Universidad del País Vasco | Euskal Herriko Unibertsitatea

Epidemiology of *Campylobacter* infection in food-producing animals:

Distribution in domestic ruminants in the Basque Country. Effect of age, breed and diet on chicken caecal microbiota and *Campylobacter* colonization

TACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGATTATCA

AGTCTCTTGTGAAATCTAATGGCTTAACCATTAAACTGCTTGGGAAACTGATAGTCTAGAGTA

INES MEDELINA OCEJO SIANTURI

PhD Thesis

2019

Supervisor:
Dr. Ana Hurtado

Universidad del País Vasco · Euskal Herriko Unibertsitatea

Epidemiology of *Campylobacter* infection in food-producing animals:

Distribution in domestic ruminants in the Basque Country. Effect of age, breed and diet on chicken caecal microbiota and *Campylobacter* colonization.

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at the Department of Animal Health, NEIKER – Basque Institute for Agricultural Research and Development

Author:
Ines Medelina Ocejo Sianturi

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Supervisor:
Dr. Ana Hurtado

Universidad del País Vasco · Euskal Herriko Unibertsitatea

Presentation of the PhD Thesis

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To my beloved ones:
my father
my mother
my brother
my significant other

"Curiosity is the engine of achievement"
- Ken Robinson

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salam sayang

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Table of contents

Presentation of the PhD Thesis and authorization pages I
Acknowledgements........ XV
LIST OF TABLES XXIII
LIST OF FIGURES........ XXIV
LIST OF ABBREVIATIONS........ XXV
1. BACKGROUND AND OBJECTIVES 1
2. GENERAL INTRODUCTION 7
2.1. *Campylobacter* 9
2.1.1. Historical background of *Campylobacter* 9
2.1.2. Taxonomic classification and general characteristics of the genus *Campylobacter* 10
2.1.3. Campylobacteriosis in humans 11
2.1.4. *Campylobacter* in animals 13
2.1.5. Antimicrobial resistance (AMR) of *Campylobacter*........ 18
2.2. Chicken gut microbiota 21
2.2.1. Functions of gut microbiota 22
2.2.2. Factors influencing gut microbiota 24
2.2.3. Dysbacteriosis 28
2.2.4. Molecular tools for the characterization of microbiota........ 28
2.2.5. Modulation of gut microbiota 32
3. METHODOLOGY AND RESULTS 35
3.1. **STUDY I**........ 37
3.1.1. Abstract 38
3.1.2. Introduction 39
3.1.3. Materials and methods 40
3.1.4. Results 47
3.1.5. Discussion 55
3.1.6. Conclusions........ 59
3.2. **STUDY II**........ 61
3.2.1. Abstract 63
3.2.2. Background........ 65

3.2.3. Methods 66
3.2.4. Results 74
3.2.5. Discussion 80
3.2.6. Conclusion 84
3.3. **STUDY III** 87
3.3.1. Abstract 88
3.3.2. Introduction 89
3.3.3. Methods 92
3.3.4. Results 97
3.3.5. Discussion 111
4. GENERAL DISCUSSION 121
5. CONCLUSIONS 133
6. SUMMARY 137
7. RESUMEN 143
8. LABURPENA 151
9. SUPPLEMENTARY MATERIAL 157
10. REFERENCES 165
Overview of academic and research activities of the author 187

LIST OF TABLES

Table 1. Primers and probes used to detect point mutations associated to macrolide (A2075G mutation in the 23S rRNA genes) and quinolone (C257T in the *gyr*A gene, Thr-86-Ile) resistance in *C. jejuni* and *C. coli*, and *gyr*A sequencing 45

Table 2. Microbiological resistance (percentage) and distribution of MICs for the 109 *C. jejuni* and 34 *C. coli* isolates. 50

Table 3. Odds ratio (OR) of association between resistance to each antimicrobial and *Campylobacter* species for each host, determined with simple logistic regression analysis using *C. jejuni* as reference. 51

Table 4. Composition of feed ingredients (g/kg) and nutrients content (%) of the experimental diets 71

Table 5. Effect of experimental diet on productive performance of broilers 75

Table 6. Effects of dietary treatments on duodenal histomorphological variables. 76

Table 7. Assessment of *C. jejuni* colonization (caecal content) and shedding (cloacal swabs) throughout the experiment as determined by real-time PCR (Log_{10} *Campylobacter* cell equivalents per gram of caecal content or cloacal swab) 78

Table 8. Genera identified to be significantly representative of each age group in each breed as a result of combining LEfSe output and significantly differentially abundant in DESeq2 analysis results. Genera were listed in descending order according to its effect size in LEfSe. 107

Table 9. Core genera between both breeds at the end of the productive period (42-day-old broilers and 81-day-old FRC), and genera specific for each breed. Genera were sorted in alphabetical order. 109

Supplementary Table S1. Number of reads that passed through each step of the pipeline in DADA2 for broilers and free-range chickens datasets. 160

Supplementary Table S2. Proportions of taxonomic assignment of the amplicon sequence variants (ASVs) and number of unique taxa per taxonomic rank in both breeds 161

Supplementary Table S3. Average relative abundance of taxa in broilers and free-range chickens for each age group, with ASVs collapsed to species rank 161

Supplementary Table S4. Spearman's rank correlations between age (days) and relative abundance of genera in caecal microbiota of broilers and free-range chickens 162

Supplementary Table S5. Differential analysis results of differentially abundant taxa (DESeq2) and potential biomarkers of age groups (LEfSe) for broilers and for free-range chickens 163

LIST OF FIGURES

Figure 1. Prevalence of *Campylobacter*-positive herds/flocks in each host. 48

Figure 2. Proportion of isolates resistant to each antimicrobial agent tested: A) *C. jejuni*; B) *C. coli*. 49

Figure 3. Distribution of antimicrobial resistance (AMR) patterns according to *Campylobacter* species and host. 52

Figure 4. Distribution of isolates resistant to 1-4 classes of antimicrobial agents according to *Campylobacter* species and host. 53

Figure 5. Schematic representation of the experimental trial. 70

Figure 6. Dynamics of *Campylobacter* infection throughout the rearing period of 42 days, as determined by real-time PCR (Ct values) analysis of environmental (air, dust, and drinkers) and animal (faeces, cloacal swabs and cloacal content) samples. 77

Figure 7. *Campylobacter* load in experimentally inoculated (grey) *vs.* non-inoculated (white) broilers as determined by real-time PCR analysis of cloacal swabs at days 21, 28, 35 and 42 (A) and broilers' caeca at days 28 and 42 (B), representing shedding and colonization levels, respectively. 79

Figure 8. Boxplots representing alpha diversity metrics of richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson) for samples from broilers (a) and FRC (b) grouped according to age. 98

Figure 9. Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity matrix on relative abundance data in broilers (a) and FRC (b). 100

Figure 10. Hierarchical clustering analysis: Dendrogram of Bray-Curtis dissimilarity matrices between samples based on different age groups in broilers (a) and FRC (b) with UPGMA method. 101

Figure 11. Microbial community composition of chicken caecal content. Stacked barplots representing relative abundances of the different phyla in all samples in broilers (a) and FRC (b). 103

Figure 12. Relative abundance of the top 10 families and top 15 genera averaged over all samples for the age-groups for broilers (A and B) and FRC (C and D). 104

Figure 13. Venn diagram illustrating core microbiome (ASVs) in broilers (a) and FRC (b). 105

Figure 14. Heatmap representing classified genera within the core microbiome of each breed. 110

Supplementary Figure S1. Rarefaction curves plotting the number of observed ASVs over the number of sequencing reads sampled per chicken from the broilers (A) and free-range chickens (B) datasets 159

LIST OF ABBREVIATION

AGP	antibiotic growth promoters
AME	apparent metabolizable energy
AMR	antimicrobial resistance
ANOVA	analysis of variance
***asp*A**	aspartase ammonia-lyase gene (house-keeping gene)
AST	antimicrobial susceptibility tests
ASV	amplicon sequence variant
B-H	Benjamini–Hochberg
bp	base pair
Bu	diet containing 0.1% coated calcium butyrate
BW	body weight
C	concentration
CC	clonal complex
cfu	colony forming units
CI	confidence interval
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
cm	centimetre
Co	control diet
CO_2	carbon dioxide
Cq	quantification cycles
Ct	threshold cycle
d	day
DFI	daily feed intake
DNA	deoxyribonucleic acid
DWI	daily weight gain
ECDC	European Centre for Disease Prevention and Control
ECOFF	epidemiological cut-off values
EFSA	European Food Safety Authority
EPEF	European Productive Efficiency Factor
ERY	erythromycin
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FCR	feed conversion ratio
FDR	false discovery rate
FOS	fructooligosaccharides
FRC	free-range slow-growing chickens
g	gram
GEN	gentamicin
GIT	gastro intestinal tract
GLM	generalized linear model
***gln*A**	glutamine synthetase gene (house-keeping gene)
***glt*A**	citrate synthase gene (house-keeping gene)
***gly*A**	serine hydroxy methyl transferase gene (house-keeping gene)
GOS	galactooligosaccharides
***gyr*A**	gyrase A gene
h	hour
HT-NGS	high-throughput next-generation sequencing
IAC	internal amplification control
IQR	interquartile range
ISO	International Organization for Standardization
Kb	kilobase
Kcal	kilocalories
L	litre
LDA	linear discriminant analysis
LEfSe	Linear Discriminant Analysis Effect Size
LNA	locked nucleic acids

LSM	least square mean
m	metre
MDR	multi-drug resistant
MDRGI	MDR genomic island
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
MLST	multilocus sequence typing
mm	millimetre
MOS	mannanoligosaccharides
N_2	nitrogen
NA	not applicable
NAL	nalidixic acid
ng	nanogram
NGS	next-generation sequencing
nM	nanomolar
NMDS	non-metric multidimensional scaling
NS	not significant
NSPs	non-starch polysaccharides
nt	nucleotide
O_2	oxygen
OR	odds ratio
OR_{adj}	adjusted odd ratio
OTU	operational taxonomic unit
p.i.	post-inoculation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
PERMDISP	permutation multivariate analysis of dispersion
pgm	phospho glucomutase gene (house-keeping gene)
Q	copy numbers
Q1	first quartile
Q2	second quartile
qPCR	quantitative PCR
RFOs	raffinose family oligosaccharides
rRNA	ribosomal ribonucleic acid
S	survival rate
s	seconds
SCFA	short chain fatty acids
SE	standard error
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SSU	small subunits
ST	MLST sequence type
STR	streptomycin
SUS	susceptible
TET	tetracycline
tkt	transketolase gene (house-keeping gene)
TLR2	toll-like receptor 2
***unc*A**	ATP synthase alpha subunit gene (house-keeping gene)
UPGMA	unweighted pair-group method with arithmetic mean
v/v	volume/volume
VNC	viable but non-culturable
Wh	diet containing 6% dry whey powder
WhBu	diet containing 6% dry whey powder and 0.1% coated calcium butyrate
WHO	World Health Organization
µL	microlitre
µm	micrometre

1. Background and objectives

Campylobacter infection is recognized as the main cause of sporadic food-borne enteritis in humans in developed countries (Humphrey, O'Brien and Madsen, 2007). The high incidence of *Campylobacter*-associated diarrhoea, as well as its duration and possible complications (reactive arthritis and neurological disorders such as Guillain-Barré), make it a serious Public Health problem with highly important socio-economic implications (Moore *et al.*, 2005). In Europe, predominance of *Campylobacter* over other food-borne pathogens has been reported and despite efforts aimed at improving control measures, campylobacteriosis incidence continued to show a significant increasing trend between 2008 and 2017 (EFSA and ECDC, 2018b). *Campylobacter* asymptomatically colonizes the gastro-intestinal tract (GIT) of mammals and birds, and food-producing animals such as poultry and domestic ruminants constitute the principal reservoirs. Poultry meat is considered the predominant source for human infection, but attribution studies suggested that this might be the case in urban, but not necessarily in rural settings where ruminants might also play an important role (Mughini Gras *et al.*, 2012; Roux *et al.*, 2013). Yet, *Campylobacter* studies in ruminant livestock are still limited compared to those carried out in poultry. Another concerning aspect of *Campylobacter* is the widespread of antimicrobial resistance (AMR) in strains isolated from livestock (Luangtongkum *et al.*, 2009; Wieczorek and Osek, 2013), and again most available data concerns isolates from poultry.

Pre-harvest interventions to reduce intestinal carriage of *Campylobacter* in food-producing animals would drastically reduce the risk of human infection. In the GIT, *Campylobacter* is one of the members of a complex and highly diverse microbial community called microbiota, which is in close relationship with the host physiology. This community is dynamic, which means that it is constantly changing in response to external and internal factors. Any intervention that results in modulating its composition by changes in the occurrence (presence/absence) and number (relative abundances) of its members would have an effect in the host health. One of the most commonly

used strategies to attempt to reduce intestinal carriage of food-borne pathogens is through modulation of the intestinal microbiota by means of feed supplementation with additives such as prebiotics, probiotics, organic acids, exogenous enzymes and plant-based essential oils (Yang, Iji and Choct, 2009). This is more easily done in poultry, where lifespan production is short and individual variation small (highly selected commercial hybrids have reduced genetic diversity within the population). Nevertheless, feed constitutes almost 70% of the cost of chicken production (Zhang and Aggrey, 2003), so selection of the additives must be carefully done to avoid compromising the productive performance (weight gain and feed conversion ratios) and/or the chickens′ health, both directly linked to the economic efficiency of the farm. Therefore, any effect on these traits has to be carefully assessed. In addition, being aware that besides diet, other factors like breed and management may also play an important role in the shaping of the gut microbiota, investigating different chicken breeds and production systems is needed to assess the effect of such factors. Broilers are the chicken type most frequently used for intensive meat production, whereas free-range slow-growing chickens are reared under less intensive and more welfare-friendly management practices. Free-range production of chicken meat normally requires slow-growing breeds and age at slaughter is normally not less than 12 weeks, in contrast to the *ca.* 6 weeks rearing period of broilers (Lewis *et al.*, 1997). The different periods of the productive life of chickens need to be evenly covered to capture the taxonomic composition of the diversity of the chicken caecal microbiota at different time points of their lifespan.

In this realm, the objectives of this PhD Thesis were:

1. to estimate the herd-level prevalence of *C. jejuni* and *C. coli* in healthy ruminants in the Basque Country.

2. to determine the AMR profiles of *C. jejuni* and *C. coli* based on the phenotypic determination of Minimum Inhibitory Concentration (MIC) values and to compare the profiles with those obtained in a similar study carried out ten years earlier in the region.

3. to evaluate, by an *in-vivo* experimental study in broilers (Ross-308), the effect of two compounds; dry whey powder (a prebiotic) and calcium butyrate (a salt-form organic acid), in the colonization and shedding levels of *Campylobacter*.

4. to investigate the possible effects of the two feed additives on the broilers' performance and their intestinal integrity by evaluating the productive parameters and histomorphological metrics of the duodenal villi.

5. to evaluate the effect of the feed additives administered on the microbial community of the broiler's caeca using 16S rRNA amplicon high-throughput sequencing.

6. to characterise the temporal development of chicken caecal microbiota in two chicken breeds; Ross-308 broilers (fast-growing breed used for intensive production) and Sasso-T451A (slow growing breed reared in free-range system), and to compare the taxonomic profile of both breeds and identify the core and specific taxa for the different breeds and/or age-groups.

7. to assess the co-existence of *Campylobacter* and the other members of the caecal microbiota in each chicken breed.

2. General Introduction

2.1. *Campylobacter*

2.1.1. Historical background of *Campylobacter*

The first description of bacteria that might belong to the *Campylobacter* genus dates back to 1886 when Theodor Escherich described a non-culturable spiral-shaped bacteria in the faeces of children who had died of what was then called *'cholera infantum'* (Escherich, 1886; Kist, 1986). Then, there were repeated findings of spiralled-form bacteria associated with sheep and cattle abortions named *Vibrio fetus* in 1919 because of their morphology and source (McFadyean and Stockman, 1913; Smith and Taylor, 1919). Years later, similar microorganisms were related to dysentery in calves and pigs and two new species were described: *V. jejuni* and *V. coli* (Jones, Orcutt and Little, 1931; Doyle, 1948). So, by then the three vibrio microorganisms *V. jejuni*, *V. coli*, and *V. fetus* were named in association with specific diseases in animals (cited in (Butzler, 2018)). However, it was not until 1938 that the first food-borne human campylobacteriosis case was reported. An outbreak in two penal institutions in Illinois putatively associated to raw milk consumption contaminated with microaerophilic vibrios caused diarrheal disease with symptoms of vomiting, abdominal cramps, diarrhoea, headache and fever (Levy, 1946).

The new genus *Campylobacter* (derived from the Greek words for curved - *Campylo* and rods - *bacter*) was proposed in 1963 and several former *Vibrio* species were transferred to the genus *Campylobacter* based on their low DNA base composition, non-fermentative metabolism and microaerobic growth (Sebald and Veron, 1963). The crucial step for the description, culture and isolation of *Campylobacter* species from patients stool or blood samples was the use of filtration and selective culture media described in the 1970´s (Butzler *et al.*, 1973; Skirrow, 1977). In the late 1980´s, the first *Campylobacter* phylogenetic data were reported after comparing their partial 16S rRNA gene

sequences (Lau *et al.*, 1987; Romaniuk and Trust, 1987), and a novel genus, *Helicobacter*, was proposed to accommodate the gastric species formerly known as *C. pylori* and *C. mustelae* along with *Wolinella* (Goodwin *et al.*, 1989).

2.1.2. Taxonomic classification and general characteristics of the genus *Campylobacter*

Currently the *Campylobacter* genus is classified in the phylum Proteobacteria, the class Epsilonproteobacteria, and the order Campylobacterales. With the genera *Arcobacter* and *Sulfurospirillum* they form the family Campylobacteraceae. The genus *Campylobacter* alone contains around 24 species, though classifications continue to be redefined as new species are described and more genomic information is available. Isolates from several species within the *Campylobacter* genus have been associated to the pathogenesis of various diseases, however not all members of this genus can cause disease in humans (Lastovica and Allos, 2008). The three most frequently isolated campylobacters are *C. jejuni*, *C. coli* and *C. fetus*. The first two are responsible for human enteric infections while the latter is mostly involved in septic abortion in cattle and sheep and infertility in cattle, as well as a range of human intestinal and extra intestinal illnesses/systemic diseases.

The characteristics of the *Campylobacter* genus were redefined in 2010 after the incorporation of *Bacteroides ureolyticus* (now called *Campylobacter ureolyticus*) to the genus. *Campylobacter* spp. are slim, non–spore–forming Gram–negative bacteria with size ranging from 0.2 to 0.8 µm wide and 0.5 to 5 µm long. Their form can be spiral, curved or sometimes straight rods, and when two cells form short chains, they can have S- or gullwing-shape. Most species are motile thanks to a single, polar, unsheathed flagellum at one or both ends of the cell that allows the characteristic rapid corkscrew-like motion when observed under dark field or phase-contrast microscopy. Exceptions in flagella presentation may occur in some species; for example, *Campylobacter*

gracilis is non-motile, *Campylobacter showae* has multiple flagella, and different cells from a single culture of *Campylobacter hyointestinalis* can present different numbers of flagella (Vandamme *et al.*, 2010). Cells in old cultures or under stressed conditions may form spherical or coccoid bodies. Thus, under unfavourable conditions, *Campylobacter* is able to alter its morphology, transforming the usual spiral shape to a spherical/coccoid form. In this form, described as viable but non-culturable state (VNC), they are metabolically active by showing signs of respiratory activity and still maintain the capacity of infection, but the detection is difficult using current conventional culture methods (Moore, Caldwell and Millar, 2001; Murphy, Carroll and Jordan, 2006; Cox, Richardson and Harrison, 2015). Most *Campylobacter* species are microaerophilic organisms, meaning that they require microaerobic conditions to multiply. They grow best in an atmosphere containing 3-10% O_2 and 5-10% CO_2 levels and the optimum temperature for their growth ranges from 30 to 42°C (Kelly, 2008). They do not ferment carbohydrates and oxidase activity is present in all species except *C. gracilis* and only sporadic in some isolates of *C. concisus* and *C. showae*.

2.1.3. Campylobacteriosis in humans

Campylobacteriosis is the leading cause of food-borne bacterial diarrheal gastroenteritis worldwide (World Health Organization - WHO, 2018). In the EU, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in 2017 and has been so since 2005. In 2017, the number of reported confirmed human cases in the EU was 246,158 with a notification rate of 64.8 per 100,000 population (EFSA and ECDC, 2018b). In the Basque Country, notification rate in the same year was 104.2/100,000 inhabitants, mainly concentrating among young patients (40.6% in <5 years-old and 20.6% in 5-14 years-old) (Anon, 2018). Despite this high prevalence, case reporting is believed to be widely underestimated because many infections result in mild self-limiting gastroenteritis.

Consequently, true incidence rates may be significantly higher than recorded. Unlike *Salmonella*, most *Campylobacter* infections are sporadic and outbreaks are rather uncommon (Havelaar *et al.*, 2013). The principal cause of campylobacteriosis in industrialized countries are thermophilic members of *Campylobacter* (Humphrey, O'Brien and Madsen, 2007), *C. jejuni* being the most common species reported (84.4%) followed by *C. coli* (9.2%), *C. lari* (0.1%), *C. fetus* (0.1%) and *C. upsaliensis* (0.1%) (EFSA and ECDC, 2018b). Thus, hereafter the term *Campylobacter* will refer to zoonotic thermophilic campylobacters, specifically *C. jejuni* and *C. coli*.

Symptoms of campylobacteriosis differ depending on several factors like infectious dose, virulence of isolates, age and immune status of the patient, and history of infection. The major clinical manifestations of campylobacteriosis include acute gastroenteritis, abdominal cramps and fever, but patients may previously manifest non-specific symptoms like headache, myalgias, nausea and chills that can last for more than 24h. The diarrhoea can last for 3-4 days and may vary from mild/watery to severe/haemorrhagic. The illness is usually self-limited and after a week, patients generally recover with no medical intervention or antimicrobial treatment (Blaser, 1997). The most severe cases, however, might need hospitalization. Occasionally, campylobacteriosis may lead to more severe and rare complications, such as Guillain-Barré syndrome (Allos, 1997), reactive arthritis (Hannu *et al.*, 2002), and bacteraemia (mainly in immune-compromised patients). While infection can occur in patients of all ages, it is more prevalent in young children and young adults than in other age groups (Tauxe, 1992).

The epidemiology of human campylobacteriosis is complex, with numerous reservoirs and potential sources of infection due to the high adaptability of *Campylobacter* to asymptomatically colonize the intestinal tract of warm-blooded animals (Skelly and Weinstein, 2003). Infection occurs through the faecal-oral route mainly by the consumption of contaminated food

or water, but humans can also acquire the infection by accidental oral ingestion of the bacteria through contact with animals, people and the environment. Poultry is considered the principal reservoir and source of human infection (Newell and Fearnley, 2003). However, increasing evidence suggests that the contribution of ruminant *Campylobacter* to campylobacteriosis in humans is also considerable. In fact, source attribution studies suggested that the most frequent source of human infection varies depending on residency location. The majority of recorded cases in urban areas were infected with chicken-associated genotypes, while genotypes related to ruminants predominated in cases from rural areas. Thus, urban cases are principally attributed to poultry meat consumption whereas ruminant-associated infections in rural areas with high livestock density are mainly acquired through faecal contamination of the environment (Mullner *et al.*, 2010; Mughini Gras *et al.*, 2012).

2.1.4. *Campylobacter* in animals

The intestinal tract of warm-blooded animals is the primary habitat of thermophilic campylobacters due to their growing and multiplication requirements, and both *C. jejuni* and *C. coli* have been identified as normal colonizers of the guts of healthy livestock, wild animals and pets. *Campylobacter* has been isolated from a wide variety of hosts such as livestock (poultry, cattle, sheep, pigs), wild animals (deer, foxes, rabbits, and badgers) and even insects (flies, darkling beetles, and cockroaches), though the latter act as vectors rather than reservoirs (Newell and Fearnley, 2003). However, their most preferred hosts are avian species because their body temperature of 41–42 °C provides them with the optimal growth conditions. *C. jejuni* and *C. coli* can be considered commensal inhabitants of the gut of these animals and rarely cause disease. An exception is the increasing number of reported cases of abortion in sheep in the USA, mainly associated with a hypervirulent tetracycline-resistant *C. jejuni* clone (Wu *et al.*, 2014; Sahin *et al.*, 2017).

2.1.4.1. Poultry

According to Food and Agriculture Organization (FAO), poultry was the most widely produced meat in 2018 reaching 123.9 million tonnes (Food and Agriculture Organization of the United Nations – FAO, 2019). Poultry, defined as domestic fowl reared for their eggs, their meat, or both, includes chickens, turkeys, ducks, geese, guinea fowl, pheasants, quail and pigeons. Of them all, broiler chickens are by far the most consumed poultry meat. *C. jejuni* is frequently isolated from chickens and ingestion of as low as 35 colony-forming units (cfu) of this microorganism can be sufficient for successful colonization and establishment in the chicken caeca within 24 hours (Stern *et al.*, no date; Coward *et al.*, 2008). Since there is no clear evidence of vertical transmission, chicks are thought to be negative at hatching, but most flocks became *Campylobacter*-positive only at 2-4 weeks of age (Van Gerwe *et al.*, 2009) and once detected, almost the entire flock is colonized within several days (Stern *et al.*, 2001) and stays so until slaughter (Coward *et al.*, 2008). The reason for the initial colonization in the second week of age is unclear, but it was suggested that presence of maternally-derived antibodies in young chicks might protect them and influence the onset of colonization (Sahin *et al.*, 2003). Nevertheless, younger chicks might be colonized under high environmental exposure pressure (Cawthraw and Newell, 2010). Transmission between animals is primarily horizontal as shedding birds contaminate the litter and their coprophagic behaviour contributes to the infection of other chickens. Contaminated feed and water may be sources of recurring infections (Van Gerwe *et al.*, 2009). Co-colonization of *C. jejuni* and *C. coli* within one flock is frequent, as it is multi-strain presence (Petersen, Nielsen and On, 2001).

High carriage load of *Campylobacter* in broiler chickens (generally around 10^6 to 10^8 cfu/g of caeca (Beery, Hugdahl and Doyle, 1988) and high flock prevalence (approximately 70% flocks in the EU (EFSA, 2010) have been

reported). Furthermore, due to the large-scale mechanized processing during slaughtering and carcass handling, the carcasses of non-contaminated birds can easily become cross-contaminated during the process (Althaus, Zweifel and Stephan, 2017). Therefore, even though *Campylobacter* is not able to grow and multiply outside the host, contamination levels in the meat that reach the consumer are still high and pose an important risk for human infection, either by consumption of undercooked meat, or by cross-contamination of other ready-to-eat products through handling of the contaminated raw meat. This would explain why poultry is recognized as the most important source of infection in human *campylobacteriosis* (Silva *et al.*, 2011) and it is estimated to be responsible for up to 80% of human campylobacteriosis cases (EFSA Panel on Biological Hazards (BIOHAZ), 2010).

Campylobacter can also be frequently found on the shells of table eggs mainly due to contamination with faecal material during oviposition. However, the risk of transmission through this via is rare because despite the capacity of *Campylobacter* to penetrate the eggshell, its survival is compromised at room temperature or lower. Besides, albumin has many protective enzymes with antimicrobial properties such as lysozyme, avidin, ovoflavoprotein, and ovotransferrin that make the environment unfavourable for microbial growth (Cogan *et al.*, 2001) and *Campylobacter* was never isolated from the yolk (Fonseca *et al.*, 2014). Still, contamination of unpasteurized egg-content derivatives has been reported, probably caused by cracking of the eggshell during processing rather than being the result of the colonization of the egg content itself (Messelhäusser *et al.*, 2011).

Campylobacter is a highly adapted microorganism that can survive in a wide range of environmental conditions. This may explain the successful transmissibility of the bacteria despite its fragility outside the host. It has been observed that strains may lose virulence and consequently reduce their colonization potential after repeated laboratory passages compared to wild-

type isolates. Interestingly, the colonization ability can be recovered after *in-vivo* passage through the chicken gut. The laboratory conditions may reflect environments outside the host where the strains might presumably loose colonization potential. However, once the bacteria are ingested by chickens and pass the favourable gut environment their colonization ability is enhanced and these now efficient colonizers can rapidly spread in the flock (Cawthraw *et al.*, 1996).

2.1.4.2. Ruminant livestock (cattle and sheep)

In ruminant husbandry, two species of *Campylobacter*, *C. jejuni* and *C. fetus,* are known to cause reproductive problems such as abortions and infertility and are therefore particularly important in the health status of the herd. Nevertheless, thermophilic campylobacters are also commonly detected in the intestinal tract of healthy ruminants (Stanley and Jones, 2003).

Campylobacter shedding in cattle is significant, and different studies have reported herd prevalence rates ranging from 23% to close to 90% (Kaakoush *et al.*, 2015). Unlike in poultry, where most animals within positive flocks are colonized, within-herd prevalence in cattle varies across farms and the proportion of individual shedder animals per herd ranges around 40-60% (Wesley *et al.*, 2000; Besser *et al.*, 2005; Milnes *et al.*, 2008). It has been suggested that different factors might account for those differences. For example, higher *Campylobacter* prevalence was reported in animals kept in feedlots compared to those raised on pastures (Garcia *et al.*, 1985; Bailey *et al.*, 2003). Similarly, prevalence is often higher in dairy cattle than in beef cattle, possibly due to the longer productive span before going to slaughter, exposing animals to a longer period of reinfections (Stanley and Jones, 2003). Other influencing factors may be herd size/stocking density, season, age, sample site, isolation method, geography and diet (Garcia *et al.*, 1985; Bailey *et al.*, 2003; Vanselow, Hornitzky and Bailey, 2006). Incidence of *Campylobacter* infection in sheep herds is also notable, though lower than in cattle. Another difference

with cattle is the predominance of the different *Campylobacter* species. Surveys in sheep farms revealed that *C. coli* is the dominant species after *C. jejuni*, in contrast to cattle farms where *C. jejuni* predominates (Wesley *et al.*, 2000; Oporto *et al.*, 2007; Fernández and Hitschfeld, 2009; Sheppard *et al.*, 2009; Roux *et al.*, 2013). Animals can be infected through contaminated pastures, feed or water supplies. Wildlife, specially avian hosts, are also believed to play an important role in the transmission of *Campylobacter* to domestic livestock, by promoting their persistence in the environment and disseminating the bacteria to other farms (Sanad *et al.*, 2013). Insects (flies, beetles and cockroaches) can also act as vectors ((Sproston *et al.*, 2010) and cited in (Newell and Fearnley, 2003)).

