CEU CARDENAL HERRERA UNIVERSITY

FACULTY OF VETERINARY MEDICE

Campylobacter epidemiology in broiler production in Eastern Spain

Thesis submitted to the CEU Cardenal Herrera University of Valencia in fulfilment of the requirements for degree of Doctor of Philosophy in the Faculty of Veterinary Medicine by:

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CERTIFICAN:

Que la memoria titulada **"*Campylobacter* epidemiology in broiler production in Eastern Spain"**, que, para aspirar al grado de Doctor Internacional en Veterinaria presenta Dña. Sofía Ingresa Capaccioni, realizada bajo nuestra dirección en la Universidad CEU Cardenal Herrera, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que,

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TABLE OF CONTENT. Page

LIST OF FIGURES. **V**

LIST OF TABLES. **VII**

LIST OF ABBREVATIONS. **IX**

ABSTRACT. **XI**

RESUMEN. **XVII**

CHAPTER I. LITERATURE REVIEW. **1**

I.1 General aspects of *Campylobacter* 3

I.1.1. Historical background of *Campylobacter* 3

I.1.2. General characteristics 5

I.1.3. Nomenclature 7

I.2. *Campylobacter* epidemiology in humans 8

I.2.1. Campylobacteriosis in humans 8

I.2.2. Human clinical aspects.. 10

I.2.3. Main sources of human campylobacteriosis 11

I.3. *Campylobacter* epidemiology in broiler production 16

I.3.1. Avian campylobacteriosis 16

I.3.2. Colonization and immunology against *Campylobacter* 17

I.3.3. *Campylobacter* in primary production 19

I.3.3.1. Prevalence in broiler batches 19

I.3.3.2. Risk factors and sources of contamination at broiler farms 20

I.3.4. Transport and slaughter of broiler flock 26

I.3.4.1. Risk factors at transportation and before slaughter 26

I.3.4.2. Prevalence on broiler carcasses 27

I.3.4.3. Risk factors during slaughter, dressing and processing 28

I.4. Identification and characterization of *Campylobacter* 31

I.4.1. Sampling methods 31

I.4.1.1. Farm level 31

I.4.1.2. Slaughterhouse 32

I.4.2. Identification of *Campylobacter* spp. 32

I.4.2.1. Bacteriological isolation and identification 32

I.4.2.2. Molecular techniques 36

CHAPTER II. STUDY OBJECTIVES. **39**

General objective 41

Specific objectives 41

CHAPTER III. MATERIAL AND METHODS. **43**

III.1. Experiment 1: *Campylobacter* epidemiology from breeders to their progeny in Eastern Spain 45

III.1.1. Study sample 45

III.1.2. Environmental sample collection 46

III.1.3. Sample collection in breeder flocks (parents) 47

III.1.4. Sample collection in broiler flocks (offspring) 48

III.1.5. Sample collection at slaughter 49

III.1.6. Bacteriological analysis 49

III.1.7. Statistical analysis 51

III.2. Experiment 2: Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp. 52

III.2.1. Study sample 52

III.2.2. Sample collection and processing 52

III.2.3. Bacteriological analysis 54

III.2.4. Statistical analysis 54

III.3. Experiment 3: Molecular detection of *Campylobacter* spp. in day-old chicks 55

III.3.1. Sample collection 55

III.3.2. Bacterial culture method 55

III.3.3. Detection and quantification of *Campylobacter* spp. by qPCR method 56

III.3.4. Molecular cloning of *C. jejuni* and *C. coli* PCR products 57

CHAPTER IV. RESULTS. **59**

IV.1. Experiment 1: *Campylobacter* epidemiology from breeders to their progeny in Eastern Spain 61

IV.1.1. Environmental samples 61

IV.1.2. Breeders (Parents) 61

IV.1.3. Broilers (Offspring) 62

IV.2. Experiment 2: Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp. 64
IV.2.1. Comparisson of sampling types 64
IV.2.2. Slaughterhouse 68
IV.3. Experiment 3: Molecular detection of *Campylobacter* spp. in day-old chicks 69
CHAPTER V. GENERAL DISCUSSION. **71**
CHAPTER VI. CONCLUSIONS. **87**
REFERENCES. **91**
ANNEX I. **147**
ANNEX II. **149**

LIST OF FIGURES. **Page**

Figure 1. Publication and original drawing of the described spiral-shape bacteria in the colons of children who had died of "cholera infantum". Münchener Medizinische Wochenschrift, (Escherich *et al*., 1886) **3**
Figure 2. *Campylobacter jejuni* forms: a) spiral form. b) coccoid form. (Pead, 1979) **7**
Figure 3. Reported numbers and notification rates of confirmed human zoonoses cases in the EU, 2014 (EFSA and ECDC, 2015b) **9**
Figure 4. Distribution of food vehicles in strong-evidence outbreaks caused by *Campylobacter* (excluding strong-evidence water-borne outbreaks) in the EU in 2013 (EFSA and ECDC, 2015a) **12**
Figure 5. Comprehensive overview of *Campylobacter* source attribution studies published between 2010 and 2015 (Skarp *et al*., 2016) **13**
Figure 6. Prevalence (%) of *Campylobacter* spp. colonised broiler batches in the EU, 2008, EFSA (EFSA, 2010) **20**
Figure 7. Prevalence of *Campylobacter*-contaminated broiler carcasses in the EU in 2008. (EFSA 2010) **28**
Figure 8. Schematic illustration of the samples collection to determine vertical transmission of *Campylobacter* passage from breeder hens to broiler progeny. For the breeder flocks, during rearing period, samples were collected at 0, 4, 8, 12, 16, and 20 weeks and during laying period samples were collected at 26, 31, 48 and 60 weeks. For the broiler flocks, during the fattening period, samples were collected at 7, 14, 21, 28, 35 and 42 days **45**
Figure 9. Environmental sample collection. a) Water sample collected from a water tank. b) Water sample collected from final dispenser lines. c) Feed sample collected from feeders. d) Dust samples from broiler houses. e) Farmer boot samples **46**
Figure 10. Caeca sample collection **47**
Figure 11. Cloacal swab sample collection (Cary Blair transport sterile swabs, DELTALAB®) **47**
Figure 12. Carcasses sampling (neck skin) **49**
Figure 13. ISO 10272-2:2006 (Annexe E) scheme for the detection of *Campylobacter* spp. mCCDA: Modified Charcoal Cefoperazone Deoxycholate agar. Preston: Preston agar. a) Cellular morphology and motility. b) Oxidase and catalase tests. c) Planting at different temperatures and in distinct atmospheres onto Columbia blood agar **50**
Figure 14. Sampling strategies. a) Boot swabs sample. b) Caecal content sample. c) Faeces sample. d) Cloacal swab sample **53**
Figure 15. *Campylobacter* isolation during the breeding period (rearing and laying period). Each animal was sampled from 0 to 60 weeks of age. [a,b,c,d,e]Different superscripts represent significant differences ($P < 0.05$). Data are presented as least squares means ± standard error of the least squares means **62**

Figure 16. *Campylobacter* isolation during fattening period. Each animal was sampled just before placing day-old chicks (day 1) and at weekly intervals until slaughter day. [a,b,c,d,e,f] Data with uncommon letters are different (P < 0.05). Data are presented as least squares means ± standard error of the least squares means **63**

Figure 17. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing. [*, #] Different superscripts represent significant differences (P < 0.05). Data are presented as least squares means ± standard error of the least squares means ... **68**

LIST OF TABLES. **Page**

Table 1. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing **65**
Table 2. Number of *Campylobacter*-positive samples and species recovered from different sample types and dynamic aspects **66**

LIST OF ABBREVIATIONS.

a_w	Water activity
BOE	Boletín Oficial del Estado (Official Spanish State Gazette).
ºC	Degrees Celsius
CDC	Centre for Disease Control and Prevention
CFU	Colony-forming units
CI	Confidence interval
CO_2	Carbon Dioxide
d	Days
DNA	Deoxyribonucleic acid
€	Euro
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
***fla*A**	Flagellin gene A PCR/restriction fragment length polymorphism
FBP	Sodium pyruvate
g	Grams
g	Gravitational acceleration
GBS	Guillain-Barré syndrome
h	Hours
HACCP	Hazard Analysis and Critical Control Points
ISO	International Organization for Standardization
LOS	Lipooligosaccharides
LOD	Limit of detection
mCCDA	Modified Cefoperazone Charcoal Deoxycholate agar
min	Minutes
mL	Millilitres
MLST	Multilocus Sequence Typing
mol	Mole
MS	Member States
n	Number of observations
N_2	Nitrogen

NaCl	Sodium chloride
ng	Nanograms
0_2	Oxygen
P	Probability value
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
ppm	Parts Per Million
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
s	Seconds
spp	Species (plural)
USA	United States of America
VBNC	Viable but non-culturable
vol/vol	Volume to volume
w/v	Percent weight/volume
µL	Microlitres
µm	Micrometers

ABSTRACT.

Campylobacter is the most common bacterial cause of human gastrointestinal disease in most developed countries. It is generally accepted that handling or consuming contaminated poultry meat is the commonest source of foodborne *Campylobacter* infections in humans. Contamination and subsequent colonization of broiler flocks at the farm level often lead to transmission of *Campylobacter* along the poultry production chain and contamination of poultry meat at retail. Therefore, reducing the prevalence of *Campylobacter* at the primary production level is expected to result in a low concentration or absence of this pathogen on the final product, and consequently in a reduction of human exposure. Yet *Campylobacter* prevalence in poultry, as well as the contamination level of poultry products, varies greatly between different countries, so there are differences in the intervention strategies that need to be applied. Although poultry are considered the major reservoir for this human pathogen, the ecology of *Campylobacter* in chicken flocks is poorly understood, hampering the design of effective intervention strategies at the pre-harvest stage. Horizontal transmission of *Campylobacter* through different sources has often been identified as the major source of flock colonization, while the vertical transmission from parent flocks and their progeny remains still unclear.

Thus, the objective of the first experiment was to investigate the epidemiology of *Campylobacter* in broiler production system of the Valencia Region, and the possibility of vertical transmission. From January 2012 to August 2013, a longitudinal and vertical study of the whole poultry production cycle was carried out in the Valencia region (eastern Spain). Breeder birds were monitored from the time just before housing the day-old chicks in the houses (rearing), then throughout the laying period (0 to 60 wk), and throughout their progeny (broiler fattening, 1 to 42 d) until slaughter. To that aim, all breeder farms belonging to the two poultry companies that handle the majority of the poultry reared in the Valencian region were investigated. Then, in order to assess the possibility of vertical transmission, samples from 21 broiler flocks corresponding to their progeny were collected and analysed for *Campylobacter* isolation. All samples were analysed according with official method ISO 10272:2006. Results revealed that on breeder farms, *Campylobacter* isolation started from week 16 and reached its peak at week 26, with 57.0% and 93.2% of positive birds, respectively. After this point, the rate of positive birds decreased slightly to 86.0% at 60 wk. However, in broiler production all day-old chicks were found negative for *Campylobacter* spp., and the bacteria was

first isolated at d 14 of age (5.0%), with a significant increase in detection during the fattening period with 62.0% of *Campylobacter* positive animals at the end of the production cycle. Moreover, non-positive sample was determined from environmental sources. These results could be explained because *Campylobacter* may be in a low concentration or in a non-culturable form at the environment, as there were several studies that successfully detected *Campylobacter* DNA, but failed to culture. This form can survive in the environment and infect successive flocks; consequently, further studies are needed to develop more modern, practical, cost-effective and suitable techniques for routine diagnosis.

Assessing the effectiveness of any potential intervention at farm level requires monitoring of the *Campylobacter* status of broiler flocks using appropriately sampling methods. Therefore, the aim of the second experiment was to assess the influence of the sample type across the rearing period for the detection of *Campylobacter* spp. at farm level. During this study, 21 commercial broiler farms were intensively sampled. Each farm was visited and sampled at weekly intervals during the rearing period (day 1, 7, 14, 21, 28, 35 and 42). On the first day of rearing, the status of the house and the day-old flock was evaluated, collecting environmental samples and caecal samples, respectively. During rearing, four different sample types were collected, including faeces with sock swabs (sock swabs), faeces directly from the litter (faeces), cloacal swabs and caecal content. All samples were analysed according to ISO 10272:2006 (Annex E) and also by direct culture. The results of this study showed that *Campylobacter* spp. was detected in all of the sample types on day 14 of rearing. From this point on, the detection increased significantly during rearing, with a maximum detection rate by the end of rearing, regardless of the sample type. All samples that were negative for direct culture were also negative after pre-enrichment. At the end of rearing, the percentage of *Campylobacter* spp. positive samples was 71.4% for caecal samples, 61.9% for cloacal swabs, 45.2% for sock swabs and 69.1% for faecal samples. *C. jejuni* was detected in all the sample types, with positive rates ranging from 67.1% to 76.0% for caecal samples and cloacal content, respectively. Caecal, cloacal swabs and faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the same sensitivity for detection of *Campylobacter* spp. in broiler flocks independently of the day of rearing.

There is not yet an acceptable standard method for the detection and isolation of *Campylobacter* spp. at farm level. For food legislation purposes, the ISO 10272:2006 is the official method for detection and enumeration of *Campylobacter* spp., while the molecular methods are not considered "confirmatory" tests. However, culture-based methods are labour intensive and time consuming, taking four or more days for completion and require robust bacterial growth, which may limit the detection of stressed bacteria that do not grow well but are still infective. The development of molecular methods constitutes an especially important breakthrough in reducing the time required and specific for the identification of *Campylobacter* spp. combined with a lower detection limit. Thus, the third experiment was carried out to investigate the occurrence of *Campylobacter* in day-old chicks using molecular methods to examine vertical transmission in poultry production. A total of 12 broiler flocks were monitored from the time of housing day-old chicks (day 1) and at the end of the rearing period (day 42). Samples were culture according with official method ISO 10272:2006 and analysed using reverse transcription quantitative real-time PCR method. Our results revealed that no evidence of *Campylobacter* was found in the day-old chicks by bacterial culture method. Nevertheless, 4 flocks out of 12 were found to be positive by the molecular method. Real-time PCR identification revealed that *C. coli* was detected in all 4 flocks, while *C. jejuni* was identified in 3 flocks. No presence of *Campylobacter* spp. was observed in the environmental samples. These results reflect the evidence for vertical transmission of *Campylobacter* spp.

While studies do not definitively rule out the detection problems and an accepted standard method will be developed for the detection and isolation of *Campylobacter* spp. at farm level, no standard measure may be successfully implemented in broiler production and therefore, from a public health point of view, strategies to reduce the number of human campylobacteriosis cases will not be efficient.

RESUMEN.

Campylobacter es la principal causa de gastroenteritis humana en la mayoría de los países industrializados, siendo la carne de pollo producto más frecuentemente implicado en la campylobacteriosis humana. El estatus sanitario que presentan los pollos de engorde al final del ciclo productivo esta estrechamente relacionado con la contaminación de las canales durante el faenado en el matadero, y por lo tanto con calidad microbiológica del producto final. Se estima que la reducción de *Campylobacter* a nivel de la producción primaria sería una herramienta esencial para disminuir o eliminar la presencia de este patógeno en la carne de pollo, y por consiguiente reducir el riesgo de exposición en las personas.

La prevalencia de *Campylobacter* en las aves de corral, así como el nivel de contaminación de los productos avícolas, varía mucho entre los diferentes países, por lo que las estrategias de intervención que deben aplicarse no son las mismas en todos los casos. Se han descrito numerosas vías de entrada de *Campylobacter* en los lotes comerciales de pollo de engorde, incluyendo la transmisión a partir del huevo. Sin embargo, la contaminación a partir del ambiente de la explotación es a menudo citada como la única fuente, restándole importancia a la posibilidad de la transmisión vertical. Además, pese a ser una bacteria objeto de estudio de numerosos trabajos de investigación aún hoy en día se desconoce su epidemiología, lo que dificulta el diseño de estrategias de intervención eficaces a nivel de campo. En este contexto, el objetivo del primer experimento fue investigar la dinámica de colonización de *Campylobacter* en el sector avícola de engorde de la Comunidad Valenciana (España), desde los reproductores (recría y puesta), así como a lo largo de su progenie (broilers), para evaluar la importancia de la transmisión vertical. Con esta finalidad durante el periodo comprendido entre enero de 2012 y agosto de 2013 se llevó a cabo un estudio longitudinal y vertical en el sector avícola de engorde de la Comunidad Valenciana de diferentes lotes de aves, desde la entrada de los pollitos y pollitas a día uno de vida en la etapa de recría, siguiéndolos por la etapa de puesta y durante el engorde y por último, durante el procesado de las canales en matadero. Todas las muestras se analizaron según la Norma ISO 10272:2006 (Anexo E) para el aislamiento de *Campylobacter*. Los resultados obtenidos revelan que el inicio de la colonización por *Campylobacter* en los lotes de reproductores no se detectó hasta las 16 semanas de vida (57,0%), con un pico máximo de prevalencia a las 26 semanas (93,2%), coincidiendo con el inicio de la puesta. Tras este pico, la prevalencia disminuyó ligeramente hasta el 86,0% al final del

ciclo productivo (60 semanas). A pesar del elevado porcentaje de aves positivas durante la etapa de puesta, todos los pollitos de un día fueron negativos a *Campylobacter* al inicio del engorde, y la bacteria se detectó por primera vez a los 14 días de edad (5,0%). A partir de ese momento la excreción aumentó significativamente a lo largo de toda la crianza, alcanzándose un máximo de 62% de aves positivas al final del ciclo (42 días). Por otro lado cabe destacar que no se aisló *Campylobacter* a partir de ninguna muestra ambiental. Este resultado podría explicarse por la baja resistencia de *Campylobacter* en el ambiente, así como por la presencia de formas viables no cultivables, que no se detectan mediante los métodos oficiales de cultivo (ISO 10272:2006). Estas formas de la bacteria pueden sobrevivir en el medio ambiente y tienen capacidad infectiva para colonizar lotes sucesivos. Por este motivo resulta imprescindible el desarrollo de nuevos métodos de diagnóstico más sensibles y rápidos, que permitan tener un mayor conocimiento de los factores de riesgo que intervienen en la colonización de los pollos de engorde por *Campylobacter* spp. para poder desarrollar medidas efectivas para su control.

La evaluación de la eficacia de cualquier medida de control microbiológico en las explotaciones avícolas exige la monitorización del estado de *Campylobacter* de los pollos de engorde, utilizando métodos de muestreo apropiados. Por lo tanto, el objetivo del segundo experimento fue valorar la influencia del tipo de muestra en el aislamiento de *Campylobacter* spp. a nivel de campo durante la etapa de engorde. Durante este estudio se muestrearon de manera intensiva 21 granjas de pollo de engorde. Las muestras se tomaron a intervalos semanales durante a lo largo de la crianza (días 1, 7, 14, 21, 28, 35 y 42). Coincidiendo con la llegada de las aves a las explotaciones, se tomaron muestras ambientales de las naves y de los pollitos para determinar su estatus frente a *Campylobacter*. En cada visita se recogieron cuatro tipos de muestras: hisopos cloacales, calzas, heces de la cama y contenido cecal. Todas las muestras se analizaron según la Norma ISO 10272:2006 (Anexo E) y mediante cultivo directo. No se aisló *Campylobacter* en ninguna muestra hasta el día 14 del ciclo. A partir de ese momento se produce un incremento significativo en la excreción de la bacteria, con un pico máximo al final de la etapa de engorde, independientemente del tipo de muestra analizado. Todas las muestras que resultaron negativas por cultivo directo lo fueron también tras el pre-enriquecimiento. Al final del engorde, el porcentaje de muestras positivas a *Campylobacter* spp. fue de 71,4% para las muestras cecales, 61,9% para los hisopos

cloacales, el 45,2% para las calzas y el 69,1% para las muestras fecales. *C. jejuni* se aisló a partir de todos los tipos de muestras, con porcentajes que oscilaron entre el 67,1% y el 76,0%, para contenido cecal e hisopos cloacales, respectivamente. La tasa de detección de *Campylobacter* varió de manera significativa según el tipo de muestra analizada y la sesión de muestreo (7, 14, 21, 28, 35, y 42 días). Sin embargo, la interacción entre el tipo de muestra y el día del muestreo no fue significativa, por lo que se eliminó del análisis. El cultivo directo de las muestras de ciegos, heces, e hisopos cloacales, sin enriquecimiento previo, tuvo la misma sensibilidad de detección para *Campylobacter* spp., independientemente del momento del ciclo productivo. Sin embargo, se detectó una disminución significativa en la tasa de aislamiento de la bacteria a partir de las muestras de calzas, comparándola con la dinámica de detección a partir de los otros tipos de muestras, coincidiendo con el día 28 de crianza.

Hasta la fecha no existe ningún método oficial para la detección y aislamiento de *Campylobacter* a nivel de campo. Actualmente, se utiliza la norma ISO 10272:2006 como método oficial para el aislamiento y recuento de *Campylobacter* en alimentos. Sin embargo, los métodos oficiales se basan en el diagnostico microbiológico de la bacteria, y resultan demasiado lentos y laboriosos para ser utilizados en estudios a gran escala. Otra de las principales limitaciones de estos métodos es que no son capaces de detectar la presencia de formas viables no cultivables o de bacterias dañadas que no crecen correctamente pero que siguen siendo infecciosas. Por este motivo, el desarrollo de técnicas moleculares de detección permitiría un avance especialmente importante respecto a la reducción del tiempo necesario para la identificación de *Campylobacter* spp., además de presentar un límite de detección inferior. En este contexto, el tercer experimento tuvo como objetivo investigar la presencia de *Campylobacter* en los pollitos de un día mediante la utilización de técnicas moleculares, para explorar la posibilidad de la transmisión vertical como fuente de colonización de los lotes de pollos de engorde. Se monitorizaron un total de 12 lotes de pollo de engorde desde la llegada de las aves a las explotaciones (día 1) hasta el final del ciclo productivo (día 42). Todas las muestras se analizaron de forma paralela según el método oficial que marca la Norma ISO 10272:2006 y mediante PCR a tiempo real. Todas las muestras de pollitos de un día analizadas mediante el método oficial resultaron negativas a *Campylobacter*. Sin embargo, 4 de los 12 lotes analizados mediante PCR a tiempo real resultaron positivos a la bacteria. *C. coli* fue aislada en 4 lotes, mientras que *C. jejuni* se identificó

en 3 lotes. Los resultados obtenidos ponen en evidencia el papel de la transmisión vertical como vía de infección de los lotes de pollo de engorde por *Campylobacter*.

En conclusión, es necesario conocer en profundidad la epidemiología de la infección en el pollo de engorde, para de esta manera establecer las medidas de control más apropiadas para su reducción o eliminación. Sin embargo, para lograr este objetivo es imprescindible continuar con la investigación sobre la mejora de los métodos de detección para *Campylobacter* spp., así como el desarrollo de un protocolo armonizado para la detección de esta bacteria en las explotaciones avícolas.

CHAPTER I. LITERATURE REVIEW.

I.1. General aspects of *Campylobacter*.

I.1.1. Historical background of *Campylobacter*.

Non-culturable spiral-shape bacteria were first noted in 1886 by Theodor Escherich, who published series of articles in the Münchener Medizinische Wochenschrift (Escherich *et al*., 1886) in which he described spiral-shape bacteria in the colons of children who had died of what he called "cholera infantum" (Figure 1). However, all attempted cultures on solid medium were unsuccessful. In the following years till the end of the century, a number of mainly german language publications appeared, describing the occurrence of such "spirilla" in cases of "cholera-like" and "dysenteric" disease. These organisms were found mainly in the colon or associated with mucous in diarrheal stool specimens. Growth on solid medium was unsuccessful, although living bacteria could be kept in liquid culture medium for a few days. All the following points suggest that the microorganisms described were probably *Campylobacter* ssp.: typical morphology association with enteritis in neonates, infants and kittens, failure to grow on solid medium despite microscopic detection, and the fact that to date no other bacteria with comparable morphology have been associated with human enteric infections (Kist, 1986).

