respectively were also studied. More detailed information about the function of each selected gene can be found in table 3.

The set of virulence associated genes of each genome was recovered using comparative tools by BLASTP (http://rast.nmpdr.org) against an in-house database created from the virulence factors found on the literature (table 3) with the protein sequence found on NCBI (https://www.ncbi.nlm.nih.gov/). The best reciprocal hit approach using BLASTP was implemented to recover shared genes for each pair of genomes, for each reciprocal hit with query coverage > 95 % and identity > 50 %, as it is described by Iraola et al. (2014).

2.5. Statistical analysis

All individual results were recorded using MS EXCEL, version 15.41 software (Microsoft Corporation; Redmond, WA, USA) and statistical analyses were performed with the software program Statgraphics Centurion XVI. At least three biological replicates (each with three technical replicates) were used to calculate means and the standard errors of the mean. The effect of two variables: atmosphere with two levels, aerobic and microaerobic, and temperature with three levels, 37 ºC, 30 ºC, 25 ºC, on the ability of *C. jejuni* to form biofilms was determined by multifactor analysis of variance (multifactor ANOVA). Main effect of atmosphere was not statistically significant ($p < 0.05$). On the contrary, main effect of temperature was significant ($p < 0.05$). The ability to form biofilms of the different PFGE/ST-CC of *C. jejuni* compared to positive and negative controls were statistically analysed using one-way analysis of variance (ANOVA). A Fisher LSD (Least Significant Difference) test was applied to determine group differences at 95 % significance level.

Table 3. *Campylobacter jejuni* virulence gene and predicted function selected in this study.

	Bacterial factor	Predicted funtion	References
Responses to stress			
	ppk1, ppk2	Starvation	Candon et al. (2007), Gangaiah et al. (2010)
	cstA	Starvation response	Brownoski et al. (2014)
	spoT	Starvation	Gaynor el al. (2005), Brownoski et al. (2014)
	hspR	Heat shock	Brownoski et al. (2014)
	htrA, htrB	Heat shock	Brownoski et al. (2014)
	sodB	Oxidative stress/aerobic response	Brownoski et al. (2014)
	katA	Catalase	Bingham-Ramos and Hendrixson (2008)
	perR	Oxidative stress/aerobic response	Brownoski et al. (2014)
	ahpC	Oxidative stress/aerobic response	Atack et al. (2008), Brownoski et al. (2014)
	dnaJ	Heat shock response	Bolton (2015)
	cosR	Oxidative stress response	Grinnage-Pulley et al. (2016)
	cprS	Osmotic stress response	Svensson et al. (2015)
	nuok	Oxidative stress/aerobic response	Yahara et al. (2017)
Motility and chemotaxis			
	flaA, flaB, flaC	Flagellin protein	Nachamkin et al. (1993), Koolman et al. (2015)
	fliA	Flagella biosintesis sigma factor	Bolton (2015)
	rpoN	Transcription of flagella rotation	Hendrixson and DiRita (2003),Dastietal. (2010)
	luxS	AI-2 biosynthesis enzyme	Quiñones et al. (2009), Bolton (2015)
	cheA	Chemotaxis protein	Bolton (2015), Koolman et al. (2015)
	cheY	Flagella rotation	Yao et al. (1997), Koolman et al. (2015)
Adhesion factors			
	cadF	Binding outer membrane protein	Kondel et al. (1997), Bolton (2015), Koolman et
	capA	Adhesion protein A	Bolton (2015), Koolman et al. (2015)
	peb4	Chaperone	Kale et al. (2011), Bolton (2015)
	pgp1	Peptidoglycan peptidase	Frirdich et al. (2012)
	waaF	Lipooligosaccharide	Naito et al. (2010)
	ilvE	Branched chain aminotransferase	Ribardo and Hendrixson (2011)

Table 3. (continuation) *Campylobacter jejuni* virulence gene and predicted function selected in this study.

	Bacterial factor	Predicted funtion	References
Invasion			
	ciaB	Adhesion and invasion	Konkel et al. (1999), Bolton (2015)
	ciaI/ciaC	Required for full invasion	Buelow et al. (2011), Bolton (2015)
	iam	Invasion associated protein	Yasser et al. (2011), Bolton (2015)
	ceuE	Lipoprotein	Bolton (2015), Koolman et al. (2015)
Capsule			
	kspM	Capsular polysaccharide	Bolton (2015)
	kpsE	Capsule biosynthesis gene	Bolton (2015)
	pgld	N-linked glycosylation	Morrison et al. (2010)
Toxins			
	cdtA,cdtB,cdtC	Cytolethal distending toxin	Dasti et al. (2010)
	cgtB, wlaN	1,3galactosyltransferase	Bolton (2015)
Iron uptake system			
	fur	Ferric uptake regulator	Bolton (2015)
	cfrA, cfrB	Outer membrane ferric enterobactin	Xu et al. (2010), Bolton (2015)
T6SS	*tssJ*, *tssL*, *tssM*	Membrane associated protein	Ugarte-Ruiz et al. (2014)
T4SS		DNA export, bacterial conjugation	Batchelor et al. (2004)

3. Results

3.1. Biofilm formation

The intensity of biofilm formation of the different MLST profiles in comparison with the control strain *C. jejuni* NCTC 11168 that was used as positive control is reflected in table 4. Production of biofilms under microaerobic and aerobic conditions did not differ significantly ($p < 0.05$). Results show that ST 904; ST 607 CC (PFGE C) forms biofilm at 37 ºC, 30 ºC and 25 ºC, and in both materials (polystyrene and stainless steel), being the biofilms produced at 37 ºC more robust than the produced at lower temperatures. ST 3769; ST 21 CC (PFGE G) has shown to form biofilm, also in both materials and atmospheres. However, in that case, all biofilms are more labile and has a clear dependence on the temperature (table 4). In both cases, no statistical differences have been observed within the different strains belonging to the same MLST profile. Moreover, ST 443; ST 443 CC (PFGE A) only formed biofilms on stainless steel coupons. On the contrary, the other genotypes ST 775; ST 52 CC (PFGE B); ST 464; 464 CC (PFGE D) and ST 1074; ST 460 CC (PFGE F) did not form biofilms in the analyzed conditions.

Table 4. Results of biofilm formation by different *C. jejuni* types on stainless steel and plastic surfaces comparing to negative and positive controls.

	Stainless steel			Plastic		
MLST	**37 ºC**	**30 ºC**	**25 ºC**	**37 ºC**	**30 ºC**	**25 ºC**
ST 443; 443 CC	-	++	+	-	-	-
ST 775; 52 CC	-	-	-	-	-	-
ST 904; 607 CC	+++	+	+	+++	++	+
ST 464; 464 CC	-	-	-	-	-	-
ST 1074; 460 CC	-	-	-	-	-	-
ST 3769; 21 CC	-	+	-	+	+	-

(-) = No biofilm formation; not significant different from negative control ($p < 0.05$).
(+) = Weak biofilm formation; significantly higher than negative control and lower than positive control ($p < 0.05$).
(++) = Moderate biofilm formation; not significant different from positive control ($p < 0.05$).
(+++) =Strong biofilm formation; significantly higher than positive control ($p < 0.05$).

3.2. Antimicrobial, cleaner and disinfectants susceptibility.

As shown in table 5, the forty-five isolates tested presented resistance to two classes of antimicrobials. The resistances were detected to (fluoro)quinolones: ciprofloxacin (100 %) and nalidixic acid (100 %) and also to tetracycline (100 %) On the contrary, all isolates were sensitive to the other two classes of antimicrobial tested; macrolide (erythromycin and azithromycin) and aminoglycoside (gentamycin). Although no differences were observed in MIC values within the same MLST profiles, some differences were observed among the different MLST genotypes.

Table 5 also shows the relation between the phenotypical behavior of the different MLST profiles and the specific antimicrobial resistance genes detected by ResFinder. All isolates tested resistant to the fluoro(quinolones) harbor a mutation in Thr86Ile of DNA gyrase conferring this resistance. Similarly, all the genotypes resistant to tetracycline, carried the *tet(O)* gene. On the contrary, none of the strains presented any resistance genes to the macrolides and aminoglycosides tested. Additionally, ResFinder found that the majority of MLST profiles have the *bla*OX resistant gene to beta-lactam in their genome. The gene *bla*OX A-184 is present in profiles ST 904 (PFGE C) and ST 443 (PFGE A) and *bla*OX A-61 in ST 3769 (PFGE G), ST 775 (PFGE B), and ST 464 (PFGE D). On the contrary, ST 460 (PFGE F) does not bring any resistant gene to this class of antibiotics. Moreover, all STs harbor the genes encoding the multidrug resistance efflux pump CmeABC. However, differences between the six STs have been found respective to the mutations present in the gene encoding for the regulator protein, CmeR, ranging from no mutations in ST 3769 (PFGE G) till the presence of eight in ST 775 (PFGE B). Some of these mutations appear in different STs (table 5).

Table 5. Antimicrobial susceptibility (MIC) and their relation with resistance genes in *C. jejuni* types.

	Quinolones			Tetracycline		Macrolides			Aminoglycoside		B-Lactam	Pump Efflux CmeABC			
MLST	**NAL**	**CIP**	**R[a] Thr86Ile**	**TC**	**R[a] *tet(O)***	**ERY**	**AZT**	**R[a]**	**GM**	**R[a]**	**R[a]**	**A**	**B**	**C**	**CmeR (Mutation)**
ST 443; 443CC (PFGE A)	R (128) [b]	R (8)	+	R (128)	+	S (0.25)	S (0.03)	-	S (0.25)	-	+	+	+	+	G144D, S207G
ST 775; 52 CC (PFGE B)	R (128)	R (32)	+	R (12)	+	S (0.5)	S (0.06)	-	S (0.5)	-	+	+	+	+	I100V, A108T, V109I,I115V, G144D, N150D, E189K, S207D
ST 904; 607 CC (PFGE C)	R (128)	R (32)	+	R (128)	+	S (2)	S (0.06)	-	S (0.5)	-	+	+	+	+	G144D, E189K, S207D
ST 464; 464 CC (PFGE D)	R (128)	R (8)	+	R (128)	+	S (1)	S (0.06)	-	S (0.5)	-	+	+	+	+	T6I, G144D, P183R, S207G
ST 1074; 460 CC (PFGE F)	R (128)	R (8)	+	R (128)	+	S (0.12)	S (0.0015)	-	S (0.5)	-	-	+	+	+	G144D, S207G
ST 3769; 21 CC (PFGE G)	R (128)	R (8)	+	R (16)	+	S (1)	S (0.06)	-	S (0.5)	-	+	+	+	+	No mutations found

[a]R: resistance mechanism. **Thr86Ile**: point mutation in the subunit A of the DNA gyrase gene**; gen tet(O)**; **[a]R B-Lactam:** gen blaOX-61/184 //NAL, nalidixic acid; CIP, ciprofloxacin; TC: tetracycline; ERY, erythromycin; AZT, azitromycin: GM, gentamicin; B-Lactam: Beta-lactam

[b]Interpretation of MIC for *C. jejuni* epidemiological cutoff values: NAL (≥16mg/L); CIP (≥0.5mg/L); TC (≥1mg/L); ERY (≥4mg/L); AZT (≥0.25mg/L); Gm (≥2mg/L); R= resistance; S=sensitive

Cleaner and disinfectant susceptibility analysis has shown that two cleaners and two disinfectants (C_1, C_2, D_1, and D_2) are effective against all *C. jejuni* strains tested according to concentrations recommended by suppliers. However, two cleaners (C_3 and C_4) were not effective at the recommended concentration, 3-10 % and 0.5-3 % respectively. All tested strains from the different STs were resistant to C_3 at 3 % (v/v), the lower concentration; but sensitives from 3.25 % (v/v) up. Furthermore, C_4 was the least effective in all tested strains, showing resistance at the double concentration than the lower limit recommended by the supplier (table 6).

Table 6. Percentage of cleaners & disinfectants tested in *C. jejuni*. Concentration in each *C. jejuni* pulsotype represents the limits of growing. Above these limits, bacteria were unable to grow and under these limits *Campylobacter* was able to grow.

		PFGE					
	(%)	**A**	**B**	**C**	**D**	**F**	**G**
Cleaner							
C_1	1-3 %	0.25	0.25	0.25	0.25	0.25	0.25
C_2	2-10 %	0.25	0.25	0.25	0.25	0.25	0.25
C_3	3-10 %	**3**	**3**	**3**	**3**	**3**	**3***
C_4	0.5-3 %	**1**	**1**	**1**	**1**	**1**	**1**
Disinfectant							
D_1	0.5-3 %	0.0015	0.062	0.062	0.031	0.031	0.031
D_2	1-2 %	0.0125	0.0125	0.062	0.062	0.062	0.062

(%) = Concentrations of use in cleaners of disinfectants recommended by suppliers in each product

(*) = Three strains belonged to this PFGE type was 2.75 (% v/v) resistant.

3.3. Genes associated with virulence factors.

All STs studied were positive for almost all of the 44 virulence-associated genes, with few exceptions. Only STs 904 (PFGE C), 464 (PFGE D) and 1074 (PFGE F) harbor all genes related with the presence of T6SS. Table 7 shows the differences among genotypes.

Table 7. *C. jejuni* differences in the 44 virulence-associated genes and T6SS.

		Virulence Factors				
				Adhesion		*GBS*
PFGE	**MLST**	**T6SS**	***capA***	***pgp1***	***ilvE***	***wlaN***
A	ST 443; 443 CC	-	-	-	-	-
B	ST 775; 52 CC	-	-	-	+	-
C	ST 904; 607 CC	+	-	-	-	+
D	ST 464; 464 CC	+	+	-	+	+
F	ST1074; 460 CC	+	-	-	+	+
G	ST 3769; 21 CC	-	-	-	+	+

4. Discussion

In this study, 45 *C. jejuni* strains, belonging to 6 different STs, isolated in a previous work, have been characterized from the phenotypic and genotypic point of view, in order to get insights, in their survival and persistence in the slaughterhouse environment (García-Sánchez et al., 2017). Other authors showed that some *C. jejuni* strains from abattoir environment may be less sensitive to stresses and therefore, reach the final products (Kudirkiené et al., 2011). Moreover, not all *C. jejuni* strains appear to have the same virulence, survival and host adaptation potential and certain metabolic activities appears to be strain dependent (Revez et al., 2011). In that sense, different bacteria strategies could be involved in overcome the harsh conditions that prevail in the

processing plant environment. Among these strategies, it is well known that biofilm formation, on contact surfaces, contributes to persistence of foodborne pathogens all along the food chain.

Results show that from the six STs tested in this study, only three: ST 443 (PFGE A), ST 904 (PFGE C) and ST 3769 (PFGE G) were able to form biofilms. These three STs agree with the results obtained in a previous work, where those STs were isolated at least during 4, 21 and 17 days respectively from a slaughterhouse environment, including dirty and clean surfaces and broiler meat (García-Sánchez et al., 2017). From those ST 904 (PFGE C) was able to form biofilm with a higher intensity than the others and at different temperatures and air conditions. It must be considered that in the plucking and evisceration steps of the broiler processing line, there are a high temperature and humidity (12-22 ºC and 90-100 % RH), together with a design equipment that may favor the development of biofilms. Although this temperature is lower than the one used in *in vitro* tests, it must be taken in account that these values are referred to the air, and probably this temperature will be higher on the surface of the slaughter equipment, considering water scalding temperature is around 53 °C and the friction due the plucking. Moreover, some authors have mentioned that chicken juice allows increased attachment, providing a conditioned surface for the bacteria to adhere to and prolong the viability of *C. jejuni* and other bacteria (Birk et al., 2004; Brown et al., 2014). In all STs studied the addition of chicken juice *in vitro* experiments increases biofilm production (data not shown). Additionally, several authors have described the presence of *C. jejuni* after cleaning and disinfection in the slaughter environment (Peyrat et al., 2008) and as a source of broiler meat contamination during slaughter process (Kudirkienè et al., 2011; Melero et al., 2012).

The presence and expression of antimicrobial resistance mechanisms may also favor the development of biofilms and the permanence of *C. jejuni* strains after cleaning on the processing surfaces, especially at subletal concentrations. In this study, all tested strains show phenotypically resistance to fluoro(quinolones) and tetracycline, whereas all strains were sensitive to the macrolides, erythromycin and azithromycin and to the aminoglycoside, gentamicin. Resistances to fluoro(quinolones) (FQ) are related with the presence, in all tested strains, of the substitution T86I in the protein GyrA, which is sufficient to confer high-level resistance (Zhang et al., 2017). Moreover, ResFinder found different not usual substitutions in this protein for the different STs. Among them, substitution R285K was found in all STs, and substitution N203S in five of them. However, those substitutions do not affect the quinolone resistance determining region (QRDR) of the gene *gyrA* (codons 69 to 120) (Hakanen et al., 2002), and it seems that do not have any effect in the MIC against FQ in the different STs characterized.

The *cmeABC* operon encodes a multidrug efflux pump, which plays a key role in bacterial physiology, and has been associated with intrinsic and acquired resistance to antimicrobial compounds and are required for bacteria to adapt to environmental stresses (Lin et al., 2002; Routh et al., 2009; Zhang et al., 2017). Transcription of this operon is repressed by protein CmeR. The gene (*cme*R) encoding this protein is located immediately upstream of the cmeABC operon and its expression blocks the transcription of the *cme*ABC operon. Mutations in the promoter region of this operon or in CmeR, results in an overexpression of the efflux pump. According to ResFinder, all STs harbor several substitutions in the regulatory protein CmeR, with the exception of ST 3769 (PFGE G). Among them, substitutions E189K, substitution of one glutamate to lysine and S207D, substitution of one serine to aspartate, in the C-terminal region, only appear in ST 775 (PFGE B) and ST 904 (PFGE C). Interestingly, those STs showed a higher MIC

(32 mg/mL) in comparison with the other three STs (8 mg/mL). According to several authors, amino acid substitutions in CmeR did not affect its binding ability to the cmeABC promoter, but a mutation that led to C-terminal truncation of CmeR abolished its DNA-binding activity (Grinnage-Pulley and Zhang, 2015). Although, these substitutions are not a truncation, the charge balance of the region changes. However, with data obtained in this study is not possible to check the effect of those changes in CmeR performance. In addition, CmeR functions as a pleiotropic regulator and modulates the expression of at least 28 other genes in *C. jejuni*, a part of repressing the transcription of *cme*ABC.

The different STs are also sensitive to macrolides erythromycin and azithromycin. However, ST 904 (PFGE C) again showed higher resistance to those antibiotics (2 mg/mL) than the other STs. Different point substitutions have also been identified in the ribosomal protein L22 for all STs, with the exception of ST 3769 (PFGE G), which do not show any substitution. These substitutions seem do not have any remarkable effect on macrolide resistance. However, ST 904 (PFGE C) harbor an insertion of six amino acids APAAKK between amino acids K118 and T119 in L22, that might have some influence in increased resistance in comparison with the other STs evaluated. Further studies should be done to corroborate this issue.

Presence of organic matter was observed during the sampling procedure, after cleaning and disinfection causes a decrease in the effectivity of the disinfectant used. In this situation, operators use to react increasing the application dose of chemical sanitizers. However, some authors have suggested that the increase of disinfectant doses might impose a selective pressure and contribute to the emergence of disinfectant resistant microorganisms (Langsrud et al., 2003; Peyrat et al., 2008). Moreover, some disinfectants are not fully biodegradable which may persist in sewage for long periods. For instance,

quaternary ammonium is biodegradable only under aerobic conditions, resulting in continuously fluctuating concentration gradients that might increase the bacteria resistance (Tezel and Pavlostathis, 2015). In this study, ST 904 (PFGE C), which presented the highest antibiotics resistance (MIC) and was the strongest biofilm former, was indeed the genotype that showed the highest resistance to quaternary ammonium disinfectants (0.062 in D_1 and D_2).

Therefore, this study suggests that might be a relation among strains which harbored a high antibiotic resistance and biofilm formation. As it has mentioned previously, ST 904 (PFGE C) presented the highest antibiotics resistances (MIC), while it was classified as stronger biofilm formation. When biofilms are formed it serves as a specific reservoir for genetic exchange by contact with foreign bacteria DNA, leading to mutations or incorporation into the recipient genome new properties. In that sense, Efimochkina et al. (2018) have described this phenomenon after culturing *C. jejuni* with antibiotics in subinhibitory antibiotic doses. Although in this study only has tested monospecies biofilm formation; other possibilities need to be take in to account such as biofilm formation in mixed-species, (Brown et al., 2014; Teh et al., 2016) or into preexisting biofilms; such as the ones formed by *Pseudomonas aeruginosa* (Culotti and Packman, 2015). It might lead that the rest of STs tested classified as weak, moderate or even not formed biofilm might be able to produce it.

Virulence factors also contribute that some genotypes are able to survive in harsh environment. However, there is a gap in understanding the relation among presence/absence of genes, the expression of these genes and their capability to form biofilms. All STs characterized in this work are related to chicken food chain and the production of human campilobacteriosis as it was shown in table 1. Moreover, all of them harbor 40 of the 44 virulence genes selected. Some differences between STs

appeared for a few genes related with adhesion virulence factors: *capA*, *pgp1* and *ilvE*. Surprisingly, the *Campylobacter* adhesion protein (CapA) is absent in the three ST that formed biofilms phenotypically. CapA plays a role in host association and colonization by *Campylobacter* and mutants for this protein failed to colonize and persist in chickens Ashgar et al. (2007). In addition, all three STs show an identity of 50 % in the sequence of the protein Pgp1 with the one expressed by *Campylobacter pinnipediorum* subsp. *caledonicus*, which is a peptidoglycan modifying enzyme (peptidoglycan LD-carboxypeptidase) required for helical cell shape (Frirdich et al., 2012). These authors have proposed a role of this protein, a part to confer the helical shape, roles in motility, biofilm formation, chick colonization, and stimulation of host cell proinflammatory mediators Nod1 and IL-8. Moreover, all STs harbor the gene *pgp2*, also a peptidoglycan LD-carboxypeptidase. Differences appear with the presence of the gene *ilvE*, a branched-chain amino acid aminotransferase, which is absent in STs 443 (PFGE A) and 904 (PFGE C), whereas the gene present in ST 3769 (PFGE) has 98 % identity with the gene present in *Campylobacter jejuni* subsp. *jejuni* 81-176. The gene *wlaN*, was detected in four STs, including STs 904 (PFGE C) and 3769 (PFGE G), and according to some authors this gene is related with the expression of Guillain-Barré syndrome authors (Datta et al., 2003; Koolman et al., 2015). Finally, the set of genes encoding T6SS is present in ST 904; (PFGE C), but not in the other two biofilm formers ST 443 (PFGE A) and ST 3769 (PFGE G). T6SS is implicated in adaptation, quorum and stress sensing, bacterial growth and motility, biofilm formation and destruction of competing bacteria (Lertpiriyapong et al., 2012; Ugarte-Ruiz et al., 2014).

In summary, this article reflects the complexity to relate the phenotypic features with the genetic information in the different STs characterized. According with the results it seems that the survival strategy to harsh conditions might have several expressions in

different STs. It seems clear that adaptation to the environment is obtained gathering different combinations of genetic changes that result in a more adaptable strain. In that sense, ST 904; ST 607 CC (PFGE C), which was isolated during 21 days in a previous work and shows the highest biofilm production and antimicrobial resistance, harbors all genes related with T6SS, and insertion of 6 amino acids in the L22 RNA protein, two amino acid substitutions in the C-terminal of CmeR protein and at least 40 genes related with virulence factors. Other STs that showed also a lower ability to produce biofilms present other genetic characteristics. However, further studies will be needed in order to better understanding the ecological and genetic characteristics of the different *Campylobacter jejuni* STs and prevent their persistence along the chicken food chain, decrease the antimicrobial resistance and human risk exposure.

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CHAPTER 4

VII. CHAPTER 4. Characterizacion of *Campylobacter* species in Spanish retail from different fresh chicken products and their antimicrobial resistance

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Abstract

Contaminated chicken products have been recognized as the primary vehicles of *Campylobacter* transmission to human. Pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance in *Campylobacter* isolates from fresh chicken products at retail were studied. A total of 512 samples including: thigh, breast, marinated and minced chicken were purchased from different retail stores. Half of the samples were packed and the other half were unpacked. The 39.4 % of the samples were *Campylobacter* positive; being unpacked chicken products (45.3 %) more contaminated than packed chicken (33.6 %). PFGE typing showed a high diversity among isolates; clustering 204 isolates into 76 PFGE types: 55 clusters of *C. jejuni*, 19 of *C. coli* and 2 of *C. lari. C. coli* genotypes showed higher resistance than other *Campylobacter* species. Although modified atmosphere packaging can reduce the prevalence of *Campylobacter* spp., does not avoid their presence in at least 33.6 % of packed chicken products analyzed. Some pulsotypes might persist in the processing plant or butcher shops environment for longer than previously thought. They are necessary more stringent control measures in previous steps of the chicken food chain, in order to avoid the presence of *Campylobacter* spp. strains at retail that can compromise consumer´s safety.