Source attribution studies have identified cattle as the second leading source of *C. jejuni* infections in humans (Skarp, Hänninen and Rautelin, 2016; Thépault *et al.*, 2018), and sheep, along with chicken, as the main sources for human *C. coli* infections (Sheppard *et al.*, 2009; Roux *et al.*, 2013). However, the importance of cattle and sheep as potential risks for human campylobacteriosis is not only attributed to direct contamination of food products like milk at the farm or carcass meat at slaughter, but also through indirect contamination of fresh products with surface and sub-surface water after disposal of animal slurries and abattoir effluents to land (Clark *et al.*, 2003) or direct contact with animals (Mullner *et al.*, 2009; Sheppard *et al.*, 2009). Although red meat can be contaminated with faecal material during slaughter, risk and level of carcass contamination is significantly lower in large animals than in poultry slaughtering, and contaminated raw milk is a more frequent via of food-borne infection than consumption of red meat in the case of ruminants.

2.1.5. Antimicrobial resistance (AMR) of *Campylobacter*

The discovery of antimicrobials was one of the greatest medical breakthroughs in human history. Unfortunately, microorganisms have evolved and have managed to develop mechanisms to resist them resulting in ineffective treatments. As for today, antimicrobial resistance (AMR) is considered one of the most important threats for Public Health worldwide. The pace of microorganisms developing resistance to antimicrobials is higher than the discovery of novel treatments and nowadays many bacteria are resistant to several (multi-resistant), and sometimes all (pan-resistant) antimicrobials available for treatment. Consequently, if the trend continues, the resources to fight resistant microorganisms will soon be depleted and many infectious diseases may one day become uncontrollable. It is estimated that by 2050, 10 million people would die each year due to resistant microorganisms if the problem is not tackled (O'Neill, 2014).

AMR results from the selective pressure that antimicrobials put on microbes' population; by essentially eliminating susceptible strains and empowering the proliferation of those populations capable to survive in the presence of certain levels of the drug. Resistance to antimicrobials can occur through several mechanisms like permeability changes in bacterial cell wall which restricts antimicrobial access to target sites, active efflux of the antibiotic from the microbial cell, enzymatic modifications and/or degradation of the antimicrobial agent, activation of alternative metabolic pathways to those inhibited by the drug, alteration of the antimicrobial targets and overproduction of the target enzyme (van Hoek *et al.*, 2011). Resistance occurs through genetic changes via either spontaneous gene level single nucleotide polymorphism (SNP) mutations or horizontal transfer of resistance genes from other bacteria. Nevertheless, the ability of bacteria to transfer genetic material between each other while sharing common environments, like the gastrointestinal tract of the host (human or animal) or the soil, accelerates the

spread of the resistance once the mutation occurred. Furthermore, bacteria may adopt multiple resistance traits over time and become resistant to multiple classes of antimicrobials, referred to as multi-drug resistant (MDR) strains. Transmission of co-resistance to several antimicrobials is favoured by the ability to transfer large fragments of genetic material.

Inappropriate use of antimicrobial agents, whether by abusive use, subtherapeutic dosage or incomplete treatment course, contributes to the spread of AMR (Engberg *et al.*, 2001; Nelson *et al.*, 2004). Although the selective pressure associated to the overuse of antimicrobials in humans accelerates the emergence and spread of resistance, there is accumulating evidence that veterinary use of antimicrobials also contributes to the development of AMR (Collignon, 2005). Therapeutic or prophylactic use of antimicrobial agents in livestock farming can incite a selective pressure on microorganisms regardless if they are commensals, pathogens or environmental strains, and reach human population through the food chain (van den Bogaard and Stobberingh, 2000). For example, authorization of the fluoroquinolone-analog enrofloxacin for poultry production was linked to the sharp increase in the prevalence of ciprofloxacin resistance among *C. jejuni* isolates from human infections in USA and Europe (Endtz *et al.*, 1991; Collignon, 2005). In contrast, fluoroquinolones were never used in Australia in animal production, and fluoroquinolone-resistant *Campylobacter* were not detected in animals in 2014, which was associated to the relatively low rate of fluoroquinolones resistance in human *C. jejuni* clinical isolates (Unicomb *et al.*, 2006; Cheng *et al.*, 2012; Shaban *et al.*, 2014).

In the case of campylobacteriosis, most infections are self-limiting, but antimicrobial treatment is necessary for some severe and complicated cases. Macrolides, fluoroquinolones, tetracycline and gentamicin can be used but the agents of choice are macrolides, like erythromycin, and fluoroquinolones, like ciprofloxacin. However, increasing emergence of *Campylobacter* strains

resistant to those antimicrobial agents has been observed over the past two decades (Luangtongkum *et al.*, 2009; Wieczorek and Osek, 2013). The increase in the number of highly resistant *Campylobacter* strains not only compromises therapeutic efficacy, but these bacteria would also act as reservoirs of resistance genes in the gut of animals. Resistance to fluoroquinolones is worrisome and has led the World Health Organization (WHO) to list *Campylobacter* spp. among the six high priority antimicrobial resistant pathogens in 2017 (World Health Organization - WHO, 2017). Resistance to tetracyclines is also highly present, but resistance to macrolides remains generally low (Ge *et al.*, 2013).

AMR in bacterial isolates can be evaluated by phenotypic (growth-based) and genotypic (molecular-based) methods. Phenotypic methods assess the growth of bacterial cells in the presence of the antimicrobial of interest and are commonly known as antimicrobial susceptibility tests (AST). Among AST methods, broth microdilution method is considered to be the gold standard. This technique tests a suspension of pure bacterium culture of a predetermined concentration against varying concentrations of an antimicrobial agent, usually in two-fold dilution series. Following incubation, tubes are examined for visible bacterial growth as evidenced by turbidity. This method provides quantitative information in the form of Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of an antimicrobial agent (in mg/L) that, under defined *in vitro* conditions, prevents the appearance of visible growth of a microorganism within a defined period of time (European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID), 2003). MIC values are then interpreted using clinical cut-off values to assess clinical resistance or epidemiological breakpoints (ECOFF) to define microbiological resistance (e.g., established by European Committee for Antimicrobial Susceptibility Testing – EUCAST, http://www.eucast.org – and/or Clinical and Laboratory Standards Institute – CLSI), http://clsi.org/). Whereas clinical resistance is needed to take

decisions regarding antibiotic therapy, microbiological resistance defined by ECOFF values is used in surveillance studies to identify isolates that differ from the wild-type population and possess acquired or mutational AMR (Kahlmeter *et al.*, 2003). Genotypic methods attempt to detect the presence of the specific resistance genes or genetic mutations underlying the genetic mechanisms for AMR using molecular techniques (usually DNA-based). In *Campylobacter* spp., the main mechanism for fluoroquinolones resistance is mainly due to a single point mutation in the DNA gyrase gene *gyr*A (C257T) that results in the change of codon 86 from threonine to isoleucine (Thr-86-Ile). Main molecular mechanisms underlying high-level resistance to macrolides are mediated through multiple point mutations in the peptidyl transferase region in domain V of the 23S ribosomal RNA (rRNA) gene (A2074G, A2074C, and A2075G) (Alfredson and Korolik, 2007). A commonly used method to detect these mutations is real-time Polymerase chain reaction (PCR) using specific probes that allow the detection of the SNPs associated to each mutation allele (Oporto, Juste and Hurtado, 2009). This genotypic approach is faster, but susceptibility has to be validated with phenotypic tests to determine that a particular isolate expresses the resistance.

2.2. Chicken gut microbiota

Compared to mammals, the gastrointestinal tract (GIT) of chicken is proportionally shorter in extension and presents shorter transit digestion times. Still, chickens are very efficient in converting consumed feed into meat (Tolkamp *et al.*, 2010). The extraction of energy and nutrients from food is not solely on account of the physiology of the host but it is also attributed to the symbiotic interactions with microorganisms that reside in the GIT. The chicken GIT is a complex ecological niche colonized by millions of microorganisms. This complex microbial community, defined as gut microbiota, is composed of commensal, symbiotic and pathogenic

microorganisms that co-exist in close association with the host and their importance in vital functions is such that it can be considered as an additional organ (O'Hara and Shanahan, 2006). In the gut microbiota, the three domains of life (Archaea, Bacteria and Eukarya) and Virus coexist in close interaction with the host, being bacteria the predominant member. With an estimated bacterial density ranging from 10^7-10^{11} bacteria/g of digesta, it is considered as one of the highest cell densities for any ecosystem (Apajalahti, Kettunen and Graham, 2004). Furthermore, the collective gut microbial genome, namely gut microbiome, encodes metabolic pathways with a potential capacity that surpasses that of the host's genome. Thus, the extensive studies available to date on chicken microbiota focused principally on the investigation of the bacterial population in the gut.

2.2.1. Functions of gut microbiota

Within the GIT, continuous interactions occur between host cells, gut microbiota and digesta, and these interactions highlight the exceptionally essential role of gut microbiota in the host health. For example, gut microbiota has been proved to confer protection against pathogenic microbes, promote beneficial effects in the development of the intestinal morphology and immunology, facilitate feed digestion and absorption of otherwise not digestible nutrients by the host, breakdown toxic compounds, and even provide beneficial metabolic products (Oakley, Lillehoj, *et al.*, 2014; Mohd Shaufi *et al.*, 2015; Waite and Taylor, 2015).

A balanced gut microbial community provides a protective mechanism that might prevent overgrowth of pathogenic bacteria by what is known as competitive exclusion. Gut microbiota that settles first in the epithelium will compete for space and nutrients with potential pathogens, might contribute to create an environmental atmosphere not suitable for potential pathogens or even inhibit their growth by the production of antimicrobial compounds like

for example bacteriocins (Servin, 2004; Schneitz, 2005; Garcia-Gutierrez *et al.*, 2019). Several studies have demonstrated the efficacy of competitive exclusion in conferring protection against enteric pathogens in young chicks as reviewed elsewhere (Schleifer, 1985; Mead, 2000).

Microbiota influences the maturation and functioning of the intestinal epithelium by promoting cell proliferation and differentiation, triggering enzyme and hormone secretions and stimulating the maintenance of the intestinal barrier integrity. For example, *Bacteroides thetaiotaomicron* stimulates the secretion of a protein necessary for the maintenance of desmosomes in the epithelial villus and the bacterial peptidoglycan favours the reinforcement of the tight junctions of the epithelial barrier by activating the signalling of Toll-like receptor 2 (TLR2) (Cario, Gerken and Podolsky, 2007; Lutgendorff, Akkermans and Söderholm, 2008). Studies on germfree chicks showed a reduced intestinal motility, lower body temperature and a poorly developed immune system compared to normal chicken, but their overall health status improved after the administration of normal microbiota (Niba *et al.*, 2009).

Gut microbiota is thought to stimulate immunological mechanisms by activating base level of inflammation, and influence the development of the cellular and humoral immune systems during early life (Cebra, 1999). Experimentally lacking gut microbiota animals are more prone to develop diseases and, compared to their wild type equivalents, generally exhibit deficiencies in their immunological mechanisms such as slower development of lymphoid cells or poorer development of secondary lymphoid tissues (Hooper, 2004).

Gut microbiota exhibits large metabolic activity, playing an important role in the host metabolism. By metabolizing nutrient substrates, gut microbiota produces large amounts of metabolites that aid the host in achieving energy requirements. For example, in the fermentation of

carbohydrates, certain groups of bacteria are responsible for breaking down complex substrates such as non-starch polysaccharides (NSPs) which requires very specialized hydrolytic enzymes (Józefiak, Rutkowski and Martin, 2004). Subsequently, the resulting metabolites will be available to other members of the microbiota to produce amino acids and short chain fatty acids (SCFA - primarily acetate, propionate, and butyrate) through further fermentation, these compounds becoming readily accessible for the host. These SCFA are an important energy source for epithelial cells and have anti-inflammatory and anti-oxidative effects. Certain vitamins that cannot be synthetized by the host like vitamin K and vitamin B, can be provided by members of the gut microbiota, and deficiency in vitamin K and B has been reported in animal models with absence of gut microbiota (Montalto *et al.*, 2009). By contributing in the breaking down of nutrients not digestible to the host and thus providing available nutrients, gut microbiota influences in the growth rates and performance of the host.

Nevertheless, gut microbiota can also exert certain negative effects such as competition for substrates or production of potentially harmful metabolites. For example, proteolytic fermentation by microbiota may result in the production of potentially harmful compounds such as ammonia, indoles and phenols. Additionally, the ability of certain groups of microorganism to metabolize drugs can compromise their effectivity (Swanson, 2015).

2.2.2. Factors influencing gut microbiota

Just like other ecosystems, the composition of the microbiota can be evaluated by estimating its biodiversity. However, it varies greatly depending on the anatomical site, age, breed, diet, husbandry, etc.

2.2.2.1. Spatial distribution of gut microbiota

Despite its broad diversity, chicken gut microbiota is predominantly associated to three most abundant phyla: Proteobacteria, Bacteroidetes and Firmicutes. However, their distribution varies extensively along the GIT, which sequentially consists of beak, oesophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, colon, rectum and cloaca. Furthermore, within the same area, microbiota may vary between mucosal and luminal content (Awad *et al.*, 2016). The initial segments of the GIT harbour low microbiota diversity and the microbial community of the GIT becomes more diverse as we go further to distal sections. The different characteristics of each region such as pH, digesta transit time, availability of substrates, etc., provide different environments that favour certain type of bacteria and consequently, establishment of differentiated microbial populations.

For instance, the crop and gizzard where feed is temporally stored, fermented, and mechanically grinded, have low pH and their microbiota is mainly dominated by *Lactobacillus* species, lactic acid producing bacteria (Witzig *et al.*, 2015; Borda-Molina *et al.*, 2016). The small intestine (duodenum, jejunum and ileum), where most part of nutrient enzymatic digestion and absorption occurs, is mainly colonized by *Lactobacillus*, *Enterococcus* and members of Clostridiaceae family. The presence in the duodenum of various enzymes and high concentrations of antimicrobial compounds such as bile salts coupled with the rapid passage of digesta through it, make its microbiota less rich and diverse compared to caecum or colon. It is noteworthy that for several reasons described below, caeca are by far the most studied GIT section in chickens. Caeca, situated immediately in the ileocecal junction, are more developed in poultry than in most other animals and are present in the form of two enlarged sacks that branch out forming two separate blind ended compartments. The caecum is the site of highest density and diversity of microbial population (Stanley, Hughes and Moore, 2014; Mohd Shaufi *et al.*,

2015), with studies reporting bacterial densities up to 10^{10}-10^{11} cells per gram of content. The longest retention time of digesta occurs in the caeca, allowing longer availability of fermentable substrates for different microorganisms to metabolize (12-20 hours) (Gong *et al.*, 2007; Rehman *et al.*, 2007; Yeoman *et al.*, 2012). Caecum is also the site where other important processes like recycling of urea, water regulation and fermentations mainly take place (Clench and Mathias', 1995; Sergeant *et al.*, 2014; Waite and Taylor, 2014). Furthermore, SCFAs are found at higher concentrations in the caeca than in other regions of the GIT (Józefiak, Rutkowski and Martin, 2004).

2.2.2.2. Temporal/successional development of gut microbiota

The GIT microbiota is a dynamic ecosystem that evolves over time. Through the productive lifespan of a commercial broiler (typically 42 days) the taxonomic composition of the gut microbiota changes significantly. It undergoes rapid changes in the early growing phase by gradually increasing in richness and diversity over time, characterized by a successional development. As the host grows different groups of bacteria emerge, some are replaced and disappear while others, namely core microbiota, settle and remain throughout their life (Lu *et al.*, 2003; Oakley, Buhr, *et al.*, 2014; Oakley and Kogut, 2016). Newly hatched chicks in commercial hatcheries, unlike mammals, have no further contact with adult hens' microbiota because the eggs are washed or fumigated in the incubator. Therefore, the initial colonization of their digestive tract is highly dependent on the microbiota present in the hatchery, transport or in the housing environment. Nevertheless, there is evidence that embryonic colonization does occur as 1-day-old chickens have been reported to already carry microorganisms in their digestive track possibly being acquired from the oviduct of the hen (Ballou *et al.*, 2016; Ding *et al.*, 2017). It has been suggested that early life microbiota is dominantly colonized by facultative anaerobic species and as these bacteria metabolize the available oxygen an anaerobic environment in the gut atmosphere is created and, subsequently, the anaerobic

species gradually become the predominant bacteria (Wise and Siragusa, 2007). Young birds harbour more Proteobacteria particularly of the family Enterobacteriaceae, while Firmicutes and Bacteroidetes dominance is observed as the chickens age (Lu *et al.*, 2003; Awad *et al.*, 2016).

2.2.2.3. Other factors influencing gut microbiota

Microbiota composition can be influenced by different dietary factors such as diet components, concentrations of nutrients (carbohydrate, fat, protein, water, minerals, vitamins and fibre), physical characteristics/structure of feed (type of grain, particle size) or feed supplementations (dietary enzymes, essential oils, prebiotics and probiotics) (Pan and Yu, 2014). However, the exact mechanisms of how diet induces changes in the gut microbiota profile and the metabolism changes that possibly influence the chicken health and productive performance are still unclear.

Rearing environment affects the establishment of gut microbial composition. The same chicken breed/line when raised in different husbandry practices might differ in microbiota composition. Studies comparing indoors versus free-range husbandry management of meat chickens evidenced higher microbiota diversity in the latter (Mancabelli *et al.*, 2016; Xu *et al.*, 2016). Chickens continuously acquire microorganisms from the surrounding environment, and their soil pecking behaviour facilitates the uptake of the complex microorganisms residing in the litter. Different studies evidenced the influence of litter management practices in the colonization patterns of the chicken GIT. Reusing litter is a common practice to reduce production costs and the frequency of its disposal (Coufal *et al.*, 2006). Chickens raised in reused litter showed higher bacterial diversity when compared to those kept in fresh litter (Wang, Lilburn and Yu, 2016).

Other factors, inherent to the host, like breed/line, gender and genotype, play an important role in shaping the chicken gut microbiota

(Lumpkins, Batal and Lee, 2010; Zhao *et al.*, 2013; Kim *et al.*, 2015). Though exhibiting similar microbial community patterns, some degree of individual variance in caecal microbial community composition in chickens has been observed in chickens of the same age, sex and breed reared together in the same conditions and receiving the same feed. (Van Der Wielen *et al.*, 2002; Stanley *et al.*, 2013; Sergeant *et al.*, 2014; Borda-Molina *et al.*, 2016).

2.2.3. Dysbacteriosis

Gut microbiota is dynamic but plastic, meaning that it can recover its community structure despite rapid changes in the GIT environment. Nevertheless, prolonged microbial diversity disturbance, also known as dysbiosis or dysbacteriosis, can increase susceptibility to diseases by the overgrowth of potentially harmful microorganisms and following infections (Chan, Estaki and Gibson, 2013). This condition imposes significant loss of overall host health, productive performance, animal well-being and subsequent economic loss and, in case of zoonotic pathogens, threat food safety.

2.2.4. Molecular tools for the characterization of microbiota

During long periods of time, our perception of the microbiota was limited to those microorganisms that could be isolated on growth media by labour-intensive culture-based methods. With the advent of culture-independent approaches like molecular profiling, it is now known that the majority of microorganisms inhabiting the gut is uncultivable by currently available cultivation techniques, partly related to limitations in isolating the mostly anaerobic gut bacteria (Rappé and Giovannoni, 2003). Furthermore, microbiome studies have demonstrated that a significant number of the sequences recovered from the gut belonged to not-yet-identified bacterial species (Zhu *et al.*, 2002). The past decade has been widely acknowledged by the scientific community as the decade of genome research, marked by the rapid advances in sequencing technologies, and along with it, the generation of

sequencing data at unprecedented pace, the development of modern computational/bioinformatics tools and the development of powerful computer hardware to deal with big data analysis (Pareek, Smoczynski and Tretyn, 2011). In particular, the development of high throughput next-generation sequencing (HT-NGS) technologies and the astonishing decreases in the costs of sequencing have enabled a vaster and more comprehensive vision on the complexity of the intestinal microbiota of chickens (Diaz-Sanchez *et al.*, 2013; Waite and Taylor, 2015). Among these HT-NGS technologies, DNA-based sequencing approaches that can be applied in the taxonomic profiling of the microbiome include targeted amplicon sequencing and shotgun metagenomic sequencing. With these techniques, the community profile of the gut microbiota is achieved by deciphering the sequence data stored in their DNA.

The most popular approach to characterize microbiota taxonomic composition is targeted amplicon sequencing, which consists of sequencing a target gene. Ideally, this marker gene must be present in all of the microorganisms of interest, highly conserved and evolutionarily stable. For example, the gene that encodes the small subunits of the ribosomal RNA (SSU rRNA) is considered a universal target for taxonomic classification of organisms, of which 16S rRNA and 18S rRNA are widely used for prokaryotic and eukaryotic microorganisms' classification, respectively. The 16S rRNA gene is universally present in bacteria and archaea, it is small in size (*ca.* 1.5 Kb) and is highly conserved with nine highly hypervariable regions (V1-V9) that allow differentiation between species. These nine hypervariable regions are flanked by conserved regions which can be used as targets for PCR amplification (Woese, 1987; Poretsky *et al.*, 2014). Most HT-NGS research in latest years has been performed on the Illumina MiSeq platform due to its cost-effective value, achieving high numbers of reads at lower cost than its competitors. This platform can provide reliable high-quality reads, but it can only sequence 250 and 230 base pair (bp) fragments for the forward and reverse reads,

respectively. Therefore, the taxonomic analysis is limited to the identification at the genus level at best because only one or two hypervariable regions can be sequenced.

The other popular DNA based HT-NGS approach for community profiling is shotgun metagenomics. The term shotgun refers to the strategy that involves fragmenting the DNA and sequencing the resulting small pieces. Direct sequencing prevents PCR biases and it provides classification and identification to a finer taxonomic level than targeted amplicon sequencing because it sequences the entire genome rather than a marker gene. For the same reason, this approach also offers the possibility to reveal information about functional potential of the microbiota studied (potential gene functions and metabolic pathways) and to recover whole genome sequences. However, shotgun metagenomics is still beyond the reach of most studies due to the high costs and the requirement of specialized computational facilities and bioinformatics expertise. Therefore, despite the limitations of 16S rRNA amplicon sequencing with regard to shotgun metagenomics, it can provide very valuable information of the microbial community biodiversity in a given sample and its cost-effective nature makes it a worthy choice for the analysis of large numbers of complex microbiota samples as a key step in understanding their microbial community structure. For example, it can be used to document biodiversity of unexplored microbiota or as a screening method before applying the highly priced and computationally intensive shotgun metagenomics for a deeper investigation. In fact, 16S rRNA amplicon sequencing is the most widely used method for microbiome profiling at the time of this study (Ju and Zhang, 2015; Borda-Molina, Seifert and Camarinha-Silva, 2018).

After the amplicon sequencing data has been generated, computational tools are employed to process the raw data. Bioinformatic tools are used to create readable tables containing taxonomic information associated to the

sequencing reads along with their respective abundance for each sample. These tables will be further analysed with different statistical approaches to answer the research questions raised. Bioinformatic analyses usually include the following steps: trimming of the adaptors and primers, quality assessment of the amplicon reads, filtering and trimming of the reads according to quality parameters chosen, merging of overlapping paired-end reads, clustering of similar sequences (on the basis of homology of the reads) according to a certain threshold to obtain representative sequence, dereplication of the clustered sequence to reduce runtime for subsequent analysis steps, removal of chimeras (chimeric sequences), taxonomic assignment by aligning each query sequence against a reference database and the construction of tables that contain information about the abundance (counts) of the representative sequence for each sample and taxonomic classification of the sequences detected. Then, with this table, comprehensive analysis of microbiome diversity can be conducted using various statistical calculations, to derive corresponding conclusions regarding community composition relationships with the metadata of the samples.

Special consideration should be given to how similar the sequences ought to be in the variable regions of choice to be considered as belonging to the same species. In the analysis of amplicon sequencing data, different approaches can be used to perform the clustering of the sequences to infer the smallest taxonomic unit of analysis. The customarily used Operational Taxonomic Unit (OTU) clustering, clusters sequences according to a fixed sequence similarity threshold, most commonly 97% (Westcott and Schloss, 2015). One reason for the chosen homology threshold is derived from the conventional wisdom that 97% homology corresponds approximately to the species definition (Stackebrandt *et al.*, 1994) and it is supported by an empirical study that showed that most strains within the species taxa shared ≥97% 16S rRNA sequence homology (Konstantinidis and Tiedje, 2005). Another reason is the attempt to reduce the consequence of PCR amplification

or sequencing errors by grouping errors together with the most represented sequence (considered as error-free sequence) (Eren, Sogin and Maignien, 2016). However, this method overestimates the number of taxa present as demonstrated in mock communities (Kopylova *et al.*, 2016). Therefore, denoising algorithms have been developed in recent years to resolve the error rates of Illumina amplicon data without imposing the arbitrary similarity thresholds that define OTUs. Differentiation of sequences by as little as one nucleotide was made more reliable and the term Amplicon Sequence Variants (ASVs) was proposed to replace OTUs (Callahan, McMurdie and Holmes, 2017).

Each of the bioinformatics steps in the analysis of data usually involves separate programs/algorithms and requires moderate knowledge of programming language in Linux/Unix. This is challenging for most researchers and may cause analytical bottlenecks. To cope with this issue, a growing number of bioinformatics programs has been created in the attempt to compile the different processes in the working procedures and some even incorporate multivariate statistics that can produce publishable tables and figures. By the time this thesis is published, there are only a handful of the so-called user-friendly tools available and include QIIME (Caporaso *et al.*, 2010), MOTHUR (Schloss *et al.*, 2009), MG-RAST (Meyer *et al.*, 2008), w.A.T.E.R.S (Hartman *et al.*, 2010), and RDP pyrosequencing tools (Cole *et al.*, 2009), among others. Still, certain familiarity with programming language is required.

2.2.5. Modulation of gut microbiota

The chicken meat industry has long relied on the use of antibiotic growth promoters (AGP) administered in chicken feed to improve feed conversion ratios and animal weight gain and reduce morbidity and mortality due to clinical and subclinical infections. It is believed that the benefits observed are in part due to the modulation of the host immune response

subsequent to the reduction of the total GIT bacterial load and suppression of potential pathogens (Feighner and Dashkevicz, 1987). However, the increasing spread of AMR and the associated threats to Public Health have prompted governments to limit its use at farm level. Since the prohibition on the use of AGP in livestock production in the EU (Castanon, 2007), alternative strategies have been investigated to achieve similar effects without the AGP-associated negative impacts, and interest in the modulation of gut microbiota in animal production has noticeably increased. The different approaches include the use of different feed additives such as prebiotics, probiotics, organic acids, exogenous enzymes and plant-based essential oils (Yang, Iji and Choct, 2009).

Prebiotics are compounds that pass undigested through the proximal parts of the GIT and can stimulate the growth or activity of beneficial bacteria that colonize the hindgut, thus improving host physiology (Gibson and Roberfroid, 1995). Most prebiotics belong to the categories of the so-called NSPs such as galactooligosaccharides (GOS), fructooligosaccharides (FOS), raffinose family oligosaccharides (RFOs) and mannanoligosaccharides (MOS). They are indigestible to the host but intestinal microbiota can break them down to produce SCFA like propionate, acetate and butyrate (Józefiak, Kaczmarek and Rutkowski, 2008). Probiotics are defined as viable microorganisms used as a feed supplement, which provide proven beneficial effects on health when administered in adequate amounts (Koleilat, 2012), with the ability to promote a good balance of GIT microbial populations (Holzapfel *et al.*, 2001). The benefits of the addition of prebiotics and probiotics in feed in terms of inducing changes of the GIT microbiota have been reviewed extensively in several publications (Stanley, Hughes and Moore, 2014; Ducatelle *et al.*, 2015; Pourabedin and Zhao, 2015). Organic acids and their salts have gained popularity due to their nutritional value and antimicrobial properties that can elicit positive effects in growth performance. They can be present as simple monocarboxylic acids (formic, acetic, propionic and butyric acids), carboxylic acids with hydroxyl group (lactic, malic, tartaric

and citric acid) or short chain carboxylic acids containing double bonds (fumaric and sorbic acids), and they are widely distributed in nature as normal components of plants or animal tissues. Organic acids are sometimes found as their sodium, potassium or calcium salts, which are the form of choice in the feed-manufacturing process because they are more stable, odourless and easier to handle than the more volatile acid form (Huyghebaert, Ducatelle and Immerseel, 2011). The effects of these compounds on enteric diseases, gastrointestinal tract integrity, nutrient digestibility, immunity and performance in chickens have been comprehensively reviewed (Khan and Iqbal, 2016). The reduction of gut colonization by food-borne pathogens is one of the expected effects of microbiota modulation in food animals. Increased levels of volatile fatty acid and the subsequent low pH reached in the gut following prebiotics or organic acids administration might exert bactericidal effects on potentially pathogenic bacteria such as *Campylobacter* (Gharib Naseri, Rahimi and Khaki, 2012).

3. Methodology and Results

3.1. Study I

Occurrence of *Campylobacter jejuni* and *Campylobacter coli* in cattle and sheep in Northern Spain and changes in antimicrobial resistance in two studies 10-years apart.

Ocejo, M., Oporto, B., Hurtado, A.

Submitted

Abstract

A cross-sectional survey was conducted in 2014-2016 in 301 ruminant herds to estimate *C. jejuni* and *C. coli* prevalence and investigate their susceptibility to antimicrobials. Risk of shedding *C. jejuni* was higher in cattle than sheep (81.2% vs. 45.2%; OR_{adj}=5.22, p<0.001), whereas risk of shedding *C. coli* was higher in sheep than in cattle (19.1% vs. 11.3%; OR_{adj}=1.71, p=0.128). Susceptibility to 6 antimicrobials was determined by broth microdilution using EUCAST epidemiological cut-off values. *C. coli* exhibited higher resistance (94.1%, 32/34) than *C. jejuni* (65.1%, 71/109), and resistance was more widespread in isolates from dairy cattle than beef cattle or sheep. Compared to results obtained 10-years earlier (2003-2005) in a similar survey, an increase in fluoroquinolone-resistance was observed in *C. jejuni* from beef cattle (32.0% to 61.9%; OR=3.45, p=0.020), and a decrease in tetracycline-resistance in *C. jejuni* from dairy cattle (75.0% to 43.2%; OR=0.25, p=0.026). Resistance to macrolides remained stable at low rates and restricted to *C. coli* from dairy cattle, with all macrolide-resistant *C. coli* showing a pattern of pan-resistance. Presence of the SNPs associated to quinolone and macrolide resistance was confirmed in all phenotypically resistant isolates. The increase in fluoroquinolones resistance is worrisome but susceptibility to macrolides is reassuring.