MÜNCHENER
MEDICINISCHE WOCHENSCHRIFT
(FRÜHER ÄRZTLICHES INTELLIGENZ-BLATT)
ORGAN FÜR AMTLICHE UND PRAKTISCHE ÄRZTE.
Herausgegeben von
Dr. Bollinger. Dr. H. Ranke. Dr. v. Rothmund. Dr. v. Schleiss. Dr. Seitz. Dr. Winckel.

№ 45. 1886. 9. November. — 33. Jahrgang.

Originalien.
Beiträge zur Kenntniss der Darmbacterien.
Von Dr. Th. Escherich.
III. Ueber das Vorkommen von Vibrionen im Darmcanal und den Stuhlgängen der Säuglinge.

Figure 1. Publication and original drawing of the described spiral-shape bacteria in the colons of children who had died of "cholera infantum". Münchener Medizinische Wochenschrift. (Escherich *et al*., 1886).

Unfortunately, these articles, published in german, remained unrecognised for many decades until Kist (Kist, 1985) reported Escherich's findings at the Third International *Campylobacter* Workshop held in Ottawa in 1985. Microaerophilic

"*Vibrio*-like" bacteria were first described by McFadyean and Stockman (1913) in 1913, who reported the association of these organisms with infectious infertility and abortion in cattle and sheep. This association was confirmed some years later when Smith and Taylor (1919) reported similar findings from aborted bovine tissues, and proposed *Vibrio fetus* as the name of the bacteria. In 1931, Jones *et al*. (1931) implicated another group of microaerophilic vibrios as the cause of some dysentery outbreaks in calves and they termed them *Vibrio jejuni*. In 1944, Doyle (1944) attributed swine dysentery to similar organisms they later named *Vibrio coli* (Doyle, 1948). The first association of microaerophilic vibrios with diarrheal disease in humans was described in 1946 by Levy (1946), who reported an outbreak of acute gastro-enteritis in Illinois resulting in hospitalization of 151 individuals with symptoms of vomiting, abdominal cramps, diarrhoea, fever and headache. The outbreak was associated with milk-borne organisms that resembled *Vibrio jejuni*. This incident is believed to be the first reported foodborne outbreak of *Campylobacter* spp. In 1957, King (1957) distinguished two groups of microaerophilic vibrios isolated from human blood cultures. One group corresponded closely to *Vibrio fetus* (presently called *Campylobacter fetus* subsp. *fetus*) but the other group, described as "related vibrios", were characterized by a higher optimal growth temperature. King's reports suggested that these "related vibrios" might be an important zoonotic cause of human enteritis. In 1963, Sebald and Véron (1963) found that these two groups differed from the other *Vibrio* species and proposed the genus *Campylobacter*, meaning "curved rod", in the family of *Spirillaceae.* Ten years later, Véron and Chatelain (1973) further elaborated the taxonomy of the new genus and proposed four *Campylobacter* species including *Campylobacter jejuni* and *Campylobacter coli*. The major breaktrough was achieved in the 1970s when Butzler *et al*. (1973) and Skirrow (1977) introduced more appropriate techniques for isolation of *Campylobacter* spp. From stools and conducted the first surveys in patients with diarrhea. The results of their studies and of numerous epidemiological studies that followed led to the recognition that *Campylobacter* spp. are a common cause of human diarrheal illness in many countries. *Campylobacter* currently belong to the family *Campylobacteraceae*, proposed in 1991, which includes four closely related genera; *Campylobacter, Arcobacter, Dehalospirillum and Sulfurospirillum* (Vandamme *et al*., 1991).

I.1.2. General characteristics.

Members of the genus *Campylobacter* are Gram-negative, and most are oxidase-positive (except for *C. gracilis*) and catalase-positive. Cells are S-shaped or spiral shaped (Ng *et al*. 1985) and 0.2-0.9 µm wide and 0.2-5.0 µm long (Vandamme *et al*., 1991), with single polar flagella at one or both ends, conferring a characteristic corkscrew-like motility, a feature by which their presence among other bacteria can be detected by phase-contrast microscopy (Snelling *et al*., 2005; Vandamme *et al*., 2010).

Campylobacter species have a strict respiratory metabolism and they do not form spores. They neither ferment nor oxidize carbohydrates; instead they obtain energy from amino acids, or tricarboxylic acid cycle intermediates (Vandamme, 2000). *Campylobacter* can metabolize mucin (Stahl *et al*. 2011) and is well adapted to survive in the mucus film of the caecal and cloacal crypts of the intestinal tract (Lee *et al*. 1986). All species are oxidase positive and negative for production of indole and Voges-Proskauer tests. Most species reduce nitrates and do not hydrolyse hippurate (Vandamme and De Ley, 1991).

Campylobacter are microaerophilic bacteria and require a low oxygen tension (3-6% 0_2) for growth (Smibert, 1984), but some strains also grow aerobically or anaerobically (Carlone and Lascelles 1982; Chynoweth *et al*. 1998). In addition, *Campylobacter* spp. are able to grow within a wide range of pH from 4.9 to 9.0, though the optimal bacterial growth is observed at pH 6.5-7.5 (Alter and Scherer 2006). The organism is sensitive to organic acids and particularly to lactic acid (Smulders, 1987). In addition, growth does not occur in environments with water activity (a_w) lower than 0.987 (sensitive to concentrations of sodium chloride (NaCl) greater than 2%w/v), while optimal growth occurs at $a_w = 0.997$ (approximately 0.5% w/v NaCl) (Silva *et al*., 2011).

Temperature has a significant influence on the survival of *Campylobacter* spp. in the environment and in foods. The optimal growth temperature is 42°C, and although they may grow within a wide range of temperatures (32°C to 47°C) (Alter and Scherer 2006), they are very sensitive to high temperature and the bacteria are inactivated relatively easily during the pasteurisation process (Birkhead *et al*. 1988). Although

Campylobacter spp. are unable to grow at temperatures under 30°C and a sudden growth decline near the lower temperature limit is observed, it has been noted that they survive up to 15 times longer at 2°C than at 20°C (Hazeleger *et al*. 1998). At freezing temperatures the ability of *Campylobacter* ssp. to survive decreases rapidly. In pure cultures, *Campylobacter* spp. are normally inactivated by frozen storage at −15°C in as few as 3 days (Stern and Kotula, 1982). As result, freezing appears to be an efficient way to reduce the level of *Campylobacter* in chicken meat (Georgsson *et al*. 2006; Rosenquist *et al*. 2006; Meldrum and Wilson 2007; FSA 2009). However, freezing does not eliminate the pathogen from contaminated foods (Lee *et al*., 1998). *Campylobacter* spp. may still be isolated from frozen poultry (Lee *et al*. 1998; Sandberg *et al*. 2005).

C. jejuni and *C. coli*, together with *C. lari* and *C. upsaliensis*, belong to the so-called thermophilic group of *Campylobacter* spp., which are able to grow at 42ºC (Penner, 1991). Under appropriate atmospheric and nutritional conditions, *C. jejuni* grows at temperatures between 32 and 45ºC, while the optimal growth temperature ranges between 42 and 45ºC (Doyle and Roman, 1981).

Campylobacter appears to be highly sensitive to environmental factors such as drying conditions and osmotic stress. During exposure to unfavourable environmental conditions, such as high oxygen concentration, extreme temperatures, low nutrient availability or low osmolality environments, *Campylobacter* may form coccoid cells, which have been associated with loss of culturability using traditional culture methods (Klančnik *et al*., 2013). Rosenquist *et al*. (2006) related this coccoid form as a non-viable, degenerative form, or a dormant state that is non-culturable with metabolically active, and is recoverable in a suitable animal host (VBNC, Viable But Non-Culturable) (Figure 2). The bacteria can decrease its metabolic activities and undergo morphological transformation from the motile spiral form to a coccoid form. As for other pathogens, it is still not clear whether this coccoid form retains the potential to be revived to a colonisation/infectious form (Ziprin *et al*. 2003; Oliver 2005). However, Cappelier (1997) observed under laboratory conditions, that *Campylobacter* strains, isolated from the soil around the broiler house, may have been transformed into viable but non-cultivable forms and might have become cultivable after passing through the intestinal tract of chickens.

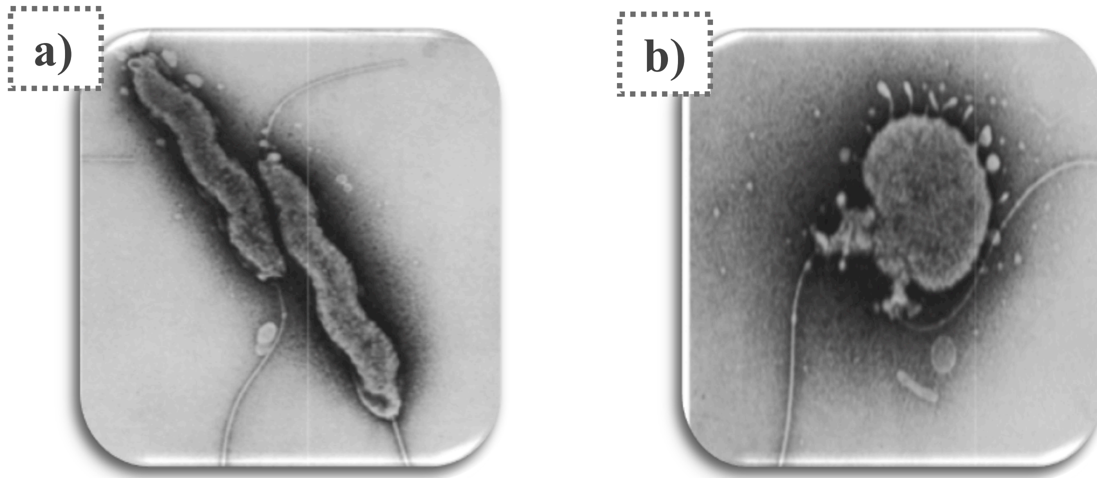

Figure 2. *Campylobacter jejuni* forms*: a)* spiral form. b) coccoid form. (Pead, 1979).

I.1.3. Nomenclature.

The genus *Campylobacter* currently comprises 34 species (http://www.bacterio.net/Campylobacter.html, last accessed 6 September 2016) and 14 subspecies. The species *C. jejuni* comprises two subspecies (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*). Currently, the family *Campylobacteraceae* consists of 3 genera: *Campylobacter*, *Sulfurospirillum*, and *Arcobacter* (Vandamme, 2000; Vandamme *et al*., 2005; Vandamme and De Ley, 1991). Within the genus, three species (*C. jejuni*, *C. coli*, and *C. lari*) are known as thermophilic members of the genus and of clinical significance as they are the dominant 2 causative agents of human campylobacteriosis. *C. jejuni* accounts for the majority of food-borne *Campylobacter* enteritis in humans, followed by *C. coli*, and to a lesser extent, by *C. lari* (Pearson *et al*., 1996; Hazeleger *et al*., 1998).

Within the past 20 years the genus *Campylobacter* has been subjected to a continuos evolution in nomenclature. In 1991, Vandamme *et al*. (1991) proposed a new family, *Campylobacteraceae*, consisting of the genus *Campylobacter*, with eleven species, and the new genus *Arcobacter*. Since *C. jejuni* and to a lesser extent *C. coli* are the most frequently isolated *Campylobacter* species both from patients with enteritis (Endtz, 1991, Skirrow and Blaser, 1992) and from poultry (Jacobs-Reitsma *et al*., 1994) the following paragraphs will focus on these two species.

Although originally placed in the genus *Vibrio*, a new genus name of *Campylobacter* was proposed (Sebald and Véron, 1963) to reflect fundamental differences from the vibrios. It was not until the 1970s before they were isolated successfully from the stools of humans with acute enterocolitis (Butzler *et al*., 1973; Skirrow, 1977). Their presence in the gut had been suspected before this time (Levy, 1946; King, 1957), but the techniques traditionally used in clinical laboratorios were not suitable for the isolation of campylobacters. Although the species names of *C. jejuni* and *C. coli* were derived from an initial association with enteric disease in animals (Jones *et al*., 1931; Doyle, 1948), they are the most important human pathogens in this genus, with the former usually responsible for the majority of enteric *Campylobacter* infections (80-90%).

I.2. *Campylobacter* epidemiology in humans.

I.2.1. Campylobacteriosis in humans

Campylobacter is a zoonotic pathogen and is the main cause of human bacterial gastroenteritis in the world (Scallan *et al*., 2011; EFSA and ECDC, 2015b). In Europe, results from the European Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Foodborne Outbreaks in 2014, revealed that *Campylobacter* has been the most commonly reported gastrointestinal bacterial pathogen in humans in the EU since 2005, with 236,851 reported confirmed cases (Figure 3). The notification rate was 71.0 cases per 100,000 of the population in European countries, with a case-fatality rate of 0.01%. In Spain, the reported incidence was higher than the average in UE accounting for 83.3 cases per 100,000 persons. However, it is well recognised that the actual numbers of human campylobacteriosis cases are underestimated as not all cases are reported in the laboratory due to the self-limiting nature of the disease and that it can be associated with mild symptoms (Allos, 2001; EFSA, 2011; Tam *et al*., 2012). In Europe, the true incidence of human campylobacteriosis is estimated to be approximately nine million cases per year (EFSA, 2011).

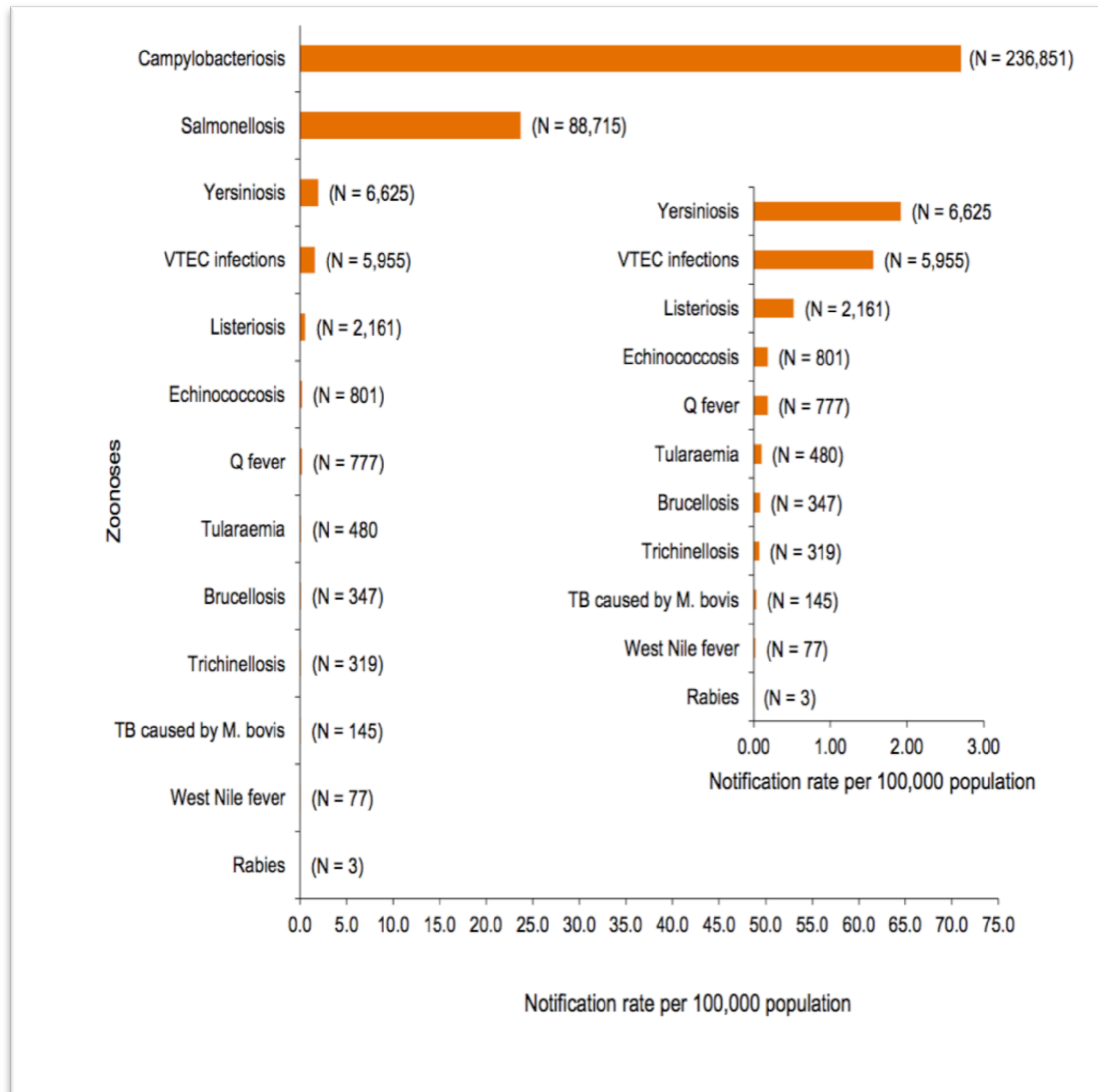

Figure 3. Reported numbers and notification rates of confirmed human zoonoses cases in the EU in 2014. Total number of confirmed cases is indicated in parenthesis at the end of each bar. Exception is made for West Nile fever where the total number of cases was used (EFSA and ECDC, 2015b).

This infection has major economic repercussions on human health care. Indeed there are direct illness costs such as health consultations, laboratory diagnosis, medical treatment or hospitalisation and indirect costs such as loss of work productivity due to sickness, product recalls and legal costs (Roberts *et al*. 2003; Bogaardt *et al*., 2004). In the EU the cost of campylobacteriosis to public health systems is estimated to be about €2.4 billion per year and the disease burden was calculated at 35,000 disability-adjusted life years (DALYs) (EFSA, 2011).

As reported above, the most frequently identified *Camplylobacter* species associated with human disease have been identified as *C. jejuni* and *C. coli* (Nachamkin and Blaser, 2000; Allos, 2001; Friedman *et al*., 2004; Lin, 2009; Hermans *et al*., 2012). In fact, it was observed that *C. jejuni* accounted for 81.8% of human campylobacteriosis cases in the EU in 2014, followed by *C. coli*, *C. lari* and *C. upsaliensis* corresponding to 7.13%, 0.13%, and 0.07% of the isolates respectively. Most *Campylobacter* infections appear to be sporadic rather than outbreak associated, and in majority of cases, the original source of infection cannot be determined (EFSA and ECDC, 2015b).

I.2.2. Human clinical aspects.

In susceptible humans, campylobacteriosis infection is associated with acute enteritis and abdominal pain lasting for up to seven days or longer. The infective dose is generally low, induced by 500-800 bacteria (Conlan *et al*., 2011). The incubation period is two to five days, but estimates have extended up to ten days. The infection results in an acute self-limiting gastrointestinal illness typically resolved in one week, characterised by mild to severe watery/bloody diarrhoea, fever, nausea, malaise and abdominal pain (Blaser, 1997). Common campylobacteriosis symptoms include acute gastroenteritis, cramping abdominal pain, fever, vomiting and headaches (WHO, 2011). Diarrhoea occurs shortly after onset of abdominal pain and varies from mild, non-inflammatory, watery to severe and bloody. The incubation period of *Campylobacter* is 3 days and falls within a range of 18 h to 8 days (Horn and Lake, 2013). Disease outcome is likely to be influenced by both host (age, health status, preexisting immunity), and pathogen specific factors, such as the virulence of the infecting strain (Altekruse et al., 1999).

The disease is self-limited in most cases in adults and non-immune-compromised individuals. However, complications may occur and include bacteraemia, irritable bowel syndrome, and reactive arthritis characterized by conjunctivitis, urethritis and/or arthritis (Havelaar *et al*., 2000; Helms *et al*., 2003; Mangen *et al*., 2005; Gradel *et al*., 2009). Guillain Barré Syndrome (GBS) is the most commonly reported chronic sequelae (Zautner *et al*., 2014). This complication is a demyelinating neuropathy (Rajabally *et al*., 2014) and is characterised by ascending paralysis (Zilbauer *et al*.,

2008). It is estimated that one in 1,000 *Campylobacter* infections leads to GBS, with 2-3% of fatal cases (Allos, 1997). The bacterium surface has lipooligosaccharides (LOS), which are important for GBS development. LOS stimulate peripheral nerve gangliosides to result in the generation of autoreactive antibodies inflammation and tissue damage (Nyati and Nyati, 2013). Miller Fisher syndrome is a non-paralytic variant of GBS and causes inability to move eyes with non-reactive pupils (Mori *et al*., 2012). Reactive arthritis is also associated with *Campylobacter* post-infection with 7 in every 100 cases. Reactive arthritis occurs mainly in joints, particularly knees and ankles (Ajene *et al*., 2013). In limited cases, *C. jejuni* has been associated with intestinal haemorrhaging (Chamovitz *et al*., 1983), toxic megacolon (McKinley *et al*., 1980), haemolytic uraemic syndrome (Shulman and Moel, 1983) and bowel syndrome (Gradel *et al*., 2009).

Human campylobacteriosis may rarely result in long-term disabilities or even death (Helms *et al*., 2003). Some persons are at higher risk of suffering severe symptoms (deriving in hospitalization and/or death) such as immunocompromised individuals, very young and very old persons (Helms *et al*., 2003; Gradel *et al*., 2009). Furthermore, *Campylobacter* strains that are resistant to the most commonly used antibiotics represent a challenge for the treatment of human campylobacteriosis (Moore *et al*., 2006).

I.2.3. Main sources of human campylobacteriosis.

The bacteria are widespread in the environment and have been detected in various animal reservoirs, including poultry, cattle, swine, and dogs (Man, 2011). Therefore, they can be a source for food or water contamination and subsequently a risk factor for human campylobacteriosis. *Campylobacter* may be transmitted from these reservoirs to humans by many different routes. Several risk factors for human campylobacteriosis have been reported in various studies conducted in developed countries, with the most common ones being: consumption and handling of chicken, and in particular undercooked chicken or commercially prepared chicken, unpasteurised milk and dairy products, consumption of untreated water, contact with domestic pets like dogs and cats, contact with farm animals, and travel abroad (Eberhart-Phillips *et al*., 1997; Studahl and Anderson, 2000; Rodrigues *et al*., 2001; Tenkate and Stafford, 2001;

Potter *et al*., 2003; Friedman *et al*., 2004; Schonberg-Norio *et al*., 2004; Stafford *et al*., 2007, Figure 4).

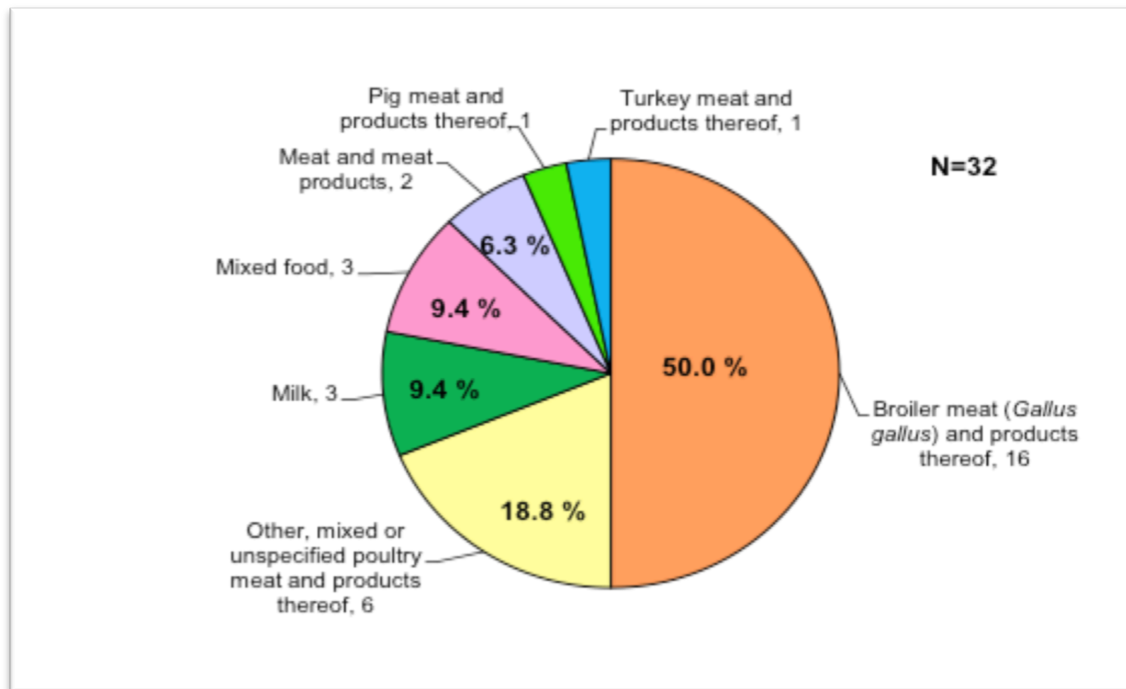

Figure 4. Distribution of food vehicles in strong-evidence outbreaks caused by *Campylobacter* (excluding strong-evidence water-borne outbreaks) in the EU in 2013. Data from 32 outbreaks are included: Austria (5), Belgium (3), Germany (1), Netherlands (1), Spain (6), and United Kingdom (16). Number after the label refers to the number of outbreaks (EFSA and ECDC, 2015a).