1. Introduction

Campylobacteriosis has been reported as the most common zoonosis, with an increase in confirmed human cases in the European Union since 2008 (EFSA, 2017). Large outbreaks are uncommon and the vast majority of human campylobacteriosis cases are sporadic (Pires et al., 2010). Most *Campylobacter* infections are self-limiting and do not require treatment with antimicrobials. However, severe and prolonged cases of campylobacteriosis and infections in immune-compromised, vulnerable populations and children may require antimicrobial therapy. In these cases, fluoroquinolones and macrolides such as erythromycin are the drugs of choice (Narváez-Bravo et al., 2017).

Several foodstuffs have been involved in the transmission of the pathogen to humans; such as, untreated water (Nilsson et al., 2017; Revez et al., 2014), milk and cheese (EFSA, 2017), salad (Calciati et al., 2012), spinach, lettuce, radish, green onion, potatoes, parsley (Park and Sanders, 1992) and fenugreek (Kumar et al., 2001). However, poultry has been identified as the natural host for *Campylobacter* species, and broilers are often colonized, especially with *C. jejuni* (Sasaki et al., 2011; Torralbo et al., 2014). Contamination of broiler flocks at farm level, often lead to transmission of *Campylobacter* along the poultry production chain and contamination of poultry meat at retail (Melero et al., 2012; Skarp et al., 2016). The role of poultry as a reservoir for this transmission has been recognized with 20-30 % of the human infections linked to handling, preparation, and consumption of broiler meat (EFSA, 2010).

C. jejuni has traditionally been categorized as a fastidious microorganism by their metabolic features, it is asaccharolytic (has limitations in the utilization of hexose sugars) and is considered to be microaerophilic and capnophilic, because requires both O_2 and CO_2 for optimal growth, preferably at 5–10% and 1–10%, respectively (Bolton and Coates, 1983; Oh et al., 2017). However, it could survive in harsh environmental

conditions, such as surfaces and equipment in broiler's slaughterhouse (García-Sánchez et al., 2017), aerobic conditions in poultry products (Di Giannatale et al., 2014) and modified atmosphere packaging (Melero et al., 2013; Meredith et al., 2014). *Campylobacter* could be recovered along all steps in the transmission through the food chain (Melero et al., 2012). Oxidative stress is one of the first obstacles that *Campylobacte*r has to face in the extra-intestinal environment (Atack and Kelly, 2009). Therefore, reduced sensitivity of some strains to oxygen would confer superior environmental resistance, increasing the likelihood of transmission between potential hosts (O´Kane et al., 2017).

Recent studies have shown a high percentage of *Campylobacter* in broiler fresh meat (36.7 %) in comparison with other poultry fresh products in the EU Member States. The highest country-specific notification rates in 2016 were observed in the Czech Republic, Slovakia, Sweden and the United Kingdom (EFSA, 2017). In the later, studies done by the Food Standards Agency corroborated that more than half of fresh chicken products, bought in retail shops and produced in UK, tested positive for *Campylobacter* in the period between 2016 and 2017 (Whitworth, 2017). In that sense, evaluation of poultry meats at retail is critical, as they really enter the consumers' kitchens (Cook et al., 2012). Cross-contamination occurs between fresh chicken meat and consumer's kitchen utensils and hands and ready-to-eat products at home (Luber et al., 2006).

The aim of this study was (i) to determinate the prevalence and genotypic profile of *Campylobacter* species in different chicken products in Spanish markets and (ii) to determine the antimicrobial Minimum Inhibitory Concentration (MIC) in different *Campylobacter* spp. populations found in retail shops.

2. Material and Methods

2.1. Study area and sampling procedure.

The study area was located in the city of Burgos in the North of Spain (107 km²) which has a total of 177,100 inhabitants. The study was carried out from 23 th February to 18 th June in 2015. During this period, a total of 512 chicken samples were purchased from 18 retail shops: 12 butcher shops and 6 markets. Thigh (n=128) breast (n=128), minced (n=128) and marinated (n=128) fresh chicken products were analyzed. Marinated products contained several spices such as, paprika, garlic, oregano and in some cases, wine. Half of the samples (n=256) were purchased in markets and correspond to products packed with modified atmosphere packaging (MAP), whereas the other half (n=256) were purchased in butcher shops in bulk without packing (aerobic or ambient atmosphere).

During this period, 13 different suppliers (A-M) of fresh chicken packed products were sampled. These were classified according to their geographical origin of manufacture in four regions: East (E), West (W), North (N), and Centre (C) (Figure 1). However, unpacked products were purchased in local butcher shops (a-l) located around the city. From these, at least four of them (a, b, c, d) received meat from the same slaughterhouse.

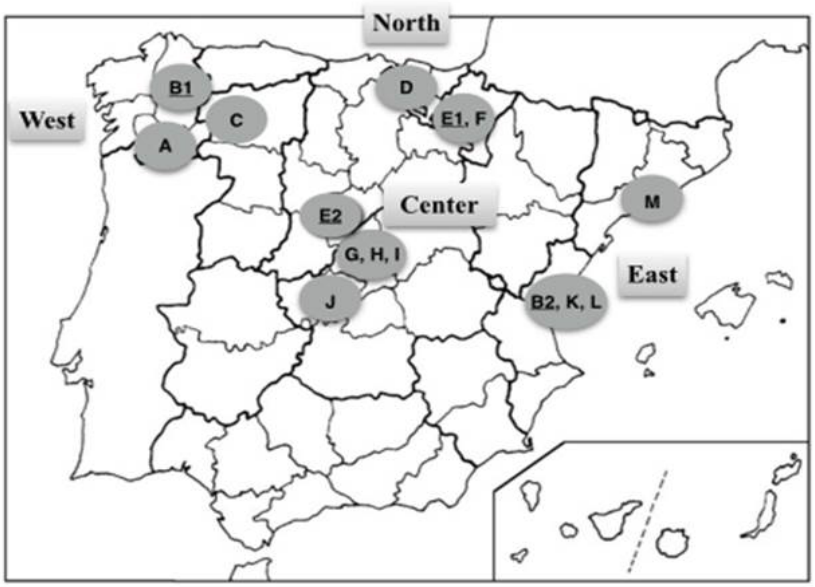

Figure 1. Geographical distribution of manufactured and fresh packed chicken products suppliers in Spain sampled in this study.

2.2 Gas and pH analysis

In MAP fresh chicken products gas analysis was carried out with a digital O_2/CO_2 analyzer (OXYBABY, WITT-Gasetechnik GmbH & Co KG, Witten, Germany). Ten milliliters gas samples were drawn from the pack headspace by the needle of the analyzer through a septum glued onto the surface of the pack.

Once opened the pack, pH analysis was measured in the product. A pH meter (micropH2001, CRISON, Barcelona, Spain) was used by inserting the pin electrode directly into the sample.

Gas and pH measures were carried out in each product in triplicate.

2.3. Isolation and identification of *Campylobacter* spp.

From each sample, 10 g were taken aseptically and placed in sterile stomacher bags for homogenize with 90 ml of sterile Preston broth made with Nutrient Broth Nº 2 (Oxoid, Basingstoke, England) supplemented with Preston Campylobacter Selective Supplement (Oxoid) and Campylobacter Growth Supplement Liquid (Oxoid). Individually, each sample was homogenized with Preston broth for 120 s and incubated microaerobically using a commercial gas-generating systems CampyGen (Oxoid) and sealed jar at 41.5 ºC for 48 h. After enrichment, a loop-full from each sample was streaked on a plate of modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) prepared with Campylobacter blood-free selective agar base (Oxoid) supplemented with CCDA selective supplement (SR0155E, Oxoid). Plates were incubated as described above for enrichment broths. From each plate, two typical isolated *Campylobacter* spp colonies were randomly selected for further analysis.

Isolated colonies from the mCCDA agar were grown on 5 mL of Brain Heart Infusion broth (Oxoid) for an overnight. DNA was extracted according to Yamada et al. 2015. Briefly, strains were suspended in 100 µL of Tris-EDTA buffer (pH 8.0) and

incubated at 95 ºC for 10 min, and centrifuged at 16,000×*g* for 1 min. The supernatants were subsequently used as templates for PCR. All isolates were analyses using multiplex PCR to identify *C. jejuni, C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus sub. fetus* as described by Wang et al. (2002). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4. Pulsed field gel electrophoresis (PFGE)

Campylobacter spp. isolates were cultured on Columbia agar (Oxoid) supplemented with 5 % defribrinated sheep blood (Oxoid) under microaerobic conditions (24 h at 41.5 ºC) for the purpose of typing. PFGE analyses were performed following the protocol according to PulseNet (www.cdc.gov/pulsenet/pathogens/pfge.html) and applying the restriction enzymes *Sma*I and *Kpn*I.*Kpn*I was used to check the diversity of all isolates with similar *Sma*I genotype.

Restricted DNA was electrophoresed for 22.5 h on 1% (w/v) SeaKem gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories). The electrophoresis conditions used consisted of an initial switch time of 5 s and a final switch time of 55 s (gradient of 6 V/cm and an included angle 120º). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories). BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0 %). The unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns. Isolates with <85 % similarity according to the dendrogram were clustered as separate pulsotypes (Boer et al., 2000).

2.5. Antimicrobial susceptibility

Campylobacter spp. isolates were sub-cultured on Nutrient agar supplemented with 5 % sheep blood (Oxoid) and incubated at 41.5 ºC for 24 h in microaerophilic conditions. After incubation, bacterial inoculum was performed into 2 mL of 0.9 % NaCl and the turbidity was adjusted to 0.5 McFarland scale to carry out the inoculation. The inoculate plates were incubated in microaerophilic conditions for 48 h at 41.5 ºC. Six antimicrobials belonging to four different classes were tested in different range concentrations: two fluoro(quinolone); ciprofloxacin (0.03-64 mg/L) and nalidixic acid (4-128 mg/L); two macrolide, erythromycin (0.12-16 mg/L) and azithromycin (0.015-1 mg/L); one aminoglycosides, gentamicin (0.12-8 mg/L) and tetracycline (0.12-128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values according to European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). One isolate was considered multi-drug resistance, when it was resistant to three or more no related antimicrobials. Strain *C. jejuni* ATCC 33560^{TM} was used as a control.

3. Results

3.1. Gas and pH analyses results

In packed fresh chicken products, atmosphere composition and pH was measured. As it can be observed in table 1, *Campylobacter* strains were isolated from samples with a pH range between 5.3-6.2 and gas composition between 11.0-72.7 % of O_2 and between 1.5 and 54 % of CO_2. Only in four out of 15 suppliers *Campylobacter* spp. was absence.

3.2. Prevalence of *Campylobacter* spp. in chicken meat.

A total of 202 out of 512 samples were *Campylobacter* spp. positive (39.4 %). In unpacked products a higher prevalence of 45.3 % (116/256) was observed in comparison with packed products, which was 33.6 % (86/256). The most contaminated products have

Table 1: Geographical origin of packed chicken product suppliers, number of samples, presence of *Campylobacter* spp. and physico-chemical parameters of packs.

Supplier	Origin	Samples	*Campylobacter*	pH	O_2 (%)	CO_2 (%)
A	West	17	-[a]	6.0	33.2	14.6
B1	West	8	37.5	5.3	21.4	54.0
B2	East	12	75	5.7	18.2	47.5
C	West	42	35.7	6.0	62.2	24.8
D	North	17	52.9	5.6	19.8	6.6
E1	North	22	50	6.0	66.9	17.2
E2	Centre	18	94.4	5.9	29.2	9.8
F	North	2	-	5.1	62.6	33.4
G	Centre	21	33.3	6.0	41.1	12.2
H	Centre	6	16.6	6.1	72.7	20.8
I	Centre	26	46.2	6.0	68.1	20.2
J	Centre	2	50	6.2	19.8	1.5
K	East	20	-	7.4	13.1	21.1
L	East	32	6.2	6.1	11.0	24.0
M	East	11	-	6.6	57.3	15.4

([a]): Absence of *Campylobacter* spp. in all samples analyzed.

been the unpacked marinated products from butcheries and packed thighs from markets with the same percentage (56.3 %), followed by 51.6 % in thigh and breast (unpacked). Minced products in both atmospheres were the least contaminated with a percentage of 21.9 % and 14 % in unpacked and packed respectively (table 2). In general, in packed products most of the strains were isolated from thigh and breast products, whereas in unpacked products isolates came from all different chicken products.

C. jejuni was the species more prevalent in all products analyzed. *C. jejuni* accounted for 77.7 % of total positive products (157/202), followed by *C. coli* 16.3 % (33/202) and *C. lari* 2.5 % (5/202). A low percentage of positives samples (3.5 %) showed a mix of species in the same product, the majority of them were a mix of *C. jejuni/C. coli* except in one that was *C. jejuni/C. lari.* Among unpacked product, *C. jejuni* represented 87.1 % of positives samples, followed by *C. coli* 11.2 % and *C. lari* 1.7 %. However, in

packed products *C. coli* were present in a higher percentage (23.3%) in several cases, taking part of the mix of species together with *C. jejuni* (table 2).

Table 2. Prevalence of the different species of *Campylobacter* in retail chicken products.

Chicken	*Campylobacter*	*C. jejuni*	*C. coli*	*C. lari*	Mix
Unpacked					
Thigh	51.6 % (33/64)	27.6 % (32/116)	0.9 % (1/116)	-	-
Breast	51.6 % (33/64)	27.6 % (32/116)	0.9 % (1/116)	-	-
Minced	21.9 % (14/64)	10.34 %(12/116)	-	1.7 % (2/116)	-
Marinated	56.3 % (36/64)	21.5% (25/116)	9.5 % (11/116)	-	-
All	**45.3% (116/256)**	**87.1% (101/116)**	**11.2 % (13/116)**	**1.7 % (2/116)**	-
Packed					
Thigh	56.3 % (36/64)	23.3 % (20/86)	12.8 % (11/86)	1.2 % (1/86)	4.6%[a](4/86)
Breast	45.3 % (29/64)	30.2 % (26/86)	1.2 % (1/86)	1.2 % (1/86)	1.2%[a](1/86)
Minced	14 % (9/64)	4.6 % (3/86)	4.6 % (4/86)	1.2 % (1/86)	1.2%[b](1/86)
Marinated	18.7 % (12/64)	8.1 % (7/86)	4.6 % (4/86)	-	1.2%[a](1/86)
All	**33.6 % (86/256)**	**65.1 % (56/86)**	**23.3 % (20/86)**	**3.4 % (3/86)**	**8.1 % (7/86)**

([a]): Mix of *C. jejuni*/*C. coli;* ([b]): Mix of *C. jejuni/C. lari*

3.3. *Campylobacter* spp. genotyping

All species of *Campylobacter* isolated from poultry products (*C.jejuni, C.coli* and *C. lari*) were typed by PFGE. From the two typical colonies isolated per product, only one was typed by PFGE in cases that both belonged to the same species. On contrary, in those products in which a mix of two different species was found, both isolates were typed. Therefore, 164 isolates from *C. jejuni*, 39 from *C. coli* and 6 from *C. lari* were selected. From these, 5 isolates were lost during the typing process (2 *C. jejuni,* 2 *C. coli* and 1 *C. lari*).

Pulse field gel electrophoresis clustered the 204 *Campylobacter* spp. isolates into 76 PFGE profiles. Among them, 55 PFGE types correspond to *C. jejuni* (162 isolates), 19 pulsotypes to *C. coli* (37 isolates) and 2 types to *C. lari* (5 isolates) as shown in figures 2 and 3 (see complete dendrogram in supplementary information). The majority of clusters are formed by one or two isolates, showing a wide diversity among chicken products. From the total of 55 *C. jejuni* types, 28 clusters (50.9 %) included one isolate, similar situation occurs in *C. coli* where 52.6 % corresponds to clusters with one isolate only.

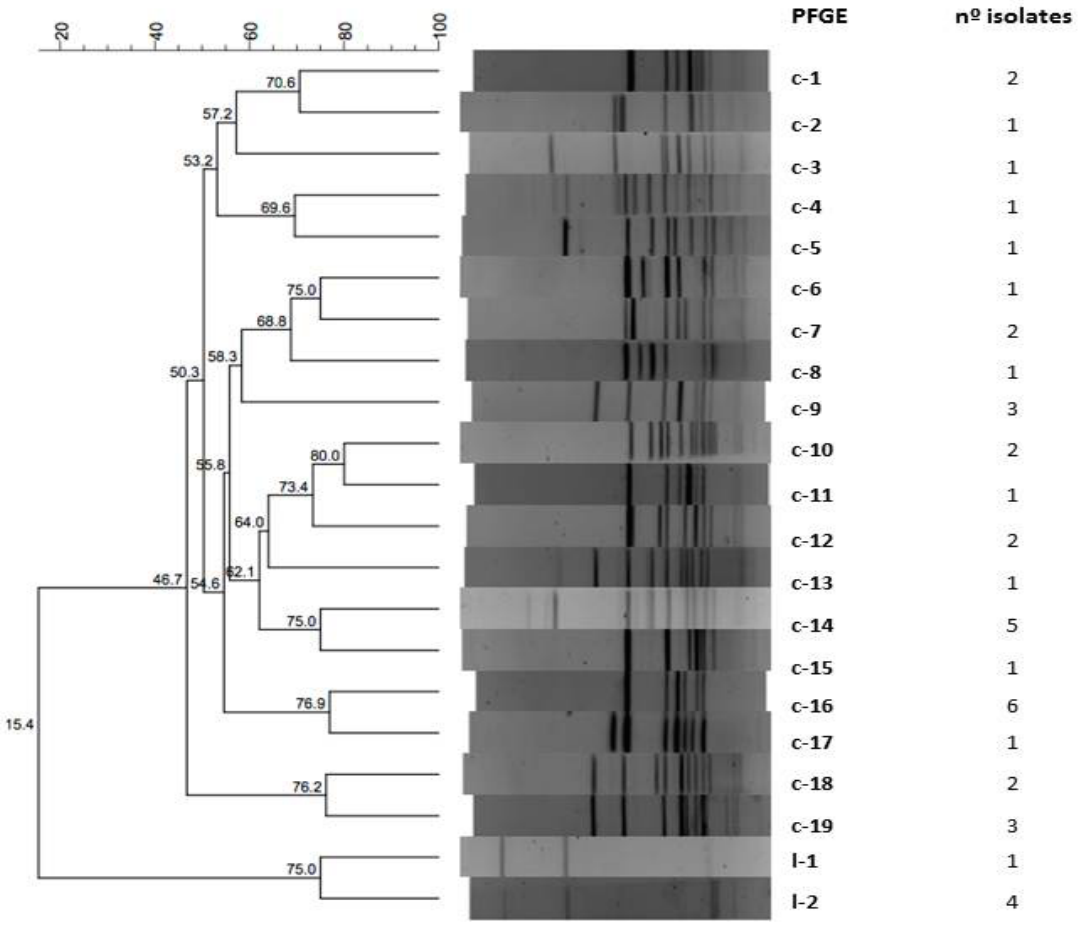

Figure 3. Dendrogram of *C. coli* and *C. lari* PFGE types.

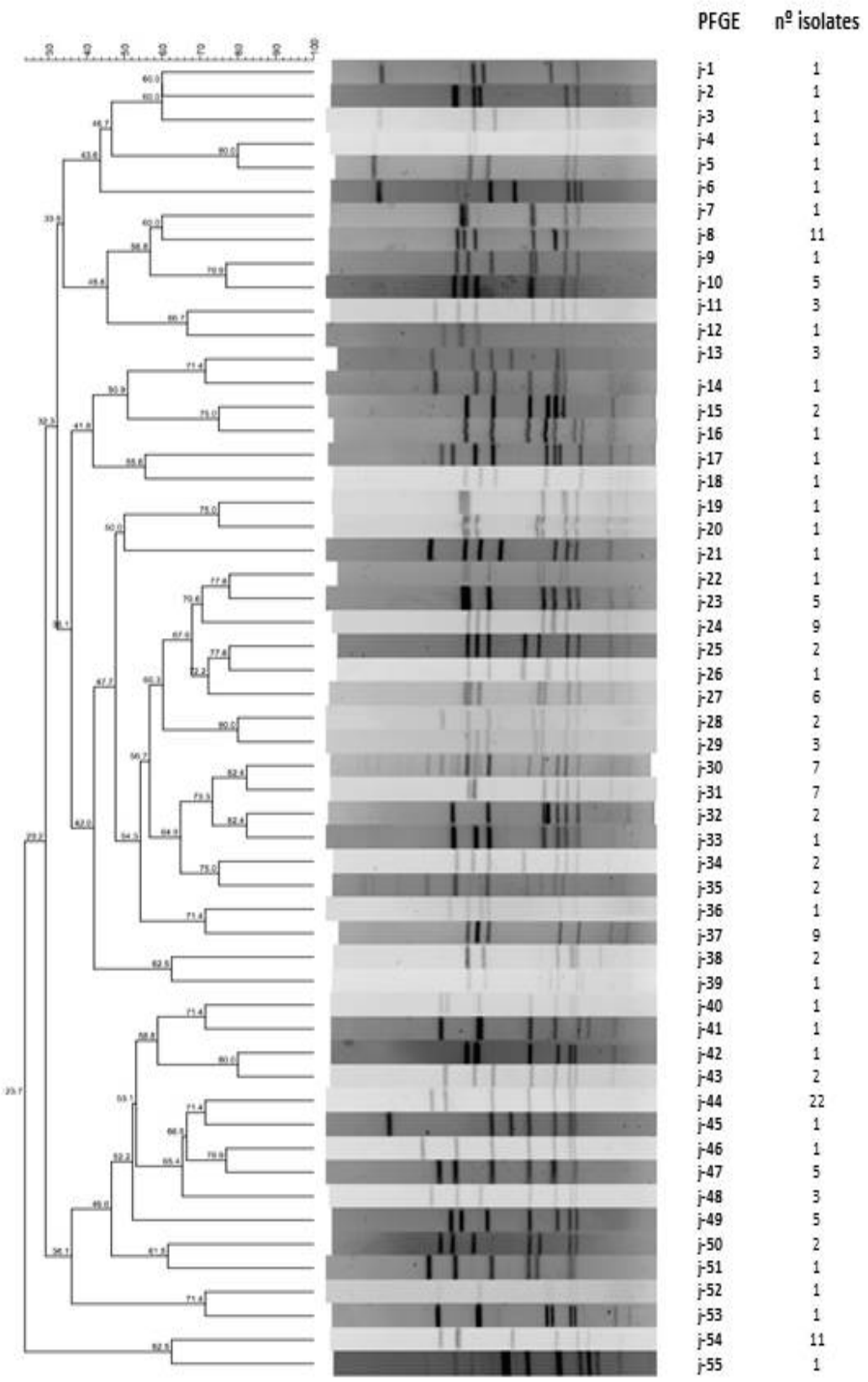

Figure 2. Dendrogram of *C. jejuni* PFGE types.

In general terms, unpacked products have shown less PFGE types although with higher numbers of isolates (9 unpacked products with 2 to 22 isolates forming the different clusters). However, packed products have shown more PFGE types although with less number of isolates (11 products from 2 to 7 isolated harbored the cluster). PFGE types sharing isolates from unpacked and packed chicken products were represented by 6 clusters with a range of 5 to11 isolates.

Clusters which harbored more than 3 isolates in the case of *C. jejuni* and *C. lari* and more than 2 in *C. coli* are shown in table 3. PFGE j-44 was the most prevalent within *C. jejuni* population (13.5 %). Isolates from this pulsotype were present only in unpacked products which were purchased in local butcher shops. The same situation happens with type j-54 and j-37 with 6.8 % and 5.5 % respectively. The PFGE type j-44 with 22 isolates together with j-8 and j-54, both with eleven isolates, were recovered during approximately 1 month in different butcher shops. A similar situation happens with cluster j-37 that was isolated during longer time, around two months. In both cases, most of the strains were isolated from samples coming from the local slaughterhouse. Moreover, j-24, j-30, j-31, j-47 and j-49 were recovered along 2 and more than 3 months from packed and unpacked chicken products. Nevertheless, clusters, with strains came mainly from packed products, were isolated during a longer period of time (table 3).

Pulsotype j-10 comprises strains from two different geographical origins from packed chicken products (C-West and I-Center). However, isolates from C-west origin appeared in samples taken with a difference of one month and a half between them. The same situation occurs with cluster c-14 from *C. coli* (c-14) appearing isolates with a difference around two months in packed products from the same supplier E2 (center). Moreover, in this case, both strains were isolated from the same type of chicken products: thigh.

Additionally, *C. lari* (type l-2) was recovered from the same supplier E1 (North) with a difference of 25 days.

Table 3. Relation between different *Campylobacter* spp. pulsotypes and suppliers of chicken products along sampling time.