Keywords: *Campylobacter jejuni*; *Campylobacter coli*; cattle; sheep; antimicrobial resistance (AMR); Minimum Inhibitory Concentration (MIC); Single Nucleotide Polymorphism (SNP).

3.1.1. Introduction

Campylobacter is the main cause of food-borne gastroenteritis in industrialized countries and the cause of the most frequently reported zoonosis in the European Union (EU). In 2017, 246,158 confirmed cases of campylobacteriosis in humans were reported in the EU, which accounted to an average notification rate of 64.8 per 100,000 population (EFSA and ECDC, 2018b). In the Basque Country, notification rate in the same year was 104.2/100,000 cases, mainly concentrating among young patients (40.6% in <5 years-old and 20.6% in 5-14 years-old) (Anon, 2018). The sporadic nature of *Campylobacter* infection causes underreporting and hampers the identification of the infection source (Havelaar *et al.*, 2013). Although poultry is the principal source for human infection, *Campylobacter* is also highly prevalent in ruminants worldwide, and there is increasing evidence that the contribution of ruminant *Campylobacter* to campylobacteriosis in humans is also considerable, with cattle being the second most important reservoir after broilers for *C. jejuni* human infection and sheep the first for *C. coli* infections in humans (Sheppard *et al.*, 2009; Roux *et al.*, 2013). Contamination of red meat is infrequent (Jacobs-Reitsma and Wagenaar, 2008) and does not seem to play a major risk for human infection, but contaminated raw milk is a frequent via of foodborne infection (Heuvelink *et al.*, 2009). In addition, *Campylobacter* from ruminant feces can contaminate water supplies and fresh products via agricultural run-off water (Clark *et al.*, 2003). Finally, humans can also acquire the infection by contact with animals. Hence, ruminants are important reservoirs for zoonotic campylobacters and source of contamination for the environment and other animals. Antimicrobial resistance is another subject of concern. Antimicrobial therapy is only recommended in systemic and severe *Campylobacter* infections or in immunocompromised patients. However, the emergence of *Campylobacter* strains resistant to the antimicrobial agents of choice (macrolides for laboratory-confirmed cases and fluoroquinolones for cases of diarrhea) compromises the therapeutic efficacy (Luangtongkum *et al.*, 2009; Wieczorek and Osek, 2013).

Despite the importance of ruminant *Campylobacter* in human campylobacteriosis (Mullner *et al.*, 2009; Mughini Gras *et al.*, 2012; Roux *et al.*, 2013), fewer studies have been conducted to estimate *Campylobacter* prevalence in ruminants (dairy cattle, beef cattle and sheep) and investigate their susceptibility to antimicrobials compared to the large number of studies carried out in poultry. In a previous study (2003-2006) carried out in ruminants in the Basque Country (Northern Spain), 62.1% of cattle herds and 55.0% of sheep flocks were *Campylobacter*-positive, identifying *C. jejuni* and *C. coli* in 21.8% and 5.9% of the farms, respectively (Oporto *et al.*, 2007). In that study, all thermotolerant *Campylobacter* species were targeted and only one isolate per positive farm was identified, so that species like *C. hyointestinalis* in cattle or *C. lanienae* in sheep contributed to the high prevalence of the genus *Campylobacter* (Oporto and Hurtado, 2011). A few *C. jejuni* isolates were then characterized for antimicrobial resistance showing high levels of resistance to tetracyclines and quinolones (Oporto, Juste and Hurtado, 2009). Now, 10 years later (2014-2016), a similar number of farms were surveyed but targeting only the main zoonotic species and performing a more exhaustive analysis of isolates with the following objectives: i) to update herd-level prevalence estimates of *C. jejuni* and *C. coli* in ruminant herds in the Basque Country; ii) to determine the antimicrobial resistance (AMR) profiles of *C. jejuni* and *C. coli*; iii) to compare antimicrobial resistance in *Campylobacter* isolated from ruminants in two studies carried out 10-years apart; iv) to investigate the potential of a highly discriminatory and rapid method for estimating the true prevalence of *Campylobacter* macrolide-resistance under an apparently low prevalence situation.

3.1.2. Materials and methods

Sampling design

A cross-sectional survey was carried out to estimate the prevalence of *C. jejuni* and *C. coli* in cattle herds and sheep flocks in the Basque Country (Northern Spain). Cattle included both dairy and beef herds, and sheep were of

the Latxa dairy breed. Details on general husbandry systems for beef cattle, dairy cattle and sheep in the region were reported elsewhere (Hurtado, Ocejo and Oporto, 2017). Briefly, while dairy cattle are mostly housed in pens, beef cattle and sheep are managed under a semi-intensive system where animals graze in farmland pastures in spring and part of the summer, and in communal mountain pastures from the middle of July until the end of November and are housed in winter. In all cases, animals of all ages are raised in the herd (suckler herds), and intensive feedlot systems are not used in the region. The census of beef cattle, dairy cattle and sheep farms was obtained from the Department of Agriculture of the Basque Government. The number of herds to sample was then calculated separately for each animal category for an expected herd prevalence of 50%, a 95% confidence level and an accuracy of 10% using Win Episcope 2.0. A sample size of 25 animals per herd was selected after estimating a within-herd prevalence of 10% and a level of confidence of at least 90% in detecting a positive.

Sampling was carried out throughout the year, and a total of 301 herds (115 dairy sheep, 104 beef and 82 dairy cattle) were visited once between February 2014 and June 2016. Rectal fecal samples from 25 animals randomly selected per herd were collected with a gloved hand, and a 25 g-pool was prepared (1 g per animal per pool) for microbiological analyses. Sample collection was carried out by veterinary clinicians as part of the usual health monitoring procedures performed on farms, strictly following Spanish ethical guidelines and animal welfare regulations (Real Decreto 53/2013). The collection of this material, being considered as routine veterinary practice, did not require the approval of the Ethics Committee for Animal Experimentation. Informed oral consent was obtained from the farm owners at the time of sample collection.

Campylobacter isolation and identification

For the isolation of thermophilic *Campylobacter* spp., 25 g of pooled rectal fecal samples were diluted 1/10 in Preston broth, homogenized and incubated for 18±2 h at 42ºC for enrichment. Suspensions (0.1 ml) were then subcultured onto a Chromogenic-*Campylobacter* Selective Agar (CASA® Agar, Biomerieux) and incubated at 42ºC in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) for 48-72 h. To confirm the presumptive *Campylobacter* and identify the species present in the pool of feces, DNA was extracted from a loopful of bacterial culture (InstaGene, BioRad, CA, USA) and screened for the presence of *C. jejuni* and *C. coli* in a multiplex real-time PCR (TaqMan® *Campylobacter* Multiplex assay, ThermoFisher Diagnostics). Individual colonies were then tested using the same multiplex real-time PCR to confirm their identity and were stored for further characterization.

Antimicrobial resistance: Broth microdilution tests and SNP discrimination by real-time PCR

Minimum inhibitory concentrations (MIC) were determined by broth microdilution using Sensititre® MIC Susceptibility Plate (ThermoFisher Scientific, Waltham, MA) containing two-fold serial dilutions of 6 antimicrobial agents (gentamicin, streptomycin, tetracycline, ciprofloxacin, nalidixic acid and erythromycin) following recommendations by the Commission Decision 2013/652/EU. MIC results were interpreted using epidemiological cut-off values (ECOFF) as developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org) to define microbiological resistance.

TaqMan real-time PCR assays were used to detect point mutations associated to macrolide (A2075G mutation in the 23S rRNA genes) and quinolone (C257T in the *gyr*A gene, Thr-86-Ile) resistance. Primers and probes used for *C. jejuni* (macrolide and quinolone) and *C. coli* (macrolide) were as

described before (Oporto, Juste and Hurtado, 2009). To detect point mutations associated to quinolone resistance (C257T in the *gyr*A gene) in *C. coli*, new primers and probes targeting a 101 bp fragment of the *gyr*A gene of *C. coli* were designed in this study (Table 1). PCR reactions and cycling conditions were as previously described (Oporto, Juste and Hurtado, 2009). The analytical specificity of the newly developed TaqMan assay to detect the point mutation C257T in the *gyr*A gene of *C. coli* was confirmed by sequencing analysis of the *gyr*A gene amplicon of control strains generated with the primers described in Table 1.

PCR-based screening method for the isolation of macrolide-resistant *Campylobacter*

In order to increase chances of isolation of macrolide-resistant *Campylobacter* (whose prevalence was expected to be very low), a PCR-based screening method followed by selective isolation in media containing erythromycin was assessed. Thus, DNA was extracted from a loopful of bacterial culture grown in CASA® agar (see section *Campylobacter* isolation and identification) from all samples identified as *C. jejuni* and/or *C. coli*-positive and screened with the macrolide SNP real-time PCR assay as described above but using only the probe that detects the resistant SNP (G in nt 2075 of the 23S rRNA gene). All samples that tested PCR-positive were then selectively isolated in Preston broth supplemented with erythromycin (4mg/L, 18±2 h at 42ºC), and individual colonies (presumptive macrolide-resistant colonies) were identified to the species level by real-time PCR and MICs were determined as above. In order to increase chances of isolation of macrolide-resistant *Campylobacter* (whose prevalence was expected to be very low), a PCR-based screening method followed by selective isolation in media containing erythromycin was assessed. Thus, DNA was extracted from a loopful of bacterial culture grown in CASA® agar (see section *Campylobacter* isolation and identification) from all samples identified as *C. jejuni* and/or *C. coli*-positive and screened with the macrolide

SNP real-time PCR assay as described above but using only the probe that detects the resistant SNP (G in nt 2075 of the 23S rRNA gene). All samples that tested PCR-positive were then selectively isolated in Preston broth supplemented with erythromycin (4 mg/L, 18±2 h at 42ºC), and individual colonies (presumptive macrolide-resistant colonies) were identified to the species level by real-time PCR and MICs were determined as above. In order to increase chances of isolation of macrolide-resistant *Campylobacter* (whose prevalence was expected to be very low), a PCR-based screening method followed by selective isolation in media containing erythromycin was assessed. Thus, DNA was extracted from a loopful of bacterial culture grown in CASA® agar (see section *Campylobacter* isolation and identification) from all samples identified as *C. jejuni* and/or *C. coli*-positive and screened with the macrolide SNP real-time PCR assay as described above but using only the probe that detects the resistant SNP (G in nt 2075 of the 23S rRNA gene). All samples that tested PCR-positive were then selectively isolated in Preston broth supplemented with erythromycin (4mg/L, 18±2 h at 42ºC), and individual colonies (presumptive macrolide-resistant colonies) were identified to the species level by real-time PCR and MICs were determined as above.

Assessment of changes in *Campylobacter* antimicrobial resistance profiles in two studies carried out 10-years apart

Antimicrobial resistance profiles were available for a 53 *C. jejuni* isolates from a similar study carried out in the region in 2003-2005 (Oporto et al., 2009). As part of this study, MIC values and presence of point mutation associated to quinolone and macrolide resistance were determined for a further 32 *C. jejuni* and 17 *C. coli* isolates from that previous study. Statistical analyses of the results from both studies were performed as described below (section Statistical analysis).

Table 1. Primers and probes used to detect point mutations associated to macrolide (A2075G mutation in the 23S rRNA genes) and quinolone (C257T in the *gyr*A gene, Thr-86-Ile) resistance in *C. jejuni* and *C. coli*, and *gyr*A sequencing

Target	Name	Sequences (5' → 3')	C (nM)	Reference
*C. jejuni gyr*A C257T	gyrCj-Fw	GGGTGCTGTTATAGGTCGTTATCA	900	Oporto, Juste and Hurtado, 2009
	gyrCj-Rv	TTGAGCCATTCTAACCAAAGCAT	900	
	Probe-gyrCj-S	HEX–CAT[+G]GAGAT[+A][+C][+A]GC[+A]GTTT–BHQ1	150	
	Probe-gyrCj-R	FAM–CATGGAGATATAGCAGTTT–MGB	150	
*C. coli gyr*A C257T	gyrCc-Fw	GAAGTGCATATAAAAAATCTGCTCGTA	400	This study
	gyrCc-Rv	TGCCATTCTTACTAAGGCATCGT	400	
	Probe-gyrCc-S	FAM–AACAGCAGTATCGCC–MGB	150	
	Probe-gyrCc-R	VIC–AACAGCAATATCG–MGB	150	
23S rRNA A2075G	23S-Fw	CAGTGAAATTGTAGTGGAGGTGAAA	900	Oporto, Juste and Hurtado, 2009
	23S-Rv	TTCTTATCCAAATAGCAGTGTCAAGCT	900	
	Probe-23S-S	HEX–CGGGGTC[+T][+T][+T]CCGTCTTG–BHQ1	100	
	Probe-23S-R	FAM–CGGGGTC[+T][+C][+T]CCGTCTTG–BHQ1	200	
*C. coli gyr*A sequencing	GZgyrACcoli3F	TATGAGCGTTATTATCGGTC	400	Zirnstein et al., 2000
	GZgyrACcoli4R	GTCCATCTACAAGCTCGTTA	400	

LNA nucleotides are indicated by a + symbol and in brackets; SNPs are underlined

Campylobacter coli strain characterization by Multilocus Sequence Typing (MLST)

C. coli DNA was extracted from pure cultures using InstaGene (Bio-Rad, CA, USA) and subjected to multilocus sequence typing (MLST) of seven housekeeping genes (*asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt* and *unc*A) as previously described (Dingle *et al.*, 2001; Miller *et al.*, 2005). The sequences obtained were queried against the *Campylobacter* MLST database PubMLST (http://pubmlst.org/campylobacter/), developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford, for type (ST) and clonal complex (CC) assignation. Novel STs were submitted to the *Campylobacter* MLST database for assignation of new numbers.

Statistical analysis

Herd-level prevalences were expressed as the percentage of herds/flocks that tested positive in each farm system out of all herds/flocks that were examined in the respective farm system, with 95% confidence intervals adjusted for the population size, using the software EpiInfo2. Variables selected to check for statistical differences in shedding prevalence of each pathogen (*i.e.*, thermophilic *Campylobacter*, *C. jejuni*, *C. coli*) were categorized as follows: host species (cattle, sheep); farm system (beef cattle, dairy cattle and sheep); sampling season (spring, summer, autumn, winter), geographical location of the farm (oceanic, continental); and presence of other species in the farm such us cattle, sheep, goats, horses (presence, absence). First, univariate logistic regressions were conducted to explore the unadjusted association between herd positivity and variables. Only significant factors ($p \leq 0.20$; likelihood-ratio test) were included for further multivariate logistic regression analysis. Test of overall significance (chunk test) was performed to assess any possible effect modifiers that could bias the magnitude of associations, and interactions with a value of $p > 0.05$ were excluded until no significant difference between the full and the reduced models was observed. To identify confounding variables, the measure of

association was estimated before and after adjusting for the potential confounder, and variables causing change of ≥ 10% in the estimated measure were retained. Adjusted odds ratios (OR_{adj}) were used as the measure of association between positivity and the explanatory variable and were expressed with their confidence interval at 95% (95% CI).

Simple logistic regression tests were performed to determine associations between resistance against the antimicrobial agents tested and host species (sheep *vs.* cattle) or farm system (beef cattle, dairy cattle and sheep). The same approach was also used to investigate whether resistance against each antimicrobial agent was more likely to be present on either *C. jejuni* or *C. coli*, stratifying the dataset by host species or farm system. Linear regression analyses were performed to compare log_2 transformed MIC values among host species and farm system, performing independent tests for susceptible and resistant isolates. Results from this study (2014-2016) and those from the study carried out 10 years earlier (2003-2005) were also compared. Qualitative assessment (resistant or susceptible outcome) was done with logistic regression and quantitative comparisons (log_2 MIC, mg/L) with linear regression. In all cases, regression analyses were performed separately for *C. jejuni* and *C. coli* and for each specific antimicrobial agent. All analyses were conducted in Stata/IC version 13.1 statistical software (StataCorp LP) and figures were elaborated with Microsoft Excel and with ggplot2 package of R statistical software (v.3.3.2).

3.1.3. Results

Campylobacter herd prevalence

The proportion of herds positive to *C. jejuni* and/or *C. coli* was 78.8% (82/104) of beef cattle, 86.6% (71/82) of dairy cattle and 54.8% (63/115) of sheep flocks, difference in proportions being statistically significant between cattle and sheep ($p < 0.001$) (Fig. 1). *C. jejuni* was the most frequently detected species, present in 85.4% of dairy cattle herds, 77.9% of beef cattle herds, and 45.2% of

sheep flocks, whereas *C. coli* was found in 17.1% of dairy cattle herds, 6.7% of beef cattle herds and 19.1% of sheep flocks. In 10.0% (30/301) of the tested herds/flocks both *C. jejuni* and *C. coli* were detected. Univariate analysis did not identify any significant explanatory variables associated to *Campylobacter* shedding. Multivariate analysis using host species (cattle *vs.* sheep) as principal explanatory variable for *C. jejuni* herd prevalence showed that cattle presented significantly higher risk of shedding *C. jejuni* than sheep (OR_{adj} = 5.22 (3.11-8.89), $p < 0.001$), and also when considering farm system as principal variable and comparing beef cattle and dairy cattle with sheep separately (dairy cattle *vs.* sheep: OR_{adj} = 7.07 (3.46-14.43), $p < 0.001$; beef cattle *vs.* sheep: OR_{adj} = 4.27 (2.36-7.70), $p < 0.001$). No associations were found between *C. jejuni* or *C. coli* shedding and any of the other variables tested. Risk of shedding *C. coli* was non-significantly higher in sheep than in cattle (OR_{adj} = 1.71 (0.86-3.40), $p = 0.128$). However, no differences were found between dairy and beef cattle.

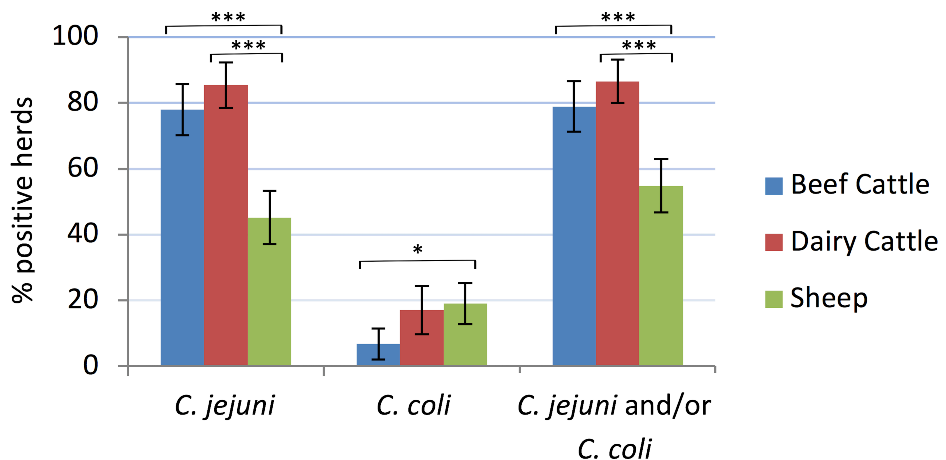

Figure 1. Prevalence of *Campylobacter*-positive herds/flocks in each host. The error bars represent the 95% confidence intervals (*, $p \leq 0.05$; ***, $p \leq 0.001$).

Antimicrobial susceptibility tests

Sensitivities to 6 antimicrobials (4 classes) were determined by broth microdilution for 109 *C. jejuni* isolates and 34 *C. coli* isolates and distributions of

MICs are shown in Table 2. Isolates susceptible to all antimicrobials tested, accounted for a total of 28.0%. Except for ciprofloxacin and nalidixic acid, resistance against all other antimicrobials was significantly more widespread among *C. coli* than *C. jejuni* isolates (Fig. 2) and the magnitude of this difference was higher in isolates from cattle than sheep, and higher in dairy cattle than beef cattle or sheep (Table 3).

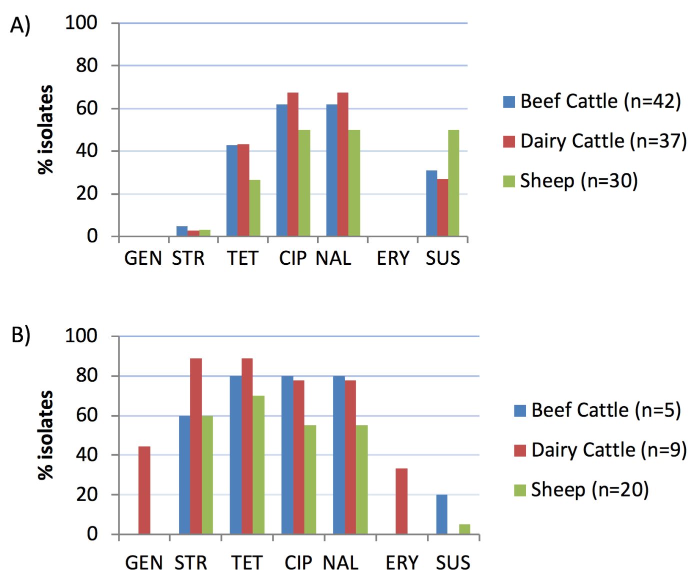

Figure 2. Proportion of isolates resistant to each antimicrobial agent tested: A) *C. jejuni*; B) *C. coli*.
CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; SUS, susceptible to all 6 antimicrobials.

Overall, 65.1% (71/109) of *C. jejuni* and 94.1% (32/34) of *C. coli* isolates showed resistance to at least one of the six antimicrobial agents tested. *C. coli* exhibited higher resistance to tetracycline (76.5%), streptomycin (67.6%) and quinolones (64.7%), whereas in *C. jejuni* resistance to quinolones (60.6%) was the most common followed by resistance to tetracycline (38.5%) (Table 2). Resistance to quinolones was always present as resistance to both ciprofloxacin and nalidixic acid. Resistance to aminoglycosides varied depending on the antimicrobial agent and the *Campylobacter* species. Thus, whereas all *C. jejuni* were susceptible to gentamicin, 3.7% were resistant to streptomycin; in the case

Table 2. *Microbiological resistance (percentage) and distribution of MICs for the 109* C. jejuni *and 34* C. coli *isolates.*

Antimicrobial class	Antimicrobial agent	*Campylobacter* species	% Resistance				No. of isolates at the indicated MIC (mg/l)												
			TOTAL	Beef Cattle	Dairy Cattle	Sheep	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Aminoglycoside	**Gentamicin**	*C. jejuni*	0.0	0.0	0.0	0.0		3	12	87	7								
		C. coli	11.8	0.0	44.4	0.0				16	11	4				**4**			
Aminoglycoside	**Streptomycin**	*C. jejuni*	3.7	4.8	2.7	3.3				1	17	84	3			**4**			
		C. coli	67.7	60.0	88.9	60.0						8	3	5	1	**17**			
Macrolide	**Erythromycin**	*C. jejuni*	0.0	0.0	0.0	0.0					109								
		C. coli	8.8	0.0	33.3	0.0					31								**3**
Quinolone	**Nalidixic acid**	*C. jejuni*	60.6	61.9	67.6	50.0						1	31	11			6	**60**	
		C. coli	64.7	80.0	77.8	55.0							3	9			3	**19**	
(Fluoro)Quinolone	**Ciprofloxacin**	*C. jejuni*	60.6	61.9	67.6	50.0		43					1	41	24				
		C. coli	64.7	80.0	77.8	55.0		11	1					6	9	**7**			
Tetracycline	**Tetracycline**	*C. jejuni*	38.5	42.9	43.2	26.7				67			2		2	6	23	**9**	
		C. coli	76.5	80.0	88.9	70.0				8						3	2	**21**	

White fields denote range of dilutions tested for each antimicrobial agent. MICs above the range are given as the concentration closest to the range and indicated in bold. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Vertical lines indicate EUCAST epidemiological cut-off values.

Table 3. Odds ratio (OR) of association between resistance to each antimicrobial and *Campylobacter* species for each host, determined with simple logistic regression analysis using *C. jejuni* as reference.

AMR	Sheep		Cattle		Beef Cattle		Dairy Cattle	
	OR (95% CI)	***p***	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
CIP/NAL	1.22 (0.39-3.80)	0.729	2.01 (0.52-7.82)	0.312	2.46 (0.25-24.02)	0.438	1.68 (0.30-9.34)	0.553
ERY[a]	NA		NA		NA		NA	
TET	6.42 (1.83-22.46)	**0.004**	7.94 (1.67-37.86)	**0.005**	5.33 (0.55-51.88)	0.149	10.50 (1.19-92.72)	**0.034**
GEN[a]	NA		NA		NA		NA	
STR	43.5 (4.89-386.74)	**0.001**	92.89 (16.62-519.09)	**<0.001**	30.00 (3.06-294.56)	**0.004**	288.00 (16.23-5108.64)	**<0.001**
MDR[b]	12.43 (1.36-113.41)	**0.025**	46.60 (9.30-223.47)	**<0.001**	30.00 (3.06-294.56)	**0.004**	72.00 (6.39-811.79)	**0.001**

[a]All *C. jejuni* were susceptible and therefore associations cannot be calculated (NA, non-applicable)
[b] Multidrug resistance pattern, defined as resistance to three or more classes of antimicrobial agents
$p < 0.05$ are considered significant and are highlighted in bold case
CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline

of *C. coli*, resistance to gentamicin was moderate (11.8%) but very high for streptomycin (67.7%). Finally, resistance to erythromycin (macrolide) was low (8.8%) and only detected in *C. coli* isolated from dairy cattle herds. No relationship was found between host species or farm system and resistance against each of the antimicrobial agents tested in neither *C. jejuni* nor *C. coli* isolates. However, MIC values for *C. jejuni* susceptible to gentamicin and streptomycin were higher in sheep than in cattle isolates (p = 0.040 and p = 0.009, respectively), whereas MICs for *C. coli* resistant to ciprofloxacin and tetracycline were higher in cattle than in sheep isolates (p = 0.011 and p = 0.029, respectively).

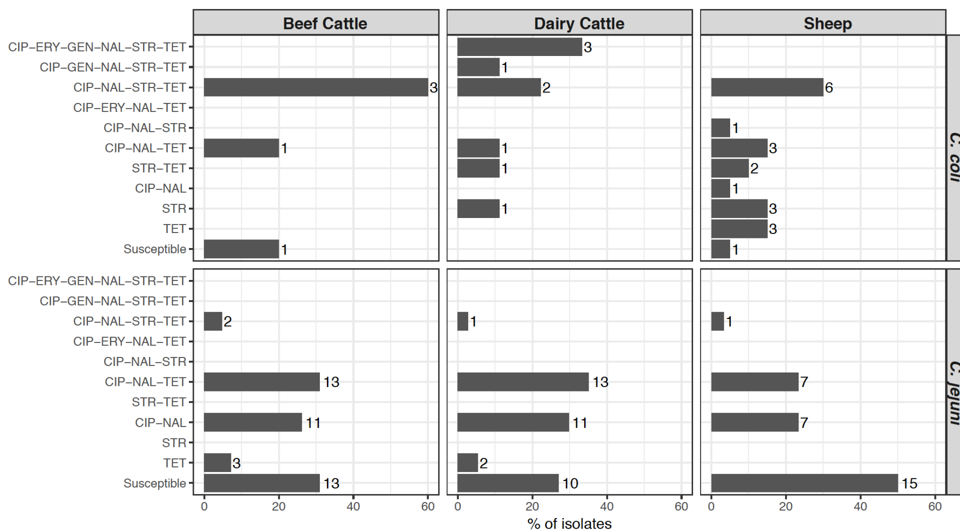

Figure 3. Distribution of antimicrobial resistance (AMR) patterns according to *Campylobacter* species and host.

Percentage of each AMR pattern was calculated for each *Campylobacter* species in each host, and numbers beside the bars represent the number of isolates. CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline

The distribution of the AMR profiles resulting from the combination of the antimicrobials tested varied according to *Campylobacter* species, but only *C. coli* isolates presented different profiles according to host (Fig. 3). The most

common combination of resistance was to quinolones and tetracycline, which was observed in 33.9% of *C. jejuni* and 58.8% of *C. coli* isolates. Multidrug resistance (MDR), defined as resistance to three or more classes of antimicrobial agents, was present in significantly higher proportions (OR= 20.72, $p < 0.001$) in *C. coli* (44.1%, 15/34) than in *C. jejuni* (3.7%, 4/109) isolates and, albeit at different levels, this trend was observed in each host (Fig. 4). Thus, MDR was more likely to occur in *C. coli* than in *C. jejuni*, being the odds ratios much higher in cattle than sheep (OR = 45.60 *vs.* 12.43), and considerably higher in dairy cattle (OR=72.00) (Table 3). Among *C. jejuni* isolates, no difference in MDR related to host was detected, and among *C. coli*, MDR was marginally more frequent in isolates from cattle than sheep (OR = 4.20 (0.98-17.95), $p = 0.053$). The most common MDR pattern was to CIP-NAL-STR-TET (Fig. 3). All three *C. coli* isolates resistant to erythromycin were also resistant to all other antimicrobials tested. Overall, dairy cattle were the host species that harboured the highest percentage of multidrug resistant *Campylobacter* isolates (15.2%), whereas sheep had the lowest (14.0%) at similar levels as beef cattle (10.6%) (Fig. 4).