Broiler meat is considered to be a major source of human campylobacteriosis, as a result of undercooking and cross-contamination, either directly onto other foods or via the kitchen environment from poultry meat during food preparation (Figure 5) (EFSA, 2010). Data from the EU summary report on zoonoses, zoonotic agents and food-borne outbreaks revealed that half of the *Campylobacter* outbreaks in the EU (16 out of 32) during 2014 were linked to broiler meat (EFSA and ECDC, 2015b). According to EFSA, handling, preparation and consumption of broiler meat is associated with 20% to 30% of human cases, while 50% to 80% could be attributable to the chicken reservoirs as a whole (EFSA, 2010). As it is well established that poultry meat is the most significant source of *Campylobacter* in the food chain (Wilson *et al*. 2008; Mullner *et al*. 2009; Sheppard *et al*. 2009), it can be predicted that a reduction of *Campylobacter* in chickens will reduce the number of cases in the human population. Therefore, implementation of *Campylobacter* control measures at the primary production level would not only reduce the contamination of broiler meat along the food chain, but also

it would lower the human exposure to the bacteria through pathways other than meat consumption (EFSA, 2010). Thus, this fact is expected to have a bigger impact on the reduction of human disease. In addition, safe handling of raw meat, thorough cooking and strict kitchen hygiene should prevent or reduce the risk posed by *Campylobacter*-contaminated broiler meat.

Country	Study period	Source attribution
Canada	2005–2007	Chicken: 64.5% Cattle: 25.8% Water: 8.4% Wild birds: 2.3%
Denmark	2007–2008	Domestic chicken: 54%/38%[a] Imported chicken: 17%/14%[a] Cattle: 17%/16%[a]
The Netherlands	Combined periods 2000–2007 and 2010–2011	Chicken: 68% Cattle: 24% Environment: 6% Sheep + pig: 2%
The Netherlands[b]	2002–2003	Chicken: 66.2% Cattle: 20.7% Environment: 10.1% Sheep: 2.5% Pigs: 0.3%
Scotland	2005–2006	Poultry: 46.3% Ruminant: 31.0% Wild bird: 1.9%
Scotland[c]	2005–2006	Ruminants: 54% Chicken: 40% Pigs: 6%
Switzerland	2002–2012	Chicken: 70.9% Cattle: 19.3% Dogs: 8.6% Pigs: 1.2%

Figure 5. Comprehensive overview of *Campylobacter* source attribution studies published between 2010 and 2015. [a]The first percentage indicates source attribution determined by asymmetric island model. The second percentage indicates source attribution by the *Campylobacter* source attribution model developed by authors. [b]Animal data supplemented with data from UK, Scotland, Switzerland, New Zealand, Curaçao, Finland and USA. [c]Only *C. coli* included (Skarp *et al.*, 2016).

Pigs seem to be a natural reservoir of *Campylobacter* spp. with prevalence between 50% and 100% and excretion levels ranging from 10^2 to 10^7 CFU/g (Munroe *et al.*, 1983; Nielsen *et al.*, 1997; Alter *et al.*, 2005; Boes *et al.*, 2005). In a national baseline survey of cattle and pigs conducted in Great Britain, the carriage rates were 54.6% and 69.3%, respectively (Milnes *et al.*, 2008). Also, recent results on the investigation of *Campylobacter* in pork meat at the slaughterhouse among European countries revealed a mean prevalence of 9.75%, ranging from 5.91 to 50.0% (EFSA and ECDC, 2015b). Despite the medium-high carriage rates, there is little information concerning the contribution of pork meat to *Campylobacter* outbreaks. Several authors found that transmission of *Campylobacter* spp. from pigs appears to be non-evident for

C. jejuni and of very low risk for *C. coli*, where only two out of 4604 incidents of infectious intestinal disease, investigated and reported to the Public Health Laboratory Service in the UK, over an eight year period, were linked to pork meat and one of these was due to cross contamination (Kramer *et al*., 2000). In contrast to the number of foodborne outbreaks attributed to consumption of undercooked poultry contaminated with *Campylobacter*, only 1% of the reported outbreaks were associated with consumption of pork meat in the EU during 2013 (EFSA and ECDC, 2015b).

Cattle are also common carriers of campylobacters (Humphrey and Beckett. 1987; Stanley *et al*., 1998; Inglis *et al*., 2004). Data for *Campylobacter* prevalence in cattle in the European Union range from 0 % to 16.5 % (EFSA and ECDC, 2015b). However, beef is not considered to be an important vehicle of transmission in human infections, because campylobacters are not commonly detected on carcasses or in beef. In surveys of retail beef only 0 to 5% of the samples have tested positive for campylobacters (Stern *et al*., 1985; Ono and Yamamoto, 1999; Whyte *et al*., 2004). Nevertheless, a recent study from the United States found that 5% (12/262) of campylobacteriosis outbreaks from 1997–2008 were due to consumption of contaminated pork, beef or game (Taylor *et al*., 2013). In addition, molecular typing studies of *C. jejuni* isolates from cattle have demonstrated a similarity with human strains (Fitzgerald *et al*., 2001; Schouls *et al*., 2003). Sporadic outbreaks of campylobacteriosis have been linked to contaminated red meat (Itoh *et al*., 1980; Inglis *et al*., 2004).

Raw milk has also been identified as a vehicle of human gastroenteritis caused by *Campylobacter* spp. in several epidemiological studies (Studahl and Anderson, 2000; Michaud *et al*., 2004). Contamination of milk with *Campylobacter* can arise from a number of different sources involving intrinsic contamination from infection in the animal prior to milking, or extrinsic contamination arising from environmental contamination of the milk with faecal material either directly from the animal at the time of milking, or indirectly from the milking equipment, farm environment or at the point of use (Rapp *et al*., 2012; Moatsou and Moschopoulou, 2014). Data from the EU summary report on zoonoses, zoonotic agents and food-borne outbreaks in 2014 revealed that the *Campylobacter* was detected in up to 16.7% of the tested units (single

or batch) of raw cow's milk intended for direct human consumption or manufacture of raw or minimal heat-treated products (EFSA and ECDC, 2015).

Raw-milk outbreaks involving *Campylobacter* have been consistently reported by several authors (Evans *et al*., 1996; Lehner *et al*., 2000; Studahl and Andersson, 2000; Schildt *et al*., 2006; CDC, 2013a; Mungai *et al*., 2015). Recently, the CDC's Emerging Infectious Disease Journal reported an increase of the outbreaks associated with raw milk from 30 (2007-2009) to 51 (2010-2012) (Mungai *et al*., 2015). Also, the European Food Safety Authority's Panel on Biological Hazards reported that 21 of the 27 raw milk-related outbreaks were attributed to *Campylobacter* spp., predominantly *C. jejuni* (EFSA and ECDC, 2015a).

Consumption of untreated water (Schorr *et al*., 1994) has also been considered as a risk factor for campylobacteriosis. In an ecological study in Sweden, positive associations were found between the incidence of *Campylobacter* spp. and the average volume of water consumed per person and similar associations were found with ruminant density. These observations suggest that drinking water and contamination from livestock might also be important factors in explaining at least a proportion of human sporadic campylobacteriosis cases (Nygard *et al*, 2004).

The faeces of livestock, domestic and wild animals, wild birds, poultry and also sewage effluents are usually the sources of *Campylobacter* in water environments (Jones, 2001). *Campylobacter* species have been described as common causative agents in waterborne gastrointestinal illness outbreaks all over the world and most of the illness cases have been associated with *C. jejuni* (Pitkänen *et al*., 2008). Untreated drinking water has been worldwide implicated in several *Campylobacter* outbreaks (Brieseman, 1987; Aho *et al*., 1989; Stehr-Green *et al*., 1991; Duke *et al*., 1996; Furtado *et al*., 1998; Miettinen *et al*., 2001; Jakopanec *et al*., 2008; Karagiannis *et al*., 2010; CDC, 2013b) and has been found as a risk factor in several case-control studies from other countries (Eberhart-Phillips *et al*., 1997; Friedman *et al*., 2004; Domingues *et al*., 2012). Specifically, *Campylobacter* was identified as the major pathogen in outbreaks traced to private water supplies in England and Wales (Said *et al*. 2003), and in Canada *Campylobacter* was the second most common pathogen in 24 waterborne disease outbreaks during 1974–2001 (Schuster *et al*. 2005). Also, in Finland, *Campylobacter*

has been the most common bacterial pathogen identified in waterborne disease outbreaks, being implicated in 11 incidents between 1998 and 2004 (Kuusi *et al*. 2005).

The proportion of *Campylobacter*-positive cats and dogs is generally low, but in two clinical investigations from the Netherlands and Norway 40.4 % and 31.2 %, respectively, of the tested dogs were found to be *Campylobacter*-positive (EFSA and ECDC, 2015a). Species information was reported by Norway, where 101 of the 119 *Campylobacter*-positive dogs were infected with *C. upsaliensis* and the rest of the findings were due to species more commonly causing human disease (*C. jejuni* in 12 dogs and *C. coli* in one dog). *Campylobacter* can be transported directly from animals (i.e. skin contaminated with faeces) to humans (petting the animal and then subsequently using the hands to touch food or mouth directly) (EFSA, 2011).

I.3. *Campylobacter* epidemiology in broiler production.

I.3.1. Avian campylobacteriosis.

Campylobacter can colonize the intestinal mucosa of most a wide range of warm-blooded host species (Newell and Fearnley, 2003). The avian species are the most common hosts for *Campylobacter* spp. probably because of their higher body temperature (Skirrow, 1977). Although all commercial poultry species can carry *Campylobacter* spp., the risk is greater from chicken because of the large quantities consumed (Humphrey *et al*., 2007). In broiler chickens *Campylobacter* is a commensal organism that establishes persistent and benign infections with colonization level up to 10^{10} colony-forming units (CFU) per gram of faeces (Sahin *et al*., 2002; Newell and Fearnley, 2003; Dhillon *et al*., 2006).

Campylobacter can be isolated from most intestinal sites of broiler chickens, but it is mainly found in the caecal and cloacal crypts, where it does not adhere to epithelial cells but is found in the mucous layer (Beery *et al*., 1988; Achen *et al*., 1998). In contrast to infection in humans, the bacteria does not induce any pathology in chickens and inhabits the lower intestine in a commensal relationship (Dhillon *et al*. 2006). Histopathological studies reveal no evidence of necrosis and no significant change in crypt architecture (Berry *et al*., 1988; Dhillon *et al*., 2006; Shaughnessy *et al*., 2011). Moreover, colonization is persistent suggesting that the immune response is ineffective

in the elimination of infection, at least under these circumstances, although older birds, e.g. layers, may have reduced colonization with time (Newell and Fearnley, 2003). In summary, this situation has large benefits for the bacterium and no detrimental effects on the host.

I.3.2. Colonization and immunology against *Campylobacter*.

Ingestion of *Campylobacter* numbers as few as 35 CFU can be sufficient for successful colonization of chicks (Stern *et al*., 1988). After ingestion, the bacterium reaches the cecum and multiplies, resulting in an established colonizing *Campylobacter* population within 24 hours after entrance (Coward *et al*., 2008). *Campylobacter* is not routinely detected in birds younger than 2-3 weeks old (Newell and Fearnley, 2003; van Gerwe *et al*., 2009). This early age-related resistance (commonly called lag phase), extended against different *Campylobacter* species, is not completely understood. *Campylobacter* specific maternal antibodies (MAB) are common in young chickens and could be involved in this protection: the high level of these antibodies observed during the first weeks, falls at 14 days, reaching minimal levels at 3-4 weeks (Sahin *et al*., 2002). Also the stage of intestinal development has been hypothesised to be involved in this age resistance, as avian intestinal niches go through physiological change during the first weeks of life (van Der Wielen *et al*., 2000). Changes in the microbial flora and competitive caecal microflora (Mead, 2002) are also considered in relation to the lag phase, together with management adjustments, such as changes in feed and medication that occur during the rearing period.

Campylobacter colonization of chickens is rapid and widespread, so that once flock colonization is detected, the majority (>95%) of the birds of that flock is colonized within several days (Stern *et al*., 2001), and stay so until slaughter (Coward *et al*., 2008; Stern, 2008). However, it has been observed that, after 8 weeks, colonisation could decrease in terms of number of bacteria and number of birds colonised which is likely to be associated with the development of an adaptive immunity and changes in the intestinal microflora (Achen *et al*. 1998; Sahin *et al*. 2003a; Vandeplas *et al*. 2010).

Although no pathology is associated with chicken colonization, an intestinal immune response to infection has been illustrated with increased cytokine expression

(Borrmann *et al*., 2007; Smith *et al*., 2005, 2008; Larson *et al*., 2008; Li *et al*., 2008) and toll-like receptor (TLR) activation (de Zoete *et al*., 2010). *Campylobacter* is able to stimulate both a systemic and mucosal immune response in chickens, as it has been shown by different studies that reported the induction of immune-associated gene and protein expression after *Campylobacter* colonization of chicken (de Zoete *et al*., 2007). However it is still largely unknown how *Campylobacter* interacts with the chicken immune system to trigger the immune response (Lin *et al*., 2009). Analysis of isolated chicken tissue displayed an increase in cytokine expression (Smith *et al*., 2008) and circulating monocytes/ macrophages (Meade *et al*., 2009), and several different types of chicken cells produce or upregulate cytokines during *in vitro* infection (Smith *et al*., 2005; Larson *et al*., 2008; Li *et al*., 2008). However, *Campylobacter*-specific antibody response is slow and moderate in chicken host because the infection does not cause a strong inflammatory response or tissue damage in intestine (Lin *et al*., 2009). In some studies, *Campylobacter* was also isolated from the bursa of Fabricius, thymus, reproductive tract, spleen, liver and blood in young chickens, suggesting that *Campylobacter* may invade intestinal epithelial cells and become systemic (Cox *et al*., 2005a, 2006, 2009; Knudsen *et al*., 2006; Lamb-Rosteski *et al*., 2008; Van Deun *et al*., 2008; Meade *et al*., 2009; Richardson *et al*., 2011). Recent studies further demonstrated that *C. jejuni* could adhere to and invade chicken intestinal epithelial cells *in vitro* and *in vivo*. However, the *C. jejuni* strains that invaded chicken epithelial cells were not able to proliferate intracellularly, but quickly evaded from the cells (Byrne *et al*., 2007; Van Deun *et al*., 2008). Therefore, Van Deun *et al*. (2008) proposed a novel colonization mechanism of *C. jejuni* by escaping rapid clearance through shortterm epithelial invasion and evasion, combined with fast replication in the mucus.

Other studies have demonstrated that the chick immune system may be inefficiently activated upon *Campylobacter* colonization and expression of several antimicrobial peptide genes may be reduced (Meade *et al*., 2009; Hermans *et al*., 2012). All these observations may indicate that *C. jejuni* is well adapted to the poultry host, and bacteria may be seen as a normal enteric flora by the host. This fact may contribute to the persistent colonization of *Campylobacter* in the avian gut.

I.3.3. *Campylobacter* in primary production.

I.3.3.1. Prevalence in broiler batches.

The prevalence in commercial broiler flocks varies greatly depending on the age of birds (Kazwala *et al*., 1990; Berndtson *et al*., 1996a, 1996b; Evans and Sayers, 2000). *Campylobacter* is rarely detected in broiler chickens less than 2–3 weeks old under commercial production conditions, although newly hatched chickens can be experimentally infected with *C. jejuni* (Shanker *et al*., 1986; Stern *et al*., 1988; Sahin *et al*., 2001). For the majority of commercial flocks, *Campylobacter* infection is usually detected after the third week of age. Once some birds become infected, *C. jejuni* spreads rapidly to most of the birds in the flock, which remain colonized up to slaughter, leading to carcass contamination at the processing plants (Jacobs-Reitsma *et al*., 1995; Berndtson *et al*., 1996a; Gregory *et al*., 1997; Evans and Sayers, 2000; Shreeve *et al*., 2000).

Commercial poultry are the major natural reservoirs of *C. jejuni*, and up to 100% of broilers at slaughter age may harbor the organism (Jacobs-Reitsma *et al*., 1995; Jacobs-Reitsma, 1997). Results from a European Union survey in member states to estimate the prevalence of *Campylobacter* in broiler batches revealed a mean prevalence of 71.2% (95% CI: 68.5; 73.7), with results ranged from a minimum of 2.0% (Estonia) to a maximum of 100.0% (Luxembourg) (Figure 6). The median of Member States prevalence of *Campylobacter*-colonised broiler batches was 57.1% (EFSA, 2010).

In addition, results from the European Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Foodborne Outbreaks in 2014, reported an overall occurrence of *Campylobacter* in fresh broiler meat, sampled at slaughter, processing and retail of 38.4% of the 6,703 tested units (single or batch, aggregated data from all sampling stages) (EFSA and ECDC, 2015b). The proportion of *Campylobacter*-positive samples of broiler meat varied greatly between reporting Member States. *Campylobacter* was detected in 35.5% of single samples at retail; six of eleven MS reporting at retail level found ≥ 50.0% positive samples. At slaughterhouse level, 44.4% of the single samples tested positive for *Campylobacter* (EFSA and ECDC, 2015b).

Shedding of *Campyloacter* by chickens varies by season, being highest in the summer (Jacobs-Reitsma *et al*., 1994; Gregory *et al*., 1997; Evans and Sayers, 2000; Newell and Wagenaar, 2000; Wedderkopp *et al*., 2000, 2001). Even though *C. jejuni* is highly prevalent in broiler chickens, some flocks remain free of *Campylobacter* throughout their lifespan (Berndtson *et al*., 1996b; Wedderkopp *et al*., 2000; Stern *et al*., 2001). *Campylobacter* is also highly prevalent in chickens raised on organic or free-range farms (Rivoal *et al*., 1999; Heuer *et al*., 2001), indicating that different production systems are equally vulnerable to invasion by this organism.

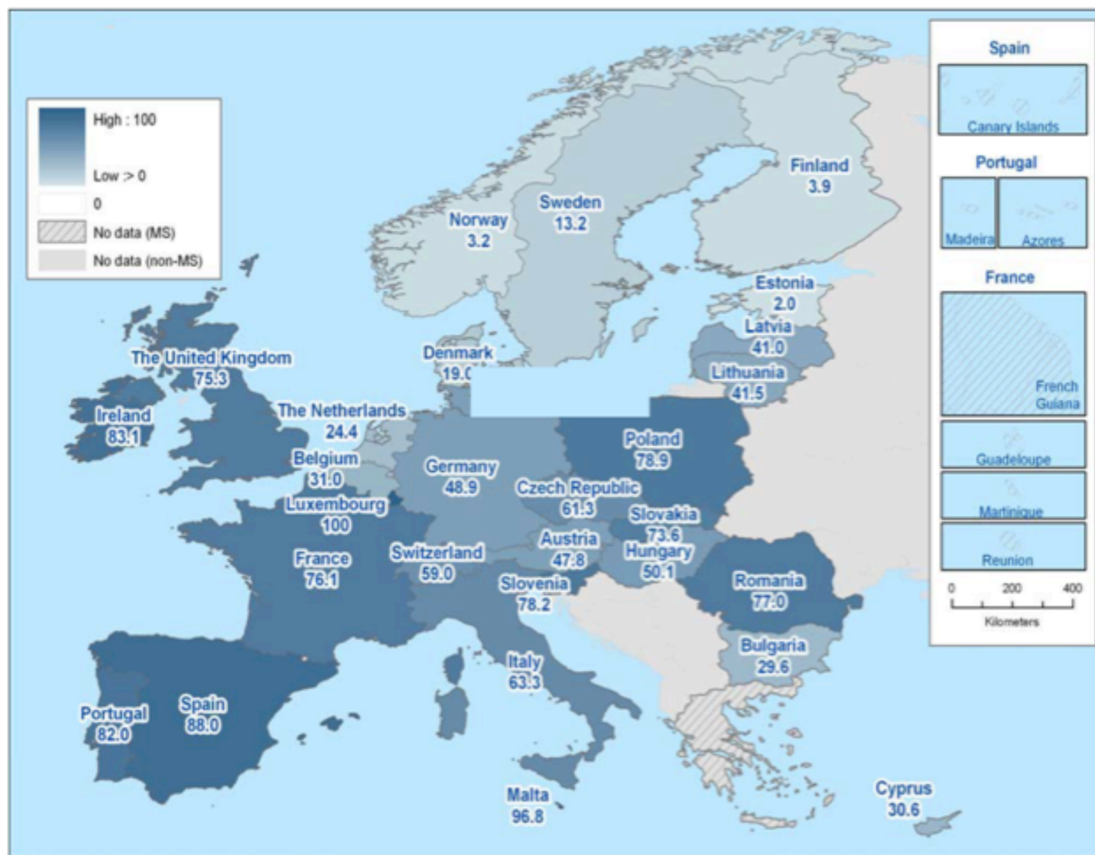

Figure 6. Prevalence (%) of *Campylobacter* spp. colonised broiler batches in the EU, 2008, EFSA (EFSA, 2010).

I.3.3.2. Risk factors and sources of contamination at broiler farms.

The possible sources and transmission routes of *Campylobacter* for poultry flocks have been investigated extensively, but no definitive factor(s) have been identified that explain the occurrence of the organism in commercial poultry flocks (Sahin *et al*., 2002; Cox *et al*., 2012). Circumstantial evidence has been accumulated in

favour of horizontal transmission from the environment as the most probable source of poultry infection by *C. jejuni* (Sahin *et al*., 2002; Newell and Fearnley, 2003; O'Mahony *et al*., 2011). Potential sources include old litter (Thakur *et al*., 2013), untreated drinking water (Pearson *et al*., 1993; Stanley *et al*., 1998; Zimmer *et al*., 2003), other farm animals or domestic pets (van de Giessen *et al*., 1996, 1998; Bouwknegt *et al*. 2004; Lyngstad *et al*., 2008; Zweifel *et al*., 2008; Ellis-Iversen *et al*., 2009), insects (Shane *et al*., 1985; Jacobs-Reitsma, 1997; Hald *et al*., 2008; Hazeleger *et al*., 2008), rodents (Gregory *et al*., 1997; McDowell *et al*., 2008), equipment and transport vehicles and farm workers (Johnsen *et al*., 2006; Lyngstad *et al*., 2008; Ridley *et al*., 2008).

Campylobacter is very sensitive to oxygen and drying, thus it is generally unable to grow in litter under normal ambient conditions (Kazwala *et al*., 1990). The organism is usually absent in fresh litter or feed samples before broilers are infected (Humphrey *et al*., 1993; Pearson *et al*., 1993; Jacobs-Reitsma *et al*., 1995; Gregory *et al*., 1997; van de Giessen *et al*., 1998; Thakur *et al*., 2013). Used litter may become contaminated by *Campylobacter* and may play a role in maintaining the bacteria in the farm environment (Montrose *et al*., 1985). In European countries, since broiler houses are usually cleaned and disinfected and the litter is changed between consecutive flocks, litter seems an unlikely source of infection in commercial broiler production (Evans, 1992). Also, a nationwide epidemiological study in the USA indicated that there were no marked differences in the prevalence and onset time of *Campylobacter* shedding among flocks on different grow-out farms having different practices of litter use (Stern *et al*., 2001).