PFGE type	Number of isolates	Chicken product[a]	Sampling time (days)	Suppliers/butcher shops[b]
C. jejuni				
j-8	11	b, t, m, n	21	a, b, c, d, g, h, i, C
j-10	5	b, t	44	C, I
j-11	3	b, m	58	j, d
j-13	3	b, t, m	16	g, e
j-23	5	b, t, m	59	a, b, f, i, I
j-24	9	b, t, m	95	B1, E2, c, j, h
j-27	6	b, t, m	14	d, f, g
j-29	3	b	1	G
j-30	7	b, t	72	B2, C, E1, E2
j-31	7	b, n	72	C, I, h
j-37	9	b, t, m, n	58	a, c, d, e, g, k
j-44	22	b, t, m, n	42	a, b, c, e, f, g, h, i
j-47	5	b, t, n	95	g, d, e, E
j-48	3	b, m, n	7	b
j-49	5	b, t, m, n	119	D, B1, h
j-54	11	b, t, m, n	31	a, b, c, d, e, f, g
C. coli				
c-1	2	m	1	D
c-7	2	m	7	k, l
c-9	3	t, b	1	E1
c-10	2	m	62	I, D
c-12	2	b, m	21	k, b
c-14	5	t	57	E2
c-16	6	t, b, m	105	C, E2, I, j
c-18	2	t	39	h, I
c-19	3	t	1	B1
C. lari				
l-2	4	t, b, n	25	E (North), c

([a]): b: breast, t: tight; m: marinated, n: minced chicken products.

([b]): from a-l: are different unpacked chicken products from butcher shops; from A-I: are different packed chicken products from industrial suppliers

3.4. Antibiotics susceptibility

The phenotypic antimicrobial susceptibility determined for the 76 types corresponded to 55 *C. jejuni* (j1-j-55), 19 *C. coli* (c-1-c-19) and 2 *C. lari* (l-1to l-2) are shown in tables 4 and 5. *C. jejuni* pulsotypes were 100 % resistant to (fluoro)quinolones, 98.2 % to tetracycline and 1.8 % to azithromycin. However, some differences were observed in types j-19 and j-21; being j-19 resistance to azithromycin and j-21 sensitive to tetracycline (table 4). Similar situation occurs with all *C. lari* pulsotypes (l-1, l-2); being resistant to quinolones and tetracycline (100%) and l-1 showing resistance to gentamycin as well (table 5).

Strains of *C. coli* were the ones who showed more resistances (table 5). All strains were resistant to tetracycline (100%). Regarding to (fluoro)quinolones, all strains showed more resistance to nalidixic acid (100 %) than to ciprofloxacin (78.9 %). Resistance to macrolides was 52.6 % in both: erythromycin and azithromycin. Only pulsotype c-19 showed resistance to gentamycin. According with these results, one pulsotype of *C. jejuni* (j-19), nine of *C. coli* (c-2, c-3, c-4, c-8, c-9, c-10, c-12, c-17, c-19) and one *C. lari* (l-1) can be considered multidrug-resistance.

Table 4. Results of antimicrobial susceptibility and MIC in *C. jejuni* PFGE types.

PFGE	CIP	NAL	TC	ERY	AZT	GM
C. jejuni						
j-1	R (16)[a]	R (64)[a]	R (2)	S (0.50)	S (0.06)	S (1)
j-2	R (8)	R (128)	R (64)	S (0.50)	S (0.06)	S (1)
j-3	R (16)	R (128)	R (64)	S (0.50)	S (0.06)	S (1)
j-4	R (8)	R (64)	R (128)	S (1)	S (0.06)	S (0.50)
j-5	R (8)	R (64)	R (64)	S (0.50)	S (0.06)	S (0.50)
j-6	R (16)	R (64)	R (4)	S (0.50)	S (0.03)	S (0.50)
j-7	R (16)	R (128)	R (128)	S (1)	S (0.06)	S (0.50)
j-8	R (8)	R (128)	R (64)	S (0.50)	R (0.03)	S (0.50)
j-9	R (16)	R (128)	R (16)	S (1)	S (0.06)	S (1)
j-10	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (1)
j-11	R (8-32)[b]	R (128)	R(64-128)[b]	S (0.50-1)[b]	S(0.03-0.06)[b]	S (0.50)
j-12	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-13	R (8)	R (128)	R (128)	S (0.50)	S (0.03)	S (0.50)
j-14	R (16)	R (128)	R (32)	S (0.50)	S (0.03)	S (0.50)
j-15	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-16	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-17	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (1)
j-18	R (16)	R (128)	R (32)	S (1)	S (0.03)	S (1)
j-19	R (8)	R (128)	R (16)	S (0.50)	R (1)	S (1)
j-20	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-21	R (8)	R (128)	S (0.50)	S (0.50)	S (0.06)	S (0.50)
j-22	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
j-23	R (8-16)[b]	R (128)	R (8-128)[b]	S (0.50)	S (0.03)	S (0.50-1)[b]
j-24	R (8)	R (128)	R (128)	S (0.50)	S (0.06)	S (1)
j-25	R (8)	R (128)	R (8)	S (0.50)	S (0.06)	S (0.50)
j-26	R (8)	R (128)	R (8)	S (0.50)	S (0.06)	S (0.50)
j-27	R (8-16)[b]	R (128)	R(64-128)[b]	S (0.50-2)[b]	S (0.03-0.06)[b]	S (1)
j-28	R (16)	R (128)	R (32)	S (1)	S (0.03)	S (1)
j-29	R (8)	R (128)	R (128)	S (0.25)	S (0.06)	S (1)
j-30	R (8)	R (128)	R (64)	S (0.25)	S (0.06)	S (0.50)

Table 4 (continuation). Results of antimicrobial susceptibility and MIC in *C. jejuni* PFGE

PFGE	CIP	NAL	TC	ERY	AZT	GM
j-31	R (8-16)[b]	R (128)	R(32-128)[b]	S (0.5-2)[b]	S (0.03-0.06)[b]	S (0.5-1)[b]
j-32	R (16)	R (64)	R (4)	S (0.50)	S (0.03)	S (0.50)
j-33	R (8)	R (64)	R (128)	S (0.50)	S (0.03)	S (0.25)
j-34	R (16)	R (128)	R (64)	S (1)	S (0.12)	S (1)
j-35	R (8)	R (128)	R (64)	S (1)	S (0.06)	S (1)
j-36	R (8)	R (128)	R (32)	S (0.50)	S (0.03)	S (0.50)
j-37	R (8)	R (128)	R (128)	S (0.50)	S (0.06)	S (0.50)
j-38	R (8-16)[b]	R (128)	R(64-128)[b]	S (1-2)[b]	S (0.06)	S (1)
j-39	R (16)	R (128)	R (128)	S (1)	S (0.06)	S (1)
j-40	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-41	R (8)	R (128)	R (16)	S (1)	S (0.06)	S (1)
j-42	R (8)	R (64)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-43	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-44	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-45	R (16)	R (128)	R (64)	S (1)	S (0.06)	S (0.50)
j-46	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-47	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-48	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-49	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.50)
j-50	R (16)	R (128)	R (128)	S (2)	S (0.06)	S (1)
j-51	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)
j-52	R (32)	R (32)	R (16)	S (0.50)	S (0.03)	S (0.50)
j-53	R (32)	R (128)	R (32)	S (0.50)	S (0.06)	S (0.50)
j-54	R (8)	R (128)	R (64)	S (0.25)	S (0.03)	S (0.25)
j-55	R (8)	R (128)	R (64)	S (1)	S (0.06)	S (1)

CIP: ciprofloxacin; **NAL**: nalidixic acid; **TC**: tetracycline; **ERY:** erythromycin; **AZT**: azithromycin; **GM**: gentamicin.

R= resistance; **S**=sensitive

([a]): Interpretation of MIC for *Campylobacter jejuni* epidemiological cutoff values: NAL (16 mg/L); CIP (0.5 mg/L); TC (1 mg/L); ERY (4 mg/L); AZT (0.25 mg/L) GM (2 mg/L).

([b]): Isolates belonging to the same cluster with different MIC values.

Tabla 5. Results of antimicrobial susceptibility and MIC in *C. coli* and *C. lari* PFGE types.

PFGE Type	CIP	NAL	TC	ERY	AZT	GM
C. coli						
c-1	R (16)	R (128)[a]	R (128)	S (1)	S (0.06)	S (1)
c-2	R (8)	R (128)	R (128)	R (16)	R (1)	S (1)
c-3	R (16)	R (128)	R (64)	R (16)	R (1)	S (0.50)
c-4	R (8)	R (128)	R (128)	R (16)	R (1)	S (1)
c-5	S (0.25)	R (16)	R (64)	S (1)	S (0.12)	S (1)
c-6	R (8)	R (128)	R (64)	S (0.50)	S (0.03)	S (1)
c-7	R (16)	R (128)	R (64)	S (0.25)	S (0.03)	S (1)
c-8	R (8)	R (64)	R (64)	R (16)	R (1)	S (1)
c-9	R (8-16)[b]	R (128)	R(64-128)[b]	R (16)	R (1)	S (1)
c-10	R (8-32)[b]	R (128)	R (128)	R (16)	R (1)	S (1)
c-11	R (16)	R (128)	R (128)	S (1)	S (0.12)	S (1)
c-12	R (8)	R (128)	R (64)	R (16)	R (1)	S (1)
c-13	S (0.25)	R (16)	R (64)	S (4)	S (0.25)	S (1)
c-14	S (0.25)	R (32)	R (32)	S (4)	S (0.25)	S (1)
c-15	R (16)	R (128)	R (32)	S (2)	S (0.06)	S (0.5)
c-16	R (16)	R (128)	R (128)	S (2)	S (0.12)	S (1)
c-17	R (16)	R (128)	R (128)	R (16)	R (1)	S (1)
c-18	S (0.25)	R (128)	R (32)	R (16)	R (1)	S (1)
c-19	R (16)	R (128)	R (128)	R (16)	R (1)	R (8)
C. lari						
l-1	R (8)	R (128)	R (64)	S (1)	S (0.06)	R (2)
l-2	R (4)	R (128)	R (64)	S (0.50)	S (0.03)	S (0.25)

CIP: ciprofloxacin; **NAL**: nalidixic acid; **TC**: tetracycline; **ERY**: erythromycin; **AZT:** azithromycin: **GM**: gentamicin.

R= resistance; **S**=sensitive

([a]): Interpretation of MIC for *Campylobacter coli* epidemiological cutoff values: NAL (16 mg/L); CIP (0.5 mg/L); TC (2 mg/L); ERY (8 mg/L); AZT (0.5 mg/L) Gm (2 mg/L).

([b]): Isolates belonging to the some cluster presented MIC differences.

4. Discussion

Monitoring *Campylobacter* prevalence and population typing in chicken products at retail level is important to assess a potential human health risk and to explore possible interventions to reduce it. Transmission along the broiler meat supply chain has been established as the main source of *Campylobacter* contamination in humans. For this reason, it is important to collect as much information as possible, to prevent and design strategies to control the presence of *Campylobacter*. According to our results the 39.4 % of chicken samples analyzed harbored *Campylobacter*. EFSA studies have shown similar percentages (39.99 %) in fresh meat from broiler at retail in European Union, reaching in Spain 50 % (EFSA, 2017).

Among the total analyzed products, packed chicken (MAP) presented less prevalence (33.6 %) than unpacked products (45.3 %). These results, agree with other studies, where *Campylobacter* was less present in MAP than in other chicken products stored under ambient or vacuum (Luber and Bartelt, 2007). How different gases concentrations used in MAP might affect *Campylobacter* survival is not yet fully understood. Due to the microaerophilic nature of *Campylobacter*, some authors state that high concentrations of O_2, above 70 %, reduce *Campylobacter* spp. counts in more than two logs (Boysen et al., 2007; Rajkovic et al., 2010). Although, it seems that those concentrations favors the growth of meat spoilage bacteria. On the contrary, other authors found some aerotolerant or even do hyper-tolerant *Campylobacter* strains able to grow in high O_2 concentrations, suggesting, that high CO_2 atmospheres can reduce or inhibit the presence of *Campylobacter* in chicken packed products (Oh et al., 2017). Moreover, Meredith et al. (2014) recommended a gas composition of 40 CO_2/30 O_2/30 N_2 as the most appropriate gaseous mixture for achieving the dual objectives of extending shelf-life while inhibiting *Campylobacter* survival. In this study, *Campylobacter* strains have

been isolated from packages with a broad range of O_2 (11.0-72.7 %) and CO_2 (1.5-54 %) composition. This fact indicates that MAP might have a positive effect against *Campylobacter,* although it is not enough to eliminate the pathogen in these products, suggesting that these strains may have greater resistance to O_2 and CO_2 than previously thought. Therefore, pre-harvest measures as biosecurity and/or post-harvest ones, such as scalding, chilling and removal of faecal residues might be considered before packing poultry product (Osimani et al., 2017).

Unpacked products presented higher *Campylobacter* spp. contamination (45.3 %). All butcher shops were positive to *Campylobacter* in each sampling day, as well as in all type of products analyzed although with some variations. Due to the fastidiousness and oxygen sensitivity, *C. jejuni* is not expected to survive efficiently during foodborne transmission in oxygen-rich, atmospheric conditions. However, our results have shown that it is evident that the pathogen can survive in aerobic environment. Some authors have mentioned that aerotolerance is one of the survival mechanisms (Bronowski et al., 2014; Rodrigues et al., 2016) and other authors have recently reported that hyper-aerotolerant *C. jejuni* are highly prevalent in retail poultry meat (Oh et al., 2017; O`Kane and Connerton, 2017).

According with our results, *C. jejuni* was the most prevalent specie in both, ambient and MAP products, representing 81.2 % of *Campylobacter* isolates. This data might be in relation with the high level of *C. jejuni* that cause infection in humans; that can reach 90 % of human infections (Skarp et al., 2016). Authors as Oh et al. (2015) found that most hyper-aerotolerant *C. jejuni* strains belong to MLST 21 CC, which is the major genotype implicated in human gastroenteritis. Therefore, it is possible that *C. jejuni* belonged to this MLST with increased aerotolerance may survive well in foods and are

more likely to reach humans, consequently causing human illnesses more frequently than aerosensitive *C. jejuni* strains (Oh et al., 2015; Oh et al., 2017).

Cross-contamination might have an important role, since it might increase *Campylobacter* prevalence. For instance, unpacked marinated product presented the highest *Campylobacter* contamination percentage (56.3 %) due to the manipulation in the butcher shops. Some authors suggested that polyphosphate used to marinade, improve the survival of *Campylobacter* species in the exudate of treated poultry products (Gunther et al., 2015). Moreover, manipulation of raw poultry before ready-to-eat food, and not washing the hands and/or the cutting board during handling of foods might be factors that increase cross-contamination (Signorini et al., 2013; Zbrun et al., 2017).

Campylobacter prevalence among products was different; being skin-on (thigh) the most packed contaminated products as it has been described in other studies (Berrang et al., 2001; Chanarapanont et al., 2003; Davis and Conner, 2007 and Stella et al., 2017). On the contrary, minced products showed low *Campylobacter* prevalence in comparison with the rest of the fresh chicken products. This fact may be explained by the use of several additives including preservatives used in the elaboration of these products. Similar results were obtained by Stella et al. (2017) in a recent study of retail poultry products in Italy.

Typing revealed a wide heterogenicity among isolates (204 isolates were clustered into 76 clusters). A similar genetic diversity has been reported by other authors (Di Giannatale et al., 2014; Pedonese et al., 2017). Moreover, our study suggests that some pulsotypes might be associated to the plant where samples come from. This is the case of pulsotypes j-37, j-44, j-54, where isolates with the same pulsotype, were recovered during 58, 42 and 31 days respectively from different butcher shops, suggesting a possible common origin, as almost all products came from the main slaughterhouse of the city.

Similar situation has been observed in cluster c-14, where strains of *C. coli* from the same product (thigh), from the same supplier (E2), were found in samples taken with a difference of 57 days. Moreover, in a previous study we found that some *C. jejuni* pulsotypes can persist in the poultry plant environment during at least 21 days (García-Sánchez et al., 2017). This hypothesis is corroborated by other authors, who identify some *C. jejuni* profiles in the same processing plant, which re-appeared at different years, suggesting that some predominant PFGE patters are associated to a given processing plant (Willians and Oyarbazal, 2012). Additionally, persistence might be observed as well inside the butcher shops. In cluster j-11 two strains, with the same pulsotype, were isolated from marinated products, in the same butcher shop with two month of difference.

Alternatively, some practices as freezing meat, in both butcher shops and industrial, might be possible in order to regulate the stock of fresh chicken products according to market demands. Freezing of *Campylobacter* positive broiler carcasses has been proposed as an intervention to reduce the incidence of *Campylobacter* in fresh poultry (Georgsson et al., 2006; Tustin et al., 2011). In that sense, Melero et al. (2013) found in chicken burgers, that freezing stress was an effective strategy to reduce *C. jejuni* counts, but only in combination with a high-O_2-MAP (50 % O_2:50 % CO_2), it was completely eliminated.

The high prevalence of *Campylobacter* in fresh chicken retail products may be considered as a public health problem, since consumer might be exposed to this biological risk. Moreover, *Campylobacter* has developed resistance to several antimicrobial agents over the years, including (fluoro)quinolones and macrolides, which are the drugs of choice in treatment (Di Giannatale et al., 2014). In this study a high resistance among different genotypes has been observed, showing severe multi-drug resistance strains, especially within *C. coli* isolates. *C.jejuni* was mainly resistance to ciprofloxacin,

nalidixic acid and tetracycline. Similar results have been described by other authors as Zhang et al. (2016) and Pedonese et al. (2017). Resistance of *Campylobacter* to fluoroquinolones was firstly reported in the late 1980s and since then, it has been increasing in many countries. An alarming situation is reported in this study, where the all C. *jejuni isolates* were resistant to this antibiotic.

5. Conclusion

This study provides information about the contamination levels and genetic diversity of *C. jejuni*, *C. coli* and *C. lari* in different fresh chicken products at retail in Spain. Results obtained suggest that some *Campylobacter* strains are more robust that previously thought, as they are able to survive in a broad range of different gas compositions and persists longer in the environment of the processing plant or butcher shop. Cross-contamination might play an important role in the high diversity of *Campylobacter* strains found at retail level. However, it is still surprisingly, that the same pulsotypes appear in different fresh chicken products coming from different suppliers and different geographical locations. This study confirms the increasing concern due to the rise in antibiotic resistance of *Campylobacter* spp. isolates in fresh chicken products at retail, which is the previous step before consumer. This fact, suggest than a bigger effort must be done in previous steps to avoid or reduce the presence of this pathogen in those products. Moreover, consumer campaigns alerting about handling of fresh chicken products at home must be conducted. Further studies, will be done to find out common metabolic characteristics among those survival strains to get insight in their adaptive mechanism that allow them to persist along the food chain till retail.

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CHAPTER 5

VIII. CHAPTER 5. Characterization of *Campylobacter* spp. isolated along the chicken food chain as a possible route of sporadic cases of campylobacteriosis in Spain

Abstract

Campylobacter jejuni and *C. coli* are one of the major causes of bacterial foodborne enteric infection. Consumption of chicken meat is considered the highest common route of human infection. Therefore, in order to relate human and chicken *Campylobacter* isolates, characterization by Pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance were performed. During years 2014 and 2015, a total of 1,039 isolates were recovered: 749 from chicken food chain (farms, slaughterhouse and retail) and 290 *Campylobacter* spp. from human infections. Among human isolates, *C. jejuni* represented 90.7 % and *C. coli* 9.3 %. Male were more affected from campylobacteriosis than female, and children between 1-5 years was the age range with highest reported cases. According to this fact, pediatric was the medical service with more cases, followed by emergency service. Hospitalization rate was under 17.8 % of the diagnosed cases, and basically affects to the age range ≥ 60 years. Genotyping by PFGE analysis has shown a clear link between isolates from chicken food chain and clinical isolates. Shared clusters were obtained for all food chain steps analyzed: 9 in farms, 4 in slaughterhouse and 22 in retail. Moreover, high antimicrobial resistance has been observed, being more than 87.5 % of human isolates ciprofloxacin resistant. Those data are of concern due to quinolones like ciprofloxacin and macrolides are the first chosen drug treatment for human campylobacteriosis.

1. Introduccion

Gastrointestinal infection with thermotolerant campylobacters, cause significant morbidity in developing and developed countries; being considered a public health problem (CDC, 2015; Damjanova et al., 2011). The number of reported confirmed cases of campylobacteriosis in the European Union during 2016 was 246,307, showing an increase of 6.1 % in comparison with 2015 (EFSA, 2017). It is characterized by watery and sometimes bloody diarrhea, fever, vomiting and abdominal cramps. Symptoms may be appeared after 1-5 days of *Campylobacter* exposure (Skarp et al., 2016). Although, it is normally a self-limiting disease, the infection may lead to Guillain-Barré and Miller Fisher syndrome. Moreover, the pathogen has been associated with a range of extra-gastrointestinal manifestations, including bacteremia, lung infections, myocarditis, brain abscesses and meningitis (Kaakoush et al., 2015).

Although, campylobacteriosis is described as sporadic cases and outbreaks are less common, many studies related the increased incidence of campylobacteriosis due to some outbreaks. In Spain, *Campylobacter* outbreak was reported in Barcelona-school affecting 75 primary school children, due to cross contamination between chicken and Russian salad (Calciati et al., 2012). An average of 28 outbreaks per year have been reported during 2003-2008 in USA (Taylor et al., 2013) like a recent outbreak associated with the consumption of milk and/or cheese (Burakoff et al., 2018; Koppenaal et al., 2017). Others have been related to commercial catering serving chicken livers preparations; which it is leading to a novel theory linking modern restaurant cooking styles with outbreaks of *Campylobacter* (Lahti et al., 2017a; Moffatt et al., 2016).

Person-to-person transmission is uncommon and human exposure can come through direct contact with animals, food or environmental reservoirs (Skarp et al., 2016). The major risk of human campylobacteriosis has generally been linked to the

consumption of poultry meat. However, practices such as eating in a restaurant, consumption of ready-to-eat sandwiches, having activities as swimming (Boorduyn et al., 2010) or having contact with pets (Iannino et al., 2017) must also be considered for an adequate risk assessment. The survival and distribution of *Campylobacter* in the environment change with both space and time, and this genetic instability will affect the human exposure to the organism (Sanderson et al., 2018) and makes more difficult to find a link between the human infection and the specific source in the epidemiological studies (Gölz et al., 2014).

Antibiotic therapy against *Campylobacter* infections is only indicated in severe and persisting infections, in sensitive populations including children, the elderly, pregnant women and immunocompromised patients, as well as in cases of extra-gastrointestinal manifestations (Luangtongkum et al., 2009). Drug of choice are fluoroquinolones as ciprofloxacin and macrolides, such as erythromycin (Skarp et al., 2016). However, these primary antimicrobials used for treatment of human *Campylobacter* infections have become less effective by resistances increasingly encountered in this pathogen, and other antimicrobial chemotherapy such as aminoglycoside should be included as possible drug for human treatment (Nielsen et al., 2010).

To fully understand the ecology and epidemiology of *Campylobacter* human infections isolates from the chicken food chain and human infections must be well characterized. In that sense, a discriminatory typing technique such as pulsed field gel electrophoresis (PFGE) has been used extensively to compare *Campylobacter* isolates from different sources (Hänninen et al., 1998). Therefore, the comparison between chicken food chain and human isolates may provide valuable information for assessing the actual risk of acquiring human campylobacteriosis. Based on this, the aims of this study were (i) to analyze epidemiological data and genetic relatedness among

Campylobacter isolates obtained from different steps of the chicken food chain and human isolates causing campylobacteriosis during the same period of time and (ii) to analyze the antibiotic resistance of human isolates.

2. Material and methods

2.1. Sampling procedure and epidemiological data

The study area was restricted to campylobacteriosis cases diagnosed in the city of Burgos during the years 2014 and 2015. Burgos is a province of Autonomous Community of Castile and Leon which is located in the North of Spain, having an extension of 107 km² and a population of 177.100 inhabitants. During this period, *Campylobacter* isolates were obtained from samples related to different steps of the chicken food chain such as farms around the city (see chapter I), slaughterhouse (see chapter II) and chicken products from different retail shops (see chapter IV). Simultaneously, isolates from human campylobacteriosis infections were collected from human fecal samples in the Microbiology Department of the Hospital University of Burgos (HUBU). The period analyzed was divided into four according to the chain step analyzed: period 1 and period 2 corresponds to farms and human isolates in autumn and spring respectively, period 3 corresponds to isolates from slaughterhouse and humans and finally the period 4 corresponds to retail and human isolates (table 1). Moreover, epidemiological data was studied, including information such as patient gender and age, date of isolation, medical service where the case was notified being Primary Health Center or Hospital (pediatric, emergency, gastroenterology, internal medicine or hematology), required or not patient hospitalization and *Campylobacter* specie. In order to assure the period of incubation, human isolates were taken one month more from the end of each food step studied as it is shown in table 1.

Table 1. Sampling scheme used in this study.