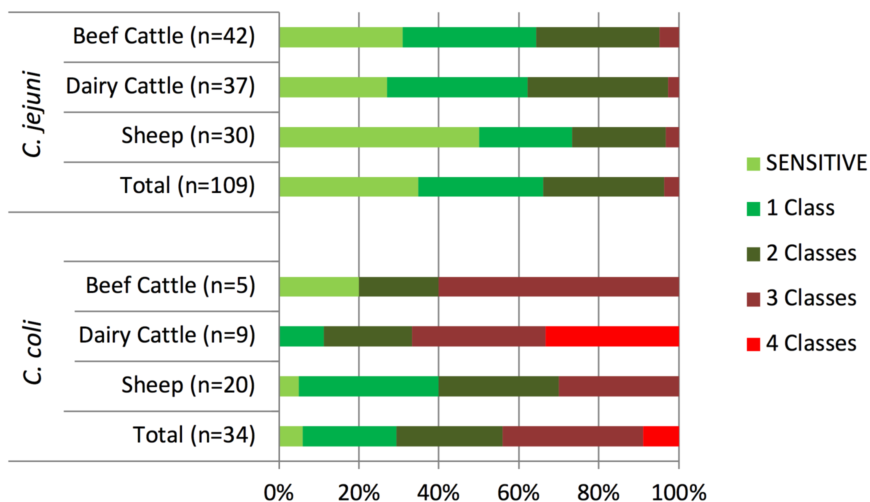

Figure 4. Distribution of isolates resistant to 1-4 classes of antimicrobial agents according to *Campylobacter* species and host.

Real-Time PCR discrimination of SNPs associated to quinolone and macrolide resistance

Sequencing analysis demonstrated the analytical specificity of the newly developed TaqMan assay to detect the point mutation C257T in the *gyr*A gene of quinolone-resistant *C. coli*. The results obtained with the real-time PCR SNP discrimination assays were in fully agreement with the phenotypic antimicrobial sensitivity test results. Thus, all *C. jejuni* (66) and *C. coli* (24) isolates that exhibited phenotypic resistance to ciprofloxacin and nalidixic acid contained the C257T mutation in the *gyr*A gene. However, one *C. coli* isolate from a sheep farm failed to give any hybridization results when analysed with the *C. coli*-specific *gyr*A SNP detection real-time PCR, but provided a resistant profile when using the *C. jejuni*-specific *gyr*A SNP assay. Sequencing analysis of the *gyr*A gene demonstrated that this isolate had a typical *C. jejuni gyr*A allele. The SNP mutation associated to resistance to macrolides (A2075G in the 23S rRNA genes) was found in the only three *C. coli* isolates that were phenotypically resistant to erythromycin. In all three isolates MIC values were far above the highest concentration tested (>128 mg/l) and therefore far higher than the MIC epidemiological cut-off value.

Isolation of macrolide-resistant *Campylobacter* using a PCR-based screening method followed by selective isolation

Macrolide-resistant *Campylobacter* was only isolated from one dairy cattle herd when directly picking colonies from the CASA® plate without selective isolation. When DNA extracted from CASA® cultures that tested *C. jejuni* and/or *C. coli*-positive were screened by real-time PCR for the macrolide-resistance associated SNP, another three dairy cattle herds tested positive, and in two of them erythromycin-resistant *C. coli* strains were confirmed after selective isolation in erythromycin-containing media; in the third herd no isolates could be recovered. Hence, prevalence of dairy cattle shedding macrolide-resistant *Campylobacter* was 3.7% (3/82) of herds.

Changes in *Campylobacter* antimicrobial resistance profiles in two studies carried out 10-years apart

Comparison of results from this study (2014-2016) and those from the study carried out 10 years earlier (2003-2005) showed a significant increase in the proportion of fluoroquinolones resistance in *C. jejuni* isolates from beef cattle (61.9% in 2014-2016 vs. 32.0% in 2003-2005; OR= 3.45 (1.21-9.83), p=0.020). However, resistance to tetracyclines in *C. jejuni* from dairy cattle decreased from 75.0% to 43.2% (OR=0.25 (0.08 – 0.85), p=0.026). In *C. coli*, no significant changes were observed in the proportion of isolates resistant to each antimicrobial, but MIC values among isolates susceptible to erythromycin were significantly lower in the present study (p<0.001), particularly in isolates from beef cattle (p=0.034).

Campylobacter coli strain characterization by Multilocus Sequence Typing (MLST)

MLST analysis of 34 *C. coli* isolates resulted in 13 ST-types, 12 of them belonging to CC-828 and another (ST-8857) not assigned to any recognized CC, and described in this study for the first time. The most common ST type (ST-827) accounted for 38.2% of the *C. coli* isolates typed, and was the most prevalent type in sheep (11/20 isolates; 55.0%) but not in cattle (2/14; 14.3%). All macrolide resistant isolates belonged to ST-2097, a ST-type associated to the three macrolide-resistant *C. coli* isolates plus another MDR *C. coli* (CIP-NAL-GEN-STR-TET) isolated also from dairy cattle.

3.1.4. Discussion

This cross-sectional study provided estimates of *C. jejuni* and *C. coli* herd prevalence in dairy cattle, beef cattle and sheep in the Basque Country, and a collection of strains representative of the region was compiled for antimicrobial resistance characterization. Results showed a widespread distribution of both

zoonotic *Campylobacter* species in ruminants, prevalence being higher than that reported in a study carried out in the same region in 2003-2005 (Oporto *et al.*, 2007), an apparent increase that can most likely be ascribed to changes in methodology rather than reflect a real increment. Still, similarly high *Campylobacter* herd level prevalence has been reported in other studies (Englen *et al.*, 2007; Rotariu *et al.*, 2009; Scott *et al.*, 2012). Also consistently with other studies in ruminants (Rotariu *et al.*, 2009; Sproston *et al.*, 2011), we found that cattle presented significantly higher risk of shedding *C. jejuni* than sheep, while risk of shedding *C. coli* was non-significantly higher in sheep than in cattle. This situation might reflect differences in the epidemiology of *C. jejuni* and *C. coli*. In this sense, increasing evidence suggests that sources and epidemiology of *C. coli* infections are different from *C. jejuni*. Thus, whereas sheep are associated to only 2.5-24% of *C. jejuni* human infections (Mullner *et al.*, 2009; Mughini Gras *et al.*, 2012), 41% of human *C. coli* clinical cases were attributed to sheep, a proportion similar to that assigned to chicken (40%) and lower than that attributed to cattle (14%) (Roux *et al.*, 2013). The higher prevalence of *C. coli* in sheep flocks as described here might explain the significant contribution of sheep to human infection with *C. coli.* On the other hand, it has been suggested that sheep-associated strains belonged to genotypes more likely to cause disease in humans. Here, half of the strains from sheep belonged to ST-827, one of the genotypes most frequently found in humans (Roux *et al.*, 2013). Our MLST results also confirmed the low diversity of *C. coli* STs of ruminant origin compared to *C. jejuni* (Oporto *et al.*, 2011) or compared to *C. coli* from swine or poultry (Roux *et al.*, 2013). All but one belonged to the same clonal complex (CC-828) but still, one novel ST was described here (ST-8857). Interestingly, one *C. coli* isolate from a sheep farm (ST-5380) showed a typical *C. jejuni gyr*A sequence suggestive of genome introgressed from *C. jejuni* as previously described for other *C. coli* strains belonging to CC-828 (Sheppard *et al.*, 2013).

Regarding AMR, differences in methodology and results interpretation hamper comparisons among studies available in the bibliography. Here, EU

harmonized methods were used, MICs were determined by broth microdilution, and EUCAST epidemiological cut-off values were used to determine microbiological resistance (Kahlmeter *et al.*, 2003). In this study, resistance against all antimicrobials tested except quinolones was more widespread in *C. coli* than in *C. jejuni* in all three host species, as was MDR. An overall higher resistance in *C. coli* than *C. jejuni* has already been reported in different hosts and for different antibiotics (Bywater *et al.*, 2004; Englen *et al.*, 2005, 2007; Châtre *et al.*, 2010; EFSA and ECDC, 2019). Overall, the highest resistance levels were found for fluoroquinolones (61.5%) followed by tetracycline (47.6%). This represented an increase in the proportion of *C. jejuni* isolates resistant to fluoroquinolones in beef cattle between the two studies carried out 10-years apart but a decrease in resistance to tetracyclines in dairy cattle. In fact, resistance to tetracycline was much lower than that reported for *C. jejuni* isolated from calves in Spain (83.3%) within the EU survey on AMR in zoonotic and indicator bacteria in 2017 (EFSA and ECDC, 2019). Although overall sales have decreased in the last years, tetracyclines have been used in livestock for many years and in 2016 still accounted for the largest sales (*ca.* 32% of total sales) in the EU and Spain (European Medicines Agency, 2018). This would explain the high levels of tetracycline resistance often reported in *Campylobacter* from food-producing animals (Cha *et al.*, 2017). The high frequency of fluoroquinolones resistance observed in this study is worrisome, while susceptibility to macrolides is reassuring. Increased resistance of *Campylobacter* to fluoroquinolones has been reported worldwide (Luangtongkum *et al.*, 2009; Sproston, Wimalarathna and Sheppard, 2018), but the levels of fluoroquinolones resistance in both *C. jejuni* and *C. coli* observed in the present study were higher than those found in ruminants in other EU countries (Jonas *et al.*, 2015; Klein-Jöbstl *et al.*, 2016; Anon, 2017), though lower than resistance rates described for poultry and pigs in Spain (EFSA and ECDC, 2018a, 2019). Use of fluoroquinolones is much higher in Spain than the European average (European Medicines Agency, 2018). In a survey carried out among veterinary clinicians in

the region (unpublished results), fluoroquinolones were mentioned to be mostly used for diarrhea and respiratory diseases in cattle, similar to data reported elsewhere for Spain (De Briyne *et al.*, 2014). Although the *gyr*A mutation associated to fluoroquinolones resistance has been described to impose certain fitness burden on *Campylobacter* (Zeitouni and Kempf, 2011), once fluoroquinolones resistant *Campylobacter* is prevalent it can persist for years even in the absence of antibiotic selection pressure so that a reversal in resistance trend might be difficult to achieve (Luangtongkum *et al.*, 2009). Resistance to macrolides was significantly lower and only detected in *C. coli* isolated from dairy cattle and at similar levels to those reported elsewhere (Bywater *et al.*, 2004; Englen *et al.*, 2007; Châtre *et al.*, 2010) and in the study carried out 10-years earlier. This is most probably due to the comparatively infrequent use of macrolides in ruminants and the highest fitness cost associated to resistance (Luangtongkum *et al.*, 2009). Also reassuring was the fact that MIC values among isolates susceptible to erythromycin were significantly lower in the present study than 10 years before. However, macrolide-resistant isolates from both studies were resistant to high concentrations of erythromycin (>128 mg/l) and in most cases co-resistant to fluoroquinolones, tetracycline and aminoglycosides. This pattern of MDR has been associated to the presence of a transferable chromosomal MDR genomic island (MDRGI) that contains the rRNA methylase *erm*(B) gene (Wang *et al.*, 2014). Resistance to aminoglycosides was found in a very small proportion of *C. jejuni* isolates, lower than that reported in the EU for cattle isolates (EFSA and ECDC, 2019). In *C. coli* resistance to aminoglycosides was higher, reaching similar levels to those reported in isolates from pigs (EFSA and ECDC, 2019). Thus, combined resistance to critically important antimicrobials was absent in *C. jejuni*, but all *C. coli* isolates resistant to erythromycin were also resistant to fluoroquinolones.

Finally, the PCR-based screening method followed by selective isolation in erythromycin-containing media developed here allowed the isolation of macrolide-resistant *C. coli* in two herds that had tested negative when using

non-selective isolation. Results demonstrated the usefulness of the method in providing more reliable estimates of macrolide-resistance than procedures that analyse a single colony per sample and likely underestimate the real prevalence. Under a low prevalence situation, PCR screening followed by selective isolation provides the processability needed to carry out extensive antimicrobial surveillance. Here it was only applied to macrolides, but a similar approach could also be used to screen for *C. jejuni* and *C. coli* quinolone resistance prevalence in regions where expected resistance levels were not as high as those found here.

3.1.5. Conclusions

This study showed a widespread distribution of *C. jejuni* and *C. coli* in ruminants, with cattle as the main reservoir of *C. jejuni* and sheep of *C. coli*, thus highlighting the importance of non-poultry reservoirs for *Campylobacter* infection. AMR was significantly more prevalent among *C. coli* than *C. jejuni* isolates, and higher in isolates from dairy cattle than beef cattle or sheep. An increase in fluoroquinolones resistance was observed, highlighting the need to promote prudent use of these antimicrobials. Resistance to macrolides, the antibiotic of choice for the treatment of diarrhoea caused by *Campylobacter* strains (especially in infants), remained stable at low rates and restricted to *C. coli*, which was reassuring. Still, active and extensive antimicrobial surveillance in campylobacters from animals is needed for early detection of emerging resistance, and the PCR-based screening method developed here proved to be a valuable tool to achieve the processability required for the analysis of large number of samples.

Author Contributions: Sample and data collection and laboratory analysis: BO & MO. Statistical analysis: MO. Interpretation of results and writing of the manuscript: AH & MO. Design and coordination of the study and final drafting

of the manuscript: AH. All authors contributed in reviewing the manuscript. All authors read and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

3.2. Study II

Effect of dry whey powder and calcium butyrate supplementation of corn/soybean-based diets on productive performance, duodenal histological integrity, and *Campylobacter* colonization in broilers.

Ocejo, M., Oporto, B., Juste, R., Hurtado, A.

***BMC Veterinary Research.* 2017; 13(1): 199.**

3.2.1. Abstract

Background: *Campylobacter* is the main cause of gastroenteritis in humans in industrialized countries, and poultry is its principal reservoir and source of human infections. Dietary supplementation of broiler feed with additives could improve productive performance and elicit health benefits that might reduce *Campylobacter* contamination during primary production. The aim of this study was to assess the effect of dietary supplementation with whey (a prebiotic) and calcium butyrate (a salt of a short-chain fatty acid) on productive traits, duodenal histological integrity, and *Campylobacter* colonization and dissemination in broiler chickens during the 42-day rearing period.

Results: Six hundred one-day-old Ross-308 chickens were placed into 20 ground pens and assigned to one of 4 corn/soybean-based dietary treatments (5 replicates of 30 chicks per treatment) following a randomized complete block design: 1) basal diet with no supplementation as the control, 2) diet supplemented with 6% dry whey powder, 3) diet containing 0.1% coated calcium butyrate, and 4) diet containing 6% whey and 0.1% calcium butyrate. At age 15 days, 6 chickens per pen were experimentally inoculated with *Campylobacter jejuni*. The results showed that supplementation of the corn/soybean-based diet with 6% whey alone or, preferably, in combination with 0.1% coated calcium butyrate improved growth and feed efficiency, had a beneficial effect on duodenal villus integrity, and decreased mortality. These favourable effects were particularly significant during the starter period. Six days after oral challenge, *Campylobacter* was widespread in the flock, and the birds remained positive until the end of the rearing period. Although *Campylobacter* was not isolated from environmental samples, it was detected by real-time polymerase chain reaction (PCR) in dust, air filters, and drinkers while birds shed culturable *C. jejuni* cells. No differences ($p>0.050$) in colonization or shedding levels that could be attributed to the diet were observed during the assay.

Conclusions: Beneficial effects on performance and intestinal health were observed, particularly during the starter period, when chickens were fed a diet

supplemented with both whey and coated calcium butyrate. However, none of the tested diets provided the chicks any differential degree of protection against *Campylobacter* infection.

Keywords: *Campylobacter jejuni*, broiler, experimental infection, dry whey powder, coated calcium butyrate

3.2.2. Background

Campylobacter infection is recognized as the main cause of sporadic food-borne enteritis in humans in developed countries (Humphrey, O'Brien and Madsen, 2007). The incidence of human *Campylobacter* infections has been steadily increasing in the last years in the EU (EFSA, 2015), and infections in children under the age of five years are especially frequent. The high incidence of *Campylobacter* diarrhoea and its duration and possible sequelae (*i.e.*, reactive arthritis and neurological disorders, such as Guillain-Barré) make it a public health problem with important socio-economic implications (Moore *et al.*, 2005). Among the over 20 species assigned to the genus *Campylobacter, C. coli* and particularly *C. jejuni* are most frequently isolated from cases of diarrheal disease in humans (Humphrey, O'Brien and Madsen, 2007).

Campylobacter asymptomatically colonizes the intestinal tracts of mammals and birds, and poultry is considered the principal reservoir and source of human infection. After infection, *C. jejuni* colonizes the chicken caeca at high levels, leading to faecal shedding of the bacterium. This high level of faecal contamination coupled with the coprophagic behaviour of chickens contribute to the rapid dissemination of the infection. Thus, when *Campylobacter* enters a flock, infection spreads rapidly, and most of the birds become colonized and remain infected throughout their productive life (Newell and Fearnley, 2003).

Because the main source of carcass contamination with *Campylobacter* is the intestinal tract, reducing its load should reduce the contamination of poultry products during slaughter and evisceration. Therefore, farm interventions to decrease the caecal colonization of broiler chickens are urgently needed. One useful intervention strategy could be supplementing chicken feed with additives that could elicit health benefits. Two such additives - the short-chain fatty acid (SCFA) butyrate and whey - have already been tested and shown promising results. Butyrate has been observed to positively affect intestinal health across species (Guilloteau *et al.*, 2010); indeed, it has anti-inflammatory properties,

increases the intestinal epithelial integrity, and elicits beneficial shifts in the composition of microbiota (Moquet *et al.*, 2016). In poultry, butyrate has also been used as a feed additive for the control of *Salmonella* Enteritidis (Fernández-Rubio *et al.*, 2009). Whey is a subproduct from the dairy industry and has interesting properties, such as high lactose content and high-quality proteins with a rich amino acid profile. Lactose cannot be efficiently hydrolysed by chicken digestive enzymes. Thus, when it reaches the lower intestinal tract, lactose included in feed is fermented by caecal microbiota, leading to the production of SCFAs and a marked reduction in the pH of the caecal contents (Morishita, Fuller and Coates, 1982). High volatile fatty acid levels and low pH in the ceca might exert bactericidal effects on pathogenic bacteria such as *Campylobacter*.

The aim of this study was to assess the effect of dietary supplementation with whey (a prebiotic), calcium butyrate (a salt of an SCFA), or their combination in terms of productive performance, duodenal histological integrity, and *Campylobacter* colonization and dissemination.

3.2.3. Methods

Birds and general management practices

In total, 600 male Ross-308 broiler chickens vaccinated against Marek's disease, infectious bronchitis virus, and coccidiosis were obtained from a commercial hatchery on the day of hatching. The chicks were reared in a controlled-environment poultry research facility on the Arkaute agricultural campus of the Basque Institute for Agricultural Research and Development (NEIKER) for 6 weeks. The temperature, ventilation, lighting schedules, and other management procedures were set according to standard Ross broiler management procedures. The floor was bedded with 5 cm of wood shaving litter, and nipple-type drinkers and cylinder feeders were used to provide water and feed, respectively, *ad libitum*.

Before the start of the experiment, the broiler house was cleaned and disinfected with formaldehyde. Subsequently, and prior to the birds' entry, environmental samples, including air, dust (from floor, walls, and fans), feeders, drinkers, litter, and feed, were collected. The presence of *Salmonella* was investigated following International Organization for Standardization (ISO) 6579:2002/Amd 1:2007 for all samples except feed, which was analysed using the VIDAS SLM test (bioMérieux, Marcy l'Etoile, France). The presence of *Campylobacter* was determined by culture and real-time polymerase chain reaction (PCR) as described below. The birds were healthy, and no disease outbreaks were observed during the experimental period.

Experimental design: dietary treatments and experimental inoculation

Chicks were allocated to 20 floor pens (2.5 × 1 m each) containing 30 birds each. Five replicate pens were allotted to each of 4 dietary treatments following a randomized complete block design. The birds were fed two-phase corn/soybean-based diets, formulated to meet Ross-308 broiler requirements during the starter (0–20 d) and grower-finisher (21–42 d) periods (FEDNA, 2008, 2010). The different diets were as follows: 1) basal diet with no supplementation as the control (Co), 2) diet containing 6% whey (Wh), 3) diet supplemented with 0.1% calcium butyrate (Bu), and 4) diet containing 6% whey and 0.1% calcium butyrate (WhBu). The whey used was a commercial sweet powder (Sueromancha S.L, Toledo, Spain; 70% lactose and 14% crude protein) consisting of a mixture of bovine and ovine cheese whey. The calcium butyrate salt used (70% butyrate and 16% Ca) was coated in a matrix of vegetable oils (Globamax Performant, Global Nutrition International, Fougères Cedex, France), and thus, a significant portion of the butyrate content will only be released when lipase is secreted in the duodenum and breaks down the lipid matrix. The four experimental diets were formulated to provide equal nutrient profiles (Table 4). The feed was commercially produced by MIBA ERKOP (Markina- Xemein, Spain) in pellet form, and no antibiotic or anticoccidial was added.

At 15 days old, 120 chicks (6 per pen) were challenged by oral gavage with 500 µL of a mixture of two *C. jejuni* strains corresponding to a dose of 10^5 colony-forming units (cfu). The two *C. jejuni* isolates used had been previously characterized and represent genotypes that are widespread in livestock and poultry in the Basque Country (Oporto *et al.*, 2011). The Campynet strain collection CNET099 (originating lab name: 81116), which was provided by the Central Veterinary Institute Wageningen UR (The Netherlands), is a human isolate that belongs to multilocus sequence type (MLST) ST267, clonal complex CC283. The complete genome of *C. jejuni* strain 81116 has been sequenced (Pearson *et al.*, 2007). This strain is infective for chickens and has been reported to be genetically stable. The second strain, CAM303, was a field strain isolated from chickens, and its MLST type is ST572, CC206. Inoculated birds were properly identified with neck tags. No mortality was observed in the inoculated chicks during the three days post-challenge.

Broiler performance

Body weight (BW) and feed intake (FI) per pen were recorded on a weekly basis to calculate the average daily weight gain (DWG) and daily FI (DFI). Chicks were inspected daily, and dead birds were removed after registering the date and dietary treatment received. Mortality was recorded daily, and the survival rate (S) was calculated. The feed conversion ratio (FCR) was calculated for the starter, grower-finisher, and the entire period as the ratio of FI to BW gain in the corresponding period after correction for BW at hatching. The European Productive Efficiency Factor (EPEF), which incorporates the BW, age, S and FCR, was calculated for the entire production period.

Sample collection

Faeces, cloacal swabs, dust, drinkers, and air samples were periodically collected (Fig. 5) throughout the trial. Faeces (fresh droppings) were collected in sterile containers from all 20 pens. Birds' cloaca (2 per pen), dust (from floor,

walls, and fans), and drinkers were sampled with sterile swabs, which were then placed in transport medium (Amies medium, SCHARLAB, Barcelona, Spain). Air samples were collected using sterile gelatine filters (80 mm in diameter and 3-μm pore size; type 17528-80-ACD; Sartorius AG, Goettingen, Germany) coupled to an air sampling device (MD8 Airscan, Sartorius) at a speed of 6 m^3/hour for 15 min. Two air samples (1.5 $m^3 \times 2$) were obtained at 10 cm above ground level at each sampling occasion: one from inside and one from directly outside the broiler house. All samples were maintained at 4°C until arrival at the laboratory. Faecal samples were analysed for the presence of *Campylobacter* by culture and real-time PCR in pools of five from each diet treatment group; swabs were pooled by pen for microbiological isolation and individually analysed by real-time PCR.

Twenty birds (1 per pen) were randomly chosen at 3, 14, 28, and 42 days of age; euthanized by exposure to carbon dioxide gas for 5 min; and immediately necropsied. No feed withdrawal was performed prior to slaughter. The caeca were aseptically dissected, and their contents were collected for DNA extraction and *Campylobacter* quantification by real-time PCR (all ages). The caecal content collected from 14-day-old chicks (one day before challenge) was also subjected to selective enrichment for *C. jejuni* isolation in 4 pools of 5 birds each (one pool per treatment). For histological examination, 2-cm sections from the apex of the duodenal loop were taken from each necropsied animal, and the lumen was rinsed with sterile phosphate-buffered saline (PBS) and fixed in 10% buffered formalin.

C. jejuni isolation

All environmental samples (*i.e.*, air filters, dust swabs, and drinker swabs) and animal samples (*i.e.*, faeces, cloacal swabs, and caecal content) collected before experimental inoculation were diluted 1:10 on Preston Enrichment Broth (Nutrient broth no. 2; Oxoid, Thermofisher Scientific, Waltham, MA, USA) supplemented with Preston *Campylobacter*-selective

supplement (Oxoid), *Campylobacter* growth supplement (Oxoid), and 5% (v/v) defibrinated lysed horse blood (Oxoid) for 18–20 h at 42°C. Then, 100 μL of enriched broth was plated on the *Campylobacter*-selective chromogenic medium CASA (bioMérieux) and incubated for 48–72 h under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) in a variable atmosphere incubator (Ruskinn Concept 400, Biotrace International, Bridgend, UK). Faecal and cloacal samples collected post-inoculation (p.i.) were plated directly on CASA agar without an enrichment step, and compatible colonies were confirmed by real-time PCR.

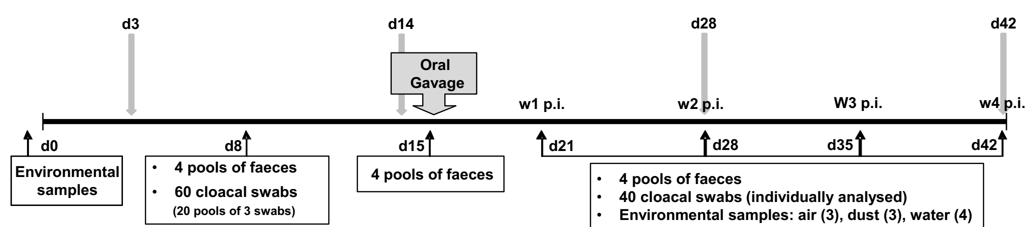

Figure 5. Schematic representation of the experimental trial.
Time values are in days. Downward solid arrows indicate the days when birds were slaughtered for collection of caecal content and duodenal tissue samples. Upward arrows show time points for sample collection as indicated in the boxes. Sampling of faeces on day 15 was carried out immediately before oral inoculation.

DNA extraction and real-time PCR

A MagMax ™ Total Nucleic Acid Isolation Kit (Ambion, Thermofisher Scientific) was used to extract genomic DNA from faecal samples and swabs, and a Power Soil Kit (MoBio Laboratories Inc., Carlsbad, CA) was used to extract DNA from the caecal contents. DNA from air filters was isolated with a QIAamp DNA mini blood kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with slight modifications, as described elsewhere (Astobiza *et al.*, 2011). At least one negative extraction control was included in each batch of samples processed.

Table 4. Composition of feed ingredients (g/kg) and nutrients content (%) of the experimental diets

Composition	**Starter diet**				**Grower-finisher diet**			
	Co	**Bu**	**Wh**	**BuWh**	**Co**	**Bu**	**Wh**	**BuWh**
Ingredients as fed (g/kg)								
Calcium Butyrate	0.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0
Whey	0.0	0.0	60.0	60.0	0.0	0.0	60.0	60.0
French corn	490.0	535.0	495.0	520.0	500.0	500.0	540.0	538.0
Soybean meal 47	297.9	302.6	294.6	279.0	315.4	315.9	317.5	317.8
Soft wheat >74	169.7	115.0	95.0	110.0	107.4	105.3	0.0	0.0
Soybean oil	10.0	10.0	21.0	9.2	42.0	42.6	50.7	51.4
Dicalcium phosphate dihydrate	3.0	5.0	5.0	3.0	20.5	20.5	19.0	19.0
Calcium carbonate	14.0	16.0	14.0	3.8	4.0	4.0	3.8	3.8
Mineral salt	3.8	3.8	3.8	2.7	3.8	3.8	2.7	2.7
Vitamin and mineral premix[1]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
DL-Methionine	0.4	0.4	0.4	1.7	1.8	1.8	1.7	1.7
L-Lysine	1.7	1.7	1.7	0.1	0.6	0.6	0.1	0.1
Salmocid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Calculated nutrients (%)								
Crude protein	22.5	22.6	22.3	22.1	20.0	20.0	20.0	20.0
AME (Kcal/kg)[2]	3152.0	3141.7	3141.5	3140.1	3100.0	3100.0	3100.0	3100.0
Digestible lysine	1.3	1.3	1.3	1.2	1.10	1.10	1.11	1.11
Digestible methionine	0.40	0.40	0.40	0.50	0.49	0.49	0.49	0.50
Calcium	1.0	1.0	0.9	0.9	0.85	0.85	0.85	0.85
Available phosphorus	0.50	0.60	0.60	0.50	0.42	0.42	0.40	0.42

Co, basal control diet with no supplementation; Bu, basal diet supplemented with 0.1% calcium butyrate; Wh, basal diet supplemented with 6% whey; BuWh, basal diet supplemented with 0.1% calcium butyrate and 6% whey.

[1] Vitamin and mineral premix providing the following (per kg of diet): vitamin A, 8000 IU; vitamin D3, 1600 IU; vitamin E, 16 mg; thiamine, 1 mg; riboflavin, 3 mg; pyridoxine, 1 mg; vitamin B12, 0.01 mg; vitamin K, 1 mg; nicotinic acid, 16 mg; pantotenic acid, 7 mg; Mn, 70 mg; ZnO, 50 mg; Fe ($FeSO_4$ H_2O), 30 mg; Cu ($CuSO_4$ $5H_2O$), 4 mg; I (KI), 1 mg; Co, 0.2 mg; Se (Na_2SeO_3), 0.1 mg; choline, 240 mg; phytase, 300 units; ethoxyquin, 110 mg.

[2]AME: Apparent Metabolizable Energy corrected by nitrogen, calculated according to de Blas *et al*. (FEDNA, 2010)

For detection purposes, a real-time PCR assay that co-amplifies a 108-bp fragment from the 16S rRNA gene of *Campylobacter* spp. and an internal amplification control (IAC) was performed as previously described (Lund *et al.*, 2004), with a minor, previously described modification to the IAC probe (Ros-García *et al.*, 2012). Ten-fold serial dilutions (10^6–1 copy) of a linearized *Campylobacter* spp. recombinant plasmid (two replicate reactions per dilution

step) were used to create a standard curve. For *Campylobacter* load quantification (cells per g of caecal content or m^3 of air), DNA extracted from caecal samples and air filters was analysed along with the standard curve. The standard linear regression equation was then used to transform the raw quantitative PCR (qPCR) data from Cq values to estimated copy numbers (Q) per reaction tube. To account for the dilutions and volume transformations performed during sample processing and the target gene copy number, the number of *Campylobacter* cell equivalents per g of caecal content or m^3 of air was calculated as follows: *Campylobacter*/g or $m^3 = Q \times (A_S/A_{EX}) \times (V_{EL}/V_T) \times (1/CN)$, where A_S is the amount of sample relative to which the results will be presented (*i.e.*, 1 g of caecal content or 1 m^3 of air), A_{EX} is the amount of sample extracted, V_{EL} is the nucleic acid extraction eluate, V_T is the amount of nucleic acid template added to the PCR, and CN is the gene copy number (3 copies per genome).