Farm animals such as cattle, pigs and other poultry have also been recognised as a potential reservoir of the *Campylobacter* (van de Giessen *et al*., 1996, 1998; Bouwknegt *et al*., 2004; Hald *et al*. 2004; Lynngstad *et al*. 2008; Zweifel *et al*. 2008). Molecular epidemiological investigations on farm livestocks showed that strains colonizing target poultry flocks can sometimes be found in adjacent livestock, including cattle and pigs (Jacobs-Reitsma *et al*. 1995; Johnsen *et al*., 2006; Ridley *et al*., 2011), and this occurrence can be detected prior to poultry flock colonization in longitudinal studies, indicating that the direction of transmission is from the livestock to the broilers. Moreover, models from the Netherlands (Katsma *et al*., 2007) indicate that removal of other livestock from a poultry farm would reduce infection only from 44% to 41%.

However, this seems to be a relatively rare event (Johnsen *et al*., 2006), and the majority of the strains in adjacent livestock are not recovered subsequently from broilers. In addition, domestic animals such as dogs and cats have been found to frequently carry and shed *C. jejuni* and *C. coli*, and their presence on farm facilities has also been recognised as a risk factor of *Campylobacter* infection of the broiler flocks (Ellis-Iversen *et al*. 2011; Torralbo *et al*., 2014).

Insects such as flies, beetles, etc., may act as mechanical vectors for *Campylobacter* transmission from various animals to chickens (Shane *et al*., 1985; Jacobs-Reitsma, 1997; Hald *et al*. 2008; Hazeleger *et al*., 2008). *Campylobacter* transmission between chicken flocks by flies under controlled laboratory conditions was first demonstrated by Shane *et al*. (1985). Thereafter, Hald *et al*. (2004) showed that *C. jejuni* most likely was carried by house flies from livestock (sheep) close to the farm and to the broiler flocks through ventilation inlets. In addition, a recent study successfully proved the effect of hygiene barriers and fly screens as a way to reduce prevalence of *Campylobacter* spp. among flocks of broiler chickens, suggesting that flies may have a linking role in *Campylobacter* epidemiology (Bahrndorff *et al*., 2013). Despite of these findings the contribution of flies as a significant source of *Campylobacter* infection of broiler flocks has been debated. Even though identical serotypes and genotypes of *Campylobacter* have been isolated from insects and broilers within broiler houses, the direction of spread was not determined (Jacobs-Reitsma *et al*., 1995; Berndtson *et al*., 1996a; Stern *et al*., 1997). In fact, the insects in a chicken house were usually not positive for *Campylobacter* until the broilers were determined to be positive, suggesting that insects might not be important as an original source of *Campylobacter* for a broiler house (Berndtson *et al*., 1996a; Nesbit *et al*., 2001). Also, some studies have shown the role of darkling beetles (*Alphitobius diaperinus*) and their larvae as potential vectors for the transfer of *C. jejuni* between successive rearing cycles (Refrégier-Petton *et al*., 2001; Hazeleger *et al*., 2008).

The evidence for rodents as infection sources for *Campylobacter* is also circumstantial (Newell *et al*., 2011). The presence of rodents on farms can have a strong association with flock positivity (Gregory *et al*., 1997; McDowell *et al*., 2008), and the efficacy of vermin control is a risk factor (Arsenault *et al*., 2007; Huneau-Salaun *et al*., 2007). Although rodents are detected within the poultry houses of some modern farms

(Evans and Sayers, 2000), the importance of this risk may be low, as *Campylobacter* carriage is detected infrequently in captured rodents (Jones *et al*., 1991).

Transmission of *Campylobacter* into a poultry house via a farm worker has been considered as one potential risk (Lyngstad *et al*. 2008, Johnsen *et al*., 2006, Ridley *et al*., 2008). Johnsen *et al*. (2006) demonstrated that transport personnel delivering day-old chicks passing through the hygiene barrier increased the risk of *Campylobacter* colonization. Human traffic is a very important vehicle for *Campylobacter* entering the poultry house from the external environment (Kapperud *et al*., 1993; Berndtson *et al*., 1996b; Evans and Sayers, 2000; Hald *et al*., 2000; Cardinale *et al*., 2004; Hofshagen and Kruse, 2005). Also, the importance of proper hygiene practices and strict hygiene barriers by farm workers has been established in many studies (Evans and Sayers 2000, Hansson *et al*., 2010). Farm staff handling of other neighboring livestock, especially poultry, increases the risk of *Campylobacter*-positive flocks, and both the number of staff members looking after the house and the number of visits they undertake per day are directly related to that risk (Refrégier-Petton *et al*., 2001; Huneau-Salaun *et al*., 2007). *Campylobacter* has been isolated from the clothes, hands, and boots of farm staff, managers, catchers, and lorry drivers (Herman, 2003; Ramabu *et al*. 2004), and molecular epidemiology provides evidence that these strains are often subsequently associated with flock colonization (Herman, 2003; Johnsen *et al*., 2006; Ridley *et al*., 2008:2011), suggesting that people entering the poultry house can track in campylobacters from the external environment.

However, farm workers may not be the only human tracking campylobacters into a poultry house. It is widely stated that thinning or partial depopulation is a significant risk factor for flock positivity, and this was confirmed by the recent European baseline survey of *Campylobacter* in poultry flocks (EFSA, 2010). Many catching crews are based within poultry company plants and, like maintenance personnel, travel from farm to farm with their own vehicles, equipment, boots, and clothing, frequently without due regard to personal hygiene or biosecurity (Newell *et al*., 2011).

Drinking water source and the method of treatment have been found to be a risk factor for *Campylobacter* colonization in many studies (Pearson *et al*., 1993; Stanley *et*

al., 1998; Zimmer *et al*., 2003). Molecular evidences of the same *C. jejuni* sequence types in water tanks and broiler farms were found by Ogden *et al*. (2007) by using multi-locus sequence typing (MLST), showing the possibility of water as a source of infection in broilers. According to those findings, Pérez-Boto *et al*. (2010) found the same *C. coli* strains in groundwater used as drinking water and chickens, and therefore concluded that drinking water was one of the sources of *C. coli* on chicken farms. However, it remains controversial if the presence of this organism in water systems is a sign of recent fecal contamination of livestock or wild birds, rather than an original source of infection (Kazwala *et al*., 1990; Berndtson *et al*., 1996b; Jacobs-Reitsma *et al*., 1995; Jacobs-Reitsma, 1997; van de Giessen *et al*., 1998). It has been found that drinking water in poultry farms usually becomes positive after chickens are colonized, questioning the role of this possible source in transmitting *Campylobacter* on poultry farms (van de Giessen *et al*., 1998, Zimmer *et al*., 2003). Therefore it is likely that contaminated water serves as a passive carrier of *Campylobacter* rather than a niche for the bacteria to grow (Sahin *et al*., 2002). Water treatments such as disinfectants might have a protective role in spreading *Campylobacter* within a flock rather than introduction into the flock (Ellis-Iversen *et al*. 2009).

Another possible path for introduction of *Campylobacter* into chicken flocks is vertical transmission from the hen through the egg to the chick. However, there has for quite some time been considerable controversy regarding the ability of *Campylobacter* to pass from one generation of poultry to the next through fertile eggs. Some studies have pointed out the possibility of vertical transmission of campylobacters to flocks via contaminated eggs. Cox *et al*. (2002b) successfully isolated the bacterium from 10% of a total of 275 semen samples collected from commercial broiler breeder roosters, with *Campylobacter* levels as high as 1,000 cells per mL. Additionally, these organisms have been recovered from various segments of the reproductive tract of breeder hens, including the oviducts (Camarda *et al*., 2000; Buhr *et al*., 2002; Hiett *et al*., 2002a; Cox *et al*., 2005b), which suggest vertical transmission as a source of *Campylobacter* infection of the flocks.

Another scenario in which the fertile egg can serve as a vehicle for passing *Campylobacter* from one generation of broilers to the next is that faeces can easily contaminate the shell surface of a fertile egg, because the egg and faeces both pass

through the cloaca (Doyle, 1984; Shane *et al*., 1986; Allen and Griffiths, 2001). *Campylobacter* can survive in the moist eggshell membranes and then, as the chick pips and emerges from the egg, it may ingest the organism entrapped in the shell membranes and become colonized, subsequently spreading this contamination to flock mates through ingestion of caecal droppings during brooding (Cox *et al*., 2012).

Molecular evidences of the occurrence of vertical transmission have also been found in different studies. First, Cox *et al*. (2002a) found identical ribotypes and *flaA* short-variable-region alleles in a commercial broiler breeder flock and its progeny broiler flock, which strongly suggest the occurrence of vertical transmission. In addition, other authors have also found amplifiable *Campylobacter* DNA in samples from hatchery fluff, intestinal tracts of developing embryos, and newly hatched chicks, which support the molecular evidence that *Campylobacter* can be present in chicks before they were delivered to the farm (Doyle, 1984; Chuma *et al*., 1994:1997b; Cox *et al*., 2002a; Hiett *et al*., 2002b; Idris *et al*., 2006).

Taking a contrary position, other authors concluded that even though the potential for vertical transmission from breeder hens to broilers exists, in practice this is thought to be at best a rare occurrence with no significant risk to commercial flocks (Shanker *et al*., 1986; van de Giessen *et al*., 1992; Chuma *et al*., 1997b; Petersen *et al*., 2001; Newell and Fearnley 2003; Callicott *et al*. 2006). In addition, some studies have consistently described the occurrence of a lag phase in the detection of *C. jejuni* colonization in chickens, which suggests that vertical transmission of this organism is uncommon (Newell and Fearnley, 2003; Calicott *et al*., 2006). However, it is possible that small numbers of organisms may be present in the hatching chick, but the growth of these organisms is constrained by environmental factors such as maternal antibodies (Sahin *et al*., 2003a). This argument is also supported by the fact that several laboratories have failed to detect *Campylobacter* in day-of-hatch chicks using routine culture methodologies (Pearson *et al*., 1996; Petersen *et al*., 2001; Stern *et al*., 2001; Herman *et al*., 2003). As it has been suggested by Agunos *et al*. (2014), there may exist an inability to culture *Campylobacter* from birds less than 2 weeks old, which presents a major barrier when researching *Campylobacter* in broilers. There is no currently available a cultural procedure that recovers and isolates *Campylobacter* from a variety of biological and food specimens on a regular basis. Most of the cultural methods were

developed for the recovery of the bacterium from fecal samples in which there are large populations of *Campylobacter*. However, these methods might be inadequate for detecting small numbers, sublethally injured or stressed cells, or viable non-culturable cells of *Campylobacter* in foods or biological samples (Cox *et al*., 2001). Because of the inability to fully validate this phenomenon culturally, there is still a strong bias against the concept that fertile eggs can be a source of introduction of *Campylobacter* into breeder and broiler flocks. As a result of this bias, many scientists have overlooked the fact that egg passage can involve much more than vertical, transovarian transmission (Cox *et al*. 2012).

I.3.4. Transport and slaughter of broiler flock.

I.3.4.1. Risk factors at transportation and before slaughter.

Broiler are loaded into crates and transported to the slaughterhouse at the age of approximately 6 weeks. During transportation, animals are under high stress conditions, such as crowding, motion, temperature fluctuations, and feed and water deprivation (Mainali *et al.,* 2009). Stress experienced by transportation from farm to processing facility results in disturbance of intestinal functions, reduced resistance of live animals, and increase of the spread of intestinal bacteria (Klančnik *et al*., 2013). As a result of stress, bacterial counts on carcasses have shown 1,000-fold increase during transportation (Altekruse *et al*., 1999).

Aside from the birds themselves, crates that are not properly cleaned could increase contamination level or introduce *Campylobacter* into free flocks (Stern *et al*. 2001; Ridley *et al*., 2011). Transport crates frequently remain microbiologically contaminated, even after cleaning and disinfecting, although they appear visually clean (Slader *et al*., 2002; Berrang *et al*., 2004; Ramabu *et al*., 2004; Allen *et al*., 2008). This was in agreement with the results of three studies that showed that 60% and 71% of the transport crates tested positive for *Campylobacter* after cleaning and disinfecting (Slader *et al*., 2002; Hansson *et al*., 2005, Rasschaert *et al*., 2007). Also, a survey conducted by Auburn University of more than 10,000 varying sizes poultry companies, discovered that 80% of poultry growers do not sanitise crates and only 18.3% sanitise trucks and trailers properly (Fielding, 2012). Remaining organic matter is detected regularly on truck crates after washing, which combats the efficacy of the sanitising

process allowing the persistence of the bacteria (O'Mahony *et al*., 2011). As a result, *Campylobacter*-negative broilers may become externally contaminated, due to transport in these crates (Stern *et al*., 1995; Hiett *et al*., 2002b; Slader *et al*., 2002; Hansson *et al*., 2005; Rasschaert *et al*., 2007). Genotypes isolated from washed crates were also identified on broiler carcasses following transport and slaughter (Hansson *et al*., 2007; Lienau *et al*., 2007). Schroeder *et al.* (2014) reported that the concentration of these pathogens on the surface of birds is related to the levels found on fully processed carcasses. Therefore, bringing down the farm prevalence of these pathogens and stress during transport is an important strategy to reduce the risk of contaminated meat products entering the food chain (McCrea *et al*., 2006; O'Mahony *et al*., 2011).

I.3.4.2. Prevalence on broiler carcasses.

In 2010, the European Food Safety Authorities reported the results of an estimation made in 2008 of prevalence of *Salmonella* and *Campylobacter*-contaminated broiler carcasses for the whole Community and for each EU Member State (EFSA, 2010). The survey was conducted at broiler-batch level in slaughterhouses and focuses on birds entering the food chain. The estimation made of prevalence of *Campylobacter*-contaminated broiler carcasses at the country-specific level took into account the proportion of carcasses contaminated with this pathogen of the total number of carcasses examined, and by also accounting for slaughterhouse clustering. The total number of sampled broiler batches in the EU was 9,324 for *Campylobacter* detection on carcass samples (EFSA, 2010).

Prevalence of *Campylobacter*-contaminated broiler carcasses in the EU is presented in Figure 7; that is, in each Member State and in both the non-EU Member States. *Campylobacter* was detected on broiler carcasses in all the participating states with a mean prevalence of 75.8%. *C. jejuni* was detected on broiler carcasses in all the participating countries, whereas *C. coli* was detected in all countries except Estonia, Finland, Sweden and Norway. *C. jejuni* was the most commonly reported species in 19 participating States, with up to 100% of this species identified among isolates in Estonia, Finland, Sweden and Norway. In contrast, *C. coli* was the most commonly isolated species in broiler batches in seven Member States (Bulgaria, Hungary, Italy, Luxembourg, Malta, Portugal and Spain).

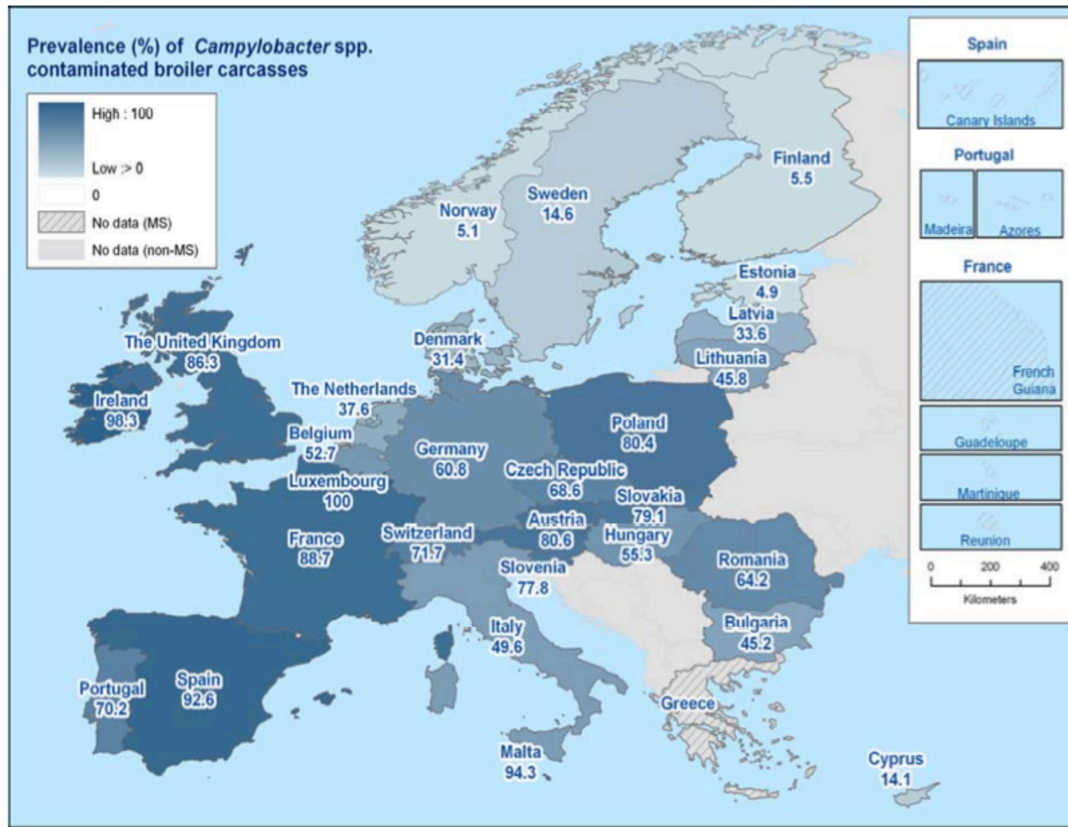

Figure 7. Prevalence of *Campylobacter*-contaminated broiler carcasses in the EU in 2008. (EFSA 2010).

I.3.4.3. Risk factors during slaughter, dressing and processing.

The high numbers of *Campylobacter* in the intestinal tract results in contamination of poultry carcasses during the slaughter process due mainly to spillage of fecal material at defeathering and evisceration, as well as to cross-contamination from the abattoir environment (Berrang *et al*., 2001:2004; Rosenquist *et al*., 2006; Allen *et al*., 2007; Johannessen *et al*., 2007; Elvers *et al*., 2011; Hue *et al*., 2011; Chokboonmongkol *et al*., 2013; Golz *et al*., 2014). However, implementation of Hazard Analysis and Critical Control Points programmes (HACCP) to reduce contamination of carcasses with *Campylobacter* is not still applied in the EU (EFSA and ECDC, 2015b).

Campylobacter is present on carcasses throughout the slaughter process, but levels may lower during scalding, chilling and freezing, and may increase during defeathering and evisceration (Rejab *et al*., 2012; Franz *et al*., 2012; Sasaki *et al*., 2013; Gónzalez-Bodí, 2015). The effect of the scalding process on *Campylobacter* carcass contamination has also been investigated in several studies. However, contradictory

results have been reported. On the one hand, some studies pointed out the scalding process as a potential cross-contamination site for *Campylobacter*. The bacterium has been isolated from the water of scald tanks before the arrival of the first birds, indicating that residual contamination persisted after sanitation (Peyrat *et al*., 2008; Rejab *et al*., 2012; Schroeder *et al*., 2014). Mean concentrations of *C. jejuni* in scald tank water have been shown to be 2.90 CFU/mL (Osiriphun *et al*., 2012). Therefore, it is certainly possible that some *Campylobacter* remained in the tank after cleaning. The survival of the bacterium in this stage has been reported previously (Rahimi *et al*., 2010). It has been suggested that feather follicles in the broiler skin may offer a protection to the bacteria, and the lost of stratum corneum at high scalding temperatures eases the attachment of the bacteria to the broiler skin (Chantarapanont *et al*., 2004). Biofilms of organic material also foster the survival of *Campylobacter* spp. on broiler skin. Furthermore, temperatures in subcutis are often 3-4ºC lower than scalding temperatures (Yang *et al*., 2001). This is in agreement with Ellerbroek *et al*. (2010), who proved an insufficient effect on *Campylobacter* elimination during the scalding process, with an isolation rate of 91.1% after scalding.

However, a reduction of the total number of bacteria on skin carcasses after scalding has been reported (Bily *et al*., 2010; Guerin *et al*., 2010; Lawes *et al*., 2012). Berrang *et al*. (2007) found a mean concentration decrease after chill of 0.43 log CFU/mL, and Guerin *et al*. (2010) saw a maximum concentration decrease after scalding of 2.9 CFU/mL and after chilling of 1.7 CFU/carcass.

The defeathering stage has also been considered to be a major site of *Campylobacter* contamination (Hue *et al*., 2011; Goddard *et al*., 2014; Duffy *et al*., 2014). During defeathering, there is a escape of fecal material through the cloaca by the action of the picker fingers pressing on the abdomen, that can lead to high broiler carcass and slaughter equipment contamination (Duffy *et al*., 2014). Furthermore, finger surfaces become rough with increasing use, favouring the colonisation of bacteria in crevices on the surface of rubber fingers and multiplying overnight if not properly disinfected. Then during the next defeathering, bacteria are transferred from rubber fingers to carcasses, and this implies cross-contamination between different flocks (Ellerbroek *et al*., 2010).

There is currently no agreement on the trend in *Campylobacter* counts after evisceration. Some authors found that the evisceration process might considerably increase cross-contamination of *Campylobacter*, especially since the rupture of viscera and the release of intestinal contents occur (Elvers *et al*., 2011; Figueroa *et al*., 2009; Ivanova *et al*., 2014; Seliwiorstow *et al*., 2015). Rosenquist *et al*. (2006) observed an increase in *Campylobacter* counts after evisceration. However, other authors reported a decrease (Allen *et al*., 2007; Hinton *et al*., 2004; Klein *et al*., 2007; Reich *et al*., 2008) or no difference in *Campylobacter* counts after evisceration (Berrang and Dickens, 2000).

Processing facilities can use two different methods to chill carcasses to reduce carcass temperature: immersion chilling or air chilling (Berrang *et al*., 2008). Immersion-chilled carcasses were found to have significantly lower *Campylobacter* numbers per milliliter than air chilled carcasses (Berrang *et al*. 2008). Some immersion-chill tanks use sanitizers such as chlorine (50 ppm maximum) to reduce other contaminants such as blood and tissue fragments (Guerin *et al*., 2010). It has been demonstrated that the use of chlorine in the chill tank significantly reduced *Campylobacter* numbers, but does not completely eliminate bacteria (Berrang *et al*. 2007). However, *Campylobacter* has been recovered from water chilling tank samples suggesting that the chill tank may represent a major area where cross-contamination can occur (Wempe *et al*. 1983; Karolyi *et al*., 2003; Lindbla *et al*., 2006). Carcasses that entered the chill tank without *Campylobacter* may become contaminated, whereas carcasses that were heavily contaminated with *Campylobacter* may show a reduced concentration of organisms upon exiting the chill tank. Despite the fact that immersion chilling may lead to cross-contamination, it removes *Campylobacter* from the surface of the carcasses, thus reducing the overall persistence (Wempe *et al*., 1983; Northcutt *et al*., 2003; Bashor *et al*., 2004). For air chilling, results have shown either no microbiological reductions (Abu-Ruwaida *et al*., 1994; Fluckey *et al*., 2003), or a slightly lower post-chill *Campylobacter* numbers (Rosenquist *et al*., 2006).

Finally, cross-contamination along the production line is an important risk factor that should be taken into account for this pathogen. Carcasses come into close contact with the surfaces of equipment, which develop residual tissue debris that contain *Campylobacter,* which leads to the contamination of subsequent carcasses (Guerin *et*

al., 2010; Kudirkienė *et al*., 2011). Furthermore, potential cross-contamination between carcasses occurs also when the external surface of birds comes into contact with other carcasses, personnel's hands, and trimming mesh gloves and knives (de Perio *et al*., 2013). Once employees in the slaughter facilities have come into contact with livestock, they become a vehicle to spread both pathogens. Ellerbroek *et al*. (2010) also studied the fact that processing equipment and workers are a source of cross contamination, and reported that staff's hands, slaughtering equipment and transport boxes become contaminated by *Campylobacter*. Many authors have also shown cross-contamination between batches from different flocks and the contamination of non-infected batches from previous slaughtered batches. Gloaguen *et al*. (2010) indicated that *Campylobacter*-positive batches especially contaminate the first carcasses of subsequent negative batches. Excessive use of water during slaughter also produces lots of aerosols and droplets in the hanging, defeathering and evisceration stages, which may also be a potential source of cross-contamination (Peyrat *et al*., 2008).