Period	Food chain	n º	period of time		Hospital	nº	Period of time	
Period 1	Farm	193	24/10/2014	09/12/2014	**Human**	76	15/10/2014	09/01/2015
Period 2	Farm	116	05/05/2015	02/07/2015	**Human**	72	08/05/2015	01/08/2015
Period 3	Slaughterhouse	238	02/06/2014	27/06/2014	**Human**	45	01/06/2014	25/07/2014
Period 4	Retail	202	23/02/2015	18/06/2015	**Human**	97	24/02/2015	20/07/2015
Total		**749**				**290**		

(nº): number of isolates recovered in each period.

2.2. Isolation and identification of *Campylobacter* spp.

2.2.1. From chicken food chain: farms, slaughterhouse and retail.

In chapter I, II (García-Sánchez et al., 2017) and IV a detailed description is given about *Campylobacter* isolation from farms, slaughterhouse and retail respectively. The species identification was carried out using multiplex PCR to identify *C. jejuni, C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* sub. *fetus* as described by Wang et al. (2002). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.2.2. From human infections.

Campylobacter spp. were isolated from human feces samples and were kindly provided by the Microbiological Service of the HUBU to further genotyping. *Campylobacter* strains were identified by MALDI-TOF mass spectrometry. Briefly, a part of a colony from each isolate was taken directly from the agar plate after 18-24 h of incubation to obtain fresh bacteria, and deposited on a microtiter plate ground steel in a single spot and allowed to dry at room temperature. After drying, the target plate was loaded into the matrix-assisted laser desorption ionization-time of flight mass spectrometry-based Vitek MS v2 system (bioMérieux, France). The mass spectral fingerprint was generated and compared automatically to the Vitek MS database version 2.0. Raw spectra of the strains were analyzed by MALDI Biotyper 2.0 software using the

default settings. The whole process from MALDI-TOF mass spectrometry measurement to identification was performed automatically without any user intervention. The software generated a list of peaks that were used for matching against the reference library, by directly using the integrated pattern-matching algorithms of the software. Spectra were obtained and a score was attributed to each identification.

2.3. Pulsed field gel electrophoresis (PFGE)

Campylobacter spp. isolates from the chicken food chain were typed by PFGE in the previous chapters (I, II, IV). Isolates from human origin were cultured on Columbia agar (Oxoid, Basingstoke, England) supplemented with 5 % defibrinated sheep blood (Oxoid) under microaerobic conditions (24 h at 41.5 ºC) for the purpose of PFGE typing. PFGE analyses were performed applying the restriction enzymes *Sma*I and *Kpn*I, according to the PulseNet International standardized protocol (www.cdc.gov/pulsenet/pathogens/pfge.html). *Kpn*I was used to check the diversity of all isolates with similar *Sma*I patterns.

Restricted DNA was electrophoresed for 22.5 h on 1 % (w/v) SeaKem gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S). The electrophoresis conditions consisted in an initial switch time of 5 s and a final switch time of 55 s (gradient of 6 V/cm and an included angle 120º). Gels were stained with ethidium bromide solution and photographed with Gel Doc XR System (Bio-Rad Laboratories). BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0 %). The unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns. Isolates with <85 % similarity, according to the dendrogram, were clustered as separate pulsotypes (Boer et al., 2000).

2.4. Antimicrobial susceptibility

2.4.1 Minimal Inhibitory Concentration (MICs)

Campylobacter isolates from the chicken food chain were sub-cultured on Nutrient agar supplemented with 5 % sheep blood (Oxoid) and incubated at 41.5 ºC for 24 h in microaerobic conditions. After incubation, bacteria was collected with a sterile cotton and suspended in 2 mL of 0.9 % NaCl and the turbidity was adjusted to 0.5 McFarland scale to carry out the inoculation. The inoculum was spread onto Nutrient agar plates supplemented with 5 % sheep blood (Oxoid) and was incubated in microaerobic conditions for 48 h at 41.5 ºC. Six antimicrobials belonging to four different classes were tested in different range of concentrations: two fluoro(quinolone); ciprofloxacin (0.03-64 mg/L) and nalidixic acid (4-128 mg/L); two macrolides, erythromycin (0.12-16 mg/L) and azithromycin (0.015-1 mg/L); one aminoglycosides, gentamicin (0.12-8 mg/L) and tetracycline (0.12-128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values according to European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). Multidrug resistance (MDR) was defined as simultaneous resistance to three or more unrelated antimicrobials (Mäessar et al., 2016). *C. jejuni* ATCC 33560^{TM} was used as a positive control as it was described by Cantero et al. (2017).

2.4.2. Disk diffusion test.

Antimicrobial resistance from *Campylobacter* human isolates was tested in the HUBU, according to their routine protocol. Briefly, isolates were sub-cultured on Mueller-Hinton (MH) agar supplemented with 5 % defibrinated sheep blood (Oxoid). The inoculation was adjusted to 0.5 McFarland scale. Discs with different concentrations according to the antibiotic tested: erythromycin (ERY, 15 μg); azithromycin (AZM, 15 μg), ciprofloxacin (CIP, 5 μg), gentamycin (GEN, 10 μg) and the betalactam imipenem

(IMP, 10 μg) were deposited on the different plates. Inhibition halos were read after 24 h of incubation at 41.5 ºC for 24 h in microaerobic conditions. Cutoffs values were performed according to CLSI protocols (www.clsi.org).

3. Results

3.1 Prevalence and epidemiological data of human isolates

A total of 1039 *Campylobacter* spp. were isolated during the period studied. From these, 749 isolates were collected from the chicken food chain: 315 from broiler's farm samples, 238 from the slaughterhouse environment and 202 from chicken products purchased from retail shops. The remaining 290 isolates were obtained from human infections occurred simultaneously during the same period of time as described in table 1. From the total of human isolates, *C. jejuni* was the predominant specie, represented by 90.7 % (263/290), and followed by *C. coli* 9.3 % (27/290). Results of the four periods studied are presented in the different paragraphs:

Period 1

In this period, farm samples were taken during autumn 2014 (see chapter I) and simultaneously isolates from human infections were collected. Moreover, the human sampling was extended by one month after the farm sampling finished due to the incubation time of campylobacteriosis (table 1). During this period, 76 human cases were reported by the Hospital. Epidemiological data showed that the highest number of infections were in males (64.5 %) and ages from 1 to 4 years (31.6 %) (table 2). Regarding the service, the majority of cases were notified by Primary Health Center (67.1 %) while the other 32.9 % was diagnosed in different Hospital departments as shown in table 3; with the pediatric service as the one that declared the highest number of cases. Hospitalization was required only in 13.2 % (10/76) of the total diagnosed cases diagnosed, from which a total of 60 % of the patients was in age ≥ 60.

Period 2

This period covers the farm sampling during spring 2015 (see chapter I) and the simultaneously period, including one extended month, for human isolates (table 1). Seventy-two isolates were recovered from human campylobacteriosis during this period. As shown in table 2 male was the sex more affected (63.9 %) like children between 1 and 4 years. During this period, similar percentage of cases was recovered from Primary Health Center (58.3 %) and Hospital departments (41.7 %); however, some differences were observed. The 88.1 % of cases in Primary Health Center were reported from the pediatric service while the emergency service reported the majority of cases in the Hospital (43.3%). The other rates are shown in table 3. In this period only 11.1 % (8/72) of patients required hospitalization and again patients over 60 were the most hospitalized (62.5 %).

Period 3

Samples from a chicken slaughterhouse were taken during one month in 2014 (see chapter II) and simultaneously samples from human campylobacteriosis with a month extended (table 1). Forty-five campylobacteriosis were diagnosed. Epidemiological data showed that during this period the infections were balanced among males (53.3%) and females (46.7 %). As well as in the other periods, ages of 1-4 years were the most affected by the infection. The 68.9 % of cases was detected by Primary Health Center and the pediatric service in the Hospital which declared 83.9 % of the infections among other services (table 3). During this period, hospitalization reached the highest rate (17.8 %) among all the periods analyzed, however, in this case the ages more compromised were between 6 and 37 years old and only one case was reported in a patient over 60.

Table 2. Epimemiological data of sex and age parameters from the total of campylobacteriosis human reported cases during the four period analyzed.

	Sex		Age					
Periods	**Male[a]**	**Female**	**<1[b]**	**1-4**	**5-9**	**10-34**	**35-39**	**≥ 60**
Period 1	49/76 (64.5 %)	27/76 (35.5 %)	10/76 (13.2 %)	24/76 (31.6 %)	12/76 (15.8 %)	15/76 (19.7 %)	6/76 (7.9 %)	9/76 (11.9 %)
Period 2	46/72 (63.9 %)	26/72 (36.1 %)	5/72 (6.9 %)	30/72 (41.7 %)	12/72 (16.7%)	6/72 (8.3 %)	7/72 (9.7 %)	12/72 (16.7 %)
Period 3	24/45 (53.3 %)	21/45 (46.7 %)	3/45 (6.6 %)	15/45 (33.3 %)	9/45 (20 %)	10/45 (22.2 %)	4/45 (8.9 %)	4/45 (8.9 %)
Period 4	60/97 (61.9 %)	37/97 (38.1 %)	8/97 (8.2 %)	41/97 (42.3 %)	18/97 (18.6 %)	14/97 (14.4 %)	5/97 (5.1 %)	11/97 (11.4 %)
Total	**179/290 (61.7 %)**	**111/290 (38.3 %)**	**26/290 (8.9 %)**	**110/290 (37.9 %)**	**51/290 (17.6 %)**	**45/290 (15.5 %)**	**22/290 (7.6 %)**	**36/290 (12.4 %)**

[a]Number of males or femalediagnosed/total ofcampylobacteriosis cases per period analyzed.

[b]Number of campylobacteriosis diagnosed in different groups of ages / total of campylobacteriosis cases per period analyzed.

Table 3. Number of campylobacteriosis reported according to the department which diagnosed the case during the four period analyzed.

	Primary Health Center			*Hospital*					
	Reported cases[1]	**P[2a]**	**M[2b]**	**Reported cases[1]**	**P[2a]**	**M[2c]**	**E[2d]**	**G[2e]**	**H[2f]**
Period 1	51/76 (67.1 %)	42/51 (82.4 %)	9/51 (17.6 %)	25/76 (32.9 %)	8/25 (32 %)	5/25 (20 %)	7/25 (28 %)	3/25 (12 %)	2/25 (8 %)
Period 2	42/72 (58.3 %)	37/42 (88.1 %)	5/42 (11.9 %)	30/72 (41.7 %)	8/30 (26.7 %)	5/30 (16.6 %)	13/30 (43.3 %)	2/30 (6.7 %)	2/30 (6.7 %)
Period 3	31/45 (68.9 %)	26/31 (83.9 %)	5/31 (16.1 %)	14/45 (31.1 %)	4/14 (28.6 %)	1/14 (7.1 %)	4/14 (28.6 %)	4/14 (28.6 %)	1/14 (7.1 %)
Period 4	64/97 (66 %)	55/64 (86 %)	9/64 (14 %)	33/97 (34 %)	10/33 (30.3 %)	4/33 (12.1 %)	13/33 (39.4 %)	5/33 (15.2 %)	1/33 (3 %)
Total	**188/290 (64.8 %)**	160/188 (85.1 %)	28/188 (14.9 %)	**102/290 (35.2 %)**	30/102 (29.4 %)	15/102 (14.7 %)	37/102 (36.3 %)	14/102 (13.7 %)	6/102 (5.9 %)

[1]Number of campylobacteriosis reported by Primary Health Center or Hospital/total number of campylobacteriosis reported.

[2]Number of campylobacteriosis reported by the different service/the total of campyobacteriosis reported in the Primary Health Center or Hospital.

P[a]: pediatric; **GM[b]**: general medicine; **IM[c]**: internal medicine; **E[d]**: emergency; **G[e]**: gastroenterology; **H[f]**: hematology.

Period 4

The last period covers those *Campylobacter* isolates from retail chicken products in 2015 during 4 months as well as those from human infections with a month extended as shown in table 1. A total of 97 campylobacteriosis were diagnosed during this period. Regarding epidemiological data, males were more affected (61.9 %) than females (38.1%) as well as children between 1 and 4 years (37.9 %). The 66.0 % of cases was detected in Primary Health Center and 34.0 % in Hospital. Almost all the cases (86.0 %) declared by the Primary Health Center came from the pediatric service and from the emergency service in the Hospital (39.4 %, table 3). From the total of cases low percentage (10.3 %) of patients had to be hospitalized from which 50.0 % were over 60. In all the periods, although patients over 60 were the less infected; they were the most hospitalized.

3.2 Genotyping

Genotyping results are focused only on the overlapping pulsotypes obtained from each period between the campylobacters isolated in the different steps in the chicken food chain and from human infections. In order to simplify the cluster analysis, one isolate from each PFGE type obtained in the different steps of the chicken food chain was used to compare with those of human infections in the corresponding period:

3.2.1. Period 1: Farms-Hospital

During this period, 76 human cases were detected but only 68 isolates were typed. PFGE analysis clustered the 68 isolates into 39 PFGE types; 34 *C. jejuni* and 5 *C. coli* (table 4). From the total of 10 PFGE types obtained in farms, five *C. jejuni* PFGE types formed clusters with human isolates. These were P_1-16, P_1-18, P_1-27, P_1-30 and P_1-36 as shown in table S1 (supplementary data). In general, all the overlapping PFGE types, except P_1-30, caused campylobacteriosis at least one month before detected in the farm

Table 4. *Campylobacter* PFGE clusters formed during each period in the chicken food chain and within human isolates and those overlapping in each period.

		CHICKEN FOOD CHAIN					HOSPITAL				
		Number PFGE	**PFGE *C.jejuni***	**PFGE *C. coli***	**PFGE *C. lari***		**Number PFGE**	**PFGE *C. jejuni***	**PFGE *C. coli***	**PFGE *C. lari***	**Shared PFGE[a]**
Period 1	**Farms**	10	8	2	-	**Human**	39	34	5	-	5
Period 2	**Farms**	11	11	-	-	**Human**	38	37	1	-	4
Period 3	**Slaughterhouse**	7	7	-	-	**Human**	33	29	4	-	4
Period 4	**Retail**	76	55	9	2	**Human**	100	77	21	2	22

[a]: Number of clusters that share isolates from the chicken food chain and humans in each period.

sampled. Moreover, all of them were diagnosed in human cases after detection in the farm at a maximum of 71 days in the case of P_1-16. Almost all the human isolates belonging to the P_1-18 were detected before its presence in the farm with the exception of one human case that appeared one month later. P_1-16 and 18 caused the hospitalization of a patient over 60 while a female of 19 years old was hospitalized in the case of P_1-36 (data is shown as supplementary in table S1).

3.2.2. Period 2: Farms-Hospital

From this period only 53 out of 72 human isolates could be typed by PFGE. Results shown that the isolates were clustered in 37 *C. jejuni* PFGE types and 1 *C. coli* PFGE type (table 4). From the 11 *C. jejuni* PFGE types found in farms four were clustered together with human isolates (P_2 -5, P_2-8, P_2-9 and P_2-28. In general, all the shared PFGE types caused campylobacteriosis in humans, at least 28 days before to be detected in the farm sampled. Moreover, all of them were diagnosed in human cases after its detection in the farm at a maximum of 26 days in type P_2-28. No hospitalization was required due to the infection with these PFGE types (data is shown as supplementary table S2).

3.2.3. Period 3: Slaughterhouse-Hospital

During this period, 45 isolates from humans were clustered into 33 PFGE types, from which 29 were for *C. jejuni* and 4 for *C. coli* (table 4). From the 7 *C. jejuni* PFGE types found in slaughterhouse, four were clustered with human isolates (P_3-A, P_3-B, P_3-C, P_3-G). P_3-A and P_3-B were first recovered in the slaughterhouse and afterwards in humans while P_3-C and P_3-G were recovered during the time of appearance in the slaughterhouse. All of the pulsotypes except P_3-C were diagnosed in human cases between 13 days and one month after its last detection in the slaughterhouse. Three human isolates belonging to P_3-B (2) and P_3-G (1) caused the hospitalization of two males (27

and 82 years old) and a female patient in pediatric serviced respectively (supplementary data are shown in table S3).

3.2.4. Period 4: Retail-Hospital

During this period, from the 97 human cases diagnosed only 69 isolates were typed. Isolates from retail samples were clustered in 100 PFGE types (77 *C. jejuni*, 21 *C. coli* and 2 *C. lari*). From these, 22 clusters were formed with isolates shared from humans and chicken products, belonging 20 clusters to *C. jejuni* and 2 to *C. coli*. No relation was observed between *C. lari* and human infections. According to the isolation date, six PFGE types were recovered during the same period of time, thirteen appeared first in chicken products and after in humans and three appeared before in humans and then in chicken products (supplementary data is shown in table S4). It is remarkable that in 8 clusters were unpacked chicken products are present there is a coincidence with the zip code of the butcher shop where sample was isolated and the zip code of the medical services of pediatric or emergency. In this chicken food chain step only four pulsotypes types caused hospitalization: P_4-53 and P_4-62 in patients over 70 years, and P4-1 and P4-6 in a male of 20 years and a female of 43 years old, respectively (supplementary data is shown in table S4).

3.3 Antimicrobial susceptibility

Results previously presented in chapters I, III, IV showed that *Campylobacter* isolates from the chicken food chain had a high rate of antibiotics resistance. In these previous food steps, all *C. jejuni* strains were 100 % resistant to fluoro(quinolones) and tetracycline, whereas *C. coli* was less resistant to fluoro(quinolones) (78–100 %), 100 % resistant to tetracycline, but more resistant to macrolides (9.5-52 %) depending of the food step analyzed. Only some multidrug resistant strains were found in *C. coli* isolates. Likewise, human isolates also presented high resistances rates. Table 5 shows the

Table 5. Resistance to different antibiotics used in human treatment for campylobacteriosis associated in each period.

Resistance	Period 1	specie[a]	Period 2	specie	Period 3	specie	Period 4	specie	Total Period
CIP	72/76 (94.7 %)	(66/6)	60/72 (83.3 %)	(56/4)	39/45 (86.7 %)	(36/3)	82/97 (84.6 %)	(78/4)	253/290 (87.2 %)
CIP+GEN	1/76 (1.3 %)	(1/-)	2/72 (2.8 %)	(1/1)	-	-	1/97 (1.0 %)	(-/1)	(4/290) (1.4 %)
CIP+ERY+AZY	1/76 (1.3 %)	(-/1)	1/72 (1.4 %)	(-/1)	1/45 (2.2 %)	(-/1)	2/97 (2.0 %)	(-/2)	5/290 (1.7 %)
Total (%)	**74/76 (97.3)**	**(67/7) (90.5/9.5)**	**63/72 (87.5)**	**(57/6) (90.5/9.5)**	**40/45 (88.9)**	**(36/4) (90/10)**	**85/97 (87.6)**	**(78/7) (91.8/8.2)**	**262/290 (90.3 %)**

CIP; ciprofloxacin;**CIP+GEN;** ciprofloxacin+gentamycin; **CIP+ERY+AZY;** ciprofloxacin+erythromycin+azithromycin.
[a]**species** (*C. jejuni/C. coli*).

distribution of antibiotic resistance found in the 290 human isolates. A total of 262 isolates (90.3 %) presented resistance at least to one antibiotic class. The majority of them, 96.6 % (253/262) showed a resistance to ciprofloxacin only, followed by 1.9 % (5/262) resistant to ciprofloxacin, erythromycin and azithromycin and 1.5 % (4/262) resistant to ciprofloxacin and gentamycin (1.5 %). Regarding *Campylobacter* species, resistances in *C. jejuni* was represented by 90.5 % (238/263) for ciprofloxacin, followed by 0.8 % (2/263) to gentamycin. On contrary, *C. coli* presented resistances to three antibiotics classes; 88.9 % (24/27) to ciprofloxacin, 18.5 % (5/27) to macrolides (azithromycin and erythromycin) and 7.4 % (2/27) to gentamycin. Table 6 shows the relation between the isolates resistant to two classes of antibiotics and the epidemiological data. Among the 5 isolates resistant to ciprofloxacin and macrolides, which are the two drugs of choice for human treatment, 2 patients were hospitalized.

Table 6. Epidemiological data of isolates resistant to two classes of antibiotics.

Periods	Specie (n)*	Age	Sex	Medical Service	PH/H**	Hospitalization
Period 1						
CIP+GEN	*C. jejuni* (1)	2	Male	Pediatric	PH	NO
CIP+ERY+AZY	*C. coli* (1)	2	Male	Pediatric	H	NO
Period 2						
CIP+GEN	*C. jejuni* (1)	7	Male	Pedriatric	PH	NO
	C. coli (1)	84	Male	Emergency	H	NO
CIP+ERY+AZY	*C. coli* (1)	68	Male	Gastroenterology	H	YES
Period 3						
CIP+ERY+AZY	*C. coli* (1)	15	Female	General Medicine	PH	NO
Period 4						
CIP+GEN	*C. coli* (1)	84	Male	Emergency	H	NO
CIP+ERY+AZY	*C. coli* (1)	20	Male	Gastroenterology	H	YES
	C. coli (1)	61	Female	General Medicine	PH	NO

(n)*; number of isolates. ****PH**: Primary Heath Center; **H**: Hospital

4. Discussion

This study has evaluated the epidemiology of human isolates and genetic relatedness with isolates from the chicken food chain to try to link the campylobacteriosis occurred in Burgos (Spain) to chicken consumption or manipulation during an equivalent period of time. Preventing campylobacteriosis depends on a thorough understanding of its epidemiology. Transmission along the chicken food chain (from broiler farms-slaughterhouse-retail) is considered as a major potential source for *Campylobacter* human infection (Ramonaite et al., 2017; Yahara et al., 2017). However, the ecology and epidemiology of *Campylobacter* is complex due to the wide range of hosts it can colonize, the high genetic diversity of the bacterium (Griekspoor et al., 2013), the ability to enter a viable but not culturable state (Bronowski et al., 2014) and its sensitivity to environmental stress (Wassenaar and Newell, 2000).

Although, campylobacteriosis is the zoonosis with the highest incidence in the European Union, the notification is not mandatory in all European Members. For instance, the notification is based on a voluntary system in Belgium, France, Italy, Luxembourg and the Netherlands or other systems has been stablished in Spain and the United Kingdom (EFSA, 2017). In Spain, campylobacteriosis data collected by Spanish Epidemiology National Survillance (RENAVE) come from Microbiology laboratories that voluntarily notify the disease to the Microbiology Information System (SIM) according to their protocols (Berradre et al., 2017). This might be one of the reasons to explain why the epidemiology of campylobacteriosis in humans and its route of transmission are still poorly understood. In that sense, epidemiological information such as gender, age, date, diagnostic service and hospitalization was recovered in this study. According to gender, males had higher infection rates than females (ratio =1.61) and the incidence was high in children under 5 years of age. This trend has been maintained

during the period 2008-2015 in Castile and Leon (Spain) according to Berradre et al. (2017). Similar gender incidence has been described in other areas of Europe as Germany or England; however, these studies reported lower male/female ratio, 1.15 and 1.14, respectively (Nichols et al., 2012; Schielke et al., 2014).

Moreover, this study has shown that the highest infection rate was found in children under 5 years (46.8 %), from which children between 1 and 4 years were the most affected (37.9 %). These data are in agreement with other studies such as Kuhn et al. (2017) and Samuel et al. (2004). However, there is a gap in understanding the link between the source and infection in this early age. According to literature, cross-contamination and poor kitchen hygiene within the household is considered to play a major role in the transmission of *Campylobacter* (Doorduyn et al., 2010; Guyard-Nicodeme et al., 2013). Other possibilities are the lack of hygiene measures in some daily activities. For instance, not washing hands after some practices; having outdoor activities, playing in parks with mud (Stuart et al., 2010), having school farm visits (Lahti et al., 2017b), contact with pets or puppies (Campagnolo et al., 2018) or contact with contaminated water surfaces as fountains (Szczepanska et al., 2017).

This study has shown that the pediatric service in the Primary Health Center reported most of the campylobacteriosis cases in children (85.1 %) in comparison with the pediatric service in the Hospital (29.4 %). It might be related with the fact that parents go first to the Primary Health Center when symptoms such as diarrhea, abdominal pain, fever and vomiting appear during the first hours of illness to avoid dehydration. However, when bloody diarrhea appears or the symptoms do not subside children are taken to the Hospital. In that sense, in the hospital the emergency service was the one that reported the highest number of cases (36.3 %). Apart from children, elderly people over 60 could also contribute to this situation, mainly when they show acute dysentery and severe

diarrhea, probably with an immunocompromised system, extra intestinal infections or when symptoms are severe or persistent.