Real-time PCR was performed in MicroAmpTM Fast optical 96-well reaction plates covered with thermo-sealing 4titude Clear Seal adhesives (Surrey, UK) at 180°C for 2 s in a 4 s2™ Thermal Sealer (4titude Ltd., Surrey, UK). The PCR volume was 20 μL, and an ABI Prism 7500 Fast Real Time PCR System (Applied Biosystems, Thermofisher Scientific) was used. The reaction consisted of 2× Premix Ex Taq (TaKaRa Bio, Mountain View, CA, USA), 50× ROX Reference Dye II (TaKaRa Bio), 600-nM *Campylobacter* primers campF2 and campR2, 200-nM *Campylobacter* probe, 500-ng/μL bovine serum albumin (BSA) (Roche Applied Science, Penzberg, Germany), and 5 μL of sample DNA. To control inhibition in detection real-time PCR assays, 100 copies of the IAC plasmid and the IAC primers (200 nM) and probe (150 nM) were also added to the reaction mix. The cycling conditions were as follows: denaturation for 10 min at 95°C, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Samples with Ct <38 were considered positive, and values in the range of 35–38 were considered positive but non-quantifiable. All samples were run

accompanied by positive controls, negative extraction controls, and at least 2 PCR-negative controls per plate.

Intestinal morphometric measurement

Formalin-fixed duodenal segments were trimmed and embedded in paraffin. Transversal sections (4 μm) were cut and stained with standard haematoxylin and eosin stain. Slides were then examined under a light microscope (Nikon Eclypse E400; Nikon Europe B.V., Amsterdam, The Netherlands) equipped with a digital network camera (Nikon DN100; Nikon Europe B.V.). The villus height (the distance from the apex of the villus to the junction of the villus and crypt) and crypt depth (the distance from the junction to the basement membrane of the epithelial cells at the bottom of the crypt) were measured on well-oriented and intact villi (i.e., both the tip and the base of the villus were in the plane of the section). All morphological variables were measured using the ImageJ software package (http://rsb.info.nih.gov/ij/). At least 5 replicate measurements for each variable studied were taken from each sample, and the average values were used for the statistical analysis.

Statistical analysis

The statistical analysis was performed according to the GLM procedure of SAS statistical package version 9.3 (SAS Institute, Cary, NC, USA) to identify any significant differences among birds fed the 4 diets in terms of their productive performance (*i.e.*, in the starter, grower-finisher, and/or entire period), *C. jejuni* colonization, *C. jejuni* shedding, and duodenal histomorphometric traits. For productive performance analysis, the pen was defined as the experimental unit, whereas for the analyses of the remaining variables, individual birds were considered as the experimental unit. Bacterial load data were converted to $\log_{10}$ cfu/g before analysis and expressed as the log of the cfu per g of caecal content or cloacal swab. Any statistical differences in productive performance, $\log_{10}$ cfu/g, and duodenal histomorphometric

measurements among treatments were determined by one-way analysis of variance (ANOVA). Whenever the overall effect was significant, Tukey's test was conducted to make pairwise comparisons between group means. Probability values less than 0.050 (p<0.050) were considered significant. The results were expressed as the least square means ± the standard error of the mean (SEM).

3.2.4. Results

Performance

The effects of the tested diets on productive performance are shown in Table 5. During the starter period (1–21 d), among the different diets tested, the highest BW and DWG were observed in birds fed the WhBu diet (p<0.001). In contrast, the Bu diet had poor effects on BW, DWG, and DFI (p<0.001) compared with all other diets tested. Additionally, birds fed the WhBu diet showed better FCR than the other treatment groups (p<0.050). During the grower-finisher phase (22–42 d), diet type exerted no effect (p>0.050) on any of the productive performance parameters. During the entire productive period (1–42 d), DFI was lower in birds fed the Bu diet (p<0.050), and both WG and DWG were poor in birds fed the Bu diet compared to those fed the Wh or combination BuWh diet. Although no differences (p=0.200) in FCR were observed among diets during the entire productive period, the values were better for the 3 supplemented diets than for the Co diet.

Although the effect of diet on survival was not significant (p>0.050), the highest mortality was recorded in the control group, and lower rates were observed in groups fed diets supplemented with whey alone or in combination with calcium butyrate. In all treatments, losses (24/35, 68.6%) were more prevalent in the last two weeks of the rearing period. Independent of the diet treatment, mortality in the two weeks after oral gavage was slightly lower than that recorded during the two previous weeks (0.08% vs. 0.10%), and no apparent negative effects, such as declining activity, decreased DFI, or diarrhoea,

were observed in the days following oral challenge. A positive effect (*p*<0.01) on the EPEF was observed in birds fed diets containing whey alone or in combination with butyrate.

Table 5. Effect of experimental diet on productive performance of broilers

	Co	Bu	Wh	BuWh	SEM[1]	*p*-value[2]
Starter period (0-21 days)						
Body Weight (g)	635.6[b]	544.2[c]	627.7[b]	681.3[a]	10.8	***
Daily Weight Gain (g/d)	29.9[b]	25.3[c]	29.5[b]	32.2[a]	0.5	***
Daily Feed Intake (g/d)	46.8[a]	41.3[b]	46.3[a]	49.1[a]	0.9	***
Feed Conversion Ratio[3]	1.57[ab]	1.64[a]	1.57[ab]	1.53[b]	0.02	*
Survival (%)	97.3	98.0	99.3	100.0	1.2	NS
Grower-finisher period (22-42 days)						
Daily Weight Gain (g/d)	99.1	99.5	102.4	100.5	1.4	NS
Daily Feed Intake (g/d)	160.6	154.4	162.8	159.2	2.2	NS
Feed Conversion Ratio[3]	1.62	1.55	1.59	1.58	0.02	NS
Survival (%)	93.3	94.7	97.3	96.7	2.3	NS
Overall production period (0-42 days)						
Body Weight (g)	2716.0[ab]	2633.1[b]	2778.7[a]	2791.9[a]	34.0	*
Daily Weight Gain (g/d)	65.3[ab]	63.3[b]	66.8[a]	67.2[a]	0.8	*
Daily Feed Intake (g/d)	105.1[a]	99.2[b]	106.0[a]	105.5[a]	1.4	*
Feed Conversion Ratio[3]	1.61	1.57	1.59	1.57	0.01	NS
Survival (%)	90.7	92.7	96.7	96.7	2.5	NS
European Production Efficiency Factor[4]	364[c]	370[bc]	403[ab]	409[a]	8.4	**

Co, basal control diet with no supplementation; Bu, basal diet supplemented with 0.1% calcium butyrate; Wh, basal diet supplemented with 6% whey; BuWh, basal diet supplemented with 0.1% calcium butyrate and 6% whey.

Each value represents the least square mean of five replicates (30 birds per replicate).

[a,b,c] Means in a row not sharing a common superscript are significantly different.

[1]Pooled standard error of mean.

[2]Significance of the effect of Diet (NS: not significant; *: p <0.05; **: p <0.01; ***: p <0.001).

[3]Feed Conversion Ratio: feed intake (g)/weight gain (g).

[4]European Production Efficiency Factor: ((Survival rate [%] x Body weight [kg]) / (age [d] x Feed Conversion Ratio)) x 100

Histology

The villus heights, crypt depths, and villus height-to-crypt depth ratios in the duodenum of broilers fed the different diets are shown in Table 6. On day 14, just before *Campylobacter* challenge, the duodenal histological development (*i.e.*, villus height) was not affected by the treatments (p>0.050), although chicks given the Co diet had deeper crypts than other birds (p<0.001). Two weeks after *Campylobacter* challenge, on day 28, the villus height increased in birds fed all three supplemented diets compared to those fed the Co diet, and chicks given the BuWh diet had the highest villi and a higher villus height-to-crypt depth ratio (p<0.001). On day 42, the villus height-to-crypt depth ratio in birds fed the BuWh diet was higher than in any other diet group (p<0.001). Overall, the addition of either of the supplements to the diet resulted in a higher villus height and/or villus height-to-crypt depth ratio in the duodenum compared to the Co diet.

Table 6. Effects of dietary treatments on duodenal histomorphological variables.

Age (days)	Variables	Co	Bu	Wh	BuWh	P^1
14	Villus height (µm)	1288.5 ± 24.5	1276.3 ± 26.9	1289.8 ± 25.4	1267.4 ± 26.9	NS
	Crypt depth (µm)	176.8 ± 4.0^a	146.3 ± 4.4^b	145.0 ± 4.2^b	150.7 ± 4.4^b	***
	Villus/Crypt ratio	7.4 ± 0.3^b	9.1 ± 0.33^a	9.1 ± 0.3^a	8.5 ± 0.3^a	***
28	Villus height (µm)	1561.6 ± 43.5^c	1748.9 ± 44^b	1792.9 ± 51.9^b	2179.9 ± 45.8^a	***
	Crypt depth (µm)	153.5 ± 4.2^b	167.4 ± 4.3^{ab}	180.0 ± 5.0^a	172.9 ± 4.4^a	***
	Villus/Crypt ratio	10.4 ± 0.4^b	10.8 ± 0.4^b	10.1 ± 0.5^b	12.9 ± 0.4^a	***
42	Villus height (µm)	1409.6 ± 26.9^b	1552.1 ± 26.4^a	1611.4 ± 28.6^a	1647.0 ± 38.3^a	***
	Crypt depth (µm)	182.2 ± 3.8^{ab}	173.0 ± 3.7^{bc}	190.3 ± 4^a	159.1 ± 5.4^c	***
	Villus/Crypt ratio	7.9 ± 0.2^c	9.1 ± 0.2^b	8.7 ± 0.2^{bc}	10.5 ± 0.3^a	***

Co, basal control diet with no supplementation; Bu, basal diet supplemented with 0.1% calcium butyrate; Wh, basal diet supplemented with 6% whey; BuWh, basal diet supplemented with 0.1% calcium butyrate and 6% whey.
Values were expressed as least square means ± SEM representing 5 birds/group.
^{a,b,c} Means in a row not sharing a common superscript are significantly different.
^{1}Significance of the effect of Diet (NS: not significant; *: p <0.05; **: p <0.01; ***: p <0.001).

Microbiology

Prior to the birds' entry into the broiler house, environmental samples, such as air, dust (from floor, walls, and fans), feeders, drinkers, litter, and feed, were confirmed to be free of *Salmonella* and *Campylobacter* by culture. Faeces, cloacal swabs, dust, drinker swabs, and air samples collected before the experimental inoculation remained negative for *C. jejuni* by both microbiological culture and real-time PCR. Similarly, *Campylobacter* was not isolated from the caecal content collected from chicks one day before the challenge (14 days old). At day 21 (six days p.i.), *Campylobacter* was isolated from all faeces and cloacal swab samples, and the DNA from faeces and most cloacal swabs (56/60) were positive on real-time PCR. *Campylobacter* was not isolated by culture from environmental samples but was detected by real-time PCR in dust samples, air filters, and drinker swabs. The same pattern was observed during the remaining three weeks of the experiment, with *Campylobacter* isolated from pooled faeces and cloacal swabs and real-time PCR detection in both animal samples (all faeces and cloacal swabs) and environmental samples (dust, air, and drinkers) (Fig. 6).

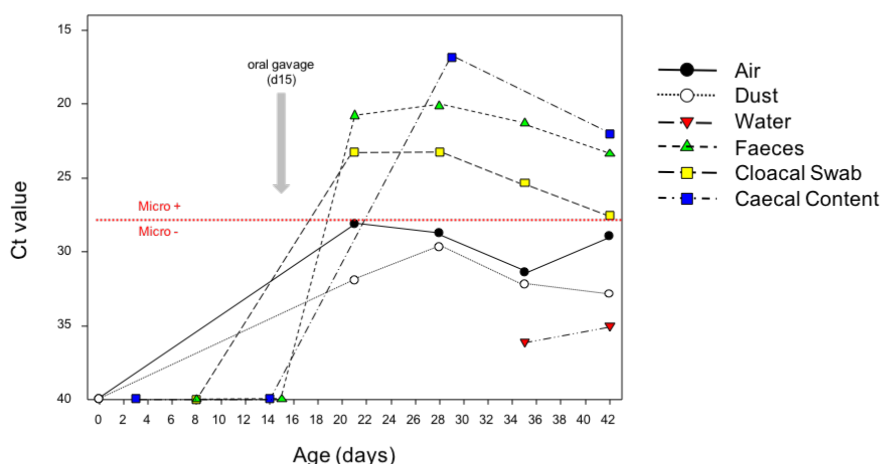

Figure 6. Dynamics of *Campylobacter* infection throughout the rearing period of 42 days, as determined by real-time PCR (Ct values) analysis of environmental (air, dust, and drinkers) and animal (faeces, cloacal swabs and cloacal content) samples. Samples above the red line were also positive by microbiological culture, whereas those below were negative.

Shedding trends, which were monitored through weekly analyses of cloacal swabs by real-time PCR, showed that the highest levels of campylobacter were detected in cloacal samples during weeks 1 and 2 p.i., when the birds were 21 and 28 days old, respectively (Table 7). Subsequently, the *Campylobacter* loads started to decrease in all diet treatments. Differences in shedding loads among birds fed the four different diets were only observed at age 35 days, when the birds fed the Bu diet excreted higher levels of *Campylobacter* than those fed the Co diet (5.0 vs. 4.1 Log *Campylobacter* cell equivalents; pooled SE=0.2, $p<0.050$).

Table 7. Assessment of *C. jejuni* colonization (caecal content) and shedding (cloacal swabs) throughout the experiment as determined by real-time PCR (Log_{10} *Campylobacter* cell equivalents per gram of caecal content or cloacal swab)

Diet	Sample	Age at sampling				*p*-value[1]
		21 days	28 days	35 days	42 days	
Co	Cloaca	5.0 ± 0.2^{a}	5.0 ± 0.3^{a}	4.1 ± 0.3^{ab}	3.7 ± 0.3^{b}	***
	Caeca	NA	9.1 ± 0.2^{a}	NA	7.7 ± 0.1^{b}	***
Bu	Cloaca	5.0 ± 0.2^{a}	5.3 ± 0.3^{a}	5.0 ± 0.3^{a}	3.8 ± 0.2^{b}	***
	Caeca	NA	8.9 ± 0.2^{a}	NA	7.6 ± 0.2^{b}	***
Wh	Cloaca	5.0 ± 0.2^{a}	5.0 ± 0.2^{a}	4.4 ± 0.2^{ab}	3.9 ± 0.2^{b}	***
	Caeca	NA	9.2 ± 0.1^{a}	NA	7.7 ± 0.1^{b}	***
BuWh	Cloaca	5.2 ± 0.2^{a}	5.6 ± 0.2^{a}	4.4 ± 0.2^{b}	3.7 ± 0.2^{b}	***
	Caeca	NA	8.9 ± 0.3^{a}	NA	7.6 ± 0.3^{b}	*

Co, basal control diet with no supplementation; Bu, basal diet supplemented with 0.1% calcium butyrate; Wh, basal diet supplemented with 6% whey; BuWh, basal diet supplemented with 0.1% calcium butyrate and 6% whey.

Values were expressed as least square means ± SEM representing 5 birds/group for caecal content samples and 10 birds/group for cloacal swabs.

[a,b,c] Means in a row not sharing a common superscript are significantly different.

[1]Significance of the effect of age at sampling (NS: not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

No differences in shedding were observed between orally challenged birds (seeders) and non-inoculated birds at days 21, 28, or 35 ($p>0.050$), but at the end of the rearing period (42 days old), the *Campylobacter* loads in cloacal swabs were higher in orally inoculated birds than in horizontally infected birds

(4.1 vs. 3.4 Log_{10} *Campylobacter* cell equivalents; pooled SE=0.2, p<0.050) (Fig. 7A). Similarly, caecal colonization was confirmed in all birds sacrificed after experimental inoculation (*i.e.*, at days 28 and 42). *Campylobacter* load ranged from 6.8 to 9.4 Log_{10} *Campylobacter* cell equivalents per g of caecal content, and the colonization levels were higher at day 28 than at day 42 (mean difference: 1.4 ± 0.1; *p* <0.001 Log_{10} *Campylobacter* cell equivalents per g of caecal content) (Table 7, Fig. 7B). No differences in colonization related to the diet were observed at any age. Similarly, no differences in colonization levels between orally inoculated birds and unchallenged birds were found within the age groups (*p*>0.050) (Fig. 7B).

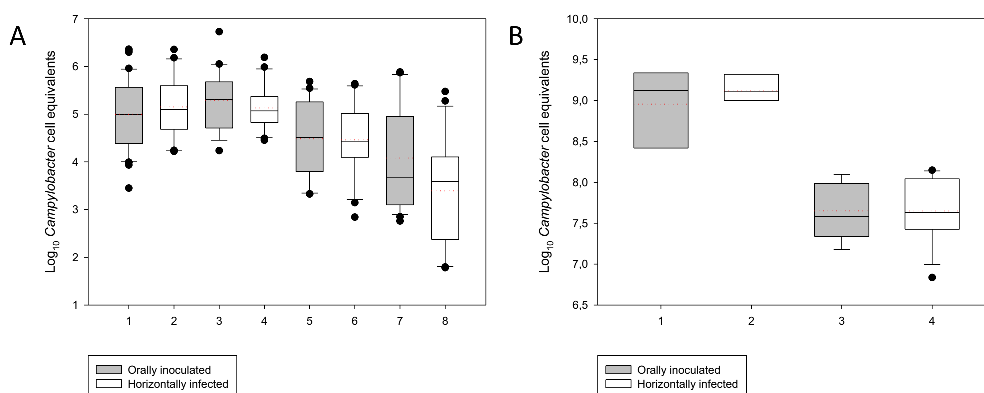

Figure 7. *Campylobacter* load in experimentally inoculated (grey) *vs.* non-inoculated (white) broilers as determined by real-time PCR analysis of cloacal swabs at days 21, 28, 35 and 42 (A) and broilers' caeca at days 28 and 42 (B), representing shedding and colonization levels, respectively.
Real-time PCR Ct values were transformed into Log_{10} *Campylobacter* cell equivalents per cloacal swab (A) or per g of caecal content (B). The boundary of the box closest to zero indicates the 25th percentile, the continuous line within the box marks the median, a dashed line marks the mean and the boundary of the box farthest from zero indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles. Outlying points (5th and 95th percentiles) are represented as closed dots. Significant differences between orally inoculated and horizontally infected are marked with an asterisk.

According to the real-time qPCR results, the contamination levels of air samples collected inside the broiler house ranged from 2.5 to 3.4 Log_{10} *Campylobacter* cell equivalents per m^3 of air, and the lowest value was measured in week 3 p.i. Air samples collected outside the broiler house were always

negative for *Campylobacter* by both culture and real time-PCR. *Campylobacter* DNA was also detected in samples collected from drinkers on days 35 and 42, but it was always below quantifiable levels.

3.2.5. Discussion

Several productive benefits have been attributed to chicken diet supplementation with whey or butyrate, but the reported outcomes of such supplementation are sometimes unapparent or inconsistent. Any positive effect of diet supplementation on performance traits would likely be more evident when the birds are subjected to a stressor, such as *Campylobacter* infection, which is a common occurrence in poultry production and is also a concerning food safety issue. Here, to simulate common conditions in broiler production, birds were experimentally inoculated with *C. jejuni* at 15 days of age, when natural infections are usually reported (Evans and Sayers, 2000; Bull *et al.*, 2006). The chickens were fed the supplemented diets throughout the production period and monitored until slaughter at 42 days of age.

From the perspective of production, during the entire 42-day period, FCR improved, albeit not significantly, in the birds fed the 3 supplemented diets compared to the Co diet. Moreover, the diet supplemented with both dried whey powder and coated calcium butyrate exerted a significant beneficial effect during the starter period. In this period, although the group fed the Bu diet had poor BW and DWG values compared to those fed all other treatments, a positive synergistic effect of the combination of 6% whey and 0.1% calcium butyrate (*i.e.*, the BuWh diet) was observed. Indeed, the BuWh diet resulted in increased weight gain without increasing the FI compared to the Co diet. Additionally, the EPEF for the entire production period, which considers not only growth and FCR but also survival, was higher among chicks fed the BuWh diet compared to all other diets. The impact of nutrition at an early age on chicks has been studied intensively, and early growth has been demonstrated to be directly correlated with the final BW and feed efficiency (Noy and Uni, 2010). Therefore, the

improved performance and survival during the starter period associated with the addition of both dried whey and coated calcium butyrate to the chicken diet is an interesting early feeding strategy that warrants further investigation.

The positive effect of whey on broiler productivity had been reported previously (Gülşen *et al.*, 2002; Kermanshahi and Rostami, 2006; Szczurek *et al.*, 2013), but the effect of the Bu diet was not as strong as expected. Dietary butyrate supplementation has been shown to improve growth performance by enhancing digestibility and absorption, promoting the structural integrity of intestinal villi, and contributing to pathogen control (Fernández-Rubio *et al.*, 2009; Guilloteau *et al.*, 2010; Kaczmarek *et al.*, 2016; Moquet *et al.*, 2016). However, the reported effects of butyrate supplementation are contradictory and can be affected by a variety of factors, such as the additive inclusion level, the precise gastrointestinal tract (GIT) segment wherein the butyrate is released, diet composition, and the age and health status of the bird (Moquet *et al.*, 2016). Previous studies (van den Borne *et al.*, 2015) showed that fatty acids must be coated to reach the lower GIT in their active form. Here, although the calcium butyrate used was coated, improvements were only observed when it was combined with whey supplementation. The improvement in protein digestibility associated with butyrate (Kaczmarek *et al.*, 2016; Moquet *et al.*, 2016), combined with the high biological value of the protein contained in whey (Szczurek *et al.*, 2013), likely contributed to the observed improvements in performance traits.

Good intestinal health is highly important for the achievement of target growth rates and high feed efficiency. Other studies have reported increased intestinal tract integrity associated with greater villus height after broiler diet supplementation with butyrate, lactose, or whey (Gülşen *et al.*, 2002; Panda *et al.*, 2009; Szczurek *et al.*, 2013). In this study, supplementation with either additive (*i.e.*, butyrate or whey) also resulted in a significantly greater villus height compared to the Co diet at 28 and 42 days. This increase in the villus height should increase the absorptive surface of the small intestine, leading to

better nutrient utilization and, thus, the observed beneficial effects on bird performance. Yet, the best outcome was observed in birds fed the BuWh diet; indeed, at the end of the experiment, these birds had the longest villi and the shortest crypts.

Microbiological and real-time PCR results revealed successful colonization and rapid spreading of the infection. At day 21, six days after oral challenge, *Campylobacter* was widespread in the flock, and nearly all birds in the various diet treatment groups were shedding *C. jejuni* at similar levels. Notably, the shedding levels of orally challenged and horizontally infected birds were indistinguishable at six days p.i. This finding indicates a very short delay time between the inoculation of the seeders and the occurrence of the first contact infections. In their modelling study, Conlan *et al.* (Conlan *et al.*, 2007) found that the mean delay was 0.9 days. Additionally, based on field data from commercial broiler flocks in Australia, Van Gerwe *et al.* (Van Gerwe *et al.*, 2009) estimated the number of secondary infections caused by one colonized bird per day to be 2.37 $\pm$ 0.295 day^{-1}; thus, one colonized bird could, on average, infect 2.37 birds per day. This finding is consistent with our observation that, in our flock of 600 broilers, in which 120 birds were orally challenged, nearly 100% of the birds were colonized by the first sampling, which was carried out 6 days p.i. The highest levels of colonization and shedding were found during the first two weeks after oral challenge, when the birds were 21 and 28 days old, respectively, and these values subsequently decreased in all diet treatments. Although *Campylobacter* was not isolated by culture from environmental samples, it was detected by real-time PCR in dust, air filters and drinkers while birds shed culturable *C. jejuni* cells. Although an enrichment culture method, which may be better able to recover damaged cells, was used, *Campylobacter* was not isolated from any environmental samples. Olsen *et al.* (Olsen *et al.*, 2009) also failed to cultivate airborne *Campylobacter* during rearing, but they were able to detect it by PCR in air samples, even before it could be detected in sock samples. In the study herein, the detection of *C. jejuni* DNA in dust and air samples collected

inside the broiler house correlated with the shedding of culturable *C. jejuni* cells. Failure to isolate *C. jejuni* from the environment would imply that culturable airborne *C. jejuni* were absent or present at negligible levels even when infection was widespread in the flock. However, non-culturable coccoid forms of *C. jejuni*, which would not be detected by conventional culture methods, cannot be ignored. Alternatively, higher sampling volumes might be required to isolate airborne culturable *C. jejuni*. The contamination levels in air samples were highest within the first 2 weeks after inoculation; during this period, shedding through cloacal swabs was also the highest, and a mean of 9.0 Log_{10} *Campylobacter* cell equivalents per g of caecal content was detected in 28-day-old birds.

Despite the beneficial effects of dietary supplementation on intestinal health and productive variables, none of the diets appeared to affect *Camplylobacter* colonization or shedding. SCFAs are known to exhibit antibacterial activities, and several studies have tested the effects of SCFAs, including butyrate; however, the results are contradictory. Van Deun *et al.* (Van Deun *et al.*, 2008) observed no reduction in *Campylobacter* colonization in 2-week-old broilers when 0.05% calcium butyrate was added to the feed in a seeder model, despite the bactericidal effect of calcium butyrate toward *C. jejuni in vitro*. Insufficient butyrate concentration in the mucous layer, where *C. jejuni* localizes, was cited as one possible reason for this failure; indeed, the rapid absorption of butyrate by enterocytes could reduce the butyrate concentration to below the bactericidal level. However, in the assay herein, a two-fold-higher concentration (0.1%) of the same additive resulted in a similar outcome. Van Deun *et al.* (Van Deun *et al.*, 2008) also showed that the effects of butyrate were dependent on the acidity of the medium. A neutral environment favours the dissociation of butyrate, but the undissociated form is the one that penetrates the bacterial membrane. Once inside the bacterial cell, where the pH is higher, butyrate dissociates, leading to a lethal accumulation of anions (Russell, 1992). In our assay, the whey in the BuWh diet reduced the intestinal pH because of

the fermentation of lactose by chicken gut microbiota, thereby increasing the bactericidal activity of butyrate. However, no differences in *Camplylobacter* colonization were found. Van Deun *et al*. (Van Deun *et al.*, 2008) argued that the close association of *C. jejuni* with the mucous layer could protect *C. jejuni* from the bactericidal effects of butyrate. More recently, Guyard-Nicodème *et al*. (Guyard-Nicodème *et al.*, 2016) reported significant reductions at day 14 (3 days after oral inoculation) in birds fed two coated sodium butyrate-based products; this reduction remained significant at days 35 and 42 when a high dose (0.3%) was used. In addition to the butyrate concentration, other factors, such as the *C. jejuni* inoculation dose and the strains used, could account for these discrepancies between studies.

3.2.6. Conclusion

Feeding chickens a corn/soybean-based diet containing dry whey powder and coated calcium butyrate improved their growth and feed efficiency, exerted a beneficial effect on intestinal health, and decreased mortality. These favourable effects were particularly significant during the starter period, when the chicken gut microbiota is still developing. However, none of the tested diets provided the chicks any differential degree of protection against *Campylobacter* infection. Further studies are currently in progress to investigate the effects of the different diets on the gut microbiome structure and the possible influence of diet-induced microbial shifts on bird performance and *Campylobacter* survival.

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Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: Execution of the animal experiment: AH, BO & MO. Sample and data collection and microbiological analysis: BO & MO. Necropsies and histological analysis: MO. Statistical analysis: MO & RAJ. Design and coordination of the study, interpretation of results and final drafting of the manuscript: AH. All authors contributed in reviewing the manuscript. All authors read and approved the final version of the manuscript.

Competing interests: The authors declare that they have no competing interests.

Consent for publication: Not applicable

Ethics approval: The protocols for this study were approved by the local Animal Ethics Committee (Diputación Foral de Álava, Register no. 1821/12.05.2014) and handling of the birds was carried out in compliance with European Community Directive 2010/63/EU and its transposition into national legislation through the Royal Decree 53/2013 on the protection of birds used for scientific purposes.

3.3. Study III

16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan.

Ocejo, M., Oporto, B., Hurtado, A.

***Scientific Reports.* 2019; 9(1): 2506.**

3.3.1. Abstract

Gut microbiota affects health, metabolism and immunity of the host, and in the case of livestock, also food-safety. Here, 16S rRNA gene high-throughput Illumina sequencing was used to describe the microbiome of chicken caeca in two different breeds and management systems throughout their whole productive lifespan. Broilers (Ross-308), as a fast-growing breed reared in an intensive system for 42-days, and a slow-growing breed of chicken (Sasso-T451A) reared in an extensive farming system with outdoor access for 86-days, were compared. The core microbiome and differentially abundant taxa, as well as taxa associated with age were identified. Age was identified as the strongest influencing factor in caecal microbiota composition, and, in general, each age-group showed an age-associated community profile, with a transition period at the middle of their lifespan. However, substantial differences were observed in the composition of caecal microbiota of both chicken breeds, microbiota being richer and more complex in free-range chicken than in broilers. Several taxa positively/negatively correlated with *Campylobacter* relative abundance were also identified. Especially noteworthy was the identification by microbial community comparison of microbiota profiles suggestive of dysbiosis in several free-range chickens, probably associated to the typhlitis later observed in the lumen of their caeca.

3.3.2. Introduction

Gut microbiota is considered as an additional organ due to its vital importance on the physiological, metabolic, immunological and digestion and nutritional uptake functions of the host (O'Hara and Shanahan, 2006). Considering its implication in food safety and public health (DuPont, 2007; Yeoman *et al.*, 2012), gut microbiota of food-producing animals has become a topic of interest and the subject of extensive studies. Among food-producing animals, poultry is the source of one of the most consumed meat worldwide. While conventionally raised poultry continues to dominate the EU poultry industry, there is an increasing demand for what is considered less intensive and more welfare-friendly management practices. In this regard, free-range production is an attribute highly appreciated among consumers who are looking for value-added food products. Free-range production of chicken meat normally requires slow-growing breeds and age at slaughter is normally not less than 12 weeks (Lewis *et al.*, 1997). Fast-growing commercial hybrids are not suitable for these production systems, which are rather used for intensive broiler production, slaughter age varying between 5 and 7 weeks.