I.4. Identification and characterization of *Campylobacter*.

I.4.1. Sampling methods.

I.4.1.1. Farm level.

Several sampling methods are in use to detect *Campylobacter* in broiler houses, including cloacal swabs (Hansson *et al*., 2004), faecal samples (Sandberg *et al*., 2006), caecal contents (Allen *et al*., 2007; Rosenquist *et al*., 2009) and boot swabs or the equivalent boot sock model (Bull *et al*., 2006; Ellis-Iversen *et al*., 2011; Ridley *et al*., 2011). However, there is not yet an accepted standard method for the detection and isolation of *Campylobacter* spp. at the farm level (Vidal *et al*., 2013).

First, faecal samples are easier to gather, but it is generally assumed that caecal samples are more appropriate, since *Campylobacter* mainly colonizes the cecum and, consequently, faecal samples often contain a lower number of bacteria per gram than caecal samples (Rudi *et al*., 2004). However, the collection of caecal contents on farm is more difficult and requires culling and post-mortem necropsy of the birds (Vidal *et al*., 2013). Concerning the specificity, both the caecal and fecal culture showed high values, because growth of *Campylobacter* in selective culture media is usually considered as unambiguous demonstration of infection (Woldemariam *et al*., 2008).

Also, cloacal swabs have been used to detect *Campylobacter* in broiler flocks (Hansson *et al*., 2004; Vidal *et al*., 2013). Several authors found that cloacal swabs are a sensitive sampling method for *Campylobacter* detection at farm level (Urdaneta *et al*., 2015).

Boot swabs are a widely used sampling method to test broilers, turkeys and laying hens on floor systems and for the statutory monitoring of chicken breeding flocks for other pathogens such as *Salmonella* (Mueller-Doblies *et al*., 2009). They are a convenient way to collect faecal material from a large number of birds, they can be used easily by farmers and could provide a standardized sampling method. Vidal *et al*. (2013) carried out a study to compare the sensitivity of different sample types on broiler farms, and concluded that boot swabs were the most sensitive sample type. However, they cannot be used to accurately determine the within-flock prevalence, and the amount of material collected could be variable according to the nature of the litter.

I.4.1.2. Slaughterhouse.

Caecal sampling is the standard method for sampling at the abattoir level to assess *Campylobacter* positivity of the flocks (EC, 2007). Samples to be collected shall be intact caeca at the time of evisceration (EC, 2007). Evaluation of carcasses contamination is made using neck skin samples from carcasses collected immediately after chilling, but before further processing such as freezing, cutting or packaging (EC, 2007).

I.4.2. Identification of *Campylobacter.*

I.4.2.1. Bacteriological isolation and identification.

The International Organization for Standardization (ISO) has developed a standardised method for the detection and enumeration of *Campylobacter* (ISO 10272-1:2006, Part A: Detection; and Part B: Colony-count technique) (ISO, 2006). The bacteriological isolation of *Campylobacter* depends on the methods used:

Enrichment.

Prior enrichment is developed which it is presumed to harbour small numbers of *Campylobacter*, or samples that contain a relatively large fraction of injured cells due to processing or unfavourable environmental conditions (Richardson *et al*., 2009). The use of an enrichment broth prior to plating normally provides better recovery when target cells are small in number, injured or stressed (Williams *et al*., 2009). Some of the most frequently used enrichment broth media for *Campylobacter* are Bolton, Preston, Park-Sanders and Exeter. Since *Campylobacter* is sensitive to peroxides, radical scavengers like horse/sheep blood and charcoal are often included in these enrichment broths, as well as growth promoting reagents, like ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP). Enrichment broths operate with various selective systems to reduce the growth of accompanying flora, and combinations of cefoperazone, vancomycin, polymyxin B, amphotericin B, colistin, trimethoprim and rifampicin are used. In order to permit recovery of damaged cells, the incubation temperature may also be gradually increased from 37ºC to the final incubation temperature of 41.5ºC in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), (Potturi-Venkata *et al*., 2007; Altekruse *et al*., 1999; ISO, 2006). Bolton broth is currently recommended for enrichment in ISO 10272-1 standards, and it has also proven to be superior to Preston and Mueller Hinton broth in supporting the growth of a test panel of relevant *Campylobacter* strains for food safety.

Isolation and selection for confirmation.

Following enrichment, or directly from samples with presumably large numbers of *Campylobacter*, samples are spread onto selective agar plates. Once again a large number of solid media exist for *Campylobacter*, and modifications to existing selective agars are also numerous. Some of the commonest ones are: modified charcoal cefoperazone deoxycholate (mCCDA), Skirrow, Karmali, Preston, Abeyta-Hunt-Bark (AHB), Campy-cefex and Butzler. The use of two selective agars with different selective principles in parallel to increase yield is recommended, but currently ISO 10272-1 is the only standard culture method (Potturi-Venkata *et al*., 2007). Solid media

for *Campylobacter* enumeration should always be dried to avoid excessive moisture and to obtain single colonies.

Confirmation of *Campylobacter* presumptive colonies.

It is performed by subculturing five colonies from selective media onto blood agar plates and examining for morphology and motility under a microscope. A number of tests can also be performed in all the cultures in which curved bacilli with a spiralling "corkscrew" motility are found to confirm the identification and to determine species: growth at 25ºC (microaerobic) and at 41.5ºC (aerobic), catalase, oxidase, glucose utilisation, hippurate hydrolysis, latex agglutination, etc.

Following isolation, identification of *Campylobacter* spp. can be based on typical colony and cell morphology and a positive oxidase reaction. Further differentiation into species is base on biochemical tests, growth temperatures and testing for susceptibility to nalidixic acid and cephalotin (Goossens and Butzler, 1991; Vandamme *et al*., 1991). However, the usefulness of some of these tests has been questioned. For example, the distinction between *C. jejuni* and *C. coli* is based on hippurate hydrolysis, but for both species, strains yielding divergent results in this test have been reported (Roop *et al*., 1984; Hebert *et al*., 1984). Further, with the increase of *Campylobacter* spp. strains resistant to nalidixic acid testing for this parameter has become of less value (Jacobs-Reitsma *et al*., 1994).

Phenotyping methods.

Serotyping.

Serotyping has a long-standing history of use in *Campylobacter* typing. Two serotyping systems have been developed and differ depending on whether heat-labile (HL) (Lior *et al*., 1982) or soluble heat-stable (HS) antigens (Penner and Hennessy, 1980; Penner *et al*., 1983) are used. According to Penner and Hennessy (1980), schemes are generally accepted and well evaluated. The major disadvantages of both techniques are the large number of untypeable strains, and time-consuming and technically demanding requirements. The antiserum reagents required for serotyping are not widely

available (Wassenaar and Newell, 2000). Serotyping does not exhibit strong discriminatory power, but can be improved in combination with a DNA-based method (Fussing *et al.,* 2007).

Phage typing.

Given the poor resolving power of serotyping, phage typing has been used as an extension to serotyping to further characterise *C. jejuni* and *C. coli*. There are currently 76 recognised phage types (Hopkins *et al.,* 2004). This method employs a set of virulent phages that may, or may not, have specificity for cell-surface receptors on the bacterial host. If the bacteriophage is able to attach and infect, cell lysis will result, seen as plaque formation on Petri dish cultures (Grajewski *et al*., 1985). Like serotyping, the main limitations of phage typing include the occurrence of non-typeable strains and problems with cross-reactivity. Furthermore, large panels of specialised reagents and a high skill level are required to perform phage typing, which limits the use of this method to reference laboratories (Sails *et al*., 2003). Consequently, phage typing has been largely replaced by more rapid, sensitive and cost-effective genotyping methods.

Hippuricase speciation.

The hippuricase biochemical test has been extensively used to differentiate *C. jejuni* from *C. coli* and *C. lari* (Nicholson and Patton, 1995). The basis of this test relies on the specific ability of *C. jejuni* to hydrolyse hippuric acid using N-benzoylglycine amidohydrolase (hippuricase), an enzyme encoded by the hipO gene (Hani and Chan, 1995). The hippuricase test offers a success rate of approximately 90%. Both the false-negative atypical *C. jejuni* strains that harbour a truncated or poorly expressed hipO gene (Totten *et al*., 1987), and the non-*C. jejuni* false-positives, have been documented (Nicholson and Patton, 1995). As with most phenotypic-based methods, the hippuricase test has been converted into PCR-based speciation methods with higher success rates (Linton *et al.,* 1997; Bae *et al.,* 2005).

Although these culture-based methods are relatively cost effective and require no sophisticated equipment, they have several limitations. Most significant drawbacks include the time required to obtain the final results and the limited response of

Campylobacter to biochemical tests. Moreover, these techniques are labour intensive and have lower sensitivity compared to serological and molecular methods. There is also the possibility of *Campylobacter* cells entering the viable but not culturable (VBNC) state under unfavourable conditions, thus providing false negative results.

I.4.2.2. Molecular techniques.

DNA technology can be applied for both detection and identification of *Campylobacter* species (Linton *et al*., 1997, Vandamme *et al*., 1997, Klena *et al*., 2004, Miller *et al*., 2007). Conventional Polymerase Chain Reaction (PCR) was first developed for *C. jejuni* and *coli* in 1992, and it is able to detect chromosomal gene sequences and cells in small numbers (Moore *et al*., 2005). The PCR has successfully been applied to detect *Campylobacter* spp. from various sources and several primer sets from sequences in the 16S and the 23S rRNA genes, the Fla genes, or other genes that have been used for specific detection of both the genus *Campylobacter* and of *Campylobacter* species (Eyers *et al*. 1993; Linton *et al*. 1996:1997; Rasmussen *et al*. 1996; Gonzalez *et al*. 1997; Hurtado and Owen 1997; Lamoureux *et al*. 1997; Fermer and Engvall 1999; Metherell *et al*. 1999; Vanniasinkam *et al*. 1999).

However, the presence of inhibitory compounds, such as those present in faceal material, may affect the PCR reaction and give false negative results (Wilson, 1997). Furthermore, direct isolation of DNA from faecal material often requires the use of extraction steps with organic solvents or several centrifugation steps, which make handling of many samples cumbersome. Moreover, PCR methods for detection of pathogens in food generally are preceded by enrichment to increase the number of microorganisms and reduce the influence of components in the food matrix (Giesendorf *et al*., 1992).

Real-time PCR (RT-PCR) assays are becoming of increasing importance since they assess the level of contamination with a given pathogen (Lübeck *et al*., 2003). It is based on the principles of conventional PCR but with continuous monitoring of product accumulation (Higuchi *et al*., 1992). This technique yields highly sensitive and specific results while avoiding manipulation of PCR products after amplification, thereby reducing the risk of cross-contamination; it can be used for rapid quantitative screening

of samples (Debretsion *et al*., 2007; Botteldoorn *et al*., 2008; Melero *et al*., 2011). Olsen *et al*. (2009) successfully used RT-PCR to detect *Campylobacter* from airborne samples in a processing facility.

The PCR reaction has been successfully combined with immunoseparation, detecting low numbers of the organism in 6 hours (Docherty *et al*., 1996; Waller and Ogata, 2000). However, some components of both food samples and selective broths can be inhibitory to the PCR reaction. More recently RT-PCR methods have been developed showing the potential of detecting as few as 1 CFU in chicken samples, and in less than 2 hours (Debretsion *et al*., 2007).

Genotyping methods.

Pulsed-field gel electrophoresis (PGFE).

As mentioned earlier, PGFE is generally considered a gold standard. Many epidemiological typing studies have successfully applied this method as a basis for identifying *Campylobacter* strains. It has been used extensively for typing *Campylobacter* in poultry studies (Posch *et al*., 2006; Klein *et al*., 2007; Lienau *et al*., 2007). The disadvantages of PFGE consist in high costs and time requirements; it is also a technically demanding method. The comparison of PFGE profiles from different laboratories and between studies has also proved difficult. Distinct electrophoretic conditions may influence the obtained profiles, different restriction enzymes are used to digest DNA, and some *Campylobacter* isolates cannot be typed by PFGE (Wassenaar and Newell, 2000). The widely used restriction enzyme SmaI generates four to ten fragments. The KpnI digest has more fragments than SmaI and is, thus, more discriminatory. It is often used as a secondary enzyme but, has also been suggested as a primary choice for epidemiological studies (Michaud *et al.,* 2001).

CHAPTER II. STUDY OBJECTIVES.

The **overall objective** of this thesis was to study *Campylobacter* epidemiology in broiler production in the Valencian Region.

The **specific aims** of this thesis were to:

(i) Assess *Campylobacter* spp. isolation from breeders and throughout their progeny (broiler flocks) in the Valencian Region.

(ii) Determine the importance of vertical transmission.

(iii) Assess the influence of the sample type across the rearing period for the detection of *Campylobacter* spp. at farm level.

(iv) Investigate the occurrence of *Campylobacter* in day-old chicks using real-time PCR to examine indications of vertical transmission in poultry production.

CHAPTER III. MATERIAL AND METHODS.

The Ethics and Animal Welfare Committee of the Universidad CEU Cardenal Herrera approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013).

III.1. Experiment 1: *Campylobacter* epidemiology from breeders to their progeny in Eastern Spain.

III.1.1. Study sample.

From January 2012 to August 2013, a longitudinal and vertical study of the whole poultry production cycle was carried out in the Valencia region (eastern Spain). Breeder birds were monitored from the time just before placing the day-old chicks in the houses (rearing), then throughout the laying and fattening period (broiler) until slaughter. Samples from 7 breeder flocks and 21 broiler flocks were analysed for *Campylobacter*. The sample collection scheme is shown in Figure 8.

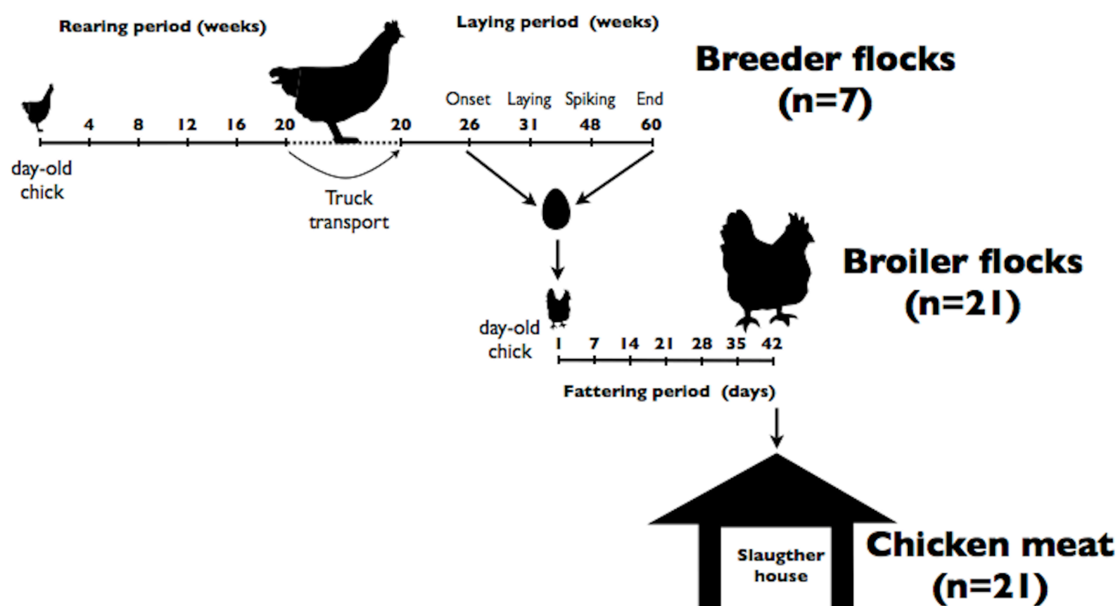

Figure 8. Schematic illustration of the samples collection to determine vertical transmission of *Campylobacter* passage from breeder hens to broiler progeny. For the breeder flocks, during rearing period, samples were collected at 0, 4, 8, 12, 16, and 20 weeks and during laying period samples were collected at 26, 31, 48 and 60 weeks. For the broiler flocks, during the fattening period, samples were collected at 7, 14, 21, 28, 35 and 42 days.

III.1.2. Environmental sample collection.

To assess the *Campylobacter* status of the houses, at the beginning and at the end of the production period (breeder and fattening), environmental samples (water, dust, surfaces, feed, and farming boots) were taken. Each sample was taken using different strategies (Figure 9).

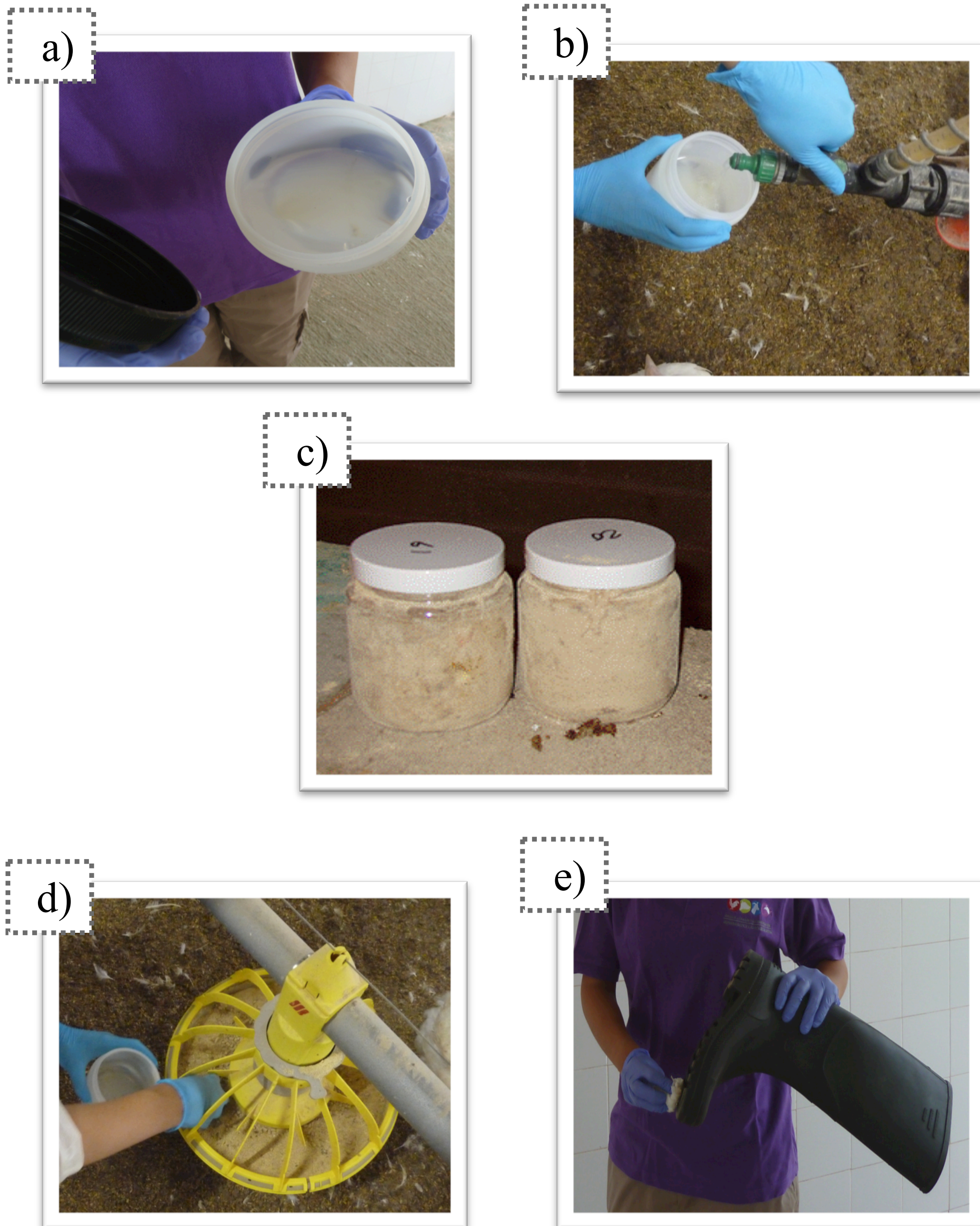

Figure 9. Environmental sample collection. a) Water sample collected from a water tank. b) Water sample collected from final dispenser lines. c) Dust samples from broiler houses. d) Feed sample collected from feeders. e) Farmer boot samples.

First, house surfaces and farmer boot samples were taken with sterile wet gauze pads (AES laboratories, Bruz Cedex, France). Feed samples were collected from the truck and feeders (about 500 g) and water was sampled from the tank and final dispenser lines (500 mL). Then, 100 g of dust (250 mL) were collected from different points of the house. The sample was homogenized in the laboratory and 25 g were analysed.

III.1.3. Sample collection in breeder flocks (parents).

A total of seven breeder flocks were visited and sampled at different times throughout productive life (rearing period: 0 to 20 week; laying period: 20 to 60 week). Each flock was located on one farm. A total of 12 and 25 houses were sampled during the rearing and laying period, respectively. The first visit occurred just before placing the day-old chicks in the houses. To assess *Campylobacter* status of the animals upon the arrival at the farm, 10 birds were randomly selected and euthanized by cervical dislocation. After necropsy, the pair of caeca were removed and placed in an individual sterile jar (Figure 10).

Figure 10. Caeca sample collection.

Caeca samples were pooled into a composite simple for the detection of *Campylobacter*. During rearing period, each flock was sampled collecting 10 cloacal swabs at 4, 8, 12, 16, and 20 week. Cloacal samples were taken individually using

sterile swabs (Cary Blair transport sterile swabs, DELTALAB®) (Figure 11). At the end of the rearing period (20 wk), the animals were transported to the laying farms. To assess the *Campylobacter* status of the slaughter truck, when the truck arrived at the farm, containers and platform were sampled with sterile wet gauze pads with disinfectant neutralizer (AES Laboratories, Bruz Cedex, France). Then, after transport, birds' cloacal samples were also taken. Finally, during the laying period, 10 cloacal samples per flock were also collected at 26 week (onset of laying period), 31 week (peak of lay), 48 week (spiking), and 60 week (end of laying period).

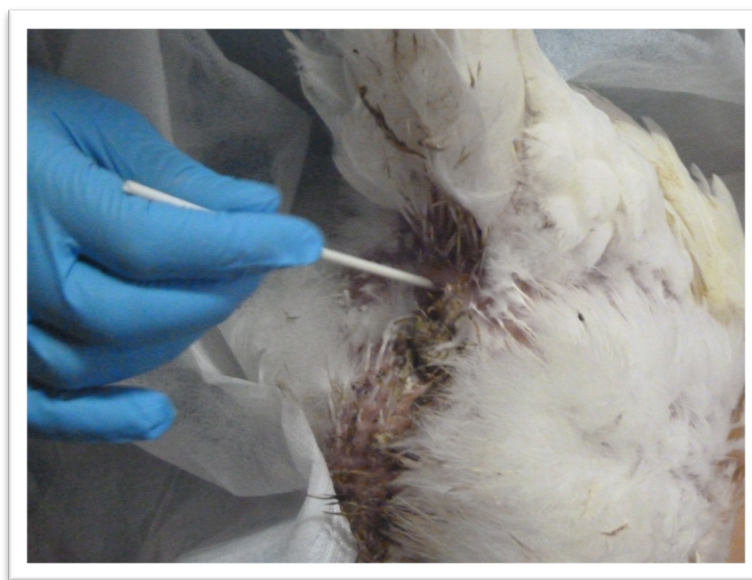

Figure 11. Cloacal swab sample collection (Cary Blair transport sterile swabs, DELTALAB®).

III.1.4. Sample collection in broiler flocks (offspring).

In this study, a total of 21 broiler flocks corresponding to the progeny of the breeder flocks during productive life were evaluated. Each flock was sampled just before placing day-old chicks (d 1), collecting the pair of caeca, as described for the breeder flocks. Then, cloacal samples were collected at weekly intervals during the fattening period (7, 14, 21, 28, 35 and 42 d), when 10 cloacal swabs were collected from each flock, as described previously.

III.1.5. Sample collection at slaughter.

All broiler flocks were monitored at the slaughterhouse. For this purpose, 3 carcasses from each broiler flock were tested. A neck skin sample was collected from each carcass after chilling (Figure 12).