Campylobacter jejuni and *C. coli* are the most frequent species that causes campylobacteriosis worldwide. However, differences in the incidence have been described among these species. This study has shown that *C. jejuni* caused more human infections (90.8 %) during the period analyzed than *C. coli* (9.2 %). Similar results for the later has been published by other studies in England and Wales (7.0 %) and in The Netherlands (8.0 %) (Doorduyn et al., 2010; Gillespie et al., 2002). Chicken food chain is the major source for *C. jejuni,* but not for *C. coli* as some authors have reported (Damjanova et al. 2011; Gillespie et al., 2002). Although, both species are associated with several hosts; *C. jejuni* is usually more abundant in chickens, cattle and wild birds and *C. coli* dominates in pigs (Sheppard et al., 2013). In that sense, other sources have been linked to *C. coli* human infections. Bessède et al. (2014) showed higher *C. coli* rates (15.25 %) probably linked to travelling abroad or differences in eating habits. It has been reported that eating specific type of meats (Halal meat, meat pies, consumption of offal, and pâté), food bought from a stall and drinking refilled bottled water have been more associated with *C. coli* infections (Gillespie et al., 2002).

Due to most human infections are sporadic and outbreaks are not common, epidemiological tracking of infection sources is complex. However, our study has shown a relation among some *C. jejuni* strains isolated from the chicken food chain with some strains that caused human infections in the same period of time. These strains were genetically related to clinical isolates recovered from human infections and were grouped in 35 clusters (similarity ≥ 85 %). Among these, 22 clusters (62.8 %) corresponded to the period 4, where isolates from retail and humans were studied. This finding suggests that *C. jejuni* strains from the chicken food chain could have had an epidemiological link with

clinical isolates as other authors have described before (Narváez-Bravo et al., 2017; Zoorman et al., 2006). Recently, EU has amended Regulation 2073/2005 by including a maximum contamination level of *Campylobacter* spp. in broiler carcasses of 1,000 cfu/g at slaughter (European Regulation 1495/2017) in order to regulate the incidence of *Campylobacter* in European Union. However, other environment and non-environment sources need to be also considered. In that sense, Lyhs et al. (2010) have suggested that despite of 32.0 % of human isolates had a similar PFGE type with isolates from poultry products; this type of food products is only a minor source of human campylobacteriosis in Finland. Therefore, other sources might be involved in the transmission of the pathogen.

Survival of identical *Campylobacter* genotypes in a defined geographical area during a period of time, as occurred in this study, might be explained by the fact that these strains could activate different mechanisms to overcome stress factors; not only during processing and food preparation, but also in the environment. Studies performed by Newell et al. (2001) and Alter and Fehlhaber (2003) detected specific subtypes that survived the complete processing at poultry abattoirs, whereas other subtypes presented in the slaughter were lost after chilling process. How specific genotypic could persist along the food chain is still unknown. In that sense, Alter and Scherer (2006) described that genotypic differences that result in phenotypic changes could allow the evolution of specific genetic variants or the strain selection when encountering adverse environmental situations; for instance, oxidative stress, changes in pH, osmolarity, temperature or hydrostatic pressure.

In this study, a relation between some *C. jejuni* genotype (P_3-A, P_3-B, P_3-C, P_3-G) found in slaughterhouse environment and in patients at hospital level has been occurred in our study (Table S3). From these, genotypes P_3-C and P_3-G persisted in the

slaughterhouse environment during at least 21 and 17 days respectively according to previous studies (García-Sánchez et al., 2017). Results cannot confirm that the environment has been the origin of the human campylobacteriosis, but we can confirm that the same genotype was recovered in both scenarios and what is more important that some of those genotypes caused an illness. The most prevalent *C. jejuni* genotype recovered at slaughterhouse level (P_3-G) belongs to Clonal Complex-21 (CC-21). Elhadidy et al. (2018) have shown that 72.2 % of the isolates found in their study were clustered in 35 STs sharing both chicken and human isolates, thus confirming an epidemiological relatedness. In that work, CC-21 was the main clonal complex, as has been occurred in the present study (P_3-G). This clonal complex has a relatively wide distribution in diverse geographic locations and across many host species, such as chicken and humans (Jorgensen et al., 2011; Skarp et al., 2016; Sanad et al., 2011). As it happens in our study, Elhadidy et al., (2018) found that isolates belonging to ST 3769; ST 21 CC were found in both chicken and human samples. However, unlike what occurs in our work, in which numerous isolates belonging to this STs have been found (more than 140), only 2 isolates have been found by the cited authors.

Only three pulsotypes: P_4-15, P_4-37 and P_4-66 share isolates belonging to two steps of the chicken food chain and human cases. However, those isolates do not show a logical sequence of appearance along de food chain and along the sampling time. For instance, pulsotype P_4-37 appeared in the slaughterhouse during 5 days in 2014 and one year after it was isolated from different chicken products and simultaneously in humans (data in supplementary table S3). Similar situation happens with the other two pulsotypes, P_4-15 and P_4-66. In both cases the pulsotype appeared first in retail products and/or in human cases before than the farm (data in supplementary table S4). Further studies will be needed to elucidate this paradox.

Most of the formed clusters (22/35) that shared isolates from the chicken food chain and humans correspond to the retail step. Among these, 13 clusters are characterized by the presence of isolates, which were recovered before in chicken products and after in human cases. Within them, in five clusters some of the chicken products were acquired in the same geographical area of the town, where patients were first diagnosed in the Primary Health Center. Other authors as Ramonaite et al. (2017) have also shown in a studied in retail broiler products, performed during one year, a strong link between strains from broiler products and human cases. Additionally, in some pulsotypes, it was observed that some human isolates were recovered more than a month, after the last recovered isolate from chicken products. This fact might be explained because (1) not all the products in the market were analyzed, (2) the slaughterhouse could still send to the market contaminated final products and/or (3) due to freezing of chicken products in the processing plant by suppliers or at home by consumers. At freezing temperatures, *Campylobacter* lose its viability, although some strains can still be isolated from frozen poultry meat and poultry products (Fernandez and Pison, 1996; Melero et al., 2013). Nevertheless, most of the studies investigating the susceptibility of *Campylobacter* to freezing in meat, detected decreases between 1 and 3 log units within the first days of frozen storage. However, it is likely that chicken skin provides an appropriate micro-environment to protect campylobacters (Alter and Scherer, 2006).

Campylobacter spp. has developed resistance to several antibiotics due to the misuse of antibiotic therapy in humans (Bloomfield et al., 2017) and their overuse in farm management. This developing antibiotic resistance is of principal interest worldwide not only regarding *Campylobacter*, but also with other clinical bacteria. Our study has shown a high resistance in isolates from humans during all the period analyzed (90.3 %), as it happens in the different steps of the chicken food chain (see Chapters I, III, IV). All *C.*

jejuni isolates from the chicken food chain were ciprofloxacin, nalidixic acid and tetracycline resistance. Moreover, Melero et al. (2012) reported also high resistance to ciprofloxacin (95.45 %) and tetracycline (81.82 %) in *C. jejuni* isolates from the chicken food chain in the same area, but 2-3 years before. Using antibiotics for preventive or prophylactic therapy purposes has been found to select resistant bacteria, and multidrug-resistant strains might reflect the past history of antimicrobial usage over longer periods in primary production (Mäesaar et al., 2016; Smith et al., 2017). This phenomenon might partly explain the high proportion of *Campylobacter* isolates of broiler origin that have been found to be resistant to fluoro(quinolones) in numerose studies (Chen et al., 2010; Roasto et al., 2007; Wieczorek et al., 2013). As fluoro(quinolones) and macrolide are the two drugs of choice for *Campylobacter* treatment, resistance to ciprofloxacin compromises the possibilities of the therapy used in patients, being a risk factor for human health.

5. Conclusion

This study has provide crucial information for a better understanding in the epidemiology of campylobacteriosis and its possible relation with campylobacters from the chicken food chain in a specific geographical area of Spain. Epidemiological data confirm that children under 5 years were the population more affected. Moreover, this study has shown a relation among *C. jejuni* strains isolated from the chicken food chain and human infections. Nevertheless, more information about the food products consumed by the patients before the onset of the illness is required, and other sources should be also considered for those strains that were not detected in the chicken food chain. A relation was found between farms and the slaughterhouse with human isolates indicating the ability of some *Campylobacter* genotypes to survive along the chicken food chain and finally causing an illness. Additionally, the highest number of PFGE types shared was

found at the retail level, highlighting the human risk of campylobacteriosis due to the consumption of chicken products. Moreover, the high rate of antimicrobial resistance found along the chicken food chain could enhance the potential risk to be transferred to humans as shown in this study. Thus, Governments must enforce poultry industry towards a reduction in the antibiotic use in animal production and to explore possible interventions to reduce the presence of resistant *Campylobacter* strains in poultry products. Additionally, to encourage the mainstreaming of epidemiological surveys in the Health System for a better understanding of the ecology of the illness, and finally to promote Educational Health campaigns among people, in order to reduce human risk of *Campylobacter* infections.

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CHAPTER 6

IX. CHAPTER 6. Epidemiology of Campylobacteriosis in Castile and Leon during the period 2008-2015

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Abstract

Background: *Campylobacter* is considered the most frequent agent of gastroenteritis in humans all around the world. The aim of this study was to know the incidence, trend and seasonality of notified campylobacteriosis cases in Castile and Leon, Spain, from 2008 to 2015. In addition, to describe the type of case and source of notification, and study the influence of age, sex and *Campylobacter* species on the hospitalization of cases.

Methods: Retrospective cross-sectional study with a time series analysis. Data on notified campylobacteriosis cases were collected from the Epidemiological Surveillance System of Castile and Leon (2008-2015). Incidence rates and incidence rate ratios by age, sex and year of notification were calculated. In order to estimate trend and seasonality, a time series analysis was performed using a multiplicative method and adjusted to a linear and exponential model.

Results: From 4,330 cases analyzed, 49.4 % of children aged under 5 were affected. The ratio of men to women was 1.43 and 61.8 % of cases were notified from secondary care. *C. jejuni* was isolated from 72.3 % of samples (n=2,593). Incidence rate ranged from 11.42 cases per 100,000 inhabitants in 2008 to 33.53 in 2015. The seasonality

range was from 71.97 % (13th four- week period) to 125.54 % (9th four-week period). Hospitalization reached 62.5 % for people aged 60 or over.

Conclusions: A growing trend of notified campylobacteriosis cases is observed in Castile and Leon (2008-2015), mainly in children aged under 5, with a peak in summer months. Males are more affected than females and hospitalization increases with age.

1. Introducción

El *Campylobacter* es considerado el agente causal más común en el mundo de gastroenteritis humana (WHO, 2012). La campilobacteriosis es la zoonosis con mayor número de casos declarados en la Unión Europea (UE), con tendencia ascendente desde 2008, y una tasa de notificación de 71 y 82,3 casos por 100.000 habitantes en la UE y España, respectivamente, durante 2014 (EFSA, 2015). En Estados Unidos (EEUU), la tasa de incidencia por 100.000 habitantes se situaba en 13,45 en 2014 (CDC, 2015), aunque se estima que afecta a más de 1,3 millones al año, ya que muchos casos no se notifican (Iranzo y cols., 2015). La infradeclaración en los países desarrollados y la falta de registros en los países en vías de desarrollo hacen que solo se conozca una parte de la verdadera incidencia de la campilobacteriosis (Epps y cols., 2013; KaaKoush y cols., 2015; WHO, 2012). Afecta principalmente a menores de 5 años (CNE, 2016; CDC, 2013a; Schielke et al., 2014), siendo una enfermedad endémica en la población infantil de los países en vías de desarrollo (Kaakoush y cols., 2015).

Dentro del género *Campylobacter*, *C. jejuni* y *C. coli* son responsables de la mayoría de las afecciones gastrointestinales en humanos (Iranzo y cols., 2015; Kaakoush y cols., 2015). Los síntomas más frecuentes son dolor abdominal, fiebre, diarrea (a menudo saguinolenta), náuseas y vómitos. Suele ser una enfermedad autolimitada, con una duración de entre 3 y 7 días, y solo en casos graves son necesarios los antibióticos para su resolución. Se ha asociado con otras enfermedades gastrointestinales y complicaciones postinfección, siendo las más comunes la artritis reactiva, síndrome de

Guillain-Barré y síndrome de Miller Fisher (Epps y cols., 2013; Kaakoush y cols., 2015; WHO, 2012).

El principal reservorio de *Campylobacter* son las aves y mamíferos, tanto domésticos como salvajes, aunque también se encuentra en fuentes naturales de agua. La vía de transmisión más común es a través de la ingesta de alimentos contaminados y poco cocinados, siendo la carne de pollo la más frecuentemente implicada, y la transmisión cruzada con otros alimentos crudos. Leche y agua sin tratar, el contacto con mascotas y con heces de animales contaminados, bien de forma directa o por vectores como las moscas, son otras posibles fuentes de infección (Silva y cols., 2011). Hay un claro patrón estacional en la presentación de la enfermedad, con mayor afectación en los meses de primavera y verano, por lo que se ha estudiado su posible relación con la temperatura medioambiental (Lake y cols., 2009; Tam y cols., 2006).

En 2015 se incluyó en la lista de enfermedades de declaración obligatoria (EDO) en el conjunto de todas las comunidades autónomas (CCAA) españolas (BOE, 2015), aunque en Castilla y León (CyL) se viene declarando como EDO desde 2007, tras la regulación de la Red de Vigilancia Epidemiológica (BOCyL, 2006) y el Sistema EDO de CyL (BOCyL, 2007). Los casos de campilobacteriosis recogidos en la Red Nacional de Vigilancia Epidemiológica Española (RENAVE) (BOE, 1996) hasta 2015 proceden de laboratorios de microbiología clínica que, de forma voluntaria, notifican al Sistema de Información Microbiológica (SIM) los casos de esta enfermedad. Según los criterios establecidos en el manual de notificación de CyL (DGSP, 2007), la campilobacteriosis es una enfermedad de notificación individualizada con datos epidemiológicos básicos (filiación, fecha de inicio de síntomas, tipo de presentación...), que se deben registrar en la encuesta epidemiológica por el personal médico y las personas responsables de la actividad asistencial de los centros de atención primaria y especializada, tanto públicos

como privados. Los casos a declarar se clasifican en "probables" si cumplen los criterios clínicos y al menos un criterio epidemiológico, y "confirmado" cuando además presenta un criterio de diagnóstico de laboratorio (DGSP, 2007). El Ministerio de Sanidad, Servicios Sociales e Igualdad (MSSSI) en 2013 actualizó los criterios y protocolos de declaración de las EDO (CNE, 2013) que se incorporaron en CyL, eliminándose los casos "probables" a efectos de notificación. Son pocos los estudios publicados en España sobre series temporales de campilobacteriosis y, hasta donde sabemos, este es el primero realizado en una comunidad autónoma.

El objetivo de este trabajo fue conocer la incidencia de los casos de campilobacteriosis declarados al Sistema de Vigilancia Epidemiológica (SIVE) de CyL y analizar la tendencia y los patrones de estacionalidad durante el período 2008-2015. Asimismo, describir el tipo de presentación y nivel asistencial de declaración de esta enfermedad, y estudiar la influencia de la edad, sexo y la especie de *Campylobacter* en la hospitalización de las personas afectadas.

2. Material and métodos

Estudio transversal, con análisis de la serie temporal, de los casos de campilobacteriosis declarados en CyL durante el período 2008-2015. Los datos se obtuvieron a través de los casos notificados al SIVE, durante dicho período, mediante una encuesta que recogía datos epidemiológicos básicos. Aunque la notificación de las EDO se realiza cuando existe sospecha clínica, en este estudio solo se tuvieron en cuenta los casos que, además de cumplir criterios clínicos, fueron confirmados por laboratorio. Para determinar la existencia de un brote se consideraron también los criterios epidemiológicos (CNE, 2013; DGSP, 2007).

De las variables recopiladas en SIVE, se analizó el año de declaración, edad, sexo, hospitalización, tipo de presentación (aislado o brote), especie de *Campylobacter* (*C*.

jejuni, C. coli, C. spp.) y nivel asistencial de declaración (atención primaria, atención especializada, otros declarantes). Dada la mayor concentración de casos en edades tempranas, se crearon grupos de edad más pequeños hasta los 10 años y más amplios a partir de esa edad, para optimizar el análisis de los datos, quedando de la siguiente manera: <1 año, 1-4 años, 5-9 años, 10-34 años, 35-59 años y ≥60 años.

Para cada año se calcularon las tasas de incidencia (TI) de campilobacteriosis de forma global y por grupos de edad y sexo así como la razón de tasas de incidencia (RTI). Como denominador se utilizaron las poblaciones a 1 de julio de cada año, obtenidas del padrón municipal, publicadas por el Instituto Nacional de Estadística (http://www.ine.es, consultado el 03/03/2016).

Para el análisis de la serie temporal, los casos se agruparon en cuatrisemanas a razón de 13 por año. Se analizó la tendencia y se efectuó una descomposición estacional, mediante el método multiplicativo, el cual divide la serie en tres componentes: tendencia-ciclo, irregularidad y estacionalidad. El estudio de esta última muestra el patrón de declaración de casos que se repite cada año, para lo que se calcularon los índices de estacionalidad en porcentaje, tal que un promedio estacional tendría un índice igual a 100. Para describir la serie temporal, se ajustaron los datos a un modelo lineal y a uno exponencial.

Para comparar la gravedad de la enfermedad (según hospitalización) en función de la edad, sexo y especie de *Campylobacter*, en el análisis bivariante, se utilizó el test de chi^2. Los datos se analizaron con los programas informáticos Epidat 4.1, SPSS Statistics v.23 y STATGRAPHICS Centurion v. 16.2.

3. Resultados

De los 4.330 casos confirmados de campilobacteriosis declarados en CyL, durante el período 2008-2015, el 99,4 % fueron casos aislados sin vínculo epidemiológico conoci-

do. El 49,4 % de los casos se agrupó en menores de 5 años y la ratio hombre/mujer fue de 1,43. Aunque en todos los casos se confirmó el diagnóstico de campilobacteriosis, en el 59,9 % de las encuestas se registró la especie de *Campylobacter* (n=2.593), de los que el 72,3 % fueron infección por *C. jejuni*. Con respecto a la gravedad de los casos, 1.069 (24,7 %) precisaron hospitalización. Desde atención especializada se produjo 61,8 % de notificaciones al sistema SIVE (tabla1). Del total de casos, 90 (2,1 %) fueron no autóctonos de CyL.

Tabla 1. Descripción de los casos confirmados de campilobacteriosis declarados en Castilla y León en el período 2008-2015.

N=4.330	n	%
Edad en grupos		
<1 año	446	10,3
1-4 años	1.695	39,1
5-9 años	665	15,4
10-34 años	616	14,2
35-59 años	334	7,7
≥60 años	574	13,3
Sexo		
Hombre	2.548	58,8
Mujer	1.782	41,2
Tipo de presentación		
Aislado	4.302	99,4
Brote	28	0,6
Hospitalización		
No	3.261	75,3
Si	1.069	24,7
Nivel asistencial de declaración		
Atención Especializada	2.675	61,8
Atención Primaria	1.633	37,7
Otros declarantes	22	0,5
N=2.593	**n**	**%**
Campylobacter		
C. jejuni	1.875	72,3
C. coli	271	10,5
C. spp.	447	17,2

Durante el período de estudio, la TI global de los casos confirmados y declarados de campilobacteriosis en CyL pasó de 11,42 por 100.000 habitantes en 2008 a 33,53 en 2015 (RTI=2,94; p<0,001) (tabla 2).

Tabla 2. Tasa de incidencia (TI) anual y razón de tasas de incidencia (RTI) de los casos confirmados de campilobacteriosis declarados en Castilla y León (2008-2015).

Año	TI por 100.000 habitantes (IC95 %)	RTI (IC95 %)	p-valor
2008	11,42 (10,15-12,81)	.	.
2009	11,78 (10,50-13,20)	1,03 (0,87-1,22)	0,704
2010	14,85 (13,40-16,42)	1,3 (1,11-1,52)	0,001
2011	18,21 (16,60-19,94)	1,59 (1,37-1,85)	<0,001
2012	19,17 (17,50-20,94)	1,68 (1,45-1,95)	<0,001
2013	26,67 (24,69-28,76)	2,34 (2,03-2,69)	<0,001
2014	36,12 (33,80-38,55)	3,16 (2,77-3,62)	<0,001
2015	33,53 (31,29-35,90)	2,94 (2,57-3,37)	<0,001

En la tabla 3 se recogen las TI por 100.000 habitantes, y RTI por año y grupo de edad. Las TI más elevadas se concentraron en los grupos de <1 año, única población que mostró incrementos anuales en todos los años de la serie, y de 1-4 años, con un repunte de este último en 2014 (figura 1). Además, las TI fueron más altas en el sexo masculino, encontrándose diferencias estadísticamente significativas, con respecto a la RTI hombre/mujer en todos los grupos etarios (tabla 4).

El análisis de la serie temporal evidenció una tendencia ascendente en la declaración de casos con dos ciclos diferenciados: un ascenso lineal regular desde 2008 hasta 2012 y un ascenso exponencial en 2013 y 2014 que se limitó en el último año de declaración (figura 2).

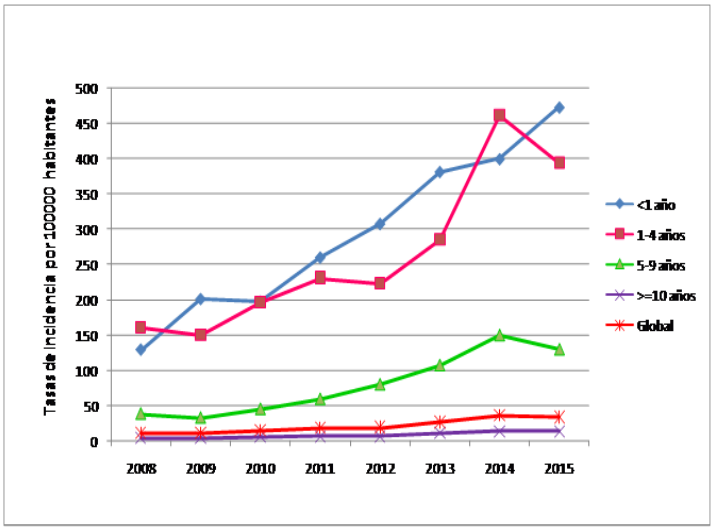

Figura 1. Tasas de incidencia, global y por grupos de edad, de los casos de campilobacteriosis confirmados y declarados en Castilla y León. Período 2008-2015.

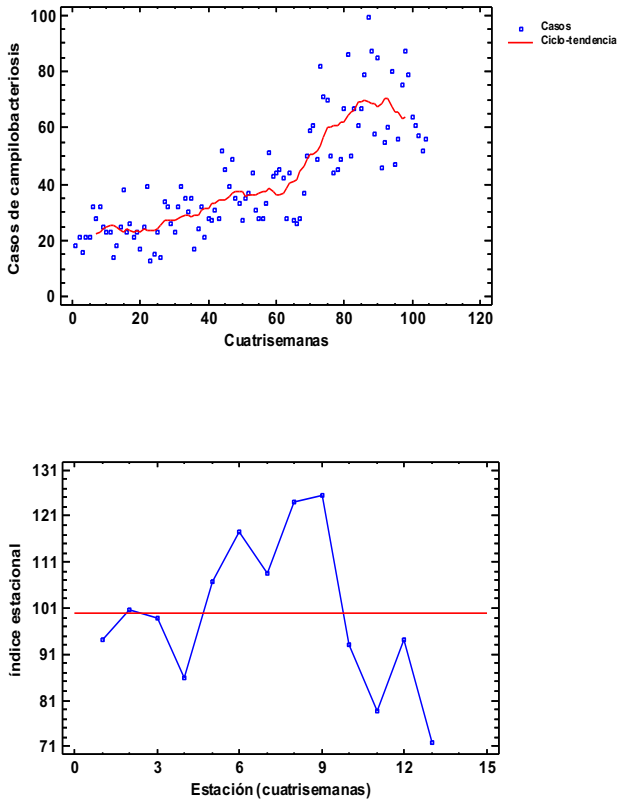

Figura 2. Tendencia y estacionalidad de los casos confirmados de campilobacteriosis declarados en Castilla y León durante el período 2008-2015.

Los índices de estacionalidad mostraron dos picos máximos en verano en las cuatrisemanas 6 y 9. El mayor valor se obtuvo en la cuatrisemana 9 cuando se declaró un 125,54 % del promedio de un ciclo completo ($p<0,05$), mientras que el valor mínimo se registró en invierno (cuatrisemana 13) con el 71,97 % ($p<0,05$) (figura 2). Ajustados los datos de la serie temporal, el modelo exponencial fue el que proporcionó el mejor resultado, R^2=67,73 % *vs* R^2=64,11 % del modelo lineal, que indicó el grado con el que reproduce adecuadamente la tendencia.

En cuanto a la hospitalización, se encontraron diferencias estadísticamente significativas en función de la edad y especie de *Campylobacter*, no así en función del sexo (tabla 5). Cabe destacar que el 33,3 % de las personas hospitalizadas pertenecían al grupo de 60 años y más. En el 82,1 % de los casos el agente causal fue *C. jejuni*. El porcentaje de hospitalización fue mayor durante el primer año de vida que en el rango de 1 a 9 años, y a partir de los 10 años aumentó con la edad, llegando a ser del 62,5 % en el grupo etario ≥60 años (figura 3).