Defining what constitutes a healthy gut microbiota is essential to help designing strategies to modulate its composition, not only in the realm of improving host performance and therefore enhance production, but also to maintain optimal host health and control zoonotic agents (Stanley, Hughes and Moore, 2014) that can contaminate food of animal origin thus posing a risk for consumers health. However, there is not clear definition of a healthy microbiota (Bäckhed *et al.*, 2012). Still, higher microbial diversity is commonly associated with a healthier host status, as the lack of sufficient diversity or evenness in a bacterial community structure appears to diminish its ability to withstand perturbation. In this sense, reduced microbial diversity has been associated with different intestinal disease states (Bäckhed *et al.*, 2012; Sommer *et al.*, 2017). Furthermore, disruption of the gut microbiota structure that results in the

elimination of subsets of beneficial bacteria, often leads to pathogen overgrowth, in conjunction with significant loss of microbial diversity (Fujimura *et al.*, 2010). Besides, factors such as diet (Apajalahti, Kettunen and Graham, 2004; Torok *et al.*, 2011; Borda-Molina *et al.*, 2016), rearing conditions (Xu *et al.*, 2016), host genetics (Zhao *et al.*, 2013) and age (Lu *et al.*, 2003; Apajalahti, Kettunen and Graham, 2004; Oakley, Buhr, *et al.*, 2014; Sergeant *et al.*, 2014; Videnska *et al.*, 2014; Ranjitkar *et al.*, 2016) can have an effect on poultry gut microbiota diversity, composition, and community structure. In addition, different regions of the bowel also harbor different microorganisms, as detailed in several reviews (Van Der Wielen *et al.*, 2002; Rehman *et al.*, 2007; Yeoman *et al.*, 2012) and recent studies (Ranjitkar *et al.*, 2016; Xiao *et al.*, 2017). The chicken caecum hosts the largest quantity and diversity of microbes along the chicken gastrointestinal tract since a prolonged retention of digesta occurs, and it is the main site of bacterial fermentation (Gong *et al.*, 2002; Apajalahti, Kettunen and Graham, 2004). Dietary interventions have been used to control gut health problems related to overgrowth of certain intestinal bacteria (dysbacteriosis) both in animals and humans, or to control zoonotic pathogens that are often found in the gut of commercially raised chickens, like *Campylobacter*, *Escherichia coli*, *Salmonella* or *Streptococcus*, as reviewed elsewhere (Oakley, Lillehoj, *et al.*, 2014; Stanley, Hughes and Moore, 2014). In a previous study, we showed the beneficial effects on performance and intestinal integrity of feeding broilers with a diet supplemented with dry whey and coated calcium butyrate (Ocejo *et al.*, 2017). Since poultry is considered the principal reservoir of *Campylobacter* and main source of human campylobacteriosis (Newell and Fearnley, 2003), in that study we also tested diet effects on *Campylobacter* colonization and dissemination but none of the tested diets provided the chicks any differential degree of protection against *Campylobacter* infection (Ocejo *et al.*, 2017). However, effects on microbiota community profiles were then not measured. With the onset of high-throughput sequencing technologies, communities as diverse and complex as gut microbiota, can be sequenced to

unprecedented depth and coverage more accurately. Targeted sequencing, also defined as amplicon sequencing, is one of the main DNA-based approaches of this technology at the time of publication (Ju and Zhang, 2015; Borda-Molina, Seifert and Camarinha-Silva, 2018). It uses the amplification of conserved regions such as 16S ribosomal RNA (rRNA) genes in the analysis of bacterial community profiling. Caecal microbial communities in chickens have been assessed in previous studies. However, most efforts have focused on broilers and studies in other breeds and management systems are scarce. Furthermore, not many studies fully covered the complete productive period (Awad *et al.*, 2016; Ballou *et al.*, 2016; Mancabelli *et al.*, 2016) or provided a deep sequencing coverage (Danzeisen *et al.*, 2011; Oakley, Buhr, *et al.*, 2014; Mohd Shaufi *et al.*, 2015; Ballou *et al.*, 2016).

The aim of this study was to thoroughly characterize the caecal microbiota in two breeds of chickens fed with different diets and bred under different production systems throughout their complete lifespan; Ross-308 broilers reared for 42 days, as the fast-growing breed used in intensive chicken meat production, and a slow-growing breed (Sasso-T451A) reared in a free-range system for 84 days. The effect of breed, diet and production system was assessed to obtain a comprehensive knowledge of how microbiota develops as chickens grow in the different phases of their productive life, and particularly the following questions were addressed: (i) How do caecal microbial communities change during the productive life of chickens? (ii) Does supplementation of diet with calcium butyrate and dry whey influence the caecal microbial community patterns in broilers? iii) Do caecal microbiome temporal dynamics vary between fast-growing and slow-growing breeds? iv) Which microbial taxa are representative of each age-group? (v) Is there a core microbiota for all age-groups in each breed, and a core microbiota for both breeds throughout their productive life? (vi) Which taxa tend to coexist with *Campylobacter* in the chicken caeca?

3.3.3. Methods

Subjects discription

Broiler chickens

Broiler (Ross-308) chickens were obtained in the frame of an experimental study carried out to assess the effect of dietary supplementation with dry whey powder (a prebiotic), coated calcium butyrate (a salt of an short-chain fatty acid - SCFA) or their combination, in terms of productive performance, duodenal histological integrity, and *Campylobacter* colonization and dissemination (Ocejo *et al.*, 2017). Briefly, 600 one-day-old Ross-308 chickens were placed into 20 ground pens (2.5 × 1 m each) and assigned to one of 4 dietary treatments (5 replicates of 30 chicks per treatment) following a randomized complete block design. The birds were fed two-phase corn/soybean-based diets during the starter (0–20 d) and grower-finisher (21–42 d) periods, with different supplementations as follows: 1) basal diet with no supplementation as control (Co); 2) diet containing 6% dry whey powder (Wh); 3) diet supplemented with coated calcium butyrate at 0.1% (Bu) and 4) diet containing 6% whey and 0.1% calcium butyrate (WhBu). The diets were formulated to meet Ross-308 broiler requirements (FEDNA, 2008, 2010) and to provide equal nutrient profiles. Chicks were reared for 42 days. Feed and water were provided *ad libitum*. To simulate a common situation in broiler production, 6 chickens per pen were experimentally inoculated with *Campylobacter jejuni* at 15 days of age, when natural infections are usually reported (Evans and Sayers, 2000; Bull *et al.*, 2006), and the infection was monitored until the end of the productive period (Ocejo *et al.*, 2017).

Free-range slow-growing chickens (FRC)

Slow-growing meat chickens (Sasso-T451A) were provided by a local commercial free-range poultry farm. Chickens were reared for 12 weeks on a

semi-extensive management system and slaughtered with average body weight of 2,235 kg. Feed and water were provided *ad libitum*. Feed consisted of grain, composed of at least 60% corn and different proportions of wheat and soya according to age requirements, as follows: 1-35 d, 5% wheat and 30% soya; 36-61 d, 11% wheat and 24% soya; and, 61-84 d, 12% wheat and 23% soya. In addition, from day 26 birds had free daytime access to grassland yards and grass became part of their diet. The flock included 3,650 birds, and stocking density was 11 chickens/m^2 within houses, and 2 m^2 per chick outdoors. No antibiotics were administered, and the only vaccine given was to control coccidiosis. The all-in all-out system was implemented. The region where the farm was located has an Atlantic climate, and during the production period (24th May – 16th August 2016), mean temperature was 19.9°C (mean maximum, 24.7ºC; mean minimum, 15.2ºC) and the total accumulated rainfall was 102.9 l/m^2.

Sample collection

Both breeds were sampled in different years but within the same period of the year. Broilers were sampled in May-June 2015 and FRC were sampled between the May and August 2016. A total of 80 broilers (1 per pen, *i.e.*, 5 replicates per diet) were randomly selected at four time points (3, 14, 29, and 42 days of age) and 38 FRC were sacrificed at five time points (4, 18, 39, 58 and 81 days of age). All chickens belonged to the same productive lot within the breed type and represented the average weight at each group. No feed withdrawal was performed before being euthanized by exposure to carbon dioxide (CO_2). Necropsy was immediately performed, and caeca were aseptically dissected, and their contents collected for DNA extraction.

Ethics statement

The protocols for the study carried out in broilers were approved by the local Animal Ethics Committee (Diputación Foral de Álava, Register no. 1821/12.05.2014). Sample collection in the free-range poultry farm was carried

out by the veterinary clinician after obtaining informed oral consent from the farm owner. In both experiments, handling of the birds was carried out in compliance with European Community Directive 2010/63/EU and its transposition into national legislation through the Royal Decree 53/2013 on the protection of birds used for scientific purposes.

DNA Isolation and next-generation (NGS) sequencing

Genomic DNA was extracted from approximately 0.15 g of caeca content using PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer´s instructions. Whereas for broilers the content of both caeca was mixed before DNA purification, for FRC caecal content from each caecum was processed individually as replicates (except for the 4-day-old group due to limited sample availability). In order to prevent repetitive freeze-thawing, two DNA aliquots were prepared; one was immediately stored at -80°C until shipping to the sequencing service and the other one was to be used for any other analyses. Concentration of DNA was determined by spectrophotometry (Nanodrop ND1000; Thermo Scientific, USA) and its integrity was visually assessed by 0.8% agarose gel alectroforesis.

Normalized DNA was submitted to the Centre for Genomic Research of the University of Liverpool (UK) for PCR amplification, library preparation and 16S high-throughput sequencing. The V4 hypervariable region of the bacterial 16S rRNA genes present in each caecal DNA sample was amplified by PCR using the universal primer set 515F/806R according to the protocol described elsewhere (Caporaso *et al.*, 2011). A dual-indexing amplification and sequencing approach was used, and the resulting amplicons were purified and subjected to paired-end sequencing (2x250bp) of one pooled amplicon sample on two runs of the Illumina MiSeq platform, one for broilers and another for FRC.

Bioinformatic Analysis | Sequence processing and data analysis

Illumina adapter sequences were trimmed from the raw fastq files using Cutadapt v1.2.1 (Martin, 2011) and reads below a window quality score of 20 were trimmed using Sickle v.1.200 (Joshi and Fass, 2011). Reads shorter than 10 bp were discarded, primers were trimmed with Cutadapt in the paired-end mode and quality of the reads was assesed using FastQC v.0.11.5 quality-control tool (Babraham Bioinformatics, Cambridge, United Kingdom)(Andrews, 2010). Subsequent analyses were performed within R environment (R Development Core Team, 2016) following DADA2 Pipeline (Tutorial (1.6) in https://benjjneb.github.io/dada2/tutorial.html) by adjusting parameters to our dataset. The end product was a non-chimeric amplicon sequence variants (ASVs) table (Callahan, McMurdie and Holmes, 2017) which records the number of times each ASV (sequence differing by as little as one nucleotide) was observed in each sample. Taxonomy assignment of representative ASVs was performed with DADA2 against GreenGenes reference database (v.13.8) (DeSantis *et al.*, 2006) with the bootstrapping threshold set to 80%. ASVs composed by less than 10 sequences in all samples were filtered out.

Ecological and statistical analyses

Downstream analyses and graphical outputs were generated with different packages in R v.3.3.2 (R Development Core Team, 2016). Continuous variables were tested for normality with the Shapiro-Wilk test. Rarefaction curves were constructed on unnormalized ASV-level data using Vegan package (Oksanen *et al.*, 2017) for each age-group to assess sequencing effort. Phyloseq v.1.19.1 (McMurdie and Holmes, 2013) was used to visualize abundance of microbial taxonomic composition, estimate biodiversity, create heatmaps and perform normalization and differential abundance tests. Both alpha and beta diversity metrics were used to estimate microbial communities' diversity. Species richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson index) were calculated for alpha diversity estimations. To compare alpha

diversity metrics among groups, non-parametric Kruskal-Wallis test was conducted, followed by subsequent pairwise comparisons with Wilcoxon rank sum test adjusted using the Benjamini–Hochberg (B-H) method. Total sum scaling was used as normalization method to account for variability in the number of reads between samples. For beta diversity analysis, dissimilarity matrix between samples was calculated with Bray Curtis method, and it was further visualized with a Non-Metric Multidimensional Scaling (NMDS) ordination technique. Bray Curtis dissimilarity was also used to perform hierarchical clustering using hclust with the unweighted pair-group method with arithmetic mean (UPGMA) method. To study the effect of age and diet on microbiota composition variability between samples based on beta diversity distance matrices, we used permutational multivariate analysis of variance (PERMANOVA) with the adonis function from R's Vegan package (Oksanen *et al.*, 2017). Pairwise PERMANOVA comparisons were done with a function written by Pedro Martinez Arbizu available in the ResearchGate questions section (https://www.researchgate.net/post/How_can_I_do_PerMANOVA_pairwise_contrasts_in_R), also adjusted with B-H method. To test for homogeneity of multivariate dispersions (*i.e.* deviations from centroids) among age-groups or diets, permutation multivariate analysis of dispersion (PERMDISP) was conducted with the function betadisper and permutest also from Vegan package.

In an attempt to look for differentially abundant taxa among the different age-groups and diets, DESeq2 (v.1.14.1) (Love, Anders and Huber, 2014) was carried out by performing the Wald significance test with a parametric fit type and multiple-inference correction by B-H method. Linear Discriminant Analysis Effect Size (LEfSe) algorithm with LDA effect size threshold of 2 (on a log^{10} scale) was applied after agglomerating data to genus level for evidencing potential biomarkers linked to age and diet. The outputs of both approaches were combined to identify potential biomarkers as those significantly representative by LEfSe and differentially abundant by DESeq2 (in all of the pairwise comparisons between the group of interest and the other age-groups).

For each breed, Spearman´s rank test was used to find taxa associated with age and taxa positively/negatively correlated with *Campylobacter* relative abundance. Multiple testing correction with false discovery rate (FDR) was performed and the correlation was considered significant if $p_{adj} < 0.05$. For *Campylobacter* correlation tests, only *Campylobacter*-positive animals were included in the analysis.

Core microbiome was identified for each breed at ASV and genus level and was defined as taxa detected in all age-groups with at least 10 reads within the group. To identify the shared and unique taxa among the age-groups, Venn diagrams were constructed with the online tool accessed through http://bioinformatics.psb.ugent.be/webtools/Venn/ link.

For the study of broilers, all analyses were done both for the whole dataset, to investigate the effect of age and/or diet, and in age-grouped subsets of data to study the effect of diet within each age-group. FRC data analyses were performed in the whole dataset to examine the effect of age. Significance ($p < 0.05$) was based on the corrected p-values.

3.3.4. Results

Sequencing output, preprocessing and taxonomic assignment

After the initial filtering and adaptors trimming process, samples from broilers accounted to a total of 9,887,438 paired-end reads with a median of 113,061 paired-end reads (IQR 95,211 – 137,688) per sample. Median length of the reads per sample was 246.1 bases (IQR: 244.5 – 247.1). After preprocessing, exclusion of ASVs classified as chloroplasts and mitochondria and removal of low-count ASVs as described in methods, the 8,879,083 amplicon reads from the 80 samples were classified into 1,163 ASVs. In free-range slow-growing chicken (FRC) samples, a total of 11,294,265 reads passed the filtering and adaptors trimming steps (median: 163,646; IQR: 116,527 – 211,636 per sample). Median

length of the reads per sample was 247 bases (IQR: 246.5 – 247.4). Two samples were discarded (<50,000), leaving 10,041,046 amplicon reads from 68 samples classified into 2,033 ASVs. Number of reads that passed through each step of the DADA2 pipeline are presented in Supplementary Table S1.

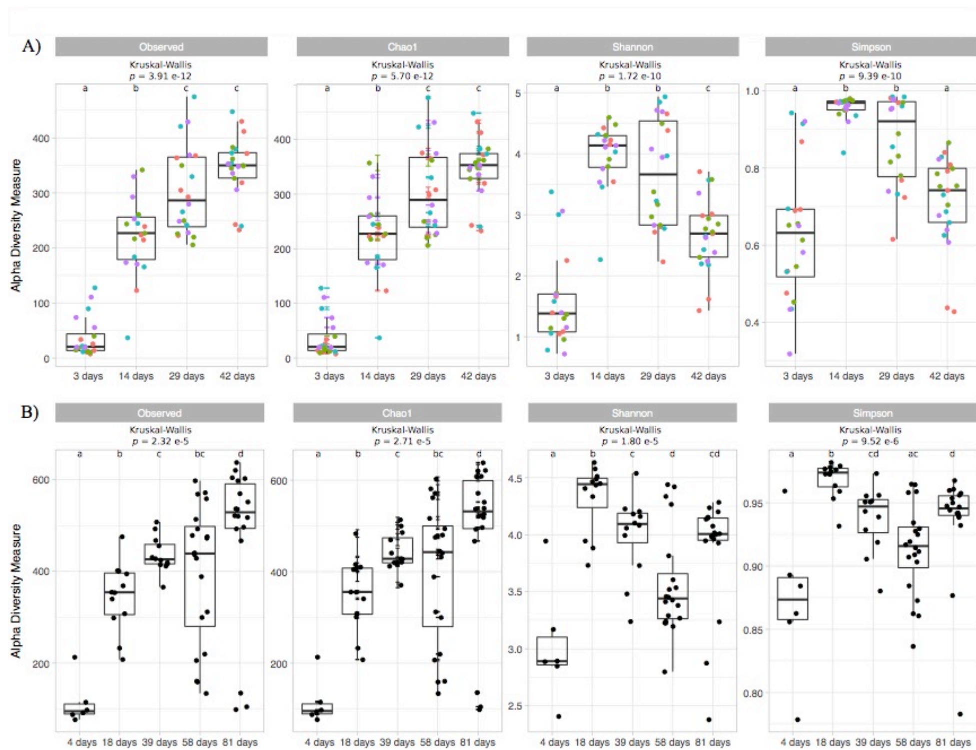

Figure 8. Boxplots representing alpha diversity metrics of richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson) for samples from broilers (a) and FRC (b) grouped according to age.

Non-parametric Kruskal-Wallis test followed by post-hoc Wilcoxon rank sum test adjusted for multiple comparisons using the B-H method was conducted. Each point represents the diversity score for a sample; broiler chickens are color-coded according to diet (a). The box represents the first (Q1) and third (Q3) quartiles of the distribution, and the line within de box marks the median. The whiskers extend from Q1 and Q3 to the last data points within 1.5×IQR and values beyond these whiskers are considered outliers. Boxplots not sharing a common letter above them are significantly different at $p_{adj} < 0.05$.

Rarefaction curves generated from the ASVs (Supplementary Figure S1) showed high sequencing coverage in all samples, and higher level of microbial diversity in FRC than in broilers. In both cases, chicken-to-chicken variation was observed in each age-group. Broilers fed different diets presented similar

rarefaction curves patterns within each age-group (data not shown). In general, proportions of taxonomic assignation were similar in both of the breeds, but the number of unique taxa identified was notably higher in FRC (Supplementary Table S2).

Alpha diversity

Alpha diversity indices of both datasets, broiler and FRC, showed similar pattern distribution with age (Fig. 12). A steady increase in species richness was observed as the chickens aged, as indicated by Observed ASV and Chao1. Both estimators showed similar patterns reassuring that the sequencing depth obtained was sufficient. Average Shannon and Simpson indices presented the lowest values in youngest chickens, indicating that the species present were not equally abundant, and reached the highest values at day 14 (broilers) and 18 (FRC), suggesting that the abundance of the different species was then more even. Both indices decreased gradually afterwards except in 81-day-old FRC, whose microbiome composition turned more evenly abundant again. Kruskal-Wallis tests of Richness, Shannon and Simpson indicated that bacterial diversity in chickens significantly differed from one age-group to another in both datasets. None of the alpha diversity indices were statistically different among diets in broilers, neither when the diet effect was analysed in the whole dataset nor restricting the dietary comparison to subsets of age-groups only and thereby controlling for the age effect. When comparing broilers and FRC, the latter showed higher values in all alpha diversity indices.

Beta diversity

In both breeds, the overall microbial community structure exhibited clear and significant shifts by age (total variance explained $R^2 = 0.45$, $p < 0.001$ in broilers and $R^2 = 0.44$, $p<0.001$ in free-range) based on PERMANOVA, and post-hoc analysis revealed significant differences between each possible pairwise comparisons between age-groups in both breeds.

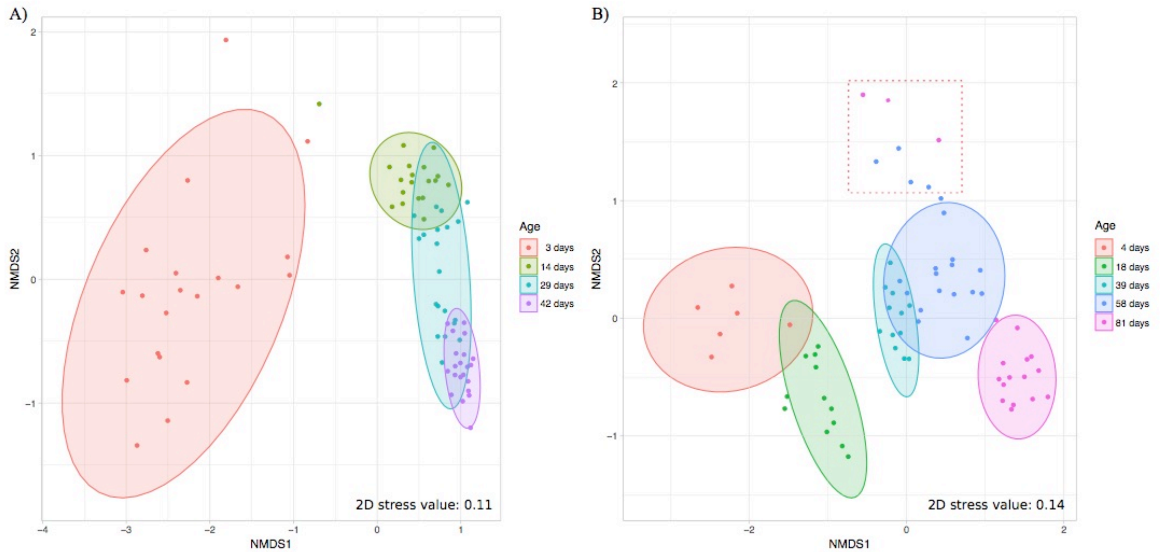

Figure 9. Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity matrix on relative abundance data in broilers (a) and FRC (b). Colors indicate age groups. Ellipses indicate 95% confidence intervals of multivariate t-distribution around centroids of the groupings with age as factor. Dots within the square area in plot B represent chickens with typhlitis, which were excluded for the estimation of the ellipses (see text).

In broilers, betadisper revealed that individual variation in the community structure was significantly greater in younger birds (3-day-old), while the lowest variance was found among 42-day-old broilers ($F=32.08$, $p<0.001$). Despite the lack of homogeneity in dispersion, the NMDS plot based on Bray-Curtis dissimilarity matrix still showed age-related clustering, with chickens of the same age clustering more closely together (Fig. 9A). Eleven of the 29-day-old broilers shared similar community structure with 14-day-old birds, while the remaining 9 chickens in the 29-day-old group were more similar in composition to 42-day-old broilers. In contrast, diet was not a significant factor neither for the whole dataset ($R^2=0.032$, $p=0.718$) nor when comparing different diets within age-groups ($p>0.05$).

In FRC, betadisper indicated that microbiome community dispersion between individuals did not vary in the different age-groups ($F=1.74$, $p=0.148$), reinforcing the age effect in PERMANOVA results. The only exception were seven samples which were clearly distant from the centroids of its age-grouping.

All these samples had in common the presence of hard caseous material in the lumen of the caeca, indicative of typhlitis. When these seven samples were excluded from their respective age-groups to calculate the 95% CI of the distribution around centroids of the ellipsoids, age clustering became tighter (Fig. 9B).

Hierarchical clustering

In agreement with the results observed in NMDS plots, dendogram of hierarchical clustering revealed that samples from 3, 14 and 42-day-old broilers (Fig. 10A) formed three separate clusters with samples from 14-day-old broilers clustering closer to 42-day-old than to 3-day-old broilers. On the other hand, samples from 29-day-old broilers grouped with both the 14-day-old and the 42-day-old broilers, with no clear predominant cluster defined.

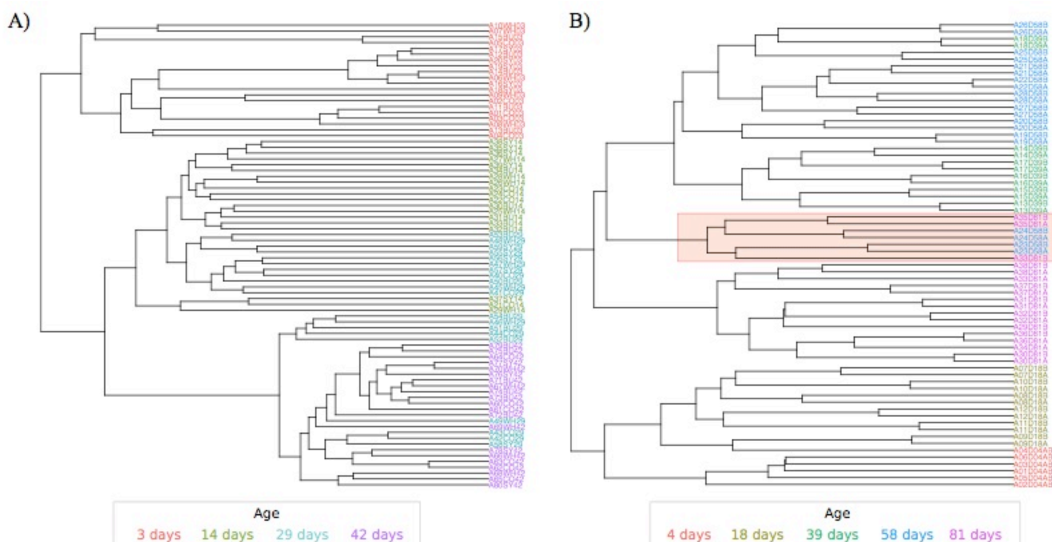

Figure 10. Hierarchical clustering analysis: Dendrogram of Bray-Curtis dissimilarity matrices between samples based on different age groups in broilers (a) and FRC (b) with UPGMA method.
The red frame in the free-range chickens dendrogram (b) indicate the samples with typhlitis

Again, no diet-related clustering was observed, neither in the whole dataset nor within subsets of age-groups. In FRC, pairs of caecal samples

collected from the same chicken always clustered together. In general, chickens clearly clustered by age-group according to their bacterial community composition. The only exceptions were one 39-day-old chicken (both caeca) that were included in the group of 58-day-old chickens, and the seven samples from four chickens with signs of typhlitis. These seven samples (three from two 81-day-old chickens and four from two 58-day-old chickens) formed a different cluster in the dendrogram independently of age (Fig. 10B).

Microbial community dynamics

A complete list of all bacterial taxa (average relative abundance) identified for each age-group and breed in the caecal content samples is provided in Supplementary Table S3. In both breeds, microbial communities displayed different relative proportions according to age even at the phylum level, and microbial community underwent dramatic shifts along time (Fig. 11 & 12). Microbial taxa consistently present over time (core microbiome) were represented by 154 ASVs within broilers and 175 ASVs within FRC (Fig. 13) and corresponded to 16 genera in broilers and 19 in FRC. Among them, 15 genera (mainly belonging to the order Clostridiales, phylum Firmicutes) were identified in every sampling point in both breeds with variations in relative abundance (Fig. 14).

Firmicutes and Proteobacteria together formed almost the entirety of the caecal microbiome of younger chickens, with Bacteroidetes reaching substantial levels in the second half of their lifespan. In both chicken breeds, Proteobacteria was a highly abundant phylum at first sampling, but proportions decreased thereafter. Thus, a 10-fold reduction in the relative abundance was observed on day 14 in broilers to 4.2% (from 54.7% on day 3) and on day 18 in FRC to 3.3% (from 31.9% on day 4).

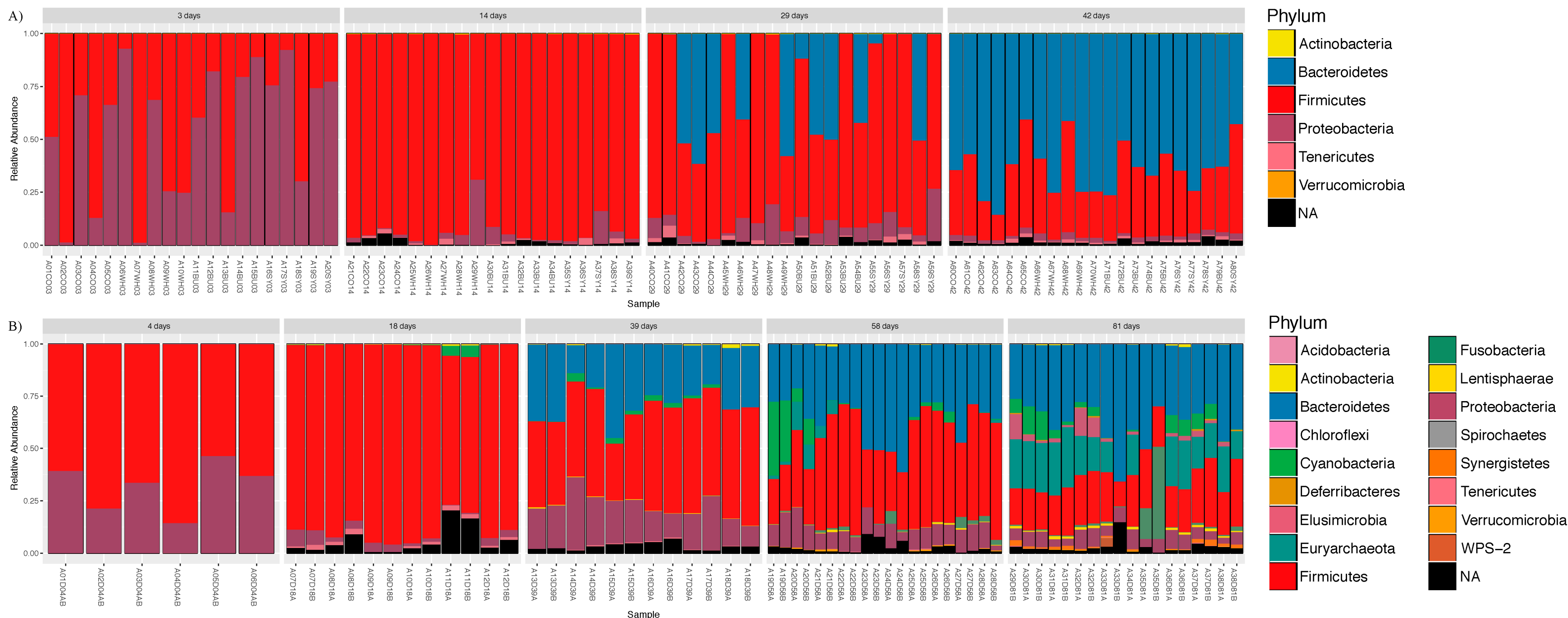

Figure 11. Microbial community composition of chicken caecal content. Stacked barplots representing relative abundances of the different phyla in all samples in broilers (a) and FRC (b).