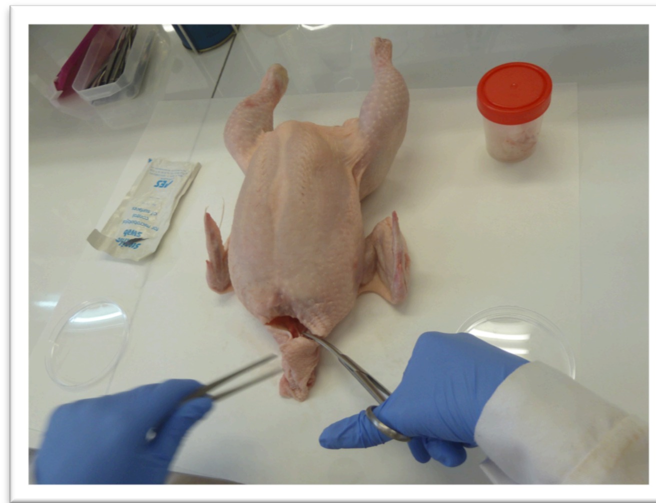

Figure 12. Carcass sampling (neck skin).

III.1.6. Bacteriological analysis.

Campylobacter isolation and speciation

The samples collected were tested by direct culture (Vidal *et al*., 2013) and enrichment culture based on official method ISO 10272:2006 (Annex E) (ISO, 2006; Figure 13), except for cloacal swabs, which were only examined by direct culture. The LOD, for the ISO method in the different kind of samples, is less than 100 CFU/sample, around 50 CFU/sample *C. jejuni* and 65 CFU/simple *C. coli*.

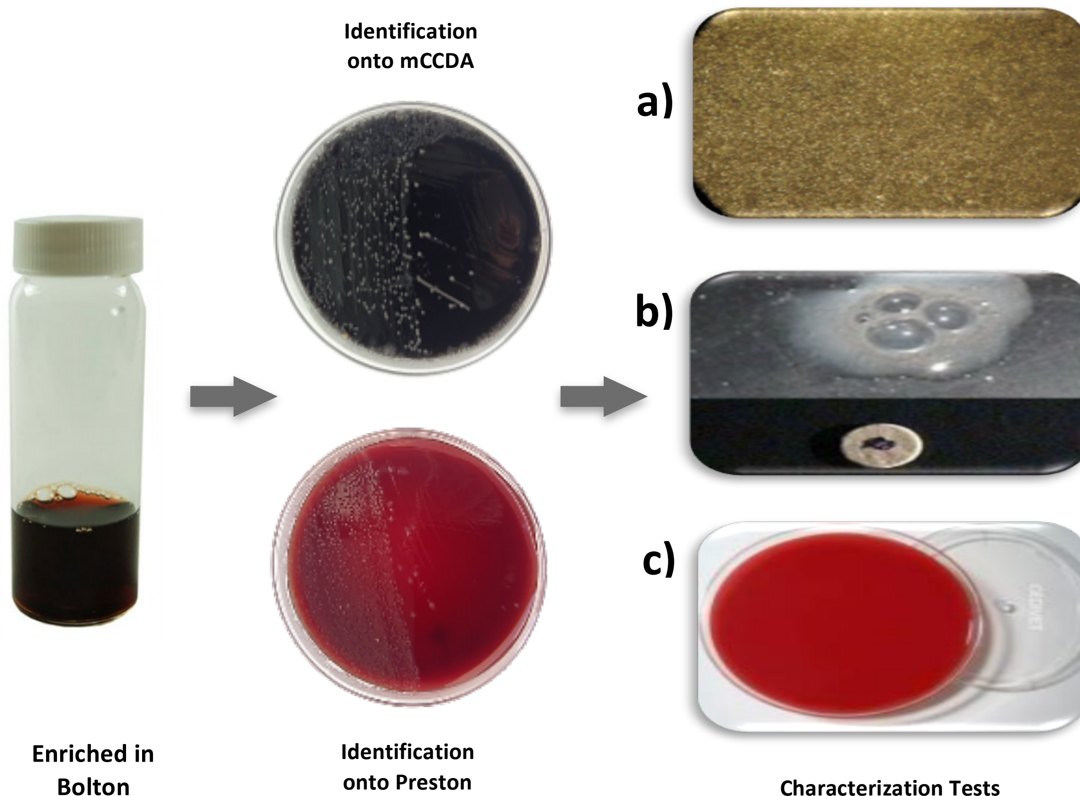

Figure 13. ISO 10272-2:2006 (Annex E) scheme for the detection of *Campylobacter* spp. mCCDA: Modified Charcoal Cefoperazone Deoxycholate Agar. Preston: Preston agar. a) Cellular morphology and motility. b) Oxidase and catalase tests. c) Plating at different temperatures and atmospheres onto Columbia blood agar.

Water samples were processed by mixing 25 mL simple with 225 mL PBS; this was then homogenized by stirring. Feed samples were processed by mixing 25 g sample with 225 mL PBS; this was then and homogenized for 60 s using a filter Stomacher bag (Separator 400; Seward, West Sussex, United Kingdom) and a Stomacher (Stomacher 400; Seward, West Sussex, United Kingdom). Surface and boot samples were processed by mixing each sterile wet gauze pad with 50 mL PBS; this was then homogenized. Sock swabs were mixed with 100 mL PBS; this was then homogenized. The faeces samples were processed mixing 25 g from each jar with 225 mL PBS; this was then homogenized. The caecal samples were processed and cultured as described by Rodgers *et al*. (2010). Briefly, a pooled caecal sample was created by homogenizing 0.02 g caecal content from one cecum from each of the 10 birds collected from the house into 2 mL PBS. From all sample types, 10 µL aliquots of each suspension were plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid, Dardilly, France) and Preston agar (CM0689, Oxoid, Dardilly, France).

Then the samples were incubated at 41.5 ± 1ºC in a microaerobic atmosphere (84% N_2, 10% CO_2, 6% O_2) for 48 h, except for the cloacal swabs, which were directly plated onto mCCDA and Preston agar and incubated as previously described. Moreover, samples were pre-enriched in 1:10 vol/vol Bolton broth (CM0983, Oxoid, Dardilly, France) and then pre-incubated at 37 ± 1ºC for 5 ±1 h. Finally, the pre-enriched broth was incubated at 41.5 ± 1ºC for 43 ± 1 h. Afterward, 100 µL sample was cultured on the 2 selective agar plates (mCCDA and Preston agar) and incubated as described above. Plates were examined for grey, flat, irregular, and spreading colonies typical of *Campylobacter*. *Campylobacter*-like colonies were purified on blood agar and identified to species level on the basis of standard procedures (ISO, 2006). One putative colony was subcultured from each plate onto sheep blood agar for confirmation as *Campylobacter* spp. *Campylobacter* confirmation was performed by a mobility test using a dark-field microscope, by oxidase and catalase biochemical test and by streaking at different temperatures and atmospheres on Columbia blood agar (AES Laboratories®, Bruz Cedex, France), because *Campylobacter* will fail to grow at 25ºC in micro-aerobic atmosphere (84% N_2, 10% CO_2 and 6% O_2) conditions and at 41.5ºC in aerobic conditions. Finally, characterization of the bacterial species was done by hippurate hydrolysis test.

III.1.7. Statistical analyses.

A generalized linear model with a binomial probability distribution and a logit link function was used to compare the isolation of *Campylobacter* in chickens throughout the productive life in breeders (0, 4, 8, 12, 16, 20, 26, 31, 48, and 60 week of productive life) and throughout the fattening period in broilers (1, 7, 14, 21, 28, 35 and 42 days of rearing period). The farm was incorporated in the model as a repeated measurement factor. For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample were assigned a 1 if *Campylobacter* was isolated from poultry or a 0 if not. A P value <0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, IL, 2002).

III.2. Experiment 2: Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp.

III.2.1. Study sample.

From March to August 2013, 21 commercial broiler farms were intensively sampled. Only one flock was studied on each farm. These farms belong to 2 companies, which handle the majority of the poultry slaughtered in Spain. To participate in the study, farms had to be commercial broiler farms with chickens reared on the floor. All the farm owners were willing to cooperate during the lifespan of the flock.

III.2.2. Sample collection and processing.

Each farm was visited and sampled at different times during the rearing period. The first visit occurred just before placing day-old chicks (d 1) and then each farm was visited at weekly intervals until the slaughter day (d 7, 14, 21, 28, 35 and 42).

Before the arrival of day-old chicks, to assess the status of the house for *Campylobacter* contamination, surface samples, water samples (one from the tank and another from final dispenser lines), feed and farmers' boot samples were taken. House surfaces and farmer boot samples were taken with sterile wet gauze pads with disinfectant neutraliser (AES laboratories, Bruz Cedex, France). Water samples (500 mL) were homogenised at the laboratory and 25 mL was analysed from each source. When the feed arrived at the farm, one sample was collected directly from feeders (500 g). Then, the feed sample was homogenised in the laboratory and 25 g was analysed. When the chickens arrived, 10 chicks per batch were slaughtered and caecal contents removed to assess the *Campylobacter* status of the batch.

During the rearing period (days 7, 14, 21, 28, 35 and 42), four different sample types were collected, including faeces with sock swabs, faeces directly from the litter, cloacal swabs and caecal content (Figure 14). To collect faeces with sock swabs, first,

the floor area of the houses was divided into two equal sectors and one pair of sock swabs was used in each sector for sampling. Samples were taken by walking over the chosen sector and each pair of sock swabs with faecal material fixed was analysed as an individual sample (Vidal *et al*., 2013). Samples of faeces were taken aseptically from the bedding with sterile gloves (two sterile pots with 500 g of faeces, approximately, Sandberg *et al*., 2006). Cloacal samples were taken using sterile swabs from 10 individuals in each house (Cary Blair sterile transport swabs, DELTALAB, Rubí, Spain). Finally, these chickens were slaughtered and each pair of caeca was obtained and placed into an individual sterile plastic pot.

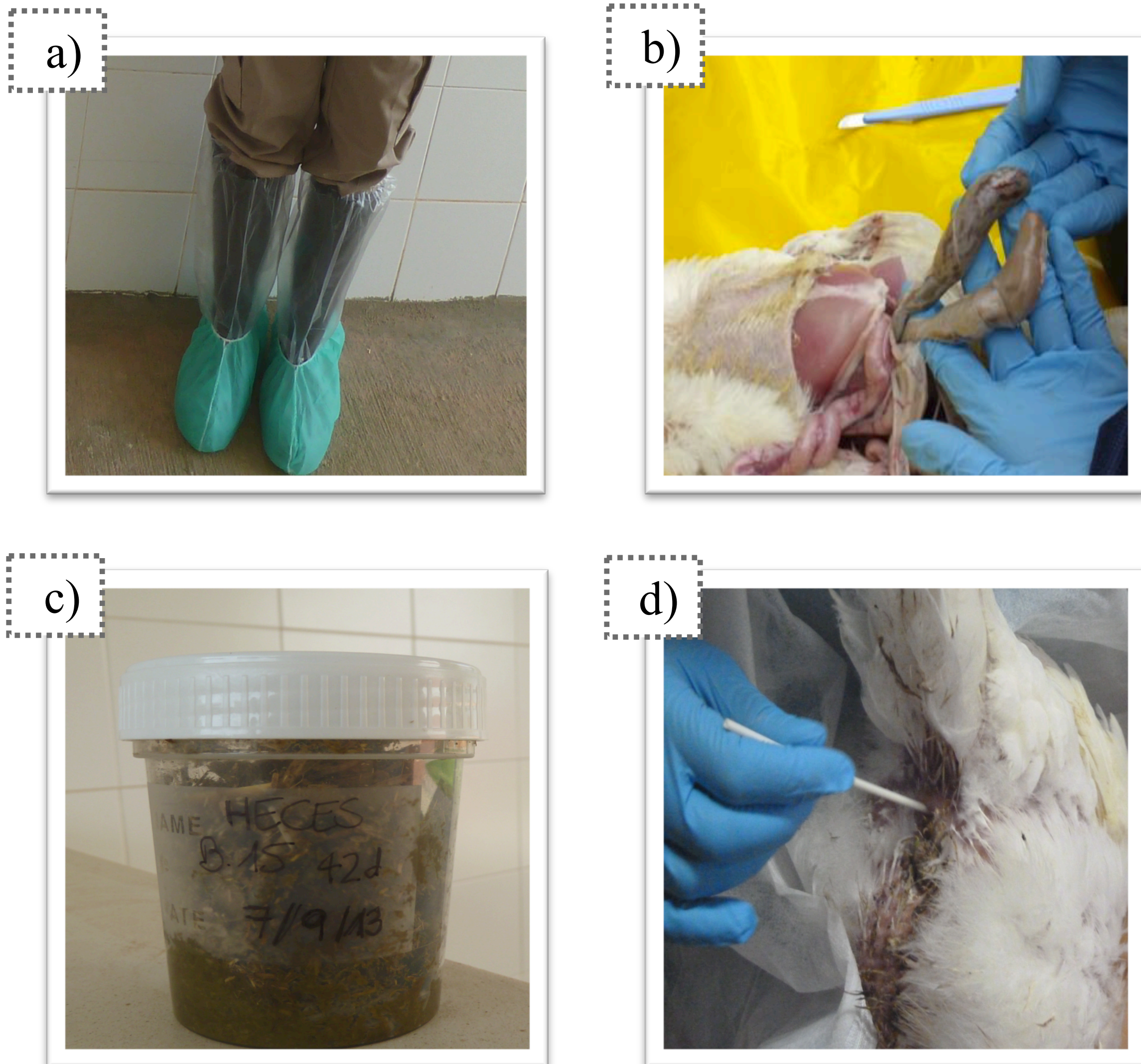

Figure 14. Sampling strategies. a) Boot swabs sample. b) Caecal content sample. c) Faeces sample. d) Cloacal swab sample.

All samples collected, with the exception of caeca, were placed in a pot with semi-solid Cary-Blair transport medium (CM0519; OXOID, Dardilly, France), then refrigerated at 5ºC and analysed within 24h of collection.

III.2.3. Bacteriological analysis.

Campylobacter isolation and speciation.

The samples collected were tested by direct culture (Vidal *et al*., 2013) and enrichment culture based on official method ISO 10272:2006 (Annex E) (ISO, 2006), except for cloacal swabs, which were only examined by direct culture, as described previously. The LOD, for the ISO method in the different kind of samples, is less than 100 CFU/sample, around 50 CFU/sample *C. jejuni* and 65 CFU/simple *C. coli*.

III.2.4. Statistical analyses.

A generalised linear model, which assumed a binomial distribution for *Campylobacter* colonising, was fitted to the data to determine whether there was an association with sample type (sock swabs, faeces, cloacal swabs and caecal content) and dynamic aspects (7, 14, 21, 28, 35 and 42 d of rearing period). For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample was assigned a 1 if it had *Campylobacter* prevalence or a 0 if it had not. A *P value* of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002).

III.3. Experiment 3: Molecular detection of *Campylobacter* spp. in day-old chicks.

III.3.1. Sample collection.

Broiler flocks samples were collected at the beginning and end of the rearing period (days 1 and 42). The first visit occurred just before placing the day-old chicks in the houses and the last just before broilers were transported to the slaughterhouse. Then, ten animals were randomly selected and caecals were obtained and processed according with Vidal *et al*. (2013). A pooled caecal sample was created by homogenizing 0.02 g of caecal content from each of ten individual caeca into 2 mL of PBS, 0.1 mol l^{-1}, pH 7.2. All samples were kept refrigerated during transport to the laboratory. Samples were immediately cultured or frozen with liquid nitrogen and stored at −80 ºC until molecular analysis after reception. Environmental samples were collected from the cleaned and disinfected broiler houses prior to chick placement. Samples from water, dust, surfaces, feed, and farming boots were taken. Each sample was taken using different strategies. First, the boots that farmers use to work during rearing period were tested. Farming boots were swabbed with sterile wet gauze pads with disinfectant neutralizer (AES Laboratories., Bruz Cedex, France). Feed samples were collected from the truck and feeders (about 500 g) and water was sampled from the tank and final dispenser lines (500 mL). Then, dust samples were also collected in different parts (25 to 30 g) of the breeder house and placed into individual sterile plastic pots. Finally, samples of surfaces from each broiler house were collected with sterile wet gauze pads (AES Laboratories., Bruz Cedex, France).

III.3.2. Bacterial culture method.

The samples collected were tested by direct culture (Vidal *et al*., 2013) and enrichment culture based on official method ISO 10272:2006 (Annex E) (ISO, 2006), except for cloacal swabs, which were only examined by direct culture, as described previously.

III.3.3. Detection and quantification of *Campylobacter* spp. by qPCR method.

After thawing the old-day chick caecal samples, 0.1 g of caecal contents was diluted in 1 mL of PBS, mixed vigorously by pipetting and centrifuged 10 min at 10.000 g. The supernatant was removed and the pellet re-suspended with 300 µL Buffer Lysis. Thereafter, the total DNA isolation followed the manufacturer's instructions for Genomic DNA from the tissue Kit (Macherey-Nagel). DNA concentration, quality, and integrity were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The extracted DNA was diluted with nuclease-free water (Ambion, USA) until 100 ng DNA µL−1 and used as a template. The results were expressed as copies per mg of DNA. The primers used to quantify *C. jejuni* (accession number: NC_002163) and *C. coli* (accession number: X88849.1) were developed by Bui *et al*. (2012) and (2011), respectively. Oligonucleotide sequences were: 16S rRNA (forward 5′-GCGTAGGCGGATTATCAAGT-3′ and Rev 5′-CGGATTTTACCCCTACACCA-3′) for *C. jejuni*, and ceuE (forward 5′ AAATTTCCGCTTTTGGACCT-3′ and Rev 5′-CCTTGTGCGCGTTCTTTATT-3′) *C. coli*. The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the qPCR products. To quantify and detect *Campylobacter* spp., qPCR assays were carried out and expression analyses performed using a model 7500 unit (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas GMBH). The PCR protocol included an initial step of 50ºC for 2 min, followed by 95ºC for 10 min, and 40 cycles of 95ºC for 1 sec, annealing at 56ºC for 20s and extended at 72ºC for 15s. To evaluate assay specificity, the machine performed a melting curve analysis directly following PCR by slowly (0.1 .C/s) increasing the temperature from 68 to 95ºC, with continuous recording of changes in fluorescent emission intensity. The DNA extracts of 10-fold dilutions from 10^8 to 10^1 number of plasmid copies/µl were used for qPCR assays to establish the standard curve and to quantify *Campylobacter* spp. in caecal samples. The total volume for every PCR reaction was 20 µL, performed from diluted (1:10) DNA template (5 µL), forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µL). The samples were run in duplicate PCR reactions, and a standard curve was included in each run. Non-template control (DNA was replaced by water) for each primer pair were run on all plates.

III.3.4. Molecular cloning of *C. jejuni* and *C. coli* PCR products.

PCR amplification was performed in an ABI GeneAmpTM system 2700 thermocycler. The reaction mixture of 25 µL contained 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µL of DNA template. The first PCR amplification was run as follows: denaturation at 94 .C for 3 min, followed by 40 cycles at 94 .C for 30 s, 56 .C for 20 s, and 72 .C for 30 s, and finally an extension step at 72 .C for 10 min. The PCR products were visualized in 2% agarose gel stained with ethidium bromide and bands of expected size were purified using a Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent *E. coli* JM109 cells (Promega). Positive colonies were isolated and plasmids extracted by a Qiagen Plasmid Mini Kit (Qiagen). Plasmids with inserts were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Universitat Politécnica de Valencia sequencing service, Valencia, Spain).

CHAPTER IV. RESULTS.

IV.1. Experiment 1: *Campylobacter* epidemiology from breeders to their progeny in Eastern Spain.

IV.1.1. Environmental samples.

A total of 580 environmental samplings were conducted in the breeder (n= 370) and broiler flocks (n= 210). No *Campylobacter* spp. was isolated in any of the environmental samples from the breeder and broiler flocks.

IV.1.2. Breeders (Parents).

During the study period, 1,040 samples out of 1,780 were found positive for *Campylobacter*. *Campylobacter* was not isolated in any of the day-old chicks sampled. The bacterium was first isolated at 16 week of the rearing period (57.0%, Figure 15). The bacteria isolation from individual breeders was homogeneous until week 26 (laying period), with a peak of 93.2% (Figure 15). After week 26, a slight decrease in *Campylobacter* isolation occurred (Figure 15). Moreover, *C. jejuni* was the most commonly identified species (67%, 77.0%, 83.0%, 88.0%, 78.0%, and 86.0%, at 16, 20, 26, 31, 48, and 60 week, respectively). All breeder flocks (n= 7) were negative for *Campylobacter* at the beginning of the rearing period. However, at the end of rearing and the beginning of the laying period, 4 of the 7 breeder flocks studied were found positive for *Campylobacter*. All breeder flocks were positive by the end of the laying period (n= 7). In addition, no differences were found between the individual positive rates before and after transport of the animals from the breeder farms to the breeder laying farms at the end of the rearing period. The percentage of positive animals before loading and after transport was 54.0% and 59.0%, respectively (Figure 15). All samples collected from transport trucks were negative for *Campylobacter* spp.

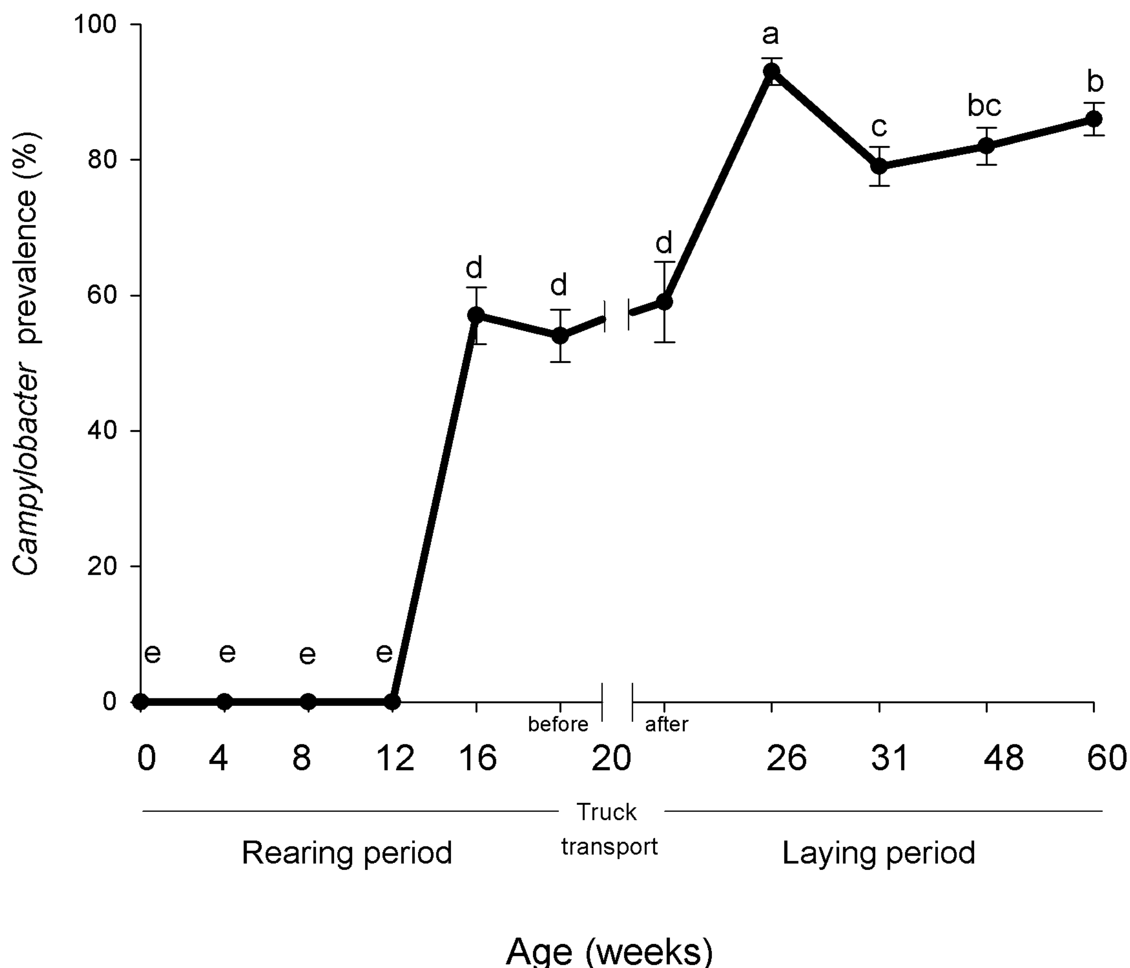

Figure 15. *Campylobacter* isolation during the breeding period (rearing and laying period). Each animal was sampled from 0 to 60 weeks of age. [a,b,c,d,e]Different superscripts represent significant differences ($P < 0.05$). Data are presented as least squares means ± standard error of the least squares means.