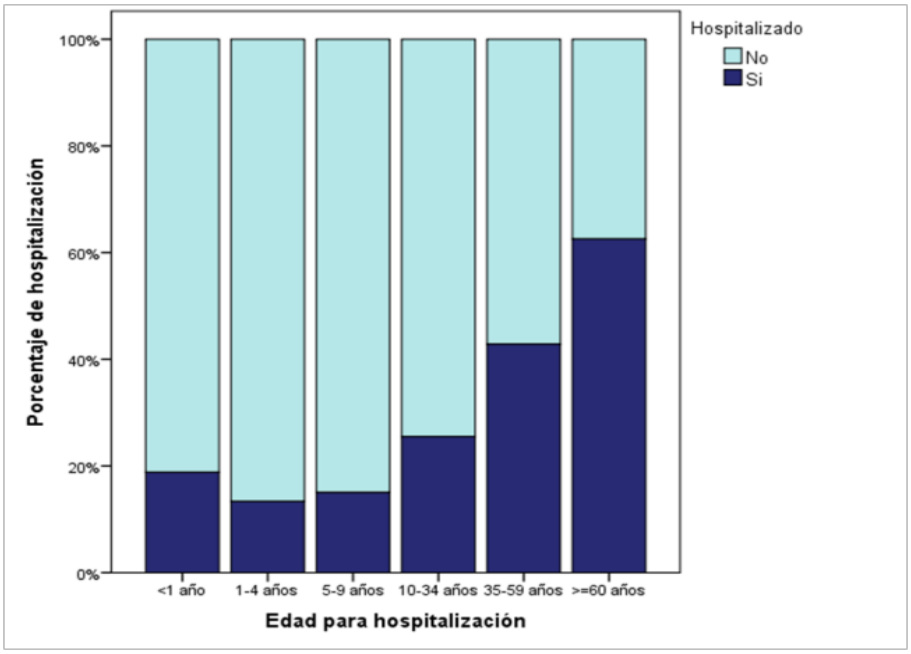

Figura 3. Porcentaje de hospitalización en cada grupo etario de los casos confirmados y declarados de campilobacteriosis en Castilla y León entre 2008 y 2015.

Tabla 3. Tasas de incidencia (TI) y razón de tasas de incidencia (RTI), por grupo de edad, de los casos confirmados de campilobacteriosis declarados en Castilla y León. Período 2008-2015.

Año	Grupos de edad (años)	TI por 100.000 habitantes (IC95%)	RTI (IC95%)	p-valor
2008	<1 año	128,63 (84,76-187,14)	.	.
	1-4 años	160,29 (134,02-190,20)	1,25 (0,82-1,96)	0,298
	5-9 años	38,37 (27,15-52,66)	0,29 (0,18-0,51)	<0,001
	10-34 años	4,4 (3,01-6,22)	0,03 (0,02-0,06)	<0,001
	35-59 años	2,2 (1,34-3,4)	0,02 (0,01-0,03)	<0,001
	≥60 años	6,21 (4,15-8,34)	0,05 (0,03-0,08)	<0,001
2009	<1 año	200,64 (144,60-271,21)	.	.
	1-4 años	150,44 (125,22-179,24)	0,75 (0,52-1,09)	0,113
	5-9 años	32,69 (22,50-45,91)	0,16 (0,1-0,26)	<0,001
	10-34 años	6,34 (4,63-8,5)	0,03 (0,02-0,05)	<0,001
	35-59 años	2,18 (1,33-3,36)	0,01(0,006-0,02)	<0,001
	≥60 años	5,17 (3,64-7,13)	0,03 (0,02-0,04)	<0,001
2010	<1 año	197,1 (140,81-268,40)	.	.
	1-4 años	196,78 (167,90-229,20)	0,99 (0,70-1,45)	0,978
	5-9 años	45,07 (32,99-60,12)	0,23 (0,15-0,36)	<0,001
	10-34 años	7,1 (5,25-9,38)	0,036 (0,02-0,05)	<0,001
	35-59 años	2,8 (1,83-4,11)	0,014 (0,01-0,02)	<0,001
	≥60 años	7,48 (5,62-9,75)	0,04 (0,02-0,06)	<0,001
2011	<1 año	259,78 (194,02-340,66)	.	.
	1-4 años	231,18 (199,80-266,10)	0,89 (0,65-1,23)	0,452
	5-9 años	59,24 (45,32-76,10)	0,23 (0,15-0,34)	<0,001
	10-34 años	8,35 (6,31-10,84)	0,03 (0,02-0,05)	<0,001
	35-59 años	3,95 (2,8-5,45)	0,015 (0,01-0,02)	<0,001
	≥60 años	9,06 (7,01-11,53)	0,03 (0,02-0,05)	<0,001
2012	<1 año	307,6 (234,73-395,94)	.	.
	1-4 años	223,62 (192,64-258,17)	0,73 (0,54-0,99)	0,036
	5-9 años	79,97 (63,70-99,13)	0,26 (0,18-0,37)	<0,001
	10-34 años	7,24 (5,32-9,63)	0,02 (0,016-0,035)	<0,001
	35-59 años	4,47 (3,22-6,04)	0,015 (0,01- 0,02)	<0,001
	≥60 años	9,57 (7,46-12,08)	0,03 (0,02-0,04)	<0,001
2013	<1 año	381,72 (297,57-482,28)	.	.
	1-4 años	286,07 (250,44-325,35)	0,75 (0,57-0,99)	0,039
	5-9 años	107,25 (88,31-129,05)	0,28 (0,21-0,38)	<0,001
	10-34 años	15,98 (13,00-19,43)	0,04 (0,03-0,06)	<0,001
	35-59 años	5,53 (4,13-7,25)	0,014 (0,01-0,02)	<0,001
	≥60 años	14,38 (11,78-17,40)	0,04 (0,03-0,05)	<0,001

Tabla 3. (continuación)				
2014	<1 año	399,88 (312,31-504,40)	.	.
	1-4 años	461,92 (415,56-512,05)	1,15 (0,89-1,51)	0,266
	5-9 años	150,15 (127,58-175,56)	0,37 (0,28-0,50)	<0,001
	10-34 años	24,65 (20,85-28,94)	0,06 (0,05-0,08)	<0,001
	35-59 años	6,6 (5,06-8,47)	0,017 (0,01-0,024)	<0,001
	≥60 años	13,48 (10,97-16,40)	0,03 (0,025-0,046)	<0,001
2015	<1 año	473,44 (377,62-586,13)	.	.
	1-4 años	394,86 (351,44-442,17)	0,83 (0,65-1,07)	0,146
	5-9 años	129,8 (108,83-153,64)	0,27 (0,21-0,36)	<0,001
	10-34 años	23,53 (19,77-27,8)	0,05 (0,04-0,06)	<0,001
	35-59 años	8,02 (6,31-10,05)	0,017(0,012-0,023)	<0,001
	≥60 años	12,99 (10,54-15,86)	0,03 (0,02-0,04)	<0,001

Tabla 4. Tasas de incidencia (TI) y razón de tasas de incidencia (RTI) por sexo y grupo de edad, de los casos confirmados de campilobacteriosis declarados en Castilla y León durante el período 2008-2015.

Grupo de edad	Año	TI Hombres	TI Mujeres	RTI Hombre/Mujer (IC95%)	p-valor
<1 año	**2008**	128,84	128,39	1,00(0,44-2,32)	0,995
	2009	279,15	117,81	2,37(1,18-5,10)	0,009
	2010	286,86	101,67	2,82(1,34-6,47)	0,003
	2011	309,54	206,63	1,49(0,83-2,76)	0,156
	2012	347,36	265,11	1,31(0,76-2,28)	0,305
	2013	439,05	319,2	1,37(0,83-2,30)	0,192
	2014	450,26	344,13	1,31(0,80-2,18)	0,267
	2015	591,2	348,51	1,70(1,07-2,75)	0,019
1-4 años	**2008**	190,12	128,62	1,48(1,03-2,14)	0,028
	2009	186,45	111,98	1,66(1,14-2,45)	0,006
	2010	214,85	177,49	1,21(0,88-1,67)	0,224
	2011	279,25	179,86	1,55(1,15-2,10)	0,003
	2012	223,7	223,54	1,00(0,74-1,35)	0,997
	2013	346,28	221,8	1,56(1,19-2,06)	0,001
	2014	502,91	417,94	1,20(0,97-1,50)	0,08
	2015	390,3	399,78	0,97(0,77-1,23)	0,835
5-9 años	**2008**	41,3	35,28	1,17(0,59-2,36)	0,635
	2009	36,67	28,5	1,28(0,61-2,77)	0,48
	2010	32,42	58,44	0,55(0,28-1,04)	0,052
	2011	75,7	41,89	1,81(1,04-3,23)	0,026
	2012	112,53	45,57	2,47(1,50-4,18)	<0,001
	2013	122,65	90,87	1,35(0,91-2,01)	0,118

Tabla 4. Continuación					
	2014	177,98	120,5	1,48(1,06-2,07)	0,016
	2015	145,4	113,2	1,28(0,90-1,84)	0,151
10-34 años	**2008**	5,05	3,71	1,36(0,64-2,99)	0,399
	2009	6,54	6,13	1,07(0,57-2,01)	0,832
	2010	7,3	6,88	1,06(0,58-1,94)	0,844
	2011	8,67	8,01	1,08(0,62-1,90)	0,772
	2012	9,56	4,77	2,01(1,05-3,98)	0,023
	2013	19,25	12,51	1,54(1,01-2,37)	0,035
	2014	30,55	18,38	1,66(1,18-2,36)	0,003
	2015	30,52	16,14	1,90(1,31-2,76)	<0,001
35-59 años	**2008**	2,77	1,58	1,75(0,65-5,17)	0,238
	2009	3,6	0,67	5,34(1,54-28,43)	0,002
	2010	2,72	2,89	0,94(0,40-2,21)	0,881
	2011	4,78	3,08	1,55(0,76-3,26)	0,197
	2012	5,39	3,49	1,54(0,80-3,07)	0,174
	2013	6,02	5,02	1,20(0,67-2,17)	0,518
	2014	7,71	5,45	1,41(0,83-2,45)	0,182
	2015	10,05	5,9	1,71(1,04-2,84)	0,025
≥60 años	**2008**	9,51	3,56	2,67(1,37-5,45)	0,002
	2009	6,89	3,78	1,82(0,90-3,77)	0,074
	2010	10,21	5,26	1,94(1,10-3,53)	0,017
	2011	10,71	5,23	2,05(1,16-3,71)	0,009
	2012	10,33	8,94	1,15(0,70-1,90)	0,548
	2013	17,16	12,11	1,42(0,95-2,12)	0,074
	2014	15,21	12,06	1,26(0,83-1,91)	0,248
	2015	15,06	11,28	1,33(0,88-2,03)	0,156

Tabla 5. Hospitalización de los casos confirmados y declarados de campilobacteriosis en función de la edad, sexo y especie de *Campylobacter* (Castilla y León). Período 2008-2015.

	Hospitalización		
	No	**Si**	
	N=3.261	N=1.069	
	n(%)		p*
Edad en grupos			<0,001
<1 año	362(11,1)	84(7,9)	
1-4 años	1.469(45)	226(21,1)	
5-9 años	565(17,3)	100(9,4)	
10-34 años	459(14,1)	157(14,7)	
35-59 años	191(5,9)	143(13,4)	
≥60 años	215(6,6)	359(33,6)	
Sexo			0,222
Hombre	1.936(59,4)	612(57,2)	
Mujer	1.325(40,6)	457(42,8)	
	N=1.849	N=744	
	n(%)		p*
Campylobacter			<0,001
C. jejuni	1.264(68,4)	611(82,1)	
C. coli	206(11,1)	65(8,7)	
C. spp.	379(20,5)	68(9,1)	

*Valor p asociado al estadístico $\chi 2$

4. Discusión

Los resultados reflejan una tendencia ascendente en los casos notificados de campilobacteriosis en CyL durante el período 2008-2015, con una clara estacionalidad en los meses de verano. La mayor afectación se produjo en los sujetos menores de 5 años y en el sexo masculino, siendo la especie *C. jejuni* la más frecuentemente implicada. La inmensa mayoría fueron casos esporádicos y la hospitalización representó la cuarta parte de las personas afectadas. La notificación fue más frecuentemente realizada desde atención especializada.

Durante los 8 años de estudio, la TI de los casos notificados de campilobacteriosis en CyL casi se triplicó. En España, en 2014 se observó un aumento con respecto a 2013

y 2012, aunque en años previos se mantuvo estable (casos declarados al SIM) (CNE, 2016). Sin embargo, en la UE la tendencia desde 2008 fue al alza (EFSA, 2015), de forma similar a nuestro estudio, así como en Alemania en un análisis realizado entre 2001-2010 (Schielke y cols., 2014). En EEUU, según los datos procedentes de la Red de Vigilancia Activa de Enfermedades de Transmisión Alimentaria (*FoodNet*), el incremento de la incidencia en 2014 fue del 13 % con respecto al período 2006-2008, sin cambios significativos en los años posteriores (CDC, 2015).

Si comparamos nuestros resultados con los registrados por la Autoridad Europea de Seguridad Alimentaria (EFSA) y el Centro Europeo para la Prevención y el Control de las Enfermedades (ECDC), las cifras difieren considerablemente. El informe de 2014 presentó una tasa de notificación para España de 82,3 casos por 100.000 habitantes (EFSA, 2015), lejos de los 36,12 de CyL hallados en nuestro trabajo. Esta diferencia podría deberse, por un lado, a que la EFSA estima que el sistema de vigilancia de la campilobacteriosis en nuestro país tiene una cobertura del 30 % y en base a ese porcentaje hace un cálculo aproximado de la tasa de notificación (EFSA, 2015) y, por otro, a que los datos remitidos desde España proceden de la búsqueda activa de casos enviados al SIM (CNE, 2016). Por el contrario, los datos de nuestro estudio provienen de los casos notificados al sistema EDO, lo cual puede llevar a una infradeclaración debido a su baja sensibilidad. Aunque algunos casos declarados proceden de los servicios hospitalarios de microbiología, aún no existe un SIM reglado en CyL.

En nuestro análisis, el mayor pico de casos se produjo entre las cuatrisemanas 6 y 9, con un descenso en la 7, de forma casi idéntica a la estacionalidad hallada en la provincia de Valladolid durante el período 2000-2004 (Luquero y cols., 2007). En la mayoría de estudios e informes también predomina la estacionalidad en verano (Bless y cols., 2014; EFSA, 2015; Schielke y cols., 2014, Spencer y cols., 2012), si bien en otros

se desplaza a mayo y junio (Ivanova y cols., 2010; Nichols y cols., 2012). En general, el cambio de hábitos durante el período estival, con mayor número de comidas y actividades recreativas al aire libre, además del aumento de la temperatura ambiente y la mayor presencia de vectores (moscas), favorecería la propagación de la bacteria y, en consecuencia, la posibilidad de infección. Dado que el consumo de carne de pollo poco cocinada es uno de los factores más implicados en la campilobacteriosis, se ha estudiado la posible relación entre la prevalencia de *Campylobacter* en los pollos y la afectación en seres humanos, con resultados contradictorios. En EEUU se observó un menor índice de casos positivos en las muestras de carne de pollo analizadas en verano (Williams y cols., 2015), mientras que en Europa se observó una asociación positiva (Jore y cols., 2010).

Hemos visto como la mayor TI en CyL se registra en los menores de 5 años, coincidiendo con los resultados de 2014 publicados en España (CNE, 2016) y en la inmensa mayoría de las series temporales consultadas (CDC, 2013a, Feierl y cols., 2009; Ivanova y cols., 2010; Schielke y cols., 2014; Spencer y cols., 2012; Weinberger y cols., 2013). En Alemania (2001-2010), aunque la mayor incidencia se registró en dicho grupo, sobre todo en menores de 1 año (igual que en nuestro estudio), el mayor incremento se produjo en las personas mayores de 50 años (Schielke y cols., 2014) de forma similar a lo hallado en Inglaterra y Gales entre 1989-2011 (Nichols y cols., 2012). Que la enfermedad sea más frecuente en niños puede deberse a la inmadurez del sistema inmunitario, y a factores como menos higiene de manos, el contacto con mascotas y las actividades en áreas de recreo contaminadas por heces de aves infectadas. En los países en vías de desarrollo, se ha sugerido la adquisición de inmunidad, tras las exposiciones repetidas a *Campylobacter* durante los primeros años de infancia, para explicar la menor incidencia en adultos (El-Tras y cols., 2015; Kaakoush y cols., 2015; Randremanana y cols., 2014).

Con respecto al sexo, encontramos una razón global hombre/mujer superior a las halladas en los mencionados estudios de Alemania (WHO, 2012, Schielke y cols., 2014) e Inglaterra y Gales (WHO, 2012, Nichols y cols., 2012), aunque más cercana a los datos de 2014 publicados en España con un 58,1 % de afectación en sexo masculino (ratio: 1,38) (CNE, 2016). Se ha argumentado que distintos comportamientos en los hábitos higiénicos y alimentarios durante la preparación de los alimentos, incluso diferencias psicológicas e inmunológicas, serían algunos de los factores de riesgo que pretenden explicar este predominio de la enfermedad en los hombres (Gölz y cols., 2014; Luquero y cols., 2007).

Los brotes de campilobacteriosis son menos comunes que en otras enfermedades de transmisión alimentaria, como la salmonelosis, aunque también se producen (Iranzo y cols., 2015; Silva y cols., 2011), hecho que se corrobora en nuestro estudio con solo 28 casos (0,6 %) asociados a brotes. En España se notificaron 13 brotes en 2014 con 93 personas afectadas, cifra pequeña en comparación con los 11.415 casos declarados (CNE, 2016). Si bien el factor más importante sigue siendo el consumo de carne de pollo y productos derivados (Calciati y cols., 2012; CDC 2013b; Kaakoush y cols., 2015; Little y cols., 2010), la ingesta de agua contaminada (Bartholomew y cols., 2014) y leche sin pasteurizar (Taylor y cols., 2013; Heuvelink y cols., 2009) son fuentes frecuentes en la aparición de brotes. En algunos estados de EEUU (Pennsylvania, Washington, California, etc.), la venta de leche cruda, que es legal, se considera el principal factor de riesgo en la aparición de brotes de campilobacteriosis en humanos (Mungai y cols., 2015).

En la mayoría de los estudios la etiología por *C. jejuni* está próxima al 90 % y por debajo del 10 % para *C. coli* (EFSA, 2015, CDC, 2013a, Schielke y cols., 2014). En nuestros resultados se mantiene mayor frecuencia de *C. jejuni* que de *C. coli*, aunque en proporciones diferentes. En Francia (2003-2010) se identificó una cifra aún superior para

este último (15,25 %) (Bessède y cols., 2014). Estos datos hay que analizarlos con precaución porque solo se registró la especie de *Campylobacter* en el 59,9 % de las encuestas, hecho que no es aislado, ya que en el informe de la EFSA de 2014, en el 52,6 % se facilitó dicha información (EFSA, 2015) y en el 34 % en EEUU en 2012 (*FoodNet*) (CDC, 2013a). Además, el 17,1 % se calificaron como *Campylobacter* spp., por lo que los resultados también podrían variar por este motivo. La falta de registros en la especie de *Campylobacter* se podría deber a varias causas. Por un lado, CyL es una comunidad autónoma muy amplia, con gran heterogeneidad en los laboratorios de microbiología. Pese a que todos cuentan con protocolos que permiten identificar al microorganismo a nivel de género, para la confirmación del caso y obtener el antibiograma, no todos disponen de la misma metodología para determinar la especie, lo que podría explicar la falta de precisión. Por otro lado, durante los primeros años de la vigilancia de la campilobacteriosis solo se recogían los datos epidemiológicos básicos, que no incluían la especie de *Campylobacter*, por lo que no existe ese dato, salvo que el responsable notificador lo conociera y lo registrara en observaciones u otro campo libre. Con la actualización de los protocolos de declaración en 2013 se incluyó este dato (CNE, 2013). Unido a todo esto, en ocasiones, es necesario tener un diagnóstico temprano para iniciar un tratamiento, generalmente en los casos más graves, y la confirmación microbiológica del caso por vía telefónica desde el laboratorio al médico responsable puede llevar a este a notificar el caso sin esperar el resultado de la especie. Si, posteriormente, no se hace una búsqueda activa para completar los datos microbiológicos se pierde esta información.

En cuanto a la gravedad de la enfermedad, casi la cuarta parte de las personas afectadas fueron hospitalizadas, cifra superior a la observada en Alemania (10 %) entre 2001- 2010 (Schielke y cols., 2014). Dado que la campilobacteriosis suele ser una enfermedad autolimitada, la mayor presentación de casos graves en nuestro estudio puede

deberse a que casi dos tercios se declararon desde atención especializada, sobreestimando los más complicados que son los que demandan asistencia sanitaria (Haagsma y cols., 2013). Por el contrario, los casos más leves no suelen demandar dicha atención y, si lo hacen, acuden a atención primaria donde no siempre se pide un coprocultivo. En caso de solicitarlo, como la evolución suele ser buena, puede que el paciente no vuelva a por el resultado y el facultativo no lo notifique, hecho que podría sugerir la infradeclaración por parte de atención primaria. La mayoría de las personas hospitalizadas correspondían a los grupos de ≥60 años y menores de 5, de forma similar a lo observado en otros estudios (CDC, 2013a; Schielke y cols., 2014). Este hecho podría reflejar que la enfermedad es más grave en edades extremas de la vida, pero también que los signos de gravedad son más alarmantes en esas edades, sobre todo en los niños, por lo que se acude más al médico. Si bien en la encuesta epidemiológica el apartado referido a la hospitalización no especifica si el ingreso hospitalario es debido a la campilobacteriosis sino que solo figura "hospitalización si/no", parece lógico pensar que en el caso de los niños que acuden a urgencias hospitalarias por una diarrea con signos de alarma (sangre, fiebre...), evolución de horas/días, si es ingresado y se confirma el caso, la causa principal de esa hospitalización sea la campilobacteriosis. En el grupo de ≥60 años, es fácil que concurran ciertas patologías crónicas que se pueden descompensar con la infección por un *Campylobacter* y que requieran hospitalización, por lo que el motivo del ingreso también se podría atribuir a esta enfermedad.

Es un hecho que la campilobacteriosis es un problema de salud pública del que solo vemos la punta del iceberg (Haagsma y cols., 2013; Kaakoush y cols., 2015). En la UE se considera que los casos podrían llegar a alcanzar los 9 millones/año si se mejoraran los registros y los sistemas de vigilancia (EFSA, 2014). Debido a la infradeclaración de casos, es difícil establecer el cálculo de gastos reales que conlleva esta enfermedad, pero

se ha estimado que en España alcanzaría los 120 millones de euros/año (Iranzo y cols., 2015), llegando a 2,4 billones en la UE (EFSA, 2014) y entre 1,2 y 4 billones de dólares/año en EEUU (Gölz y cols., 2014).

La principal limitación del estudio es la posible infrarrepresentación de casos, debido a la baja sensibilidad del sistema EDO, ya que es un sistema pasivo de búsqueda de casos que puede influir en la infranotificación de algunos casos y en la mayor representación de otros. Sirvan como ejemplo los datos no publicados referidos al área de salud de Burgos para 2015 que, con una población de 361.129 habitantes, estiman en 367 los aislamientos de *Campylobacter* frente a los 139 casos declarados, lo que supone el 37,87 % de todos los casos diagnosticados. Del total de casos declarados, el 80,58 % fue desde el ámbito hospitalario. Por otro lado, el SIM actualmente es un sistema básico de la Red de Vigilancia Epidemiológica de CyL (BOCyL, 2006), que añadiría flexibilidad y especificidad a la declaración de la campilobacteriosis, pero que en el momento de la elaboración de este estudio no se ha desarrollado regladamente. En este trabajo, tampoco se ha analizado la diferencia en el nivel de declaración de cada provincia ni se han tenido en cuenta otros factores como los geográficos (medio rural/ urbano), fuente de infección e influencia de la proximidad a granjas avícolas, que se han analizado y vinculado con la enfermedad en otros estudios (Bessell y cols., 2010; Nichols y cols., 2012; Schielke y cols., 2014). A pesar de estas limitaciones, en cuanto a la infradeclaración y representatividad de los casos, pensamos que no es un factor de confusión que haya influido en el incremento de la incidencia de la enfermedad notificada en CyL en el periodo de estudio.