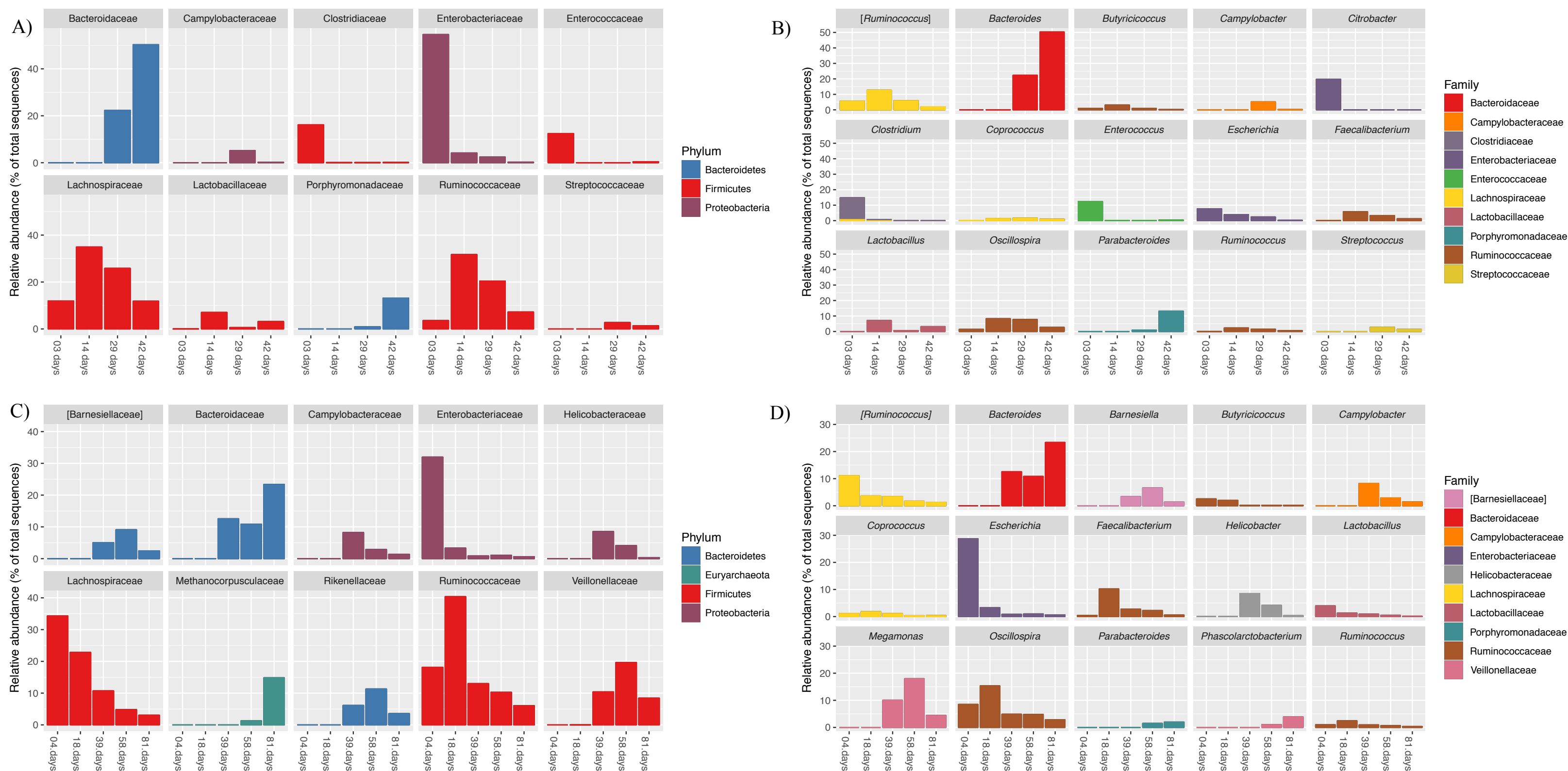

Figure 12. **Relative abundance of the top 10 families and top 15 genera averaged over all samples for the age-groups for broilers (A and B) and FRC (C and D).**

Proteobacteria shifted from a predominance of Enterobacteriales at younger ages, to Campylobacterales after the fourth week of age. Although Enterobacteriaceae were part of the core microbiome of both breeds, during the first two samplings it accounted for nearly all Proteobacteria (94-100%), but represented only 10% by the end of their productive period. Enterobacteriaceae in younger birds were mainly represented by *E. coli*, except in 3-day-old broilers in which *Citrobacter* accounted for 34% of Proteobacteria and was identified as a biomarker of that age-group (Table 8). Then, Campylobacterales became the main order within Proteobacteria. The highest abundance of *Campylobacter* was found in 29-day-old broilers (43% of Proteobacteria) and in 39-day-old FRC (67% of Proteobacteria). *Helicobacter* (45%) also accounted for the high abundance of Campylobacterales in 39-day-old FRC, but it was never found in broilers. At the end of their productive life, *Sutterella* (order Burkholderiales) became the most prevalent Proteobacteria genus in 42-day-old broilers (75% and biomarker of the group) and second in 81-day-old FRC (21%, after *Campylobacter* – 22%). In both breeds, the relative abundance of *Sutterella* was strongly correlated with time (spearman rho > 0.75) (Supplementary Table S4) and with *Campylobacter* (rho = -0.61, p<0.001 in broilers and rho = -0.42, p=0.037 in FRC). At the end of the productive period, Enterobacteriaceae represented only about 10% of all Proteobacteria.

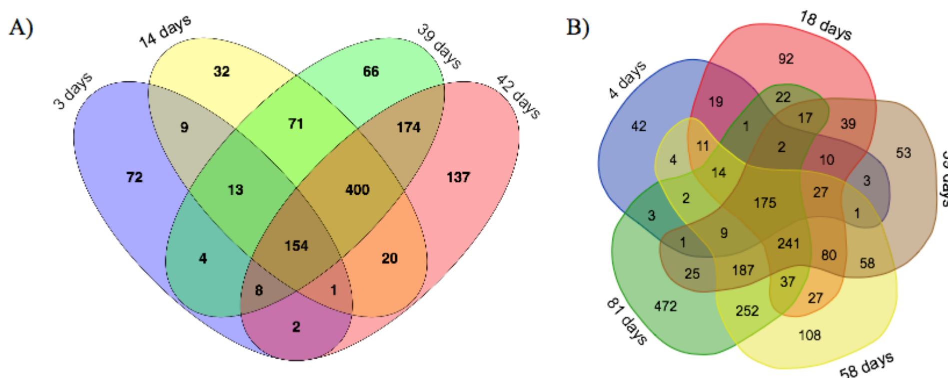

Figure 13. Venn diagram illustrating core microbiome (ASVs) in broilers (a) and FRC (b).

Firmicutes was dominated by families within the order Clostridiales (Lachnospiraceae, Ruminococcaceae and Clostridaceae) whose relative abundance varied with age and breed but formed part of the core microbiome of both chicken breeds. In fact, most genera identified in every sampling point in both chicken breeds belonged to the order Clostridiales (Fig. 7). Albeit present at important levels throughout the productive period in both breeds, highest proportions were detected in 14-day-old broilers (83% of bacteria) and 18-day-old FRC (81%). In 3-day-old broilers, this order was composed mainly by the family Clostridiaceae (50%), and Lachnospiraceae levels (37%) were three times higher than Ruminococcaceae (11%). Afterwards, Clostridiaceae family was almost depleted as Lachnospiraceae and Ruminococcaceae gained importance and the ratio between these two families became stable. Comparatively, levels of Clostridaceae in FRC were never substantial (always below 3%). Instead, Lachnospiraceae and Ruminococcaceae were found in younger animals, along with Veillonellaceae after day 39. In both cases, the presence of Clostridiaceae became irrelevant in older individuals (<0.6%). *Clostridium* genus was present as core microbiome in both breeds, was a biomarker of the youngest birds (LDA effect size >5) (Supplementary Table S5), and was negatively correlated with age, more strongly in FRC than in broilers (rho = -0.72 *vs.* -0.39) (Supplementary Table S4). However, in 3-day-old broilers, almost 70% of this genus was represented by *C. perfringens* while in FRC *C. perfringens* only represented 6%. LEfSe identified Lachnospiraceae as biomarker of 14-day-old broilers and 4-day-old FRC (LDA effect size >6) (Supplementary Table S5). *Coprococcus* was among the top genera within Lachnospiraceae family in both broilers and FRC and it was present at all sampling points. The highest levels of Ruminococcaceae were observed in the second sampling point in both breeds. *Oscillospira, Faecalibacterium*, *Ruminococcus* and *Anaerotruncus* were among the top genera and they were observed consistently in both broilers and FRC throughout their lifespan. In fact, these four genera were identified as biomarkers of 18-day-old FRC, and *Anaerotroncus* was a biomarker of 14-day-old broilers (Table 8).

Table 8. Genera identified to be significantly representative of each age group in each breed as a result of combining LEfSe output and significantly differentially abundant in DESeq2 analysis results. Genera were listed in descending order according to its effect size in LEfSe.

Age	Genera
Broiler	
3 days	*Citrobacter*, *Enterococcus*, *Clostridium* (Clostridaceae)
14 days	*Anaerotruncus*, *Candidatus_Arthromitus*
29 days	*Campylobacter*
42 days	*Parabacteroides*, *Suterella*, *Dehalobacterium*
FRC	
4 days	*Clostridium* (Clostridaceae), *Epulopiscium*
18 days	*Oscillospira*, *Faecalibacterium*, *Anaerotruncus*, *Lachnospira*, *Ruminococcus* (Ruminococcaceae)
39 days	-
58 days	*Alistipes*, *AF12*, *Pseudoramibacter_Eubacterium*
81 days	*Elusimicrobium*, *Desulfovibrio*, *YRC22*, *Succinatimonas*, *Butyricimonas*, *Mucispirillum*, *Prevotella*, *Veillonella*, *Oxalobacter*

Two families of the order Lactobacillales (Enterococcaceae and Lactobacillaceae) were also part of the core microbiome in both breeds. The genus *Enterococcus* (family Enterococcaceae) accounted for 12% of the bacteria in broilers but represented only 2% of bacteria in FRC. Lactobacillaceae decreased with age in FRC from the highest levels found at 4 days, while it reached its highest peak of abundance in 14-day-old broilers due to the increase in *Lactobacillus* abundance. Opposite associations of *Lactobacillus* with *Campylobacter* were observed in broilers (rho = -0.43, p=0.012) and FRC (rho = 0.29, p=0.124). Another Lactobacillaceae genus, *Pediococcus*, was only found in the youngest chickens and it was 10 times more abundant in FRC than in broilers. Streptococcaceae was part of the core microbiome of FRC but it never represented more than 0.3% of the bacteria, whereas in 29-day-old broilers *Streptococcus* accounted for 3% of bacteria. At the end of their productive life, Firmicutes was still an important phylum in the caecal microbiota of both broilers (42 day-old) and FRC (81 day-old), but a clear phylum predominance shift from Firmicutes to Bacteroidetes was observed and Bacteroidetes became

the main phylum (63.5% in broilers and 37.3% in FRC). In FRC, this reduction in the Firmicutes:Bacteroidetes ratio was lower than in broilers due to the presence of other phyla, in contrast to the much more limited community profile diversity found in broilers. *Parabacteroides* was identified as a potential biomarker of 42-day-old broilers whereas *Butyricimonas*, *YRC22* and *Prevotella* were biomarkers of 81-day-old FRC. In both breeds, *Parabacteroides* presented a strong correlation with age (spearman rho > 0.75) (Supplementary Table S4) and *Campylobacter* (rho = -0.59, $p<0.001$ in broilers and rho = -0.39, $p=0.039$).

Among the minor phyla, Tenericutes was present at steady levels throughout the productive period (0.002-1.5%), with *Anaeroplasma* as the main genus in broilers (Anaeroplasmataceae was a core family for broilers) and *Mollicutes* in FRC. Actinobacteria was always present except in 3-day-old broilers; levels ranged from 0.007-0.4%. In broilers, Actinobacteria was mainly composed of *Corynebacterium* and *Brachybacterium* and in FRC of *Corynebacterium*, *Bifidobacterium* and *Adlercreutzia*. Verrucomicrobia was only present in 42-day-old broilers and it was represented by only one genus, *Akkermansia,* at a very low proportion (0.0006%). In FRC, Verrucomicrobia was detected on days 58 and 81, and unassigned members of the family Cerasicoccaceae were more abundant than *Akkermansia*. A further 11 phyla were identified only in FRC, four of them reaching significant levels at the end of the productive period. Among these was the phylum Euryarchaeota (*vadinCA11* and unclassified reads from Methanocorpusculaceae family), the only archaea detected in this study, but present at high levels (17.1% in 81-day-old FRC). Also noteworthy was the presence of Cyanobacteria (4.3%), and Fusobacteriaceae (Fusobacteria) and Elusimicrobiaceae (Elusimicrobia) that reached 3.9% and 3.0%, respectively.

When chickens of both breeds were compared at the end of their productive life (42-day-old broilers *vs.* 81-day-old FRC), 30 common genera were

identified, along with 35 genera exclusive of FRC and 7 only found in broilers (Table 9).

Table 9. Core genera between both breeds at the end of the productive period (42-day-old broilers and 81-day-old FRC), and genera specific for each breed. Genera were sorted in alphabetical order.

Core Genera (n = 30)	FRC specific (n = 35)	Broilers specific (n = 7)
Akkermansia	*02d06*	*Anaeroplasma*
Anaerofustis	*Adlercreutzia*	*Anaerostipes*
Anaerotruncus	*AF12*	*Brachybacterium*
Bacteroides	*Alistipes*	*Ignatzschineria*
Blautia	*Anaerofilum*	*Jeotgalicoccus*
Butyricicoccus	*Avibacterium*	*Lachnospira*
Campylobacter	*Barnesiella*	*Staphylococcus*
cc_115	*Bifidobacterium*	
Clostridium	*Bilophila*	
Coprobacillus	*Butyricimonas*	
Coprococcus	*Candidatus Arthromitus*	
Corynebacterium	*Collinsella*	
Dehalobacterium	*Desulfovibrio*	
Dorea	*Dialister*	
Enterococcus	*Elusimicrobium*	
Escherichia	*Fusobacterium*	
[Eubacterium]	*Gallibacterium*	
Faecalibacterium	*Helicobacter*	
Holdemania	*Megamonas*	
Lactobacillus	*Mucispirillum*	
Oscillospira	*Odoribacter*	
Parabacteroides	*Oxalobacter*	
Proteus	*Paraprevotella*	
Roseburia	*Peptococcus*	
Ruminococcus	*Phascolarctobacterium*	
[Ruminococcus]	*Prevotella*	
SMB53	*Pseudoramibacter_Eubacterium*	
Streptococcus	*Rikenella*	
Succinatimonas	*Slackia*	
Sutterella	*Sphaerochaeta*	
	Turicibacter	
	vadinCA11	
	Veillonella	
	Victivallis	
	YRC22	

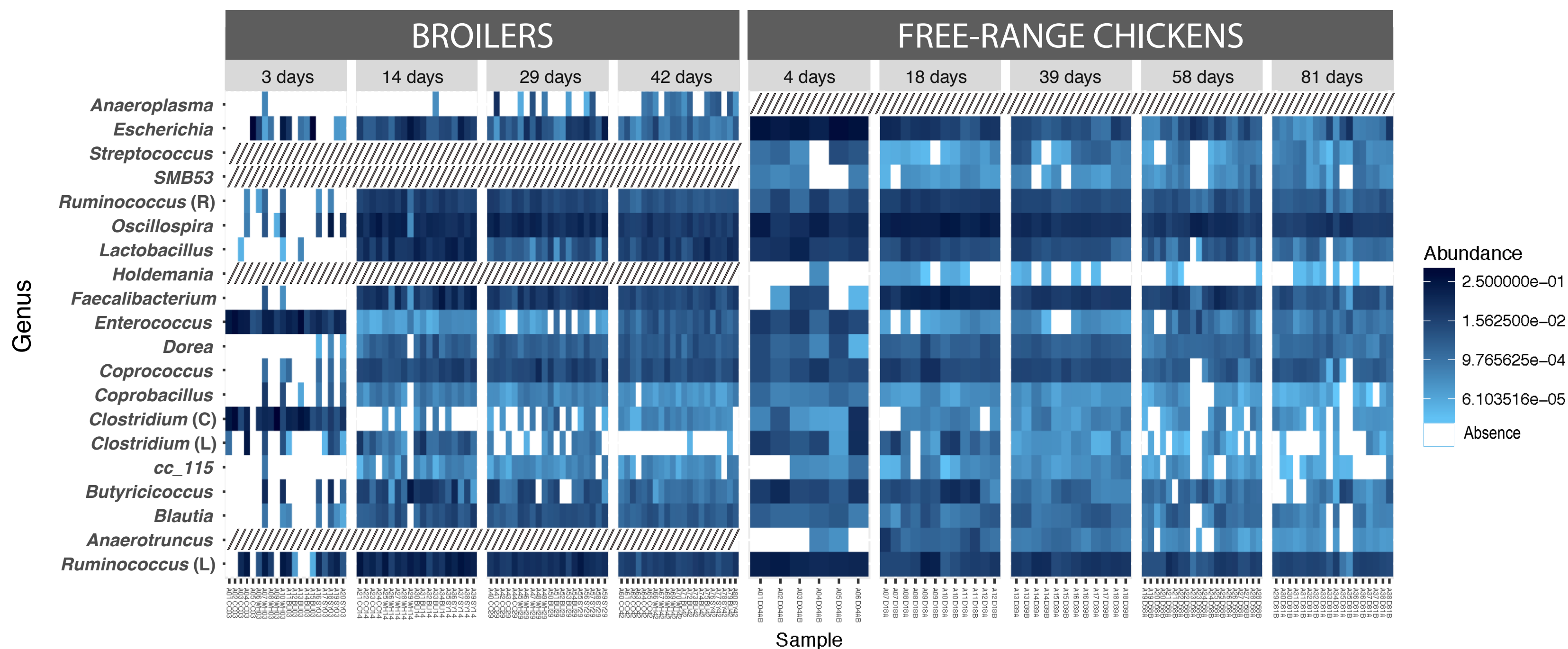

Figure 14. Heatmap representing classified genera within the core microbiome of each breed.
Darker shades of blue represent higher relative abundance as indicated in the legend; white colour represents absence. Boxes with diagonal pattern indicate that a particular genus was absent in at least one age group and therefore it was not considered as part of the core microbiome. Letter in brackets following a genus name indicates the family the genus belongs to (R – Ruminococcaceae, C – Clostridiaceae, L – Lachnospiraceae).

3.3.5. Discussion

This study provided a thorough description of the microbiome in chicken caeca in two different breeds and management systems commonly used in the chicken meat industry, throughout their whole productive period in an attempt to provide a global picture of the microbial taxonomical diversity. For this purpose, high sequencing coverage of short amplicon reads was achieved by using targeted high-throughput sequencing technology. For more powerful and reproducible analyses, instead of using the threshold of ≥97% nucleotide sequence identity in the sequenced 16S rRNA gene region for operational taxonomic units (OTUs) definition, we identified sequence variants, ASVs, differing by as little as one nucleotide using the high-resolution DADA2 method, a model-based approach for correcting amplicon errors without constructing OTUs (Callahan *et al.*, 2016). This method allows for more specific taxonomic assignation and permits future assignations when updated taxonomic information is available in the databases. By analysing biological sample replicates from each FRC (two ceca) we also demonstrated that both caeca had comparable microbiome composition, and that the analysis of a single caecum provides sufficiently representative data. This is in agreement with a previous study that analysed ten 42-day-old broilers from the same commercial flock (Sergeant *et al.*, 2014). Using these methods, broilers as a fast-growing breed raised in an intensive breeding system and a slow-growing breed of chicken grown in an extensive farming system with outdoor access were compared. Furthermore, the effect of feed supplementation with whey and calcium butyrate in broilers caecal microbiota was assessed to investigate if the beneficial effects on productive performance and duodenal histological integrity of dietary supplementation with these compounds (Ocejo *et al.*, 2017) were associated to diet-induced microbial shifts. Unexpectedly, our findings suggested that supplementation as described elsewhere (Ocejo *et al.*, 2017) did not alter the overall structure of the caecal microbiota of broilers. Other studies have shown that diet and feed additives (Apajalahti, Kettunen and Graham, 2004; Danzeisen

et al., 2011; Torok *et al.*, 2011; Stanley *et al.*, 2013) are common factors that impact gut microbiome diversity, composition, and structure, but opposite results have also been reported (Oakley, Buhr, *et al.*, 2014; Thibodeau *et al.*, 2015; Ranjitkar *et al.*, 2016). In any case, since our analyses did not find any diet-associated changes, diet was henceforth excluded as a variable and results from broilers were directly compared with those from FRC.

In agreement with other studies (Lu *et al.*, 2003; Danzeisen *et al.*, 2011; Videnska *et al.*, 2014; Ballou *et al.*, 2016; Ranjitkar *et al.*, 2016), the diversity and composition of caecal microbiome in both breeds were strongly influenced by age, increasing in complexity and biodiversity as the chickens grew. Breed was also an influential factor, with FRC presenting higher complexity in their caecal microbiome than broilers, consistent with results in a previous study (Mancabelli *et al.*, 2016). Further supporting previous reports (Stanley *et al.*, 2013; Sergeant *et al.*, 2014; Borda-Molina *et al.*, 2016), inter-individual variation was observed in the microbial community structure of birds of the same age, despite the fact that each type of chickens originated from the same breeder and flock and were reared under the same conditions. However, this variation was mainly due to differences in relative abundance rather than to taxonomic composition, which was consistent within each age-group and breed. In general, each age-group showed an age-associated community profile. Thus, three developmental stages of microbiota composition were identified in broilers during their lifespan and four in FRC, with an intermediate stage, as confirmed by UPGMA clustering and NMDS plots. The first stage, represented by 3-day-old broilers and 4-day-old FRC, showed a clearly immature microbiota dominated by Proteobacteria and Firmicutes. However, in broilers, Proteobacteria predominated (*Citrobacter* as main contributor) over Firmicutes (*Enterococcus*), and in FRC Firmicutes (*Ruminococcus*, fam. Lachnospiraceae) were more abundant than Proteobacteria (*E. coli*). In the second stage, represented by 14-day-old broilers and 18-day-old FRC, a drop in Proteobacteria proportion to less than 5% (*E. coli*) was observed resulting in an absolute dominance of the phylum

Firmicutes (around 90%) represented by the families Lachnospiraceae and Ruminococcaceae, at similar levels in broilers but enriched in Ruminococcaceae over Lachnospiraceae in FRC. The relatively high abundance of members of the families Lachnospiraceae (*Coprococcus*, *Roseburia*, *Anaerostipes*) and Ruminococcaceae (*Faecalibacterium*, *Anaerotruncus*), which have been shown to express enzymes favoring the production of butyrate over propionate (Rajilić-Stojanović and de Vos, 2014; Polansky *et al.*, 2016), is likely associated to the high nutrient requirements for growth during this phase since butyrate is the most preferred source of energy in quickly growing chickens (Onrust *et al.*, 2015). The third stage would be represented by 42-day-old broilers and 58-day-old FRC, where a replacement of members of Firmicutes with Bacteroidetes occurred, in a more gradual manner in FRC than in broilers. Firmicutes was still represented by Lachnospiraceae and Ruminococcaceae in broilers, whereas in FRC Veillonellaceae accounted for almost half of the Firmicutes, with *Megamonas* as the most abundant genus. Within the phylum Bacteroidetes, *Bacteroides* clearly prevailed in broilers (50.3%), but represented a smaller proportion in FRC (10.8%) along with members of Rikenellaceae (11.3%) and Barnesiellaceae (9.1%). Remarkably, *Megamonas* and *Bacteroides* produce propionate as the main end product of the degradation of complex plant polysaccharides (Salonen *et al.*, 2014)'(Polansky *et al.*, 2016). Although propionate is a less preferred energy source than butyrate for rapid growth, at this stage, its production as the result of digestion of complex polysaccharides might represent an efficient balance between energy acquisition from available nutrients and sustained growth (Polansky *et al.*, 2016). The very rich and diverse caecal microbiome of 81-day-old FRC would constitute stage 4, represented by 14 phyla. The most prevalent phyla were Bacteroidetes, Firmicutes and Euryarchaeota, with Bacteroidaceae, Methanocorpusculaceae and Veillonellaceae as the most representative families, respectively. The microbial community of broilers aged 29 days and FRC aged 39 days could be considered a transition between stages 2 and 3, when some of the birds still presented similar

microbiota composition to the previous age-group while others were already diverging towards a more mature microbiota structure. This situation was described elsewhere when comparing 14-day-old and 28-day-old broilers (Lu *et al.*, 2003).

Microbiota development is probably a continuum process of microbial communities' succession, where certain taxa are replaced by others as chickens grow. Based on the time points assessed, the stages described here for the whole chicken lifespan clearly showed the evolution from an early immature stage to a mature microbiota that differed between breeds. In agreement with other reports on broiler caecum microbiota, early stages were dominated by Proteobacteria (at the expense of Enterobacteriaceae), Firmicutes increased in later phases (Danzeisen *et al.*, 2011; Awad *et al.*, 2016; Ballou *et al.*, 2016) and Bacteroidetes was enriched at the end of the productive period (Xiao *et al.*, 2017). However, studies on the microbial community in the caecum of meat chickens older than 42 days are scarce (Mancabelli *et al.*, 2016). Therefore, we compared the results of FRC with those reported for laying hens (Videnska *et al.*, 2014), and found some similarities for stages 1-3. However, higher microbial diversity, particularly in the second half of the productive period, was observed in this study, and higher levels of Bacteroidaceae and lower proportions of Porphyromonadaceae were found within the Bacteroidetes. The outdoor access and the consumption of grass might be the explanation for this diverse and complex microbiota. Early microbial exposure has been described to play a major role in determining the distinctive characteristics of the microbial community (Fujimura *et al.*, 2010). In fact, the microbial community structure of FRC was notably distinct before and after outdoor access, when grass was introduced in the diet. Twelve days after outdoor grazing, five new classes emerged (Deltaproteobacteria, Epsilonproteobacteria, RF3, Fusobacteriia, and Lentisphaeria), the last two never found in broilers. In the second half of the productive period, the number of different phyla that reached important levels was far larger in FRC than in broilers, and included members of Euryarchaeota,

the only archaeal phylum detected in this study, and only in FRC. Here, these methanogenic archaea (unclassified reads of the Methanocorpusculaceae family and *vadinCA11*) accounted for 17% of the total microbial community of 81-day-old FRC. Previous studies had already described the presence of methanogenic archaea in chickens caeca (Qu *et al.*, 2008; Wei, Morrison and Yu, 2013; Mancabelli *et al.*, 2016) but always at lower abundances than those found here. Methanogens have been described to act as major consumers of the hydrogen accumulated from bacterial fermentation that leads to reduced or less energy-efficient fermentation (Macfarlane and Macfarlane, 2003). By removing hydrogen, methanogenic archaea contribute to increase microbial fermentation rates and enhance host energy capture (Hou *et al.*, 2016).

Interestingly, the above-mentioned classification into different developmental stages of microbiota composition had some exceptions. Several FRC which clearly separated from their respective age-group turned out to show signs of typhlitis in the lumen of their caeca. Microbial communities of these individuals were less diverse and more similar to each other than to other members of their respective age-groups. Studies in animals have shown that alterations in the normal physiology of the gut can decrease/alter microbial community diversity (Oakley *et al.*, 2008; Calvo-Bado *et al.*, 2011; Stanley *et al.*, 2012). Further analyses are needed to define taxa related to this dysbiosis, but these results showed that dysbiosis can be detected by microbial community profile investigation.

Despite differences associated to age and production type or breed, there was a core microbiome for both chicken breeds which was mainly composed by members of the Firmicutes phylum. In fact, the only phyla present during the whole productive lifespan of both broilers and FRC were Firmicutes and Proteobacteria. These two phyla accounted for almost the total caecal microbiome of younger chickens, and Bacteroidetes only reached important levels in the second half of their lifespan. Although this trend was common to

both breeds, relative abundances were different since the composition of caecal microbiota was richer and more complex in FRC than in broilers and a higher number of taxa was always observed. Species found in FRC but not in broilers included propionate producers like *Megamonas*, methanogenic archaea of the phylum Euryarchaeota or sulphate reducers like Desulfovibrionaceae. Besides, some taxa that have been associated with healthy gut were present exclusively or at higher levels in FRC. This was the case of *Bifidobacterium*, a genus of the phylum Actinobacteria that produces lactic acid as a major product of glucose fermentation and elicits a beneficial effect on the host intestinal ecosystem (Binda *et al.*, 2018). Actinobacteria is a phylum commonly found in the avian gastrointestinal tract (Waite and Taylor, 2014), and plays a crucial role in the development and maintenance of intestinal homeostasis (Binda *et al.*, 2018). Here, as described in other studies (Sergeant *et al.*, 2014; Awad *et al.*, 2016; Mancabelli *et al.*, 2016; Xiao *et al.*, 2017), levels of Actinobacteria in the caeca of both chicken breeds were low, but abundance and diversity of the representing taxa were always higher in FRC than in broilers. Among the minor phyla associated to the mature microbiota, it was noteworthy the presence of bacteria with potential to stimulate mucus layer formation and therefore associated with healthy gut (Derrien *et al.*, 2008; Rodríguez-Piñeiro and Johansson, 2015), like *Akkermansia* (phylum Verrucomicrobia) found at substantially higher levels in FRC than broilers, and *Mucispirillum* (phylum Deferribacteres) found only in FRC and at higher levels in 81-day-old FRC where it was a biomarker. Although further studies are needed to fully understand the role of gut microbiota in defining states of health and disease, available data suggest that richer and more complex microbial communities with a high level of functional redundancy could confer advantages under environmental changes and as thus be considered more robust (Fujimura *et al.*, 2010; Bäckhed *et al.*, 2012; Sommer *et al.*, 2017).