IV.1.3. Broiler Flocks (Offspring).

No day-old chick sampled was found positive for *Campylobacter*. During the fattening period, 329 samples out of 1,260 were positive for the bacterium. *Campylobacter* isolation of chickens differed significantly depending on the day of the fattening period (Figure 16). *Campylobacter* was first isolated in chickens at day 14 of age (5.0%, Figure 16) and the isolation increased significantly throughout the fattening period, with the highest rate at the end of fattening (62.0%, Figure 16). *C. jejuni* was the most commonly identified species (100.0%, 87.0%, 90.0%, 75.0% and 67.0%, at 14, 21, 28, 35 and 42 days of rearing period, respectively). All broiler flocks (n=21) were

declared negative for *Campylobacter* at day 0 of rearing. However, at day 42 almost all were positive for the bacteria (n= 20).

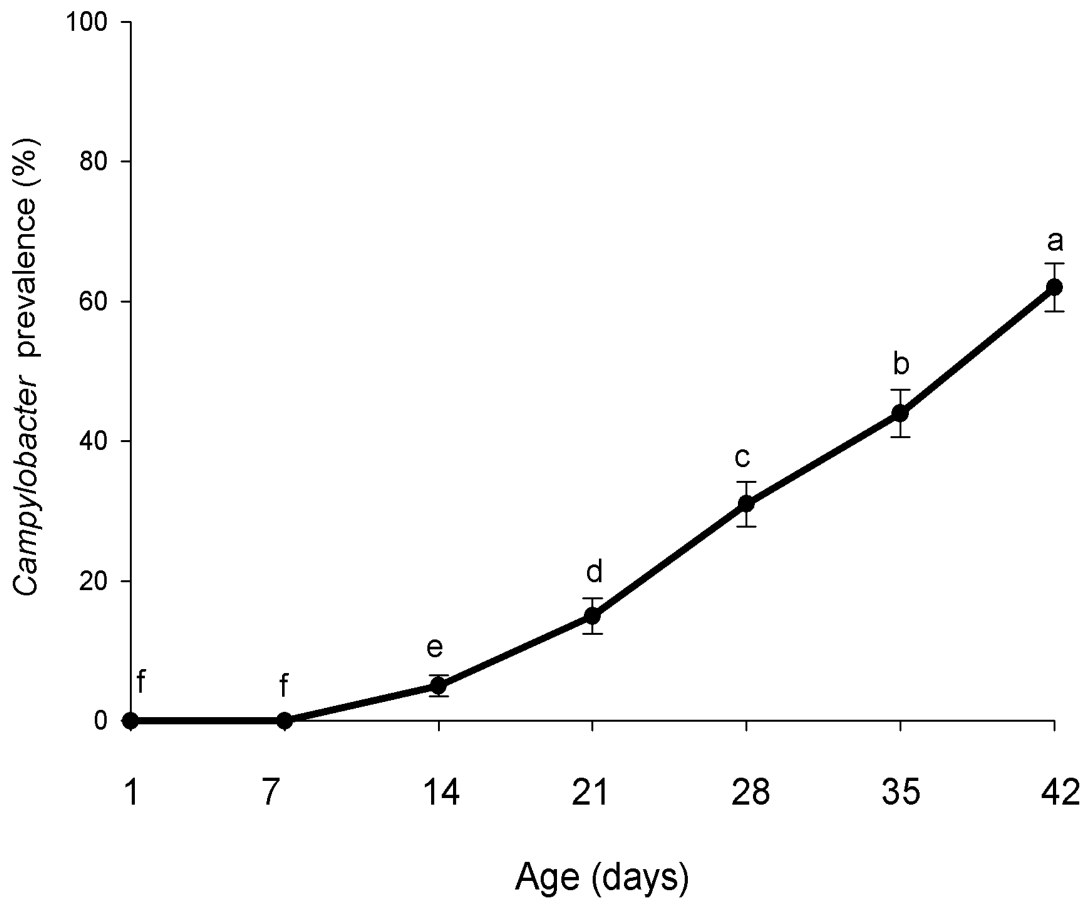

Figure 16. *Campylobacter* isolation during fattening period. Each animal was sampled just before placing day-old chicks (day 1) and at weekly intervals until slaughter day. [a,b,c,d,e,f] Data with uncommon letters are different (P < 0.05). Data are presented as least squares means ± standard error of the least squares means.

IV.2. Experiment 2: Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp.

IV.2.1. Comparisson of sampling types.

On the first day of rearing, none of the day-old chick flocks or environmental, feed and water samples were positive for *Campylobacter*. Therefore, all houses were included in the study. The number of positive flocks by each sample type during rearing is given in Table 1. In total, 20 flocks were positive for *Campylobacter* in at least one of the samples tested. The number of positive flocks detected varied between sample types and the day of rearing (Table 1). The culture of cloacal swabs allowed the detection of all the positive flocks. Caecal and faecal samples allowed the detection of 17 and 16 of the positive flocks, respectively. Nevertheless, sock swab samples failed to detect nine positive flocks. In *Campylobacter* detection, the bacteria were first detected in one of these flocks after 7 days, but *Campylobacter* spp. was detected in all of the sample types on day 14 of rearing. From this moment on, the detection increased significantly during rearing, with a maximum detection rate at the end of rearing, regardless of the sample type.

Table 1. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing.

Sample type/Positive	Total positive		Dynamics of *Campylobacter* spp. during rearing (days)											
			7		14		21		28		35		42	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Flocks	20	95.2	1	4.8	3	14.3	7	33.3	11	52.4	16	76.2	20	95.2
Caecal content	18	85.7	1	5.5	3	16.7	5	27.8	8	44.4	12	66.7	17	94.4
Cloacal swab	20	95.2	0	0.0	2	10.0	5	25.0	10	50.0	16	80.0	20	100.0
Sock swab	14	66.7	1	7.1	1	7.1	5	35.7	3	21.4	7	50.0	11	78.6
Faeces	19	90.5	1	5.3	1	5.3	6	31.6	7	36.8	9	47.4	16	84.2

n: number of positive flocks.

Table 2. Number of *Campylobacter*-positive samples and species recovered from different sample types and dynamic aspects.

Sample type	Total positive		7 d		14 d		21 d		28 d		35 d		42 d	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Caecal content (n=252)														
Total positive [a]	79	31.3	1	2.4	5	11.9	8	19.0	14	33.3	21	50.0	30	71.4
C. jejuni	53	67.1	1	100.0	4	80.0	8	100.0	9	64.3	12	57.1	19	63.3
Other	26	32.9	0	0.0	1	20.0	0	0.0	5	35.7	9	42.9	11	36.7
Cloacal swab (n=1254)														
Total positive [a]	329	26.4	0	0	10	4.8	32	15.2	65	30.9	92	43.8	130	61.9
C. jejuni	250	76.0	0	0	10	100.0	28	87.5	59	90.8	71	77.2	82	63.1
Other	79	24.0	0	0	0	0.0	4	12.5	6	9.2	21	22.8	48	36.9
Sock swab (n=252)														
Total positive [a]	48	19.1	1	2.4	2	4.8	7	16.7	6	14.3	13	30.9	19	45.2
C. jejuni	33	68.7	1	100.0	2	100.0	6	85.7	4	66.7	9	69.2	11	57.8
Other	15	31.2	0	0.0	0	0.0	1	14.3	2	33.3	4	30.8	8	42.2
Faeces (n=244)														
Total positive [a]	68	27.8	1	2.4	1	2.6	8	19.0	12	28.6	17	40.5	29	69.1
C. jejuni	49	72.1	1	100.0	1	100.0	8	100.0	10	83.3	11	64.7	18	62.1
Other	19	27.9	0	0.0	0	0.0	0	0.0	2	16.7	6	35.3	11	37.9
Total positive [a]	526	26.0	3	0.9	18	5.4	55	16.4	97	28.9	143	42.6	210	59.3
C. jejuni	387	73.6	3	100.0	17	94.4	50	90.9	82	84.5	103	72.0	132	62.9
Other	139	26.4	0	0.0	1	5.6	5	9.1	15	15.5	40	28.0	78	37.1

n: number of positive samples.

[a] Sum of positive samples by species. Only one type of species was identify in each sample.

At sample level, the number of positive samples and the species recovered are summarised in Table 2. All samples that were negative for direct culture were also negative after pre-enrichment. At the end of rearing (d 42), the percentage of *Campylobacter* spp. positive samples was 71.4% for caecal samples, 61.9% for cloacal swabs, 45.2% for sock swabs and 69.1% for faecal samples. *C. jejuni* was detected in all the sample types, with positive rates ranging from 67.1% to 76.0% for caecal samples and cloacal content, respectively. *Campylobacter* detection was significantly different between sample types collected and the day of rearing (d 7, 14, 21, 28, 35, and 42). However, the interaction was not significant, so it was removed from the analysis.

As shown in the Figure 17, *Campylobacter* could not be detected in all of the sample types until day 14. The positive results for *Campylobacter* among the analysed samples were similar until day 21, yielding 19.0% for caecal content, 15.2% for cloacal swabs, 16.7% for sock swabs and 19.0% for faeces. Moreover, isolation rates depend significantly on the rearing period time. There was also a significant effect of the sample types on *Campylobacter* isolation. After day 28, a significant decrease of *Campylobacter* isolation on sock swabs was detected (14.3%) compared with the detection in the other sample types (28.6%, 30.9% and 33.3% for caecal, cloacal swabs and faeces, respectively). These results were consistent with those for the rest of the rearing period (Figure 17). *C. jejuni* was the most commonly isolated species (73.6%) found in all sample types. significantly depending on the day of the fattening period (Table 2). *Campylobacter* was first isolated in chickens at d 14 of age (5.0%, Figure 16) and the isolation increased significantly throughout the fattening period, with the highest rate at the end of fattening (62.0%, Figure 17). *C. jejuni* was the most commonly identified species (100.0%, 87.0%, 90.0%, 75.0%, and 67.0%, at 14, 21, 28, 35, and 42 d of rearing period, respectively). All broiler flocks (n= 21) were declared negative for *Campylobacter* at d 0 of rearing. However, at d 42 almost all were positive for the bacteria (n= 20).

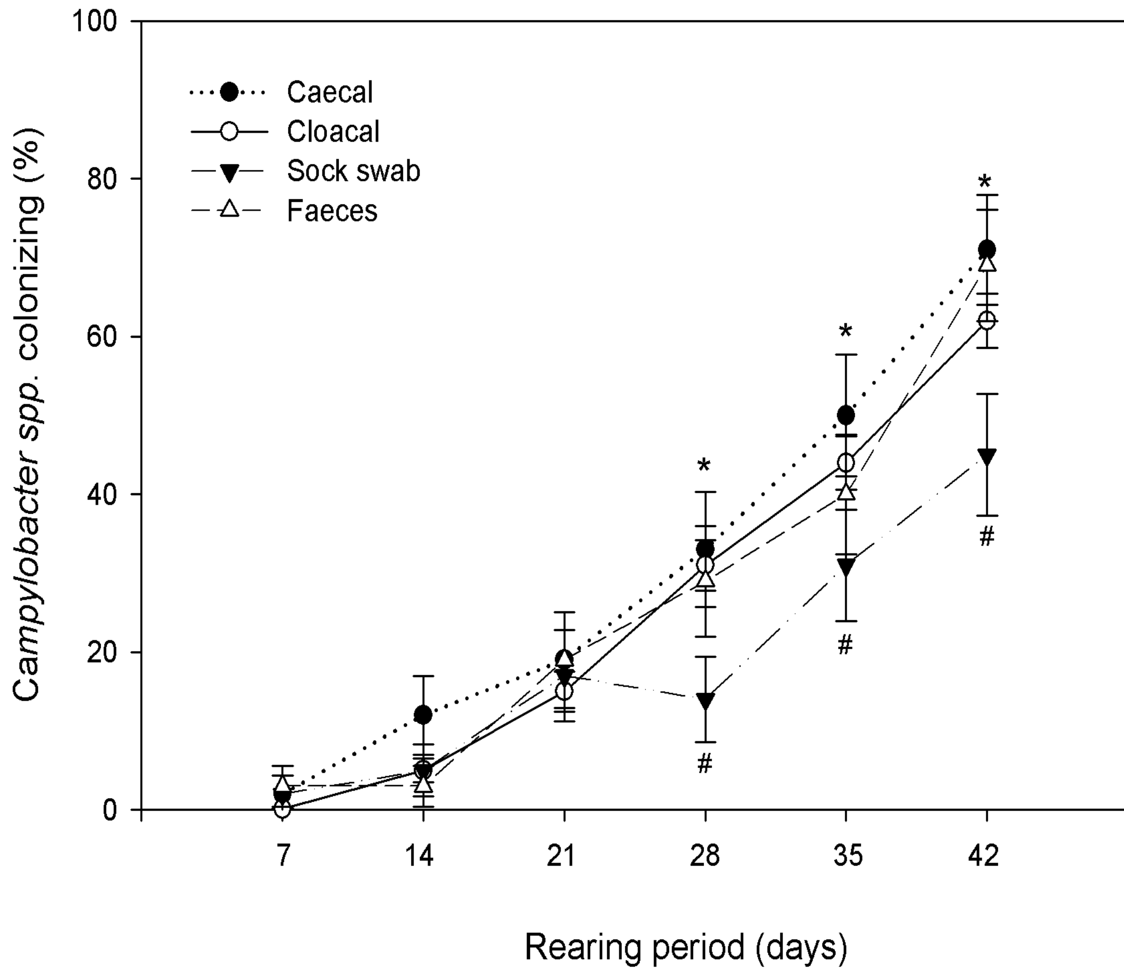

Figure 17. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing. *,# Different superscripts represent significant differences (P < 0.05). Data are presented as least squares means ± standard error of the least squares means.

IV.2.2. Slaughterhouse.

At slaughter, 3 broiler carcass samples from each flock were evaluated. A total of 52 samples out of 63 were positive for *Campylobacter* (82.5%). *C. jejuni* was the most commonly identified species (75.0%).

IV.3. Experiment 3: Molecular detection of *Campylobacter* spp. in day-old chicks.

No presence of *Campylobacter* spp. was verified in the day-old chick by bacterial culture method, while 4 flocks out of 12 were found to be positive for *Campylobacter* by molecular method and also tested positive at the end of the rearing period. Real-time PCR identification revealed that *C. coli* was detected in the 4 flocks, while *C. jejuni* was identified in 3 flocks. Quantitative data on *C. coli* were in a range of 1.3×10^2 to 4.9×10^3 CFU/mL, whereas data on *C. jejuni* were in a range of 7.7×10^4 to 3.4×10^5 CFU/mL. No presence of *Campylobacter* spp. was verified in the environmental samples. All 12-broiler flocks were found to be contaminated with *Campylobacter* by the end of the rearing period.

CHAPTER V. GENERAL DISCUSSION.

Campylobacteriosis is one of the most prevalent bacterial gastrointestinal diseases in humans worldwide, and poultry is considered the most important source for human infection (EFSA and ECDC, 2015b). It has been estimated that 20-40% of human *Campylobacter* infections are linked to the handling or consumption of chicken products (EFSA, 2010). As broiler meat is the largest identified source of human exposure to *Campylobacter*, an effective way to protect public health from *Campylobacter* foodborne infections could be the decrease of the prevalence and numbers of the bacteria in broiler chickens at the primary production stage. This control strategy will decrease the introduction of high numbers of *Campylobacter* into the slaughterhouse, and consequently on the final product (EFSA, 2010). To this aim, the prevalence of *Campylobacter* in the poultry production chain has been widely investigated in many countries over the last decade. Several studies have been conducted to identify on farm sources for infection of *Campylobacter* in broiler chickens that will help to prioritize control points and recommendations for biosecurity improvements (Agunos *et al*., 2014). Although several strategies for reducing the incidence of this pathogen in poultry and poultry products have being studied and applied, the prevalence of *Campylobacter* is still high (EFSA, 2011). Results from a European Union-wide baseline survey on *Campylobacter* in broiler batches carried out in 2008 found that the prevalence of *Campylobacter*-colonised broiler batches at community level was 71.2%, being 88.0% for Spain (EFSA, 2011), suggesting that biosecurity measures implemented at farm level are not effective in controlling this pathogen. Therefore, a more detailed understanding of the ecology of *Campylobacter* among commercial flocks is needed to aid the design of more effective intervention methods.

To date, epidemiology of *Campylobacter* spp. in Spanish broiler production is very limited. Additionally, the majority of prevalence studies have been conducted in commercial broiler flocks, that are typically slaughtered at the age of five to six weeks, so there is little information concerning the long-term colonisation of chickens with this organism since the animal arrival to the farm (Colles *et al*., 2011). Describing the natural history of infection in maturing chickens may improve our understanding of the host-colonizer relationship and guide interventions in younger birds. Thus, a longitudinal study was carried out to investigate *Campylobacter* epidemiology in broiler production and the possibility of vertical transmission in Eastern Spain. To this aim, all

breeder farms belonging to the two integrated poultry companies that handle the majority of the broiler production in the Valencian region (Eastern Spain) were evaluated. The survey of the bacterium in the breeder stage found that *Campylobacter* isolation started from week 16 and reached its peak at week 26 (onset of laying period), with 57.0% and 93.2% of colonized birds, respectively. After this point, the rate of *Campylobacter* isolation decreased slightly to 86.0% at 60 weeks (end of laying period). Despite of the fact that *Campylobacter* is typically detected at age of two weeks during the fattening period (Newell and Fearnley, 2003), this study demonstrated that during breeding there is a delay in *Campylobacter* detection until the fourth month in breeder flocks. Similarly, Menna *et al*. (2005) carried out a study about the prevalence of *Campylobacter* in poultry breeder flocks in Italy, and did not isolate the bacterium until 20 weeks of age. Also, Colles *et al*. (2011) reported that some breeder flocks could remain *Campylobacter* negative until 5 weeks of age. It has been postulated that the dynamics of initial flock colonization are age dependent, potentially related to changes in immunological maturity (Newell and Fearnley, 2003). Other possible reason that will explain the delay of infection in breeder flocks until fourth month of life, would be because breeder farms usually maintain high levels of biosecurity and used standard vaccine handling practices, while commercial fattening farms maintain basic biosecurity practices (Pérez-Boto *et al*., 2012; Mutinda *et al*., 2014). Under strict biosecurity measures it is likely that only low numbers of campylobacters from the environment will enter the house, and if these are stressed due to adverse conditions (e.g., exposure to harmful compounds, air, or low levels of nutrients) their colonization potential may be compromised (Newell *et al*., 1985).

Stress factors have been shown to increase the susceptibility of farm animals to colonization by pathogens, faecal shedding and horizontal transmission, and consequently, the contamination risk of animal products (Humphrey, 2006; Rostagno, 2009; Verbrugghe *et al*., 2012). Many recent studies have demonstrated that bacteria, such as *Salmonella* and *Campylobacter*, are capable of exploiting the neuroendocrine alterations due to the stress response in the host to promote growth and pathogenicity (Lyte, 2004; Freestone *et al*., 2008; Marin *et al*., 2009; Verbrugghe *et al*., 2012). Several management practices in poultry production, such as transportation, feeding restriction or spiking, are known to be very stressful for animals and have a big influence in pathogens epidemiology (Whyte *et al*., 2001; Lafferty and Holt, 2003;

Marin and Lainez, 2009). First, transportation has been identified as a stressor for poultry, and therefore as a major risk in relation to carcass contamination and subsequent end product safety (Whyte *et al*., 2001). Transport-induced stress may occur as a result of a combination of external factors such as crowding, motion, and temperature fluctuations together with food and water deprivation (Whyte *et al*., 2001). Such stresses can result in increased corticosteroid levels and decreased shear strength of the wall of the intestinal tract (Mulder, 1996). Moreover, stressed animals exhibited increased peristaltic movement of material through the gut and excreted pathogenic microorganisms more frequently (Linton and Hinton, 1986). During this study we evaluated the influence of transportation on *Campylobacter* prevalence in breeders but no significant increase was observed before and after transportation. Likewise, Whyte *et al*. (2001) reported that the overall *Campylobacter* spp. prevalence in faeces was unchanged after the transportation of broilers compared with pre transport levels, although a significant increase on mean counts of *Campylobacter* spp. was detected. Also, Wesley *et al*. (2005) did not find significantly changes between the prevalence of two turkey flocks before and after transportation, and they suggest that this fact that may be attributed to the already high prevalence of *Campylobacter* spp., and the resultant need for sampling more birds per flock to detect a statistically significant shift. In contrast, Stern *et al*. (1995) reported an increase in the concentration of *Campylobacter* in broiler caecal material after transport. Besides transportation, broiler breeders are subjected to other stressors during their productive life, such as severe feed restriction (D'Eath *et al.,* 2009). Broiler breeders are commonly fed a restricted diet once daily during the laying period in order to prevent health and reproductive problems associated with increased voluntary feed intake (Chen *et al*., 2006). However, it has been reported that limit-fed birds can show behavioral abnormalities, such as pecking behavior in empty feeders, more time spent on drinking, hyperactivity, and stereotyped pacing before feeding, that are characteristic of hunger and frustration and induce chronic stress (de Jong *et al*., 2002, 2003, 2005; D'Eath *et al.,* 2009; Nicol, 2015). Moreover, the laying period implies a change in the management practices, since males and females (that have been reared separately during the first five months of life) are mixed together in a new farm. Male broiler breeders have shown an increased aggression level towards female breeders, often resulting in fear and injury to the females (de Jong *et al*., 2009). It is not clear why this occurs, but is believed to be associated with management practices such as feed restriction (Shea-Moore *et al*., 1996;

Millman and Duncan, 2000). Add to this the energetic demands of high rates of lay may also cause physiological stress on matured birds (Nicol, 2015). The results on *Campylobacter* prevalence among layer farms, supplying fertile hatching eggs for the broiler flocks under investigation, clearly indicate that *Campylobacter* isolation was elevated during breeders' egg-productive lives (from 26 to 60 weeks). Finally, the spiking is a management practice that requires the introduction of new reproductive males (young, unfamiliar and inexperienced birds) into an already established flock, in order to counteract the naturally occurring decline in fertility levels at 40 weeks of age. The introduction of new birds in an already established flock may cause social stress to the birds, due to the change in social structure and the disruption of the pecking order (Rostagno, 2009). It has been reported that social stress increases susceptibility to bacterial and viral infections in chickens (Holt, 2003). Therefore, this management practice would be expected to increase *Campylobacter* positive rate. However, in this study no significant increase in the prevalence of the organism was observed after spiking in breeder flocks. Similarly, Colles *et al*. (2011) reported no discernable variation in either prevalence or genetic after changes in flock management, such as mixing together different birds, and release onto the range. Thus, results from this survey might be attributed to the high prevalence found within positive flocks, in which a 100% infection rate was common.