Esta tendencia ascendente de los casos de campilobacteriosis declarados en CyL, especialmente en menores de 5 años, plantea la necesidad de diseñar programas de educación sanitaria, dirigidos a progenitores y cuidadores, en relación con la higiene y

cuidado en la preparación de los alimentos así como en las zonas de recreo al aire libre. Por otra parte, sería aconsejable introducir mejoras en la calidad de la recogida de datos de las encuestas epidemiológicas para poder indagar la fuente de infección, disminuir la infradeclaración de los casos leves y trabajar con datos más reales que puedan ayudar a fomentar políticas globales de salud pública en torno a la campilobacteriosis. Para ello, además de fortalecer el sistema de vigilancia epidemiológica, dotándolo de suficientes recursos para poder manejar tal volumen de información, sería conveniente una colaboración multidisciplinar (autoridades sanitarias, industria alimentaria, comunidad científica, etc) con el fin de aunar fuerzas en la investigación y control de la carga de esta enfermedad en humanos que ayuden a detectar cambios en el riesgo de su adquisición y generar nuevas hipótesis de trabajo.

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DISCUSIÓN GENERAL

X. DISCUSIÓN GENERAL

En este apartado se presenta una breve discusión general, que relaciona los resultados obtenidos en los diferentes capítulos y completa la discusión que se ha realizado por separado en cada uno de ellos, proporcionando una visión global sobre el estudio realizado en la presente tesis doctoral.

La discusión general se estructura en dos partes. La primera trata del estudio de la presencia y la diversidad genética de *Campylobacter* spp. a lo de la cadena alimentaria de la carne de pollo. La segunda parte, estudia la caracterización de las cepas aisladas previamente, haciendo especial mención a las estrategias de supervivencia y persistencia, tanto en el ambiente de cada una de las etapas de la cadena alimentaria, como a lo largo de la misma, llegando hasta el propio consumidor.

1. PREVALENCIA Y GENOTIPADO DE *CAMPYLOBACTER* A LO LARGO DE LA CADENA ALIMENTARIA DE CARNE DE POLLO.

El consumo de aves, especialmente de carne de pollo es considerado como la principal ruta de infección en humanos. Debido a las enormes pérdidas económicas que supone en la Unión Europea (2,4 billones al año) y la alta tasa de incidencia en humanos (246.307 casos declarados en el año 2016, lo que representa casi el 70% de todos los casos reportados de zoonosis); recientemente, ha entrado en vigor una nueva normativa europea (Reglamento 1495/2017) que regula la contaminación de *Campylobacter* spp. a nivel de matadero. Esta normativa establece un límite máximo de 1.000 ufc/g después del enfriado de la canal. En este sentido, la presente Tesis Doctoral aporta nuevos conocimientos para poder profundizar en la epidemiología y establecer estrategias de control. En base a nuestros resultados, las prevalencias de *Campylobacter* que se han detectado a lo largo de la cadena alimentaria de la carne de pollo han sido relativamente altas como se indican en los diferentes capítulos. En granjas, fue del 42,9 % y 30,8 %,

en otoño y primavera respectivamente (capítulo 1), 48,2 % en matadero (capítulo 2), y 39,4 % en productos de pollo comerciales (capítulo 4).

Para establecer una relación genética entre todas las cepas encontradas en la cadena alimentaria, se utilizó la técnica de electroforesis en campo pulsado (PFGE). De acuerdo a esta metodología, se encontró una gran variabilidad genética, siendo esta más destacada en los productos de pollo, tanto envasados como sin envasar (capítulo 4). Además, esta relación genética se evidenció también con las cepas aisladas en los casos de campilobacteriosis. Del total de genotipos encontrados en la cadena alimentaria, 35 fueron compartidos con cepas aisladas en humanos: 9 procedentes de granjas, 4 procedentes de matadero y 22 de productos de pollo (capítulo 4).

En base a los resultados obtenidos en el capítulo 1, esta variabilidad genética se ha observado entre las diferentes granjas avícolas, las cuales presentan diferentes pulsotipos entre sí, y en la misma granja entre distintas estaciones. Sin embargo, no ocurre lo mismo cuando estudiamos cada granja de forma individualizada, existiendo en general; de uno a tres pulsotipos diferentes. Aun así, es asumible que existe una gran diversidad genética. Por tanto, cuando las aves son transportadas al matadero y sacrificadas, es lógico pensar que dicha diversidad genética se mantiene o incluso puede aumentar por contaminaciones producidas a través de las cajas de transporte o en el propio ambiente del matadero antes del sacrificio como se ha descrito en la bibliografía. Sin embargo, en base a los resultados obtenidos, en el matadero (capítulo 2), esta diversidad, se ve reducida, a pesar del número variable de granjas distintas, que aportan pollos para el sacrificio diario, identificando únicamente 7 genotipos durante un mes de muestreo: ST 443; 443 CC (PFGE A); ST 775; 52 CC (PFGE B), ST 904; 607 CC (PFGE C), ST 464; 464 CC (PFGE D), ST 3769; 21 CC (PFGE G) y PFGE E. De los cuales, hubo dos genotipos, PFGE G y PFGE C, que fueron los más abundantes; 63,1 % y 23,0 %

respectivamente. Además, estos genotipos se aislaron durante 17 y 21 días; a partir de distintos puntos de muestreo durante el sacrificio de las aves, e incluso después de aplicar los protocolos de limpieza y desinfección. Este hecho, sugería que estas cepas podían persistir en el ambiente de la planta de procesado. Para confirmar esta hipótesis, era importante comprobar que las cepas de *Campylobacter jejuni* aisladas, a lo largo de diferentes días, pertenecían a una misma población.

Para contestar a este planteamiento, se realizaron análisis genómicos mediante técnicas de secuenciación: Multilocus Sequence Typing (MLST) y whole-genome Multilocus Sequence Typying (wgMLST). La técnica MLST estudia la variación genética mediante el análisis de cambios genéticos producidos en genes altamente conservados "*housekeeping*", dando lugar a un perfil alélico o secuencia tipo (ST), que a su vez se agrupan en complejos clonales (CC). Sin embargo, wgMLST estudia las diferencias alélicas dentro de una misma secuencia tipo, siendo una herramienta muy útil para resolver la relación clonal entre cepas. En base a los análisis realizados, se determinó que las dos poblaciones anteriormente citadas, pertenecientes a ST 3769 y ST 904, eran altamente clonales con una similitud del 99,1 % y un 98,8 % respectivamente. Concluyendo, por tanto, que *Campylobacter* puede sobrevivir y persistir en el ambiente durante más tiempo de lo que previamente se pensaba, dadas sus teóricas exigentes condiciones de crecimiento (capítulo 2).

En base a los resultados de los capítulos 1, 2 y 4, se ha mostrado que *Campylobacter*, a pesar de su fragilidad y sus fastidiosas necesidades de crecimiento se ha aislado en todas las etapas, superando diferentes condiciones de estrés. *Campylobacter* es un patógeno microaerófilo y está asociado al intestino de hospedadores para poder crecer. Sin embargo, en la presente tesis, se ha aislado de ambientes que no son los más óptimos para que dicha bacteria subsista, como son el ambiente de la granja (superficies

de comederos, bebederos, agua), el ambiente del matadero (antes y después de los procesos de limpieza y desinfección); o, en diferentes combinaciones de gases (ricos en O_2 o en CO_2) presentes en las atmósferas modificadas de diferentes productos envasados de pollo analizados, como se refleja en el capítulo 4.

Una vez confirmada la presencia de diferentes especies de *Campylobacter* a lo largo de la cadena alimentaria de carne de pollo, nos planteamos las siguientes preguntas:¿Por qué los genotipos ST 3769 y ST 904 pueden persistir durante 17 y 21 días en el ambiente del matadero? ¿Cuáles son los mecanismos implicados por los que estos patógenos, con necesidades tan fastidiosas de crecimiento, son capaces de sobrevivir a lo largo de la cadena alimentaria de carne de pollo? Para contestar a este planteamiento, estudiamos la caracterización de las cepas aisladas, como se muestra en el siguiente apartado.

2. CARACTERIZACIÓN DE CEPAS DE *CAMPYLOBACTER*: ESTRATEGIAS DE SUPERVIVENCIA Y PERSISTENCIA.

Según la bibliografía descrita, existen numerosos genes implicados en estos fenómenos por los cuales *Campylobacter* puede sobrevivir. Sin embargo, existe un vacío en el conocimiento de por qué o cuándo pueden estos genes expresarse o no. Por otra parte, también está descrito que *Campylobacter* puede entrar en el estado de "viable pero no cultivable" (VBNC), mediante el cuál las bacterias no pueden ser recuperadas con los medios de cultivo convencionales.

En este sentido, el capítulo 3 muestra una relación entre los posibles mecanismos implicados en la supervivencia y persistencia del patógeno en el ambiente hostil de la planta de procesado. Entre estos mecanismos, se encuentra la capacidad de ciertas bacterias, entre las que se encuentra *Campylobacter*, para producir biopelículas (*biofilms*), ya sea sola o en combinación con otras bacterias, como estrategia de

supervivencia. Además, numerosos genes de virulencia o resistencia frente a desinfectantes y/o antibióticos están también involucrados en la supervivencia en el ambiente como se cita en el capítulo 3. En base a este capítulo, tres de las seis secuencias tipo (STs) estudiadas: ST 443 (PFGE A); ST 904 (PFGE C) y ST 3769 (PFGE G), presentaron capacidad de formar biofilms, aunque con ligeras diferencias en las condiciones analizadas, siendo las cepas del ST 904 (PFGE C), las que mostraron la formación de biopelículas más robustas en las distintas condiciones. La presencia de mecanismos de resistencia frente a diferentes compuestos antimicrobianos, también puede jugar un papel importante en la persistencia de determinadas cepas en el ambiente. La mayor parte de los genotipos estudiados, presentaron una alta tasa de resistencia frente a ciprofloxacino, ácido nalidíxico y tetraciclina; así como a dos de los detergentes evaluados. Sin embargo, fueron las cepas del ST 904 (PFGE C), las que presentaron una mayor resistencia a los diferentes compuestos antimicrobianos evaluados fenotípicamente.

El estudio genético, más detallado de los diferentes genotipos, mostró que la secuencia tipo ST 904 (PFGE C), portaba en su genoma: (1) varios genes de resistencia a compuestos antimicrobianos, algunos de los cuales incluían determinadas mutaciones e inserciones, (2) la presencia de genes que codifican el sistema de secreción T6SS, así como (3) la presencia de un gran número de genes relacionados con factores de virulencia, que en su conjunto, podrían explicar la capacidad de formar biofilms más robustos en este ST. Así pues, la suma de este conjunto de pequeños cambios, podría explicar la capacidad, para sobrevivir y persistir durante períodos de tiempo más largos, en condiciones ambientales hostiles, como puede ser el matadero. Sin embargo, la ausencia de algunos genes como *capA*, *pgp1* and *ilvE,* todos ellos relacionados con la capacidad de adhesión, muestra la complejidad de las estrategias adoptadas por algunas cepas de *C. jejuni* para

adaptarse a medios hostiles. Otros genotipos formadores de biopelículas, presentan ligeras diferencias en su genoma con respecto al ST 904, lo que nos lleva a pensar que, existen múltiples soluciones para que las cepas puedan persistir y sobrevivir a lo largo de la cadena alimentaria de carne de pollo, y por tanto, ser capaces de causar la enfermedad en humanos.

Además, otro hecho destacable en los resultados obtenidos de la presente tesis, es la alta tasa de resistencia frente a los antibióticos durante toda la cadena alimentaria, incluidas las cepas aisladas de pacientes hospitalarios. Las principales resistencias encontradas han sido, de forma general, frente a ciprofloxacino, ácido nalidíxico y tetraciclinas. En la mayoría de los casos, *C. coli* fue la especie que presentó una mayor resistencia, pudiendo clasificarse algunas cepas de esta especie como multi-resistente, entendiéndose como tal, cuando una cepa es resistente a tres o más antibióticos de clases no relacionadas; como se evidencia en los capítulos 1, 3, 4 y 5.

La gran variabilidad genética en *Campylobacter* encontrada a lo largo de la cadena alimentaria de pollo se traduce en una gran versatilidad a nivel genómico, favoreciendo la adaptación del patógeno a numerosos ambientes como se ha podido comprobar en los diferentes capítulos y dificultando, por tanto, las estrategias empleadas para reducir su presencia en las distintas etapas en la cadena alimentaria. Así pues, es necesaria una monitorización y evaluación continua de la presencia de *Campylobacter* spp. a lo largo de la cadena alimentaria de la carne de pollo, con el objetivo de tener un mayor conocimiento del comportamiento del patógeno y establecer así medidas adecuadas, que puedan reducir la presencia del mismo en las diferentes etapas analizadas, y por tanto, reducir las infecciones en humanos.

En base a los capítulos 5 y 6, se ha mostrado que las infecciones por *Campylobacter* ocurren en un alto porcentaje, tanto en la zona de Burgos como en el total

de Castilla y León, y que estas pueden estar relacionadas con cepas procedentes de la cadena alimentaria de la carne de pollo (capítulo 5). Principalmente, como resultados de ambos capítulos, se ha descrito que la población de mayor riesgo es la que afecta a pacientes con edades menores de 5 años (pediatría). Este hecho sugiere, la necesidad de realizar campañas de educación sanitaria en el ámbito doméstico, sobre la manipulación de la carne de pollo y de los alimentos en general en el hogar, para reducir la incidencia de la enfermedad en esas edades.

Los conocimientos aportados en la presente tesis doctoral proporcionan información útil para establecer una relación genotípica entre las cepas de *Campylobacter* spp. aisladas en la cadena alimentaria de la carne de pollo (granja, matadero, productos de pollo) y cepas aisladas de pacientes humanos con campilobacteriosis. Esta relación nos permite profundizar en el conocimiento y entendimiento de la epidemiología y transmisión de *Campylobacter* a través de la carne de pollo. Por otra parte, y como resultado de esta tesis, pueden establecerse diferentes estrategias de control en todas las etapas analizadas "desde la granja a la mesa". Entre estas, se pueden resaltar las siguientes: (1) implementación de medidas de bioseguridad en las granjas, (2) optimización de los protocolos de limpieza y desinfección en los mataderos y plantas de procesado, así como en carnicerías/pollerías, haciendo especial mención a zonas donde se puedan producir *biofilms* y (4) promover la educación sanitaria en el hogar. Afianzando así, las buenas prácticas de higiene tanto en operadores de industrias avícolas, manipuladores de alimentos y consumidores con el fin de reducir la presencia de *Campylobacter* a lo largo de la cadena alimentaria, y por tanto, reducir el número de casos de campilobacteriosis y evitar, de igual modo, la adquisición de resistencias antimicrobianas.

CONCLUSIONES
CONCLUSIONS

XI. CONCLUSIONES GENERALES

1. La especie de *Campylobacter* aislada con mayor frecuencia, a lo largo de toda la cadena alimentaria de carne de pollo y en pacientes diagnosticados con campilobacteriosis, ha sido *Campylobacter jejuni*.
2. Se ha constatado que hay una gran variabilidad de pulsotipos de *Campylobacter jejuni* en las granjas. Cada granja presenta un número muy limitado de pulsotipos, característicos de cada una y diferentes de las otras, y que van variando en función de la estación del año analizada.
3. A pesar de la gran variabilidad de pulsotipos encontrados en la granja, en el matadero y planta de procesado, se han encontrado, en el espacio de un mes, solo siete pulsotipos distintos. Dos, de estos siete pulsotipos, C y G, se han aislado durante 21 y 17 días respectivamente, incluso después de aplicar el protocolo de limpieza y desinfección. El análisis del genoma completo de estos pulsotipos mediante wgMLST, ha puesto de manifiesto que las poblaciones de ambas son altamente clonales. Estos hechos, sugieren que dichos pulsotipos pueden persistir en el ambiente del matadero, durante más tiempo de lo que se pensaba, y por tanto actuar como fuente de contaminación ambiental.
4. De los siete pulsotipos aislados en el matadero y la planta de procesado, sólo tres fueron capaces de formar biofilms. De ellos, el pulsotipo C (ST 904; ST 607 CC) fue el que produjo biofilms más robustos en las diferentes condiciones y superficies ensayadas, seguido del pulsotipo G (ST 3769; ST 21 CC), los dos pulsotipos que más persistieron en el ambiente de la planta de procesado.

5. Los resultados obtenidos sugieren, que la capacidad de formar biofilms más robustos por parte del pulsotipo C, puede ser debido a la suma de pequeños cambios en el genoma del mismo, como la presencia de varias mutaciones o inserciones en genes que le hacen ser más resistente frente a compuestos antimicrobianos, la presencia del sistema de secreción T6SS, y la presencia de un gran número de genes relacionados con factores de virulencia. Sin embargo, la ausencia de algunos genes como *capA*, *pgp1* and *ilvE,* todos ellos relacionados con la capacidad de adhesión, muestra la complejidad de las estrategias adoptadas por algunas cepas de *C. jejuni* para adaptarse a medios hostiles.
6. El estudio realizado a productos de carne de pollo comerciales, ha puesto de manifiesto la gran diversidad y variedad de pulsotipos de *Campylobacter* spp. en estos productos. Asimismo, se ha visto que hay una mayor prevalencia en productos no envasados frente a los productos envasados con atmósfera modificada. Sin embargo, la cantidad y diversidad de pulsotipos existentes en este último de tipo de productos, demuestra que a pesar de ser microaerófilo, *Campylobacter jejuni* tiene una capacidad de resistencia mayor de lo que se pensaba a distintas atmósferas de envasado.
7. En el presente estudio se ha comprobado que una importante variedad de pulsotipos, encontrados en diferentes etapas de la cadena alimentaria de pollo, se han aislado también en pacientes diagnosticados con campilobacteriosis. Lo que demuestra la estrecha relación entre la carne de pollo y dicha enfermedad, aunque dicha relación no explica la alta incidencia de la misma en la población.

8. La mayoría de las cepas de *Campylobacter jejuni*, aisladas a lo largo de este estudio, presentan una alta resistencia a los antibióticos: ciprofloxacino, ácido nalidíxico y tetraciclina. Algunas cepas aisladas de *C. coli* pueden ser consideradas como multi-resistentes, ya que aparte de ser resistente a los antibióticos antes mencionados, lo son también a la eritromicina y azitromicina, y algunas a la gentamicina.
9. La campilobacteriosis en Castilla y León tiene una marcada estacionalidad, siendo los pacientes menores de 5 años y en el sexo masculino los más implicados. Las hospitalizaciones suponen la cuarta parte del total de los casos declarados y *C. jejuni* es la especie más frecuentemente implicada.

Conclusión final

Esta tesis doctoral ha permitido conocer la prevalencia de *Campylobacter* spp. a lo largo de toda la cadena alimentaria de la carne de pollo, desde la granja hasta el consumidor y sus relaciones genéticas. *Campylobacter* spp. es capaz de persistir en diferentes ambientes, siendo capaz de superar ambientes hostiles para el patógeno y persistir más tiempo de lo que hasta ahora estaba descrito en la bibliografía.

Los resultados obtenidos en la presente tesis doctoral hacen énfasis en las medidas preventivas que hay que establecer desde el primer eslabón de la cadena alimentaria para reducir la presencia del patógeno. Entre estas, pueden citarse; bioseguridad en las granjas para reducir que *Campylobacter* pueda transmitirse horizontalmente, mejorar los procedimientos de limpieza y desinfección en el matadero y/o planta de procesado, realizar campañas de sensibilización y concienciación tanto en los operarios de carnicerías y/o supermercados, como el consumidor final para evitar contaminación

cruzada con otros productos alimentarios. Además, esta tesis demuestra de una forma alarmante la alta tasa de resistencia frente a los antibióticos que existe en toda la cadena alimentaria; confirmando que estamos ante un problema de Salud Pública, no solo por las altas resistencias frente a antibióticos y las altas pérdidas económicas que genera al sistema sanitario, sino porque este patógeno puede persistir en el ambiente pudiendo actuar como nuevas fuentes de contaminación, siendo aún más si cabe, difícil su eliminación.

XI. GENERAL CONCLUSIONS

1. The most commonly isolated *Campylobacter* species, along the chicken food chain and in patients diagnosed with campylobacteriosis, has been *Campylobacter jejuni*.
2. It has been found a great variability of *Campylobacter jejuni* pulsotypes in the farms. Each farm has a very limited number of pulsotypes, which are characteristic of each farm, different from other farms and different depending on the season of the year analyzed.
3. Despite the great variability of pulsotypes found on the farm, in the slaughterhouse and processing plant, only seven distinct pulsotypes have been isolated during one month. Two of these seven pulsotypes, C and G, have been isolated at least during 21 and 17 days respectively, even after applying the cleaning and disinfection protocol. The analysis of the complete genome of these pulsotypes by wgMLST, has shown that the populations of both are highly clonal. These facts suggest that these pulsotypes may persist in the slaughterhouse environment, for longer than previously thought, and therefore act as a source of environmental contamination.
4. Only three of the seven pulsotypes isolated in the slaughterhouse and the processing plant, were able to form biofilms. Pulsotype C (ST 904; ST 607 CC) was the one that produced more robust biofilms in the different conditions and surfaces tested, followed by the pulsotype G (ST 3769; ST 21 CC). These two pulsotypes were the ones that persisted longer in the environment of the processing plant.

5. The results obtained suggest that the ability to form more robust biofilms by the pulsotype C, may be due to the sum of small changes in its genome, such as the presence of several mutations or insertions in genes that make it more resistant against antimicrobial compounds, the presence of the T6SS secretion system, and the presence of a large number of genes related to virulence factors. However, the absence of some genes such as *capA*, *pgp1* and *ilvE*, all related to adhesion capacity, shows the complexity of the strategies adopted by some strains of *C. jejuni* to adapt to harsh conditions of the environment.
6. The study carried out on commercial chicken meat products has shown the great diversity and variety of pulsotypes of *Campylobacter* spp. in these products. Likewise, it has been observed that there is a higher prevalence in non-packaged products in comparison with products packaged with modified atmosphere. However, the number and diversity of pulsotypes found in packed products, shows that despite being microaerophilic, *Campylobacter jejuni* has a greater resistance capacity than previously thought to different packaging atmospheres.
7. In the present study it has been proven that an important variety of pulsotypes, found in different stages of the chicken food chain, have also been isolated in patients diagnosed with campylobacteriosis. This fact shows the close relationship between chicken meat and this disease, although this relationship does not explain the high incidence of this foodborne illness in the population.
8. Most strains of *Campylobacter jejuni*, isolated throughout this study, have a high resistance to antibiotics: ciprofloxacin, nalidixic acid and

tetracycline. Moreover, some isolates of *C. coli* can be considered as multi-resistant, since apart from being resistant to the aforementioned antibiotics, they are also resistant to erythromycin and azithromycin, and some of them to gentamicin also.

9. Campylobacteriosis in Castile and Leon has a seasonality pattern, with patients under 5 years of age and males being the most involved. Hospitalizations account for a 25 % of all reported cases, being *C. jejuni* the most frequently implicated species.

As a final conclusion and reflection

This PhD thesis has allowed knowing the prevalence of *Campylobacter* spp. along the chicken food chain, from the farm to the consumer and their genetic relationships. *Campylobacter* spp. is able to persist in different environments, being able to overcome harsh environmental conditions and persist longer than previously thought according to the literature.

The results obtained in this thesis emphasize the need to established preventive measures from the first step in the food chain to reduce the presence of this pathogen. Some of these measures are: improve biosafety strategies on farms to reduce horizontal transmission of *Campylobacter*, improve cleaning and disinfection procedures at the slaughterhouse and/or processing plant, awareness campaigns in both foods such as operators butcher shops and/or supermarkets and consumers, in order to avoid practices that favor cross-contamination with other food products. In addition, this thesis shows a great concern about the high rate of antibiotics resistance strains of *Campylobacter* spp. throughout the food chain; confirming that this is a public health problem, not only by their high resistance against antibiotics and the economic losses generated in the public

Health System, but also because this pathogen can persist in the environment acting as a source of contamination, and making its elimination even more difficult.

ANEXOS

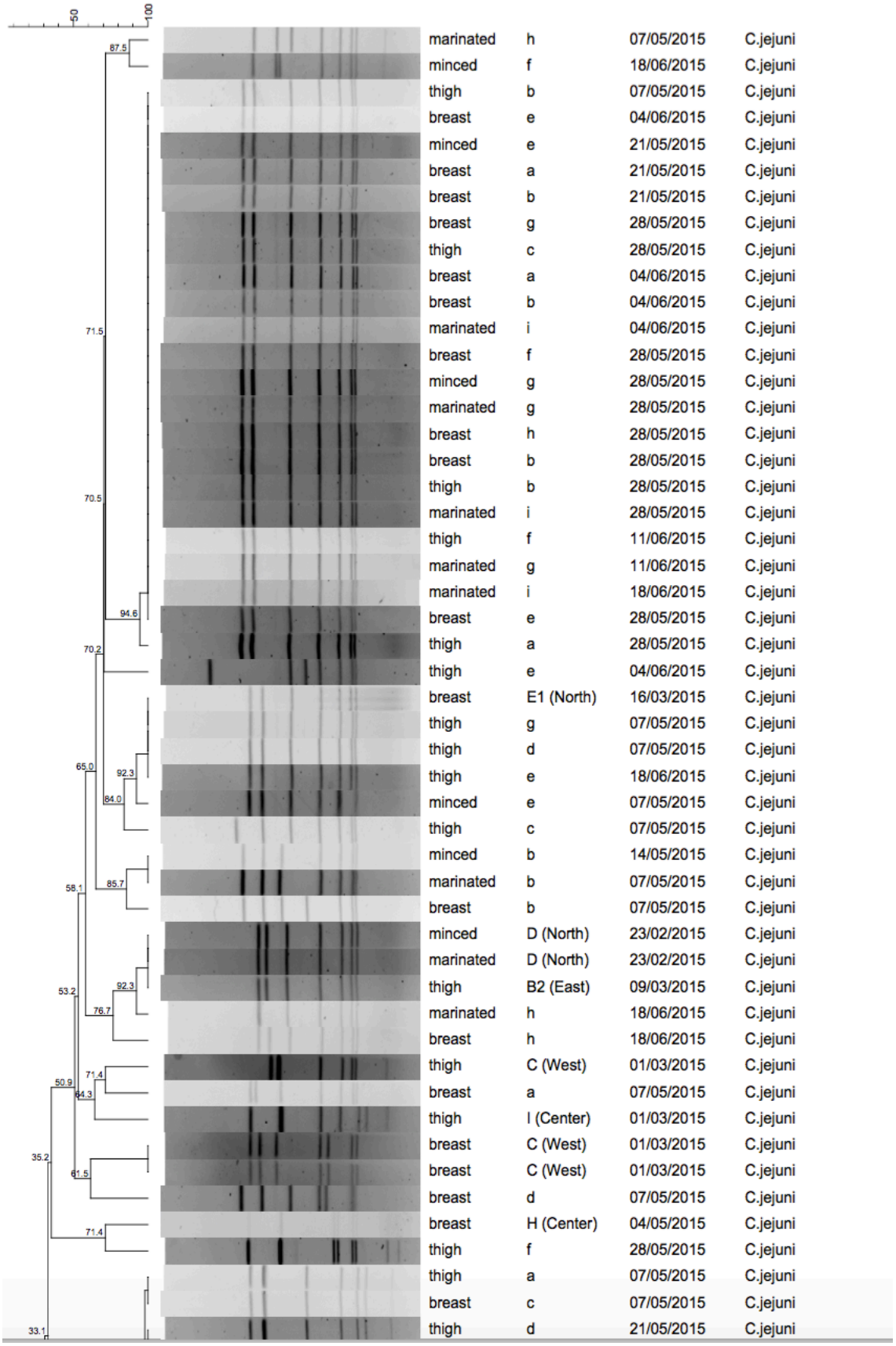
XII. ANEXOS

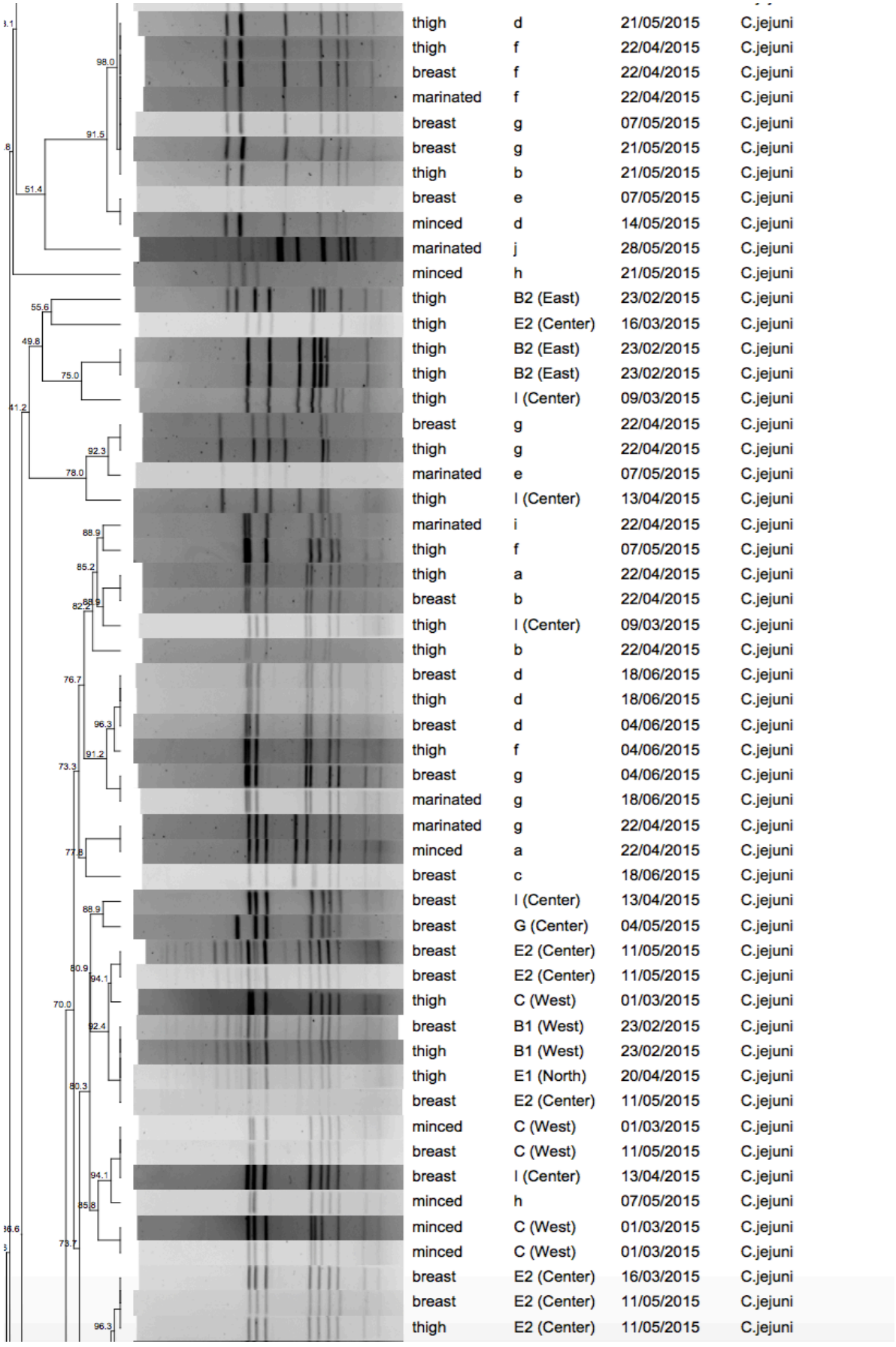
Como anexos se adjuntan los siguientes:

I. **CAPÍTULO 4.** Dendograma adicional con información complementaria al Capítulo 4 (páginas 187-208).

II. **CAPÍTULO 5.** Información adicional con información complementaria al Capítulo 5 (páginas 217-240). Incluyendo las siguientes tablas: S1, S2, S3 y S4.

III. CAPÍTULO 4. Dendograma adicional con información complementaria (páginas 187-208).





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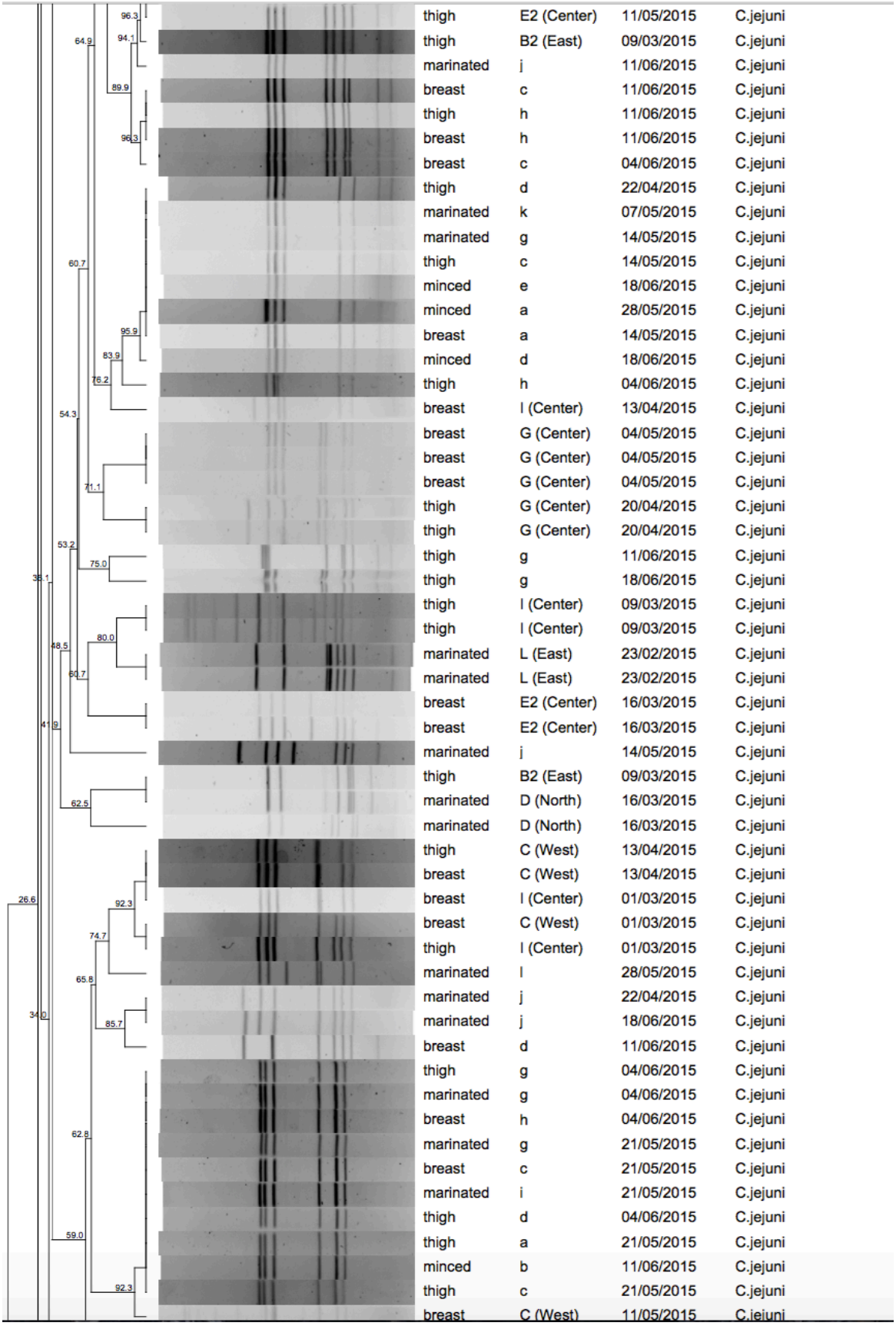
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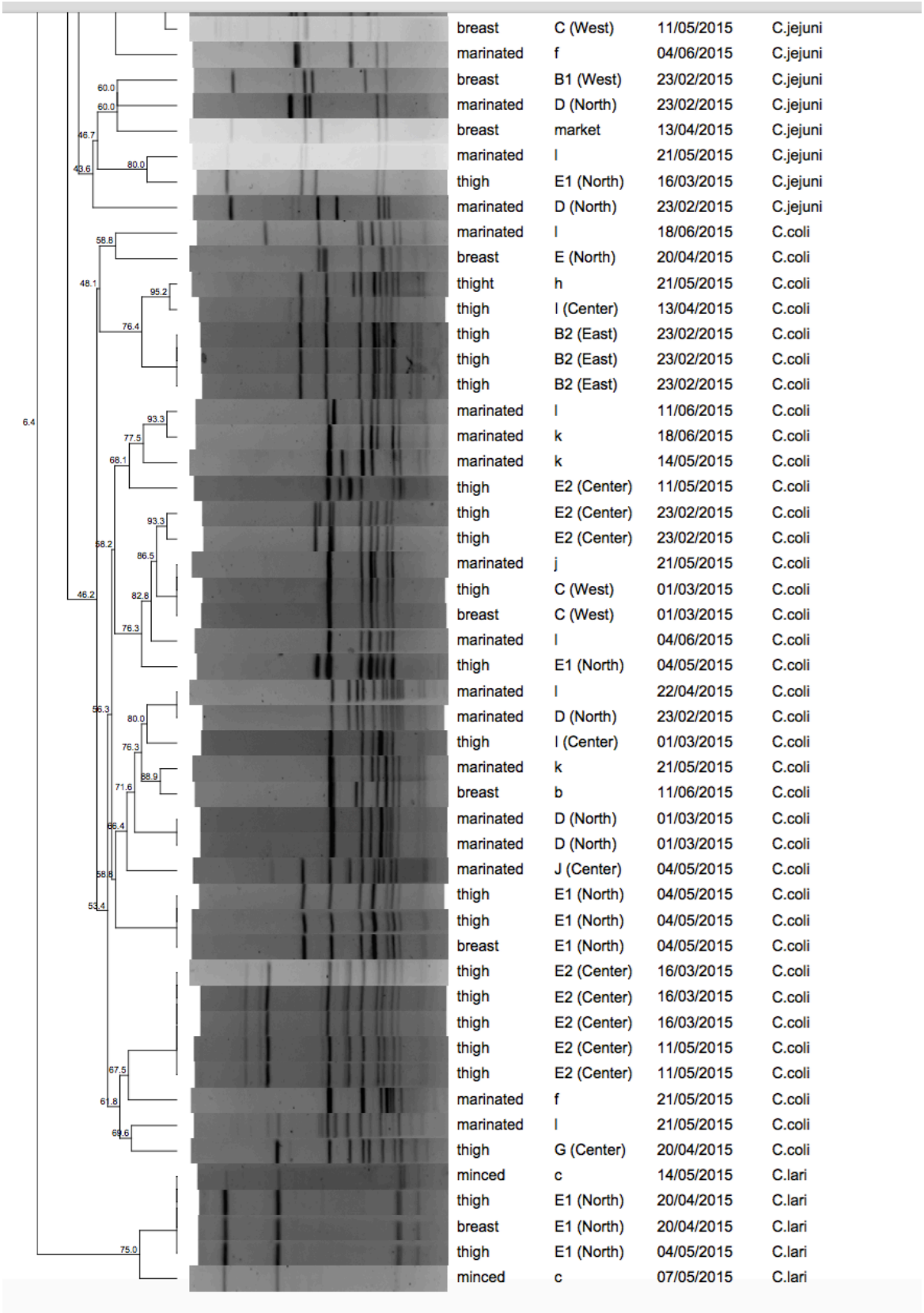

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breast	b	11/06/2015	C.coli
marinated	D (North)	01/03/2015	C.coli
marinated	D (North)	01/03/2015	C.coli
marinated	J (Center)	04/05/2015	C.coli
thigh	E1 (North)	04/05/2015	C.coli
thigh	E1 (North)	04/05/2015	C.coli
breast	E1 (North)	04/05/2015	C.coli
thigh	E2 (Center)	16/03/2015	C.coli
thigh	E2 (Center)	16/03/2015	C.coli
thigh	E2 (Center)	16/03/2015	C.coli
thigh	E2 (Center)	11/05/2015	C.coli
thigh	E2 (Center)	11/05/2015	C.coli
marinated	f	21/05/2015	C.coli
marinated	l	21/05/2015	C.coli
thigh	G (Center)	20/04/2015	C.coli
minced	c	14/05/2015	C.lari
thigh	E1 (North)	20/04/2015	C.lari
breast	E1 (North)	20/04/2015	C.lari
thigh	E1 (North)	04/05/2015	C.lari
minced	c	07/05/2015	C.lari

IV. CAPÍTULO 5. Información adicional con información complementaria al Capítulo 5 (páginas 217-240).Incluyendo las siguientes tablas: S1, S2, S3 y S4.

Table S1. *Campylobacter* PFGE clusters formed during period 1 (farms) with human isolates and the correlated epidemiological data.

PFGE	nº isolates	Date	Origin	Sex	Age	Service	ZIP Code	Hospitalization
P1-16	5	24/10/14	Poultry farm				-	
		21/10/14	Human	Male	5	Paediatric	9007	NO
		24/11/14	Human	Female	35	Emergency	9006	NO
		3/12/14	Human	Female	74	Haematology	9006	YES
		2/1/15	Human	Female	1	Emergency	9006	NO
P1-18	9	9/12/14	Poultry farm				-	
		20/10/18	Human	Female	1	Pediatric	9001	NO
		31/10/14	Human	Female	10	Emergency	9006	NO
		26/11/14	Human	Female	0	Pediatric	9003	NO
		28/11/14	Human	Male	45	Emergency	9006	NO
		29/11/14	Human	Male	1	Pediatric	9001	NO
		1/12/14	Human	Female	79	Haematology	9006	YES
		9/1/15	Human	Female	13	Pediatric	9007	NO
		20/10/14	Human	Male	1	Pediatric	9006	NO
P1-27	7	19/11/14	Poultry farm				-	
		16/10/14	Human	Male	2	Pediatric	9005	NO
		11/11/14	Human	Female	31	General	9005	NO
		2/12/14	Human	Female	5	Pediatric	9005	NO
		20/10/14	Human	Female	0	Pediatric	9006	NO
		12/11/14	Human	Male	1	Pediatric	9005	NO
		19/11/14	Human	Male	0	Emergency	9006	NO
P1-30	2	6/11/14	Poultry farm				-	
		4/12/14	Human	Male	10	Pediatric	9001	NO
P1-36	5	3/12/14	Poultry farm				-	
		6/11/14	Human	Female	19	Gastroenterology	9006	YES
		3/12/14	Human	Female	60	Gastroenterology	9006	NO
		2/1/15	Human	Male	4	Emergency	9006	NO
		2/1/15	Human	Male	7	Pediatric	9007	NO

Table S2. *Campylobacter* PFGE clusters formed during period 2 (farms) with human isolates and the correlated epidemiological data.

PFGE	nº isolates	Date	Origin	Sex	Age	Service	ZIP Code	Hospitalization
P_2-5	2	26/5/15	Poultry farm				-	
		17/5/15	Human	Male	3	Pediatric	9006	NO
P_2-8	6	9/6/15	Poultry farm				-	
		12/5/15	Human	Male	0	Pediatric	9007	NO
		15/6/15	Human	Male	2	Pediatric	9600	NO
		19/6/15	Human	Male	0	Pediatric	9006	NO
		18/5/15	Human	Female	58	Emergency	9006	NO
		25/5/15	Human	Female	2	Pediatric	9003	NO
P_2-9	4	26/5/15	Poultry farm				-	
		13/5/15	Human	Female	0	Pediatric	9007	NO
		19/6/15	Human	Male	1	Pediatric	9006	NO
		25/5/15	Human	Male	7	Pediatric	9006	NO
P_2-28	2	9/5/15	Poultry farm				-	
		21/6/15	Human	Male	5	Pediatric	9006	NO

Table S3. *Campylobacter* PFGE clusters formed during period 3 (slaughterhouse) with human isolates and the correlated epidemiological data.

PFGE	n° isolates	Date	Origin	Sex	Age	Service	ZIP Code	Hospitalization
P_3-A	4	23/06/2014-27/06/2014	S*				9001	
		9/7/14	Human	Female	2	Pediatric	9006	NO
		9/7/14	Human	Female	1	Pediatric	9001	NO
		25/7/14	Human	Female	1	Pediatric	9006	NO
P_3-B	3	27/6/14	S*				9001	
		9/7/14	Human	Male	22	Gastroenterology	9006	YES
		9/7/14	Human	Male	82	Internal Medicine	9006	YES
P_3-C	3	6/06/2014-25/06/2014	S*				9001	
		17/6/14	Human	Female	6	Pediatric	9006	NO
		21/6/14	Human	Male	1	Pediatric	9006	NO
P_3-G	6	2/06/2014-19/06/2014	S*				9001	
		26/6/14	Human	Male	1	Pediatric	9006	NO
		3/7/14	Human	Male	0	Pediatric	9005	NO
		4/7/14	Human	Male	7	Pediatric	9001	NO
		15/6/14	Human	Male	44	Emergency	9006	NO
		23/7/14	Human	Female	6	Pediatric	9006	YES

S*: Slaughterhouse

Table S4. *Campylobacter* PFGE clusters formed during period 4 (chicken products) with human isolates and the correlated epidemiological data.

PFGE	nº isolates	nºisolates /sample	Date	Sex	Age	Service	Zip Code	Hospitalization
P_4-1	4	3	23/2/15			Thigh	9005	
		1	11/4/15	Male	20	Gastroenterology	9006	YES
P_4-3	3	1	16/3/15			Thigh	9006	
		1	21/6/15	Male	0	Pediatric	9007	NO
		1	20/7/15	Male	2	Pediatric	9006	NO
P_4-4	10	8	01/03/2015-11/05/2015			Thigh/breast/ marinated/minced	9005	
		1	25/5/15	Male	7	Pediatric	9006	NO
		1	19/6/15	Female	1	Pediatric	9001	NO
P_4-5	11	6	23/02/2015-11/05/2015			Thight/breast	9005	
		1	25/5/15	Female	1	Pediatric	9006	NO
		1	31/5/15	Female	43	Internal Medicine	9006	YES
		1	3/3/15	Female	3	Pediatric	9006	NO
		1	13/3/15	Male	2	Pediatric	9006	NO
		1	29/5/15	Female	6	Emergency	9006	NO
P_4-6	13	9	09/03/2015-11/06/2015			Thight/breast/ marinated	9003/9005 9006	
		1	20/4/15	Male	3	Emergency	9006	NO
		1	13/5/15	Female	0	Pediatric	9007	
		1	13/4/15	Male	8	Pediatric	9006	NO
		1	19/6/15	Male	1	Pediatric	9006	NO
P_4-8	4	2	1/3/15			Minced	9005	
		1	4/4/15	Male	5	Pediatric	9006	NO
		1	25/5/15	Female	2	Pediatric	9003	NO
P_4-12	3	1	22/4/15			marinated/minced	9005/9004	
		2	15/4/15	Male	5	Pediatric	9006	NO
P_4-15	12	8	22/04/2015-18/06/2015			Thight/breast/ marinated/minced	9003/9004/ 9005/9006	
		1	12/5/15	Male	0	Pediatric	9007	NO
		1	18/5/15	Female	58	Emergency	9006	NO
		1	15/6/15	Male	2	Pediatric	9600	NO
		1	19/6/15	Male	0	Pediatric	9006	NO
P_4-16	2	1	4/6/15			Thigh	9006	
		1	4/5/15	Female	0	Pediatric	9006	NO
P_4-18	4	2	16/3/15			Breast	9006	

		1	19/3/15	Male	1	Pediatric	9007	NO
		1	17/3/15	Male	1	Pediatric	9006	NO
P_4-37	7	5	1/3/2015-13/04/2015			Thight/breat	9005/9006	
		1	5/4/15	Male	13	Emergency	9006	NO
		1	12/5/15	Male	7	Pediatric	9007	NO
P_4-38	13	12	11/05/2015-04/06/2015			Thight/breast/ marinated/minced	9003/9004 9005/9006	
		1	15/6/15	Male	9	Emergency	9006	NO
P_4-53	2	1	23/2/15			breast	9005	
		1	25/6/15	Male	78	Internal Medicine	9006	YES
P_4-55	23	22	07-05-2015-18/06/2015			Thight/breast/ marinated/minced	9003/9004 9005	
		1	19/6/15	Female	1	Pediatric	9003	NO
P_4-57	8	5	16/3/2015-18/06/2015			Thigh/breast/ minced	9005/9006	
		1	6/3/15	Male	10	Pediatric	9005	NO
		1	14/4/15	Female	1	Pediatric	9006	NO
		1	15/4/15	Female	5	Pediatric	9006	NO
P_4-61	4	1	1/3/15			Thigh	9005	
		1	19/5/15	Female	34	General Medicine	9001	NO
		1	31/5/15	Female	53	Emergency	9006	NO
		1	9/7/15	Female	84	Emergency	9006	NO
P_4-62	8	5	23/02/2015-18/06/2015			Thigh/breast/ marinated/minced	9005/9006	
		1	12/6/15	Female	70	Gastroenterology	9006	YES
		1	15/6/15	Male	54	Emergency	9006	NO
		1	17/7/15	Male	1	Pediatric	9005	NO
P_4-66	3	2	1/3/15			Breast	9005	
		1	21/6/15	Male	5	Pediatric	9006	NO
P_4-68	2	1	28/5/15			Thigh	9005	
		1	9/6/15	Male	1	Pediatric	9003	NO
P_4-77	13	11	22/04/2015-28/5/2015			Thigh/breast/ marinated/minced	9004/9005 9006	
		1	18/5/15	Female	4	Pediatric	9001	NO
		1	18/5/15	Male	1	Pediatric	9001	NO
P_4-88	3	2	21/05/2015-11/06/2015			Breast/marinated	9003/9005	
		1	26/6/15	Male	8	Pediatric	9007	NO
P_4-90	3	2	11/06/2015-18/06/2015			Marinated	9003	
		1	25/5/15	Female	25	Emergency	9006	NO

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