In contrast to the colonization of breeder flocks, *Campylobacter* detection in broiler flocks during the fattening period started from day 14 and reached its peak on day 42, with 10.0% and 62.0% of *Campylobacter* isolation from birds, respectively, an outcome consistent with the findings of previous studies (Jacobs-Reitsma *et al*., 1995; Evans and Sayers, 2000; Newell and Wagenaar, 2000; Shreeve *et al*., 2000; Sahin *et al*., 2002; Cox *et al*., 2012). The flock positivity rate was nearly 100%, since 20 out of 21 broiler flocks investigated were found positive by the end of rearing, with a mean flock prevalence of 62.0%. These results are also consistent with previous findings of Torralbo *et al*. (2014), who reported a flock prevalence of 66.3% in birds aged from 36 to 50 days old risen in Southern Spain. The prevalence of *Campylobacter* among broiler flocks seems to differ according to studies and locations, ranging from 34.2% in Great Britain (Ellis-Iversen *et al*., 2009) to 63.9% in Italy (Di Giannatale *et al*., 2010). Our results clearly indicate that broilers are highly contaminated with the bacterium at the end of fattening. Moreover, bacterial counts on carcasses have shown 1,000-fold

increase during transportation (Altekruse *et al*., 1999). During crating or transport, any pathogen, such as *Campylobacter*, could still colonize the ceca of birds, which would be retained during processing (Keener *et al*., 2004). Aside from the birds themselves, crates that are not properly cleaned could increase contamination levels (Stern *et al*. 2001).

As reported above, all broiler flocks were highly infected with *Campylobacter* by the end of fattening in this study. It has been shown that the status of the arriving flock to the slaughterhouse is closely related to the status of *Campylobacter* contamination at the end of processing, representing a considerably risk factor for human campylobacteriosis (Rosenquist *et al*., 2003; McCrea *et al.,* 2006; Allen *et al*., 2007; Berrang *et al*., 2007). The contamination of broiler meat occurs on the slaughter and processing chain, either at slaughter, when carcasses of colonized birds may become contaminated by fecal matter, or while passing down the line due to cross-contamination (Johannessen *et al*., 2007; Franz *et al.,* 2012). The rapid production rate, close proximity of carcasses and limitations in the design of processing equipment might all cause difficulties for washing internal cavities properly, which would provide an ideal environment for bacteria protection (Rosenquist *et al.,* 2006). *Campylobacter* contamination rates in chicken carcasses vary widely among countries. In a European Union baseline study of 26 member states, the chicken carcass contamination rate was 75.8% on average, but ranged from 4.9% to 100% (EFSA, 2010). Results of this study are consistent with the literature, showing a mean contamination rate of 82.5% in chicken carcasses after chilling. Our findings are in agreement with a previous study in which a similar prevalence (73%) was found (Rasschaert *et al*., 2007). However, contamination with *Campylobacter* varies among European countries. To give examples, Danish study showed a prevalence of 37% (Heuer *et al*., 2001), while a Dutch study indicated that prevalence ranged from 20% to 31% between 2002 and 2005 (Van Asselt *et al*., 2008). This disparity might be due to differences in sampling schemes, analytical methods, or the ages of the birds that were sampled (Hue *et al*., 2010). Nevertheless, carcass contamination is not only related to the within-flock prevalence of *Campylobacter* colonisation, but it may also occur throughout the entire slaughter process (Berndtson et al., 1996a). The count of *Campylobacter* found on the carcass varies along the processing chain, with peaks been reported at the exsanguination and de-feathering stage, followed by a reduction along the rest of the

chain, although an increase sometimes has been observed at evisceration (Guerin *et al*., 2010; Hue *et al*., 2011; González-Bodí, 2015). First, the strong possibility of contact between intestinal material and carcasses during exsanguination is considered a risk factor for *Campylobacter* carcasses contamination (Hue *et al*., 2011; González-Bodí, 2015). Also, it has been reported that during de-feathering faecal material escaped through the cloaca by the action of picker fingers pressing the abdomen, which would enhance the contamination of external and internal carcass surfaces (Rosenquist *et al.,* 2006; Chokboonmgkol *et al.,* 2013). Evisceration process has also been reported to contribute to carcass contamination (Hue *et al*., 2010). It has been shown that the machines used for evisceration may often not adapt to the natural variation of carcass sizes within a given batch. Consequently, the rupture of viscera is common and the release of intestinal contents can contaminated the carcasses eviscerated. Moreover, the equipment and the machines could also be dirtied and therefore could contaminate the following carcasses. In contrast, air-chilling conditions are related to a reduction in overall carcass contamination (Giombelli *et al.,* 2013). It has been shown that the number of contaminated carcasses can lower if sensors are used for controlling purposes, such as drying skin surfaces or cold conditions (Guerin *et al.,* 2010). Thereafter, the level of contamination generally decreases along the processing line, and is reduced even further when freezing is included (Georgsson *et al*., 2006). However, as the infective dose of *Campylobacter* is typically low (EFSA, 2010), even batches contaminated at a low level may be considered to be threats to public health. However, risk analyses indicate that highly contaminated carcasses contribute most to the risk of human illness, and that a reduction of fecal carriage would be the intervention with the best cost-utility ratio (Havelaar *et al*., 2005; Lindqvist and Lindblad, 2008).

Regarding *Campylobacter* species isolated*,* the results of this study agree with those reported by the EFSA (EFSA and ECDC, 2015b), with *C. jejuni* being the most prevalent species isolated from in all productive stages (breeders 79,8%; broilers 83,8%, and carcasses after chilling 75.0%), followed by *C. coli*. However, some authors described a higher proportion of *C. coli* in certain poultry productions types such as free-ranged chickens, laying hens or chicken breeders (O’Mahony *et al*. 2011; Patriarchi *et al*., 2011; Kalupahana *et al*., 2013; Colles *et al*., 2015; Prachantasena *et al*., 2016). The increase in the *C. coli* proportion in chickens has been associated with flock age (Colles *et al*., 2011), and/or the use of disinfectants and antibiotics on farms, that could

select for certain bacterial populations (Wales and Davies, 2015). Nevertheless, both species are the most frequent species associated with human infection, accounting for over 95% of infections (Wagenaar *et al*., 2013; and ECDC, 2015b). Despite of these findings there is still no implemented *Campylobacter* Control Programme to reduce the incidence of the bacteria in broiler flocks in Europe.

The results reported before show the dynamics of *Campylobacter* detection during the breeder, layer and fattening period. However, it is also important to understand how the bacterium is disseminated from chickens to the environment of the farm and the role of the environment in chicken infection. Horizontal transmission is often considered as the main route linked to the spread of *Campylobacter* in poultry production (Van Gerwe *et al*., 2005; Lin, 2009). Different pathways have been suggested to explain flock colonisation while rearing, including contamination from previous flocks and exposure to potential sources of the bacterium, such as the other animals on farms, insects, rodents, the environment, litter and drinking water (Wassenaar *et al*., 2011). As it has been discussed before, results from this survey revealed a high *Campylobacter* rate in breeder and broiler flocks. An inadequate cleaning and disinfection and short downtime of the broiler houses between flocks could explain these results (Rivoal *et al*., 2005; Bull *et al*., 2006, Messens *et al*., 2009; Allen *et al*., 2011). Environmental reservoirs, including water, feed, dust, and farmers' boots were tested for the presence of *Campylobacter* prior to chicks' arrival in breeder and broiler flocks. However, we ruled out this explanation, as all environmental samples (n= 580) were negative for *Campylobacter* in our study. The bacterium has been difficult to detect in the environment, and this is most likely due to desiccation or possibly because of bacterial assumption of viable but non-culturable state (VNBC), in which the bacteria cannot be detectable using the standard ISO 10272:2006 procedure (Cox *et al*. 2001; ISO, 2006).

Transmission of *Campylobacter* from breeder flocks to broiler offspring has traditionally been dismissed as a source of contamination due to the lack of culture-based detection of *Campylobacter* from newly hatched chicks (Adkin *et al*., 2006; EFSA, 2010; Newell *et al*., 2011; Cox *et al*., 2012; Hiett *et al*., 2013; Agunos *et al*., 2014). Results from this study reported that the organism was not recovered from any day-old chick sample (breeders and broilers) by using traditional culture methods (ISO

10272:2006, ISO, 2006), suggesting that vertical transmission was not involved in the colonization of broiler flocks. Similarly, Bull *et al*. (2006) found 83% of the faecal samples collected from the breeder flocks to be positive, but did not detect the bacterium in chicks that had hatched from eggs laid by *Campylobacter*-positive hens until the birds were at least 3 weeks old. However, they found indistinguishable isolates in two breeders flocks and their progeny, but they concluded that vehicles visiting both farms might have transferred *Campylobacter* from the breeders to the broilers by means other than vertical transmission. Also, Callicott *et al*. (2006) stated that they could not find any evidence for vertical transmission of *Campylobacter* from grandparent flocks in Sweden and their progeny in Iceland, despite *Campylobacter* from grandparent birds and the parent birds showing identical genetic fingerprints. The authors concluded that this was because of migrating birds rather than vertical transmission. However, broiler houses in Iceland are not accessible to the environment, so an influence by wild birds is unlikely.

As reported before, epidemiological investigations of commercial flocks indicate that newly hatched chicks appear to be free of *Campylobacter*, suggesting that vertical transmission does not play a role in *Campylobacter* infection of poultry flocks (Newell and Fearnley, 2003). However, Cox *et al*. (2012) suggested that the fact that a low transmission rate exists, along with an insensitive method of sampling a flock, more than likely explains why *Campylobacter* is not detected within a broiler flock until chicks are almost 3 weeks of age. As a result, transmission of *Campylobacter* from breeder flocks to broiler offspring has traditionally been dismissed as a source of contamination due to the lack of culture-based detection of *Campylobacter* from newly hatched chicks (Hiett *et al*., 2013). Specifically, although there was some evidence for vertical transmission or pseudo vertical transfer (Chuma *et al*., 1994:1997b; Hiett *et al*., 2002b: 2003b; Idris *et al*., 2006), it seems that this research is still hampered by the sensitivity of isolation and genotyping techniques (Cox *et al*., 2012; Agunos *et al*., 2014). Several hypotheses have been put forward to explain researchers' difficulty to isolate *Campylobacter* during the first two weeks of placement. First, protective maternal antibody effects delay *Campylobacter* colonization (Sahin *et al*., 2002, 2003a). Second, *Campylobacter* may be in a non-culturable form as there were several studies that successfully detected *Campylobacter* DNA, but failed to culture (Chuma *et al*., 1994:1997b; Sahin *et al*., 2002). Thus, there is a need to explore the use of a more

reliable molecular technique for detecting viable or "potentially infectious units" of *Campylobacter* (Kruger *et al*., 2014) from hatchery and chick samples (Agunos *et al*., 2014). Third, different isolation techniques have highly variable sensitivity that may affect results if *Campylobacter* concentration is below the detection limits (Chuma *et al*., 1997a). Because of the inherently low number of cells in eggs/eggshells, embryos, yolk sac, and neonatal intestines, enhanced recovery techniques (e.g., combining membrane filtration and enrichment) need to be explored to improve our detection limits in these samples (Jokinen *et al*., 2012). Fourth, the type of sample may influence the sensitivity for detection of Campylobacter (Vidal *et al*., 2013).

The difficult isolation of the bacterium in day-old chicks and environment using the standard culture method reinforce the need for further investigations on sampling methodology and detection techniques (Chuma *et al*., 1994:1997b; Sahin *et al*., 2002; Idris *et al*., 2006). The main reason that explains the lack of isolation from environmental samples or day-old chick samples could be explained because traditional culture methods may be inadequate for detecting small numbers, sublethally injured or stressed cells, or viable non-culturable cells of *Campylobacter* in foods or biological samples (Cox *et al*. 2001). Assessing the effectiveness of any potential intervention at farm level requires monitoring of the *Campylobacter* status of broiler flocks using appropriately structured sampling methods (Bronzwaer *et al*., 2009). To this end, the development of a harmonised protocol for the detection of *Campylobacter* at the farm level will require careful consideration of the optimum sample type, sample collection method, transport conditions and laboratory protocols (Vidal *et al*., 2013). Caecal sampling is the standard method for sampling at abattoir level (EC, 2007). However, the collection of caecal contents on the farm could be difficult and requires culling and post-mortem of the birds (Vidal *et al*., 2013). Other sampling methods are also use to detect *Campylobacter* in broiler houses, including cloacal swabs (Hansson *et al*., 2004; OIE, 2008), faecal samples (Sandberg *et al*., 2006), and sock swabs or the equivalent boot sock model (Bull *et al*., 2006; Ellis-Iversen *et al*., 2011; Ridley *et al*., 2011; Vidal *et al*., 2013). Boot swabs are a widely used sampling method for *Salmonella* testing in broilers, turkeys and laying hens on floor systems and for the statutory monitoring of chicken breeding flocks. They are a convenient way to collect faecal material from a large number of birds; they can be used easily by farmers and could provide a standardized sampling method (Mueller-Doblies *et al*., 2009). In this context,

experiment II was carried out to determine performance characteristics of four sampling methodologies (cloacal and boot swabs, caecal and faecal samples) for the detection of *Campylobacter* from broiler flocks at primary production. The bacterium was first detected in one of these flocks after 7 days in caecal, cloacal and faecal samples. Then, *Campylobacter* was isolated from all of the sample types on day 14. All sample types tested resulted in the same detection rate until 21 days of rearing. However, the sock swab samples taken between 28 to 42 days of rearing failed to detect positive samples, whilst the use of caecal, faecal and cloacal samples isolated significantly more samples. These results were not in agreement with previous findings of Vidal *et al*. (2013), who reported that sock swabs moistened in Cary-Blair medium, are a sensitive sampling method for detection of *Campylobacter* spp. in broiler flocks. Our methodology, although the samples were moistened in Cary-Blair medium, was based on the direct culture of all sampling types onto mCCDA medium without an enrichment step. Contradictory results have been reported regarding enrichment of caecal material in Bolton broth. Some authors showed that enrichment was less effective than direct culture for isolating thermophilic *Campylobacter* spp. (Musgrove *et al*., 2001; Williams *et al*., 2008). However, other studies concluded that using an enrichment step prior to plating usually provides better recovery when target cells are either low in number, injured, or stressed (Richardson *et al*., 2009; Williams *et al*., 2009). Specifically, Vidal *et al*. (2013) reported that enrichment increased the sensitivity of the sock swabs. Moreover, when analysing large numbers of samples, the workload should be minimised and avoidance of duplication of selective agar, or omission of an enrichment step, might be an attractive choice, even accepting a possible consequential lesser sensitivity (Ugarte-Ruiz *et al*., 2012). However, our results showed that a pre-enrichment step does not increase the sensitivity for *Campylobacter* detection because all samples that were negative by direct culture were also negative by pre-enrichment. Therefore, the fast, simple and cheap method of direct plating was shown to yield similar isolation efficiency for detection of *Campylobacter* in caecal, faecal and cloacal samples. Nevertheless, some authors have suggested that using both methods in parallel (direct and enrichment) could enhance the sensitivity (Hald *et al*. 2000; Maher *et al*. 2003; Habib *et al*. 2008; Rodgers *et al*., 2010). Results from experiment II proved that caecal, cloacal swab and faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the same sensitivity for detection of *Campylobacter* spp. in broiler flocks independently of the day of rearing. Nevertheless, further research into

improvement of culture procedures seems necessary to detect *Campylobacter* spp. from broilers, especially at the onset of rearing because all day-old chicks sampled (breeders and fattening broilers) were negative using the official method for detection and enumeration of *Campylobacter* spp. for food legislation purposes (ISO, 2006).

Campylobacter isolation in experiment I and II was performed following the ISO 10272:2006 standard procedure, which is the official method for detection and enumeration of *Campylobacter* spp. for food legislation purposes (ISO, 2006). The microbiological detection of *Campylobacter* is notoriously difficult and time-consuming, owing to its strict growth requirements and the means by which the organism is detected and cultured is not standardised. Several authors agree that the development of molecular methods constitutes an especially important breakthrough in reducing the time required and specific for the identification of *Campylobacter* spp. combined with a lower detection limit (Ugarte-Ruiz *et al*., 2012; Gharst *et al*., 2013). Nevertheless, the two regulatory agencies in charge of food safety in the USA, the Food and Drug Administration of the Department of Health and Human Services, and the Food Safety and Inspection Services of the U.S. Department of Agriculture, do not consider these molecular tests "confirmatory" and so the actual culture has to be obtained from presumptive positive samples for confirmation purposes (Gharst *et al*., 2013). Consequently, experiment III was conducted to compare the performance of traditional culturing method, based on the recommended ISO 10272:2006 protocol, and Real-time PCR for *Campylobacter* detection in 12 broiler flocks. The International Organization for Standardization (ISO) standard method for detection of *Campylobacter* spp. recommended enrichment using Bolton broth, followed by culture on selective modified charcoal cefoperazone desoxycholate agar (mCCDA) and one other alternative agar plate (ISO, 2006). Bolton broth is currently the medium recommended by the US Food and Drug Administration (Hunt *et al*., 2001), the International Standard Organization (ISO, 2006) and the Nordic Committee of Food Analysis (Rosenquist *et al*., 2007). It is realized that the enrichment step has to compromise between selectivity and the inhibition of competitor organisms, together with the recovery and growth of the target organism to detectable levels (Baylis *et al*., 2000).

Nevertheless, a culture-independent approach based on DNA amplification has several advantages over classical bacteriology for *Campylobacter* detection, notably a

faster performance combined with a lower detection limit (Ugarte-Ruiz *et al*., 2013). Real-time PCR may provide an alternative to culture and detection of *Campylobacter* in samples of intestinal origin (Lund *et al*., 2004; Ridley *et al*., 2008; Randall *et al*., 2010). Moreover, this technique will detect viable but not cultivable cells, for which it is unknown whether they provide a risk for consumers (Nogva *et al*., 2000; Humphrey *et al*., 2007). In this study, *Campylobacter* occurrence was investigated in caecal samples from twelve day-old broiler flocks using molecular and bacterial culture methods. The bacterium was not detected in any sample analysed following the recommendations of the standard ISO 10272-1:2006 procedure. However, four flocks were determined as *Campylobacter*-positive by Real-time PCR. These results agree with previous findings from Idris *et al*. (2006) who also failed to detect viable *Campylobacter* in the intestine of day-of-hatching chicks using a culture approach, but successfully isolated *C. coli* DNA in the ileum, cecum, and yolk contents of chicks on the day-of-hatching. Similarly, Rodgers *et al*. (2010) found that a small number of samples were negative by culture methods but positive by PCR methods, suggesting that the organisms may have been unable to grow due to the selective properties of the agar, or a loss of *Campylobacter* viability in the sample post-collection. Also, Ugarte-Ruiz *et al*. (2013) carried out a study of the effect of different isolation protocols on detection and molecular characterization of *Campylobacter* from poultry, and concluded that the maximum of detectable cells predicted by qPCR is a sensitive and powerful evaluation tool. Real-time PCR yields highly sensitive and specific results while avoiding manipulation of PCR products after amplification, thereby reducing the risk of cross-contamination; it can be used for rapid quantitative screening of samples (Debretsion *et al*., 2007; Botteldoorn *et al*., 2008; Melero *et al*., 2011). However, phenotypic expression of certain properties cannot be tested, and, without cultures, additional information such as subtyping or antimicrobial resistance testing cannot be obtained. The major limitation of the DNA-based qPCR method is the potential detection of both live and dead, or non-culturable cells, which can lead to an overestimation of the number of bacteria (Flekna *et al*., 2007; Lin, 2009). Therefore, qPCR results can be considered the theoretical maximum of detectable microorganisms, accepting that this may be an overestimate as molecular detection also reports the presence of dead cells. Thus, qPCR-positive results can be used as a maximum value to correlate culture-dependent results. According to ISO 10272:2006, these can be regarded as true positive due to the target-specific DNA probe-based PCR response (Bui *et al*., 2012). In our

study, the 4 day-old chick flocks that were detected positive by qPCR, were determined *Campylobacter* negative with ISO method. However, these flocks were *Campylobacter* positive at the end of the rearing with the ISO method. Therefore, the real-time PCR assays for *C. jejuni* and *C. coli* described in this study have several advantages over culture-based techniques. These include allowing a large increase in throughput, enabling simultaneous processing of several samples (qPCR can be run in a 96-well format and many steps in the assay can be automated), and reducing the total time required for analysis. The identification at the species level and the quantification on the entire DNA extracted from faecal, feed, and environmental samples is a new tool to enhance our understanding of the epidemiology of *Campylobacter*. In terms of risk assessment, this ability to differentiate and quantify these two species permits a more precise description of the carriage and excretion of *C. jejuni* and *C. coli* by livestock animals.

In conclusion, these results highlight our lack of knowledge of the ecology for transmission of *Campylobacter* in poultry and the fact that molecular techniques or more sensitive culture methods may be necessary to detect early colonization by *Campylobacter* in broiler chicks. Molecular detection in day-old chicks, supported by culture detection later in the life of the flock, allowed us to demonstrate that *Campylobacter* could be present in the chicks before placement.

CHAPTER VI. CONCLUSIONS.

1. In breeder production *Campylobacter* isolation started from week 16, and the isolation rate was elevated during the breeders egg-productive lives (from 26 to 60 weeks).

2. We found no evidence of *Campylobacter* transmission from vertical and environmental sources in breeder (parent) and broiler flocks (offspring) using the standard ISO 10272:2006 procedure.

3. The onset of colonization in broilers was not detected until day 14 of rearing, and the isolation increased significantly throughout the fattening period, with the highest rate at the end of fattening. The mean contamination rate in broiler chicken carcasses after chilling was elevated (82.5%), and *C. jejuni* was the most commonly identified species in all productive stages (breeders, broilers and carcasses after chilling).

4. Caecal, cloacal swabs and faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the same sensitivity for detection of *Campylobacter* spp. in broiler flocks independently of the day of rearing.

5. No presence of *Campylobacter* spp. was verified in the day-old chick by bacterial culture method, while 4 flocks out of 12 were found to be positive for *Campylobacter* by molecular method and also tested positive at the end of the rearing period.

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ANNEX I.

LIST OF ORIGINAL PUBLICATIONS.

This thesis is based on the following publications:

- **Ingresa-Capaccioni, S.**, E. Jiménez-Trigos, F. Marco-Jiménez, P. Catalá, S. Vega, C. Marin. 2016. ***Campylobacter* epidemiology from breeders to their progeny in Eastern Spain.** *Poult. Sci*. 95(3):676-83.

- Marin, C., Peñaranda, D.S., **Ingresa-Capaccioni, S.,** Vega, S., Marco-Jiménez, F. 2015. **Detection of *Campylobacter* spp in Day-Old Chick Demonstrate Vertical Transmission in Poultry Production.** Molecular *Journal of Animal and Veterinary Sciences*., 2, 32-36.

- **Ingresa-Capaccioni, S.,** S. González-Bodí, E. Jiménez-Trigos, F. Marco-Jiménez, P. Catalá, S. Vega, C. Marin. 2015. **Comparison of different sampling types across the rearing period in broiler flocks for isolation of *Campylobacter* spp.** *Poult Sci*., 94, 766-71.

ANNEX II.

LIST OF CONGRESS.

The results of this thesis have been presented on the following congress:

- **S. Ingresa-Capaccioni,** S. Sevilla, L. Montoro, E. García, D. S. Peñaranda, S. Vega, F. Marco-Jiménez, C. Marín. **"Evidencia del papel de la transmisión vertical en la infección de pollitos de un día por *Campylobacter* spp."** LIII Simposio Científico de Avicultura. Zaragoza, 28-29 de Septiembre 2016. Póster.

- **S. Ingresa-Capaccioni**, S. González, P. Catalá-Gregori, F. Marco-Jiménez, S. Vega, C. Marín. "***Campylobacter*: desde la recría a la canal".** LI Simposio Científico de Avicultura. PREMIADO. Alfara del Patriarca (Valencia, España), 2-3 Octubre 2014. Comunicación oral.

- M. Colvée-Bosh, **S. Ingresa-Capaccioni**, F. Marco-Jiménez, P. Catalá-Gregori, S. Vega, C. Marín. **"Comparación de los distintos tipos de muestras para el aislamiento de *Campylobacter* spp. durante el periodo de cría en explotaciones de broiler".** LI Simposio Científico de Avicultura. Alfara del Patriarca (Valencia, España), 2-3 Octubre 2014. Póster.

- **S. Ingresa**, Sara González Bodí, Pablo Català, Santiago Vega García y Clara Marín Orenga. **"¿Cuál es el origen de la elevada prevalencia de *Campylobacter* a nivel de campo?"** X Congreso Internacional de Estudiantes de Ciencias Experimentales y de la Salud. PREMIADO. Universidad CEU Cardenal Herrera (Moncada, España), 24, 25 y 26 de Abril 2013. Comunicación oral.