Considering the major role of poultry in human campylobacteriosis, associations of *Campylobacter* relative abundance with the different age-groups

or other taxa were investigated. When *Campylobacter* (mainly *C. jejuni* and/or *C. coli*) enters the flock, infection spreads rapidly and colonizes the caeca of most birds at high levels, and birds remain infected throughout their productive life (Newell and Fearnley, 2003). Here, *Campylobacter* was identified at differentially higher abundance levels in 29-day-old broilers and 39-day-old FRC (LDA effect size >5). This was expected for 29-day-old broilers, which had been experimentally inoculated with *Campylobacter* at 15 days of age (Ocejo *et al.*, 2017), but a high abundance of *Campylobacter* was also found in 39-day-old FRC as a consequence of a natural infection. This confirms the widespread of this genus in chicken poultry production, particularly in extensive systems where strict biosecurity measures are difficult to implement. At the end of their productive life, when Enterobacteriaceae represented only about 10% of all Proteobacteria, *Sutterella* (order Burkholderiales) became the most prevalent Proteobacteria genus in 42-day-old broilers and second in 81-day-old FRC. Spearman correlation analysis showed a negative correlation of *Sutterella* with *Campylobacter* in both breeds. *Sutterella* has been identified in humans, dogs and turkey, and it has been proposed as a direct competitor for *Campylobacter* in the intestinal ecosystem (Scupham *et al.*, 2008) and associated to low feed conversion rate broilers (Singh *et al.*, 2012) and high weight rabbits (Zeng *et al.*, 2015). Another genus negatively associated with *Campylobacter* in both breeds was *Parabacteroides* (phylum Bacteroidetes). This genus has been associated with healthy gut microbiota and certain species have been suggested to exert positive effects in the host immunity (Kverka *et al.*, 2011). On the other hand, several genera of the phylum Firmicutes (*Faecalibacterium*, *Anaerotruncus*, *Blautia*) were positively associated with *Campylobacter*. In broilers, Lactobacillaceae showed a considerable decrease in relative abundance in 29-day-old broilers, when *Campylobacter* load was the highest as determined by real-time PCR elsewhere (Ocejo *et al.*, 2017), and *Lactobacillus* abundance was negatively correlated with *Campylobacter* as confirmed here by 16S rRNA NGS. This is in agreement with studies that showed a decrease in Lactobacillaceae

abundance associated to increased levels of *Campylobacter* (Sakaridis *et al.*, 2018). However, in the case of FRC, the correlation between *Lactobacillus* and *Campylobacter* was positive but weaker and statistically non-significant. Given the association between the consumption of *Campylobacter*-infected chickens and human campylobacteriosis, these associations and the possible influence of microbial community composition on *Campylobacter* levels are of great interest and require further studies.

In conclusion, age was the strongest influencing factor in caecal microbiota composition of chicken, and, in general, each age-group showed an age-associated community profile, with a transition period at the middle of their lifespan. However, there were substantial differences in the phylogenetic composition of caecal microbiota between broilers and FRC, microbiota being richer and more complex in FRC, which had access to grass and soil outside, than in broilers reared intensively on a more limited diet. Broilers are under higher pressure than FRC to grow rapidly and need a microbial community that provides optimal recovery of energy from food, whereas FRC grow slowly and have contact with complex microbial ecosystems in the environment. The higher diversity in microbial community and the presence exclusively or at higher levels in FRC of some taxa that have been associated with healthy gut, suggested that FRC harboured a healthier microbiota. Finally, by microbial community comparison we were able to identify a group of animals with dysbiosis, *i.e.*, a group of FRC with signs of typhlitis in the lumen of their caeca which stood out after showing a microbial community less diverse and more similar to each other than to other members of their respective age-groups.

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4. General Discussion

Campylobacteriosis is the most frequently reported food-borne zoonosis in the European Union (EFSA and ECDC, 2018b). Due to the widespread carriage of *Campylobacter* in the GIT of food-producing animals, strategies aimed at reducing bacterial burden in live animals are key to reduce the load reaching the final consumers and hence, reduce the incidence of campylobacteriosis. A good knowledge of the different epidemiological aspects of *Campylobacter* in the different reservoirs is crucial to design effective interventions at the farm end of the food safety continuum. Control measures directed at preventing or reducing the colonization levels of food-producing animals, could significantly contribute to reduce the risk of human infection. Correspondingly, this PhD thesis was aimed at generating knowledge on two of the most important reservoirs of *Campylobacter*: chickens and domestic ruminants (*i.e.*, cattle and sheep). It is worth underlining that there has been relatively little literature on the occurrence of *Campylobacter* spp. in ruminants compared to poultry despite growing evidence supporting their implication in human infection. The only data on this regard available in the Basque Country dated back to a study carried out 10-years earlier (Oporto *et al.*, 2007). However, in that occasion isolation methods used were designed to favour growth of all thermophilic *Campylobacter* species, including non-zoonotic species. Here, a similar cross-sectional study (Study I) was designed to specifically obtain updated data on herd-level prevalence of *C. jejuni* and *C. coli* in healthy ruminants (dairy cattle, beef cattle and sheep) in the Basque Country, and to investigate the AMR profiles of the isolates obtained.

The results presented in Study I evidenced a high prevalence of *C. jejuni* and *C. coli* in healthy domestic ruminants in the Basque Country, apparently higher than that reported 10 years earlier (Oporto *et al.*, 2007). Nevertheless, this apparent increase in prevalence estimates can most likely be ascribed to changes in methodology rather than reflect a real increment. Here, culturing conditions were specific for *C. jejuni* and *C. coli* and presence of any of these two species was assessed by multiplex real-time PCR analysis on a loopful of bacterial

growth increasing chance of detection, whereas before, all thermotolerant *Campylobacter* species were targeted and only one isolate per positive farm was identified. Still, similarly high *Campylobacter* herd level prevalence has been reported in other studies thereby confirming the widespread distribution of these bacteria among cattle and sheep worldwide (Englen *et al.*, 2007; Rotariu *et al.*, 2009; Scott *et al.*, 2012).

Overall prevalence of *C. jejuni* was remarkably higher than prevalence of *C. coli* but differences were observed in the distribution of *Campylobacter* species between cattle and sheep. Cattle posed a higher risk of shedding *C. jejuni* than sheep, in contrast to the predominance of *C. coli* in sheep flocks over cattle herds. Though not reaching statistical significance, when cattle category was broken down according to production type, prevalence of *C. jejuni* was higher in dairy cattle than in beef cattle. It has been suggested that animals at feedlots are at higher risk of shedding *Campylobacter* spp. than animals grazing on pastures (Bailey *et al.*, 2003). In the Basque Country, animals of all ages are raised in the herd (suckler herds), and intensive feedlot systems are not used in the region. However, dairy cattle are mostly intensively housed in pens while beef cattle and sheep are kept in a semi-intensive management system where animals graze in pastures most part of the year and are only housed in winter. Conversely, a different situation was observed in the case of *C. coli*. Risk of shedding *C. coli* was higher in sheep than in dairy cattle and risk was even lower in beef cattle. Besides, and contrary to the high genetic diversity of *C. jejuni* isolated from ruminants (Oporto *et al.*, 2011), when *C. coli* isolates were characterised by MLST, half of the strains from sheep belonged to ST-827, one of the genotypes most frequently found in humans. These findings clearly suggest that the epidemiology of *C. jejuni* and *C. coli* differ, and are in line with source attribution studies that described sheep-associated isolates to be more likely related with clinical *C. coli* infections in humans (Roux *et al.*, 2013).

Though *C. jejuni* was the predominant species isolated from all farm systems, resistance to the different antimicrobials as well as MDR was more

prevalent in *C. coli*. This is in agreement with previous studies that reported an overall higher resistance in *C. coli* than *C. jejuni* in different hosts and for different antibiotics (Bywater *et al.*, 2004; Englen *et al.*, 2005, 2007; Châtre *et al.*, 2010; EFSA and ECDC, 2019). Especially noteworthy was the high rates of resistance against quinolones and tetracyclines in both *C. jejuni* and *C. coli*, and the high resistance levels of *C. coli* to streptomycin. Comparison of results from this study (2014-2016) and those from the study carried out 10 years earlier (2003-2005) showed an increment in the resistance level for most antimicrobials. The only exceptions were the decrease in resistance to tetracycline in *C. jejuni*, which was due to the decrease observed in sheep and mainly in dairy cattle. High levels of tetracycline resistance have been reported in *Campylobacter* from food-producing animals elsewhere (Cha *et al.*, 2017). In contrast, fluoroquinolones resistance increased in both *C. jejuni* and *C. coli*, being statistically significant the increase in the proportion of flouroquinolone-resistant *C. jejuni* isolates from beef cattle. Since fluoroquinolones are one of the antimicrobial classes of choice for the treatment of severe campylobacteriosis, the very high levels of resistance observed here, which are higher than those found in ruminants in other EU countries (Jonas *et al.*, 2015; Klein-Jöbstl *et al.*, 2016; Anon, 2017), is worrisome and calls for prudent use. Resistance to macrolides was low and restricted to *C. coli* isolated from dairy cattle. The proportion of resistant *C. coli* isolates was non-significantly lower than 10-years earlier, but this tendency was also supported by a significant decrease in the MIC values among isolates susceptible to erythromycin, particularly in isolates from beef cattle. Macrolide-resistance levels reported here were similar to those observed in other studies (Bywater *et al.*, 2004; Englen *et al.*, 2007; Châtre *et al.*, 2010) and these data are reassuring for clinical practice. Use of antimicrobials in livestock is particularly high in Spain compared to other European countries (European Medicines Agency, 2018) and this might explain some of the results found here. Still its use in ruminants is significantly lower than in more intensive production systems like poultry or swine. Accordingly, levels of resistance

reported here for cattle and sheep were lower than those reported in Spain for broilers and pigs (EFSA and ECDC, 2018a, 2019). However, interventions to promote prudent use of antimicrobials in food-producing animals are needed to tackle the dissemination of antimicrobial resistance since the interchange of antimicrobial resistant bacteria between the human and animal compartment occurs in both directions.

Compared to ruminants, considerable amount of literature has been published on *Campylobacter* occurrence in chickens. However, in the Basque Country chicken meat farms represent a very small proportion of the food-producing animal sector in comparison to cattle and sheep. Still, given the public health concern posed by *Campylobacter*-contaminated chicken meat and under the hypothesis that reducing caecal loads of *Campylobacter* might reduce risk of food-borne infection in humans, we attempted to delve into the complex microbial ecology of the chicken caeca and its association with *Campylobacter* colonization. To achieve this purpose, a better understanding of *Campylobacter* colonization dynamics as well as a better knowledge of chicken caecal microbiota are needed. To do so, we studied the caecal microbiota at the different phases of the productive life of two different chicken breeds with different management practices, broilers (Ross-308) reared in an intensive system during 6 weeks and a slow-growing breed of chickens (Sasso-T451A) reared in a free-range system during 12 weeks (FRC). Samples from FRC were collected from a commercial farm throughout their productive life whereas for broilers, an experimental study was designed to evaluate the effect of two feed supplements on *Campylobacter* colonization and shedding along with any effects on modulating the microbiota composition.

In the experimental study carried out in broilers (Study II), synergistic supplementation of corn/soybean-based diets with dry whey powder and coated calcium butyrate elicited beneficial effects on productive parameters as well as duodenal villi structural integrity of broiler chickens. A similar study (Pineda-Quiroga *et al.*, 2017) also reported synergistic activity of whey and calcium

butyrate by observing beneficial effects on duodenal development and increased weight. However, contrary to our results, authors reported no improvements in feed conversion ratio. The favourable effects described in our study were particularly notable during the first half of the broilers' productive life (starter period). The improvement in protein digestibility associated with butyrate (Guilloteau *et al.*, 2010; Kaczmarek *et al.*, 2016), combined with the high biological value of the protein contained in whey (Szczurek *et al.*, 2013), might have likely contributed to the observed improvements in performance traits. An added value of the use of whey powder as chicken feed supplementation, is the revalorization of a sub-product from the dairy industry by offering an alternative use for this waste product. However, the antibacterial properties expected from whey and/or butyrate did not affect *Campylobacter* colonization or shedding levels. A similar outcome was described previously (Van Deun *et al.*, 2008) and authors speculated that rapid absorption of butyrate by enterocytes could have reduced the butyrate concentration below the bactericidal level and that the mucus layer might have conferred some degree of protection to *C. jejuni* (Van Deun *et al.*, 2008). Moreover, only residual concentrations might have reached the caeca, the main site of *C. jejuni* colonization. The antibacterial effect expected from whey would be the consequence of a decrease in the caecal pH following the fermentation of the lactose contained in whey (Tellez *et al.*, 1993). This study did not measure caecal pH, but a similar feeding trial reported a reduction of caecal pH after whey supplementation (Pineda-Quiroga *et al.*, 2017).

This study also provided valuable information regarding *Campylobacter* colonization dynamics in an infected flock. Rapid dissemination and colonization of *Campylobacter* were observed following experimental inoculation. By day six post-infection, nearly 100% of the birds sampled were colonized and the shedding levels of orally challenged chickens were comparable to those found in horizontally infected birds. Moreover, *C. jejuni* DNA was widely detected in environmental samples such as air, dust and

drinkers and environmental levels correlated positively with the observed shedding levels of the birds. Nevertheless, the microbiological methods used failed to isolate any colony from environmental samples. This was expected, because *Campylobacter* is susceptible to desiccation and aerobiosis. Yet, this apparently innocuous environmental contamination cannot be neglected because there is a great chance that *Campylobacter* undertook a VNC state that could eventually reactivate its functions after reaching the favourable chicken GIT environment. Thus, they can be considered as important source of re-infections.

Even though the diet treatments evaluated in Study II did not reduce *Campylobacter* colonization levels, it was interesting to explore the gut microbiota to try to relate the observed improvements in productive traits and duodenal histological integrity to possible diet-induced microbial shifts. Furthermore, microbiota data would help to understand the microbial ecology of the intestinal site where *Campylobacter* resides alongside numerous other microorganisms. So, a next-generation sequencing technology was applied in Study III to thoroughly characterise the caecal microbiota of broilers. Unexpectedly, our findings suggested that the different feed supplementations administered did not alter the overall structure of the caecal microbiota compared to broilers fed a non-supplemented diet. Although other studies claimed that diet and feed additives influenced gut microbiome diversity, composition, and structure (Apajalahti, Kettunen and Graham, 2004; Danzeisen *et al.*, 2011; Torok *et al.*, 2011; Stanley *et al.*, 2013; Pineda-Quiroga *et al.*, 2018), opposite results have also been reported (Oakley, Buhr, *et al.*, 2014; Thibodeau *et al.*, 2015; Ranjitkar *et al.*, 2016). The absence of effect on the caecal microbiota composition together with the fact that we did not observe any reduction in neither colonization nor shedding levels of *Campylobacter* suggested that the feed supplements failed to reach effective concentration levels at the caecal environment and that any changes, if occurred, would have been more evident in upper sites of the GIT such as the small intestine.

Periodic samplings throughout the productive life permitted the characterization of the broilers caecal microbiota across time. Microbiota richness was low in young broilers and diversity increased with age. Moreover, the inter-individual variance observed in the first days of age became less evident as the birds matured. Proteobacteria, Firmicutes and Bacteroidetes were the most abundant phyla throughout the productive life, the first two being predominant in young animals and the latter becoming more abundant at slaughter age. These results were in agreement with previous studies that had reported greater influence of age over diet in broilers (Oakley, Buhr, *et al.*, 2014). In order to investigate if similar developmental patterns would apply to other breeds under a different husbandry system, the caecal microbiome of a slow growing breed (Sasso-T451A) reared in a free-range system (FRC) was also characterised in Study III. Similarly to broilers, and in agreement with other studies, age was found to be the main driver of the caecal microbiome composition, that increased in complexity and biodiversity as the chickens grew (Lu *et al.*, 2003; Danzeisen *et al.*, 2011; Videnska *et al.*, 2014; Ballou *et al.*, 2016; Ranjitkar *et al.*, 2016). Still, breed and management were proven to be important influential factors, as FRC presented higher complexity in their caecal microbiome than broilers, consistent with a previous study (Mancabelli *et al.*, 2016). Despite certain differences, caecal microbiota of both breeds displayed an age-associated community profile. Three developmental stages of microbiota composition were identified in broilers during their lifespan and four in FRC, with an additional intermediate stage. Proteobacteria and Firmicutes predominated in the first stage (3-day-old broilers and 4-day-old FRC). In the second stage, represented by 14-day-old broilers and 18-day-old FRC, Firmicutes prevailed barely leaving space for other members. Then, an intermediate transition stage was observed in broilers aged 29 days and FRC aged 39 days reflecting the dynamic process of microbial community succession where certain taxa are replaced by others as chickens grow. Forty-two-day-old broilers and 58-day-old FRC would represent stage 3, where a replacement of members of

Firmicutes with Bacteroidetes occurred. Stage 4 was solely found in 81-day-old FRC and was characterised by a very rich and diverse caecal microbiome represented by nothing less than 14 phyla. The outdoor access along with the consumption of grass, as well as a longer lifespan might be among the explanations for the more diverse and complex microbiota of FRC compared to broilers. For both broilers and FRC, certain groups of bacteria were identified as core microbiome, defined as taxa that remained present during the whole productive life. These were predominantly composed of members from the Firmicutes phylum and to a lesser extent, Proteobacteria. In addition, by microbiota comparison, we were able to identify several animals with altered caeca suggesting that dysbiosis can be detected through microbiome profiling studies.

In Study II we were able to monitor *Campylobacter* colonization and dissemination after the experimental inoculation. The highest levels of *Campylobacter* colonization and shedding were found within the first 2 weeks after inoculation when the birds were 21 and 28 days old, respectively, decreasing afterwards irrespective of diet treatments. *Campylobacter* reads obtained in Study III in broilers correlated well with this observation and a similar pattern was also observed in FRC, with a peak in *Campylobacter* reads in 39-day-old chickens that decreased afterwards until slaughtering age. These results, also confirmed by microbiological culture and real-time PCR analyses (results not shown), indicated that the FRC flock was naturally infected with *Campylobacter*. In both breeds, when present, *Campylobacter* relative abundance was positively correlated with *Faecalibacterium*, *Anaerotruncus* and *Blautia* and negatively correlated with the abundance of *Sutterella* and *Parabacteroides*. *Lactobacillus* was also negatively correlated with *Campylobacter*, but only in broilers. These associations with *Campylobacter* levels are of great interest as baseline for potential microbiota modulation studies and require further research.

It is worth remarking that even though microbiome characterization with 16S rRNA amplicon throughput sequencing outperforms other techniques such as traditional culture, there are several limitations (Poretsky *et al.*, 2014). The taxonomic analysis is limited to identification at genus level at best, but reliable estimations are recommended only down to family level because one hypervariable region was sequenced. The exponential nature of the PCR step used prior to sequencing might favour the amplification of the most abundant taxa over minor ones (Gonzalez *et al.*, 2012). Besides, primer design as well as taxonomic classification are highly dependent on available reference databases. Moreover, microorganisms can have more than one copy of the 16S rRNA gene (Acinas *et al.*, 2004). Nonetheless, this technique is currently the most cost-effective way to obtain valuable information on the taxonomic composition of the microbiota. Besides, the pitfalls described would equally affect all the samples allowing reliable comparisons between them. Furthermore, our study applied the DADA2 pipeline that classifies unique sequences as ASVs (Callahan *et al.*, 2016), a method that resolves differences in sequence variants at as low as a single nucleotide and maintains the original sequence data in the abundance table, allowing posterior analyses with updated databases. An alternative technique that overcomes all these drawbacks is shotgun metagenomics. This technique directly sequences total DNA from a sample and targeted microorganism extend beyond prokaryotes and archaea, including all DNA-harbouring microorganisms at finer resolution than 16S rRNA amplicon sequencing. Shotgun metagenomics also give the added value of determining potential functional diversity of the microbial niche as well as the possibility to discover novel microorganisms and functions. Still, as this method is DNA-based, viable microbes cannot be distinguished from dead bacteria. Therefore, its complementation with metatranscriptomic studies (RNA-based) would help to decipher the active metabolic pathways in the studied ecosystem.

These NGS techniques can also be worth implementing for the study of AMR dissemination in livestock, not only in *Campylobacter* but also in

commensal bacteria with the ability to horizontally transfer AMR genes to other microbes. Thus, metagenomics can be useful to explore the resistome (collection of total genetic determinants of resistance) in the whole sample to obtain information on their abundance, diversity and molecular structure. Even so, phenotypic characterization of pure isolates must still be used to determine whether a particular isolate expresses the resistance, as we did in this study.

In conclusion, this PhD Thesis has broadened knowledge about different aspects of *Campylobacter* infection in broilers and ruminant livestock. Updated herd-level prevalence data on *C. jejuni* and *C. coli* in ruminants in the Basque Country as well as AMR profiles in the obtained isolates has been provided and the information can be of valuable use as a starting point in order to evaluate the effectiveness of possible intervention strategies. By investigating the microbial ecology in the site where *Campylobacter* mainly resides in chickens, this Thesis has also generated a comprehensive dataset on caecal microbiota in two meat-type chicken breeds under different management practices and in different timepoints of the productive life that can be fully exploited for other purposes. It can serve as a catalogue of taxonomic composition of caecal microbiota in chickens, and can be used as reference for future caecal microbiota studies.

5. Conclusions

1. *C. jejuni* and *C. coli* are highly prevalent in ruminant herds in the Basque Country, cattle being the main reservoir of *C. jejuni* and sheep of *C. coli*. These results highlight the importance of non-poultry reservoirs of *Campylobacter* and their potential risk for human infection.

2. AMR was significantly more widespread among *C. coli* than *C. jejuni* ruminant isolates, with higher prevalence in those obtained from dairy cattle than beef cattle or sheep. A high proportion of isolates were resistant to fluoroquinolones and tetracycline, particularly in *C. coli*. Resistance to macrolides was low and restricted to *C. coli* isolated from dairy cattle, with macrolide-resistant *C. coli* showing a pattern of pan-resistance.

3. Comparison of AMR profiles observed in this study (2014-2016) and those from another study carried out 10 years earlier (2003-2005) showed an overall increase in resistance. In particular, there was a significant increase in the proportion of fluoroquinolones resistance, specifically associated to *C. jejuni* from beef cattle. Conversely, resistance to tetracyclines in *C. jejuni* decreased due to an important reduction in dairy cattle. Resistance to macrolides remained stable at low rates, which was reassuring since these are the antimicrobials of choice to treat severe human *Campylobacter* infections.

4. Feeding broilers a corn/soybean-based diet supplemented with dry whey powder and coated calcium butyrate improved chickens' growth and feed efficiency, exerted beneficial effect on intestinal health, and decreased mortality, the effects being particularly significant during the starter period. However, this supplementation did not have any effect on reducing *C. jejuni* colonization or shedding levels, or in the overall community structure of the chicken caecal microbiota.

5. The chicken caecal microbiota is a dynamic ecosystem, whose composition is influenced by age, breed and management system. Age was the strongest driving factor in shaping the chicken caecal microbiota composition, and in general, each age-group showed an age-associated community profile, with a transition period at the middle of their lifespan. In addition, the taxonomical composition of the caecal microbiota was richer and more complex in birds of a slow-growing breed of free-range chickens (Sasso-T451A) than in intensively reared broilers (Ross-308).

6. *Campylobacter* shares its niche in the chicken caeca with a complex microbial community. In both chicken breeds, correlation analysis identified a positive correlation of *Campylobacter* with *Faecalibacterium*, *Anaerotruncus* and *Blautia*, and a negative correlation with *Sutterella* and *Parabacteroides*. *Lactobacillus* was also negatively correlated in broilers. Further studies are needed to specifically assess the effect of modulating abundance levels of each of these members of the microbiota on *Campylobacter* control.

7. A group of animals with dysbiosis (typhlitis in the lumen of their caeca) showed a less diverse microbial community than healthy animals suggesting that dysbiosis can be detected by microbial community profile characterization.

6. Summary

Campylobacteriosis is the food-borne enteritis with the highest incidence in developed countries, and cause of serious socio-economic effects. It is mainly caused by *Campylobacter* spp., particularly the species *C. jejuni* (*ca.* 85% of the cases) and *C. coli* (*ca.* 10%). *Campylobacter* colonizes asymptomatically the gastrointestinal tract of mammals and birds and transmission to humans occurs via faecal-oral route. Control measures aimed at decreasing colonization in food-animals could contribute significantly to reducing the risk of human infection. Therefore, a good knowledge of the different epidemiological aspects of *Campylobacter* in the different reservoirs is crucial to design effective interventions at the farm end of the food safety continuum. In this regard, the general objective of this PhD Thesis was to broaden knowledge on different aspects of *Campylobacter* on two of its most important reservoirs for human infection: chickens and domestic ruminants (*i.e.*, cattle and sheep).

The first study of the present Thesis (Study I) was aimed at studying the herd-level prevalence of *C. jejuni* and *C. coli* in healthy ruminants in the Basque Country, and to investigate the antimicrobial resistance (AMR) profiles of the isolates obtained. For this purpose, a cross-sectional study was designed, and rectal faecal samples were collected from healthy animals in 301 ruminant herds (82 dairy cattle, 104 beef cattle and 115 sheep). *C. jejuni* was detected in 85.4% of dairy cattle herds, 77.9% of beef cattle herds and 45.2% of sheep flocks, whereas *C. coli* was found in 17.1% of dairy cattle herds, 6.7% of beef cattle herds and 19.1% of sheep flocks. Risk of shedding *C. jejuni* was higher in cattle than sheep (81.2% vs. 45.2%; $OR_{adj}=5.22$, $p<0.001$), whereas risk of shedding *C. coli* was higher in sheep than in cattle (19.1% vs. 11.3%; $OR_{adj}=1.71$, $p=0.128$). Susceptibility to 6 antimicrobials was determined by broth microdilution using EUCAST epidemiological cut-off values (ECOFF). AMR was significantly more frequent among *C. coli* than *C. jejuni* isolates (94.7% *vs.* 65.1%), with a greater prevalence in isolates obtained from dairy cattle than beef or sheep. A high proportion of isolates were resistant to quinolones and tetracycline, particularly in *C. coli* (63.2% and 76.3% respectively), whereas resistance to macrolides was low and restricted to *C. coli* from dairy cattle (8.3%). Compared to a similar study conducted 10 years earlier (2003-2005), in this study

(2014-2016) a general increase in AMR prevalence was observed. In particular, there was a significant increase in the proportion of resistances to fluoroquinolones, specifically associated with *C. jejuni* from beef cattle (32.0% to 61.9%), but a decrease in resistance to tetracyclines in *C. jejuni* due to a significant reduction in dairy cattle (75.0% to 43.2%). Resistance to macrolides remained stable at low rates, which is reassuring since they are the antimicrobials of choice to treat severe *Campylobacter* infections in humans. These results highlight the importance of non-poultry reservoirs of *Campylobacter* and their potential risk for human infection and the associated spread of AMR.

A second study (Study II) was carried out to assess the effect of diet intervention in chickens as a possible *Campylobacter* control strategy in the reduction of colonization and/or shedding levels. Thus, an experimental study was carried out with a fast-growing chicken breed (Ross-308 broilers) reared intensively for 42 days and fed conventional corn-soybean diet supplemented with: i) 0.1% calcium butyrate; ii) 6% whey; iii) 0.1% butyrate + 6% whey; and, iv) control diet without supplement (5 replicates of 30 chickens per treatment). At the age of 15 days, 6 chickens per pen were experimentally inoculated with *C. jejuni*. The results obtained showed that the diet supplemented with whey (6%) in combination with calcium butyrate (0.1%) had beneficial effects on productive performance and intestinal health especially during the starter period. However, none of the supplements added to the diets provided apparent reductions in the colonization and excretion levels of *Campylobacter*. These results suggested that the diet of conventional feed based on corn and soybeans supplemented with whey (6%) and calcium butyrate (0.1%) could be an interesting strategy for productive purposes but not for *Campylobacter* control measures.

To investigate the effect of diet as well as other factors such as age (different points of the productive life) and breed (broilers and free-range chickens - FRC) not only on *Campylobacter* but also on the total microbial composition (microbiota), high-throughput amplicon sequencing targeting the hypervariable region V4 of the 16S rRNA was used to characterize the taxonomic composition of the chicken caecal microbiome (Study III). The study was conducted on broilers from Study II (80

Ross-308 broilers, 5 replicates per diet at the ages of 3, 14, 29, and 42 days) and on FRC (38 Sasso-T451A chickens of 4, 18, 39, 58 and 81 days). Diet supplementation with whey and calcium butyrate did not have any effect on the overall structure of the microbial community of broilers' caeca, despite its beneficial effect on the productive parameters. In both broilers and FRC, age was the most influencing factor, characterized by an age-associated microbial community profile that increased in biodiversity as the chickens aged. However, differences in microbial community structure and composition were observed between breeds, as higher richness and complexity was found in the microbiome of FRC than in broilers. This might be associated to the longer productive lifespan and the outdoor access of FRC compared to broilers. This study comprehensively described the different developmental stages of the chicken caecal microbiome and how *Campylobacter* shares its niche in the chicken caeca with a complex microbial community. In both chicken breeds, correlation analyses identified a positive correlation of *Campylobacter* with *Faecalibacterium*, *Anaerotruncus* and *Blautia* and a negative correlation with *Sutterella* and *Parabacteroides*. *Lactobacillus* was also negatively correlated with *Campylobacter*, but only in broilers. Further studies will be needed to specifically assess the effect of these members of the microbiota in modulating the abundance levels of *Campylobacter*. In addition, the methodology applied to the study of microbial communities also allowed the identification of a group of animals with dysbiosis, specifically some chickens with signs of typhlitis in the lumen of their caecum. These animals showed a less diverse microbial community and more similar among them than to their respective age groups, confirming the usefulness of these techniques to identify situations of dysbiosis.

7. Resumen

La campylobacteriosis es la enteritis de transmisión alimentaria de mayor incidencia en los países desarrollados. El número de casos de campilobacteriosis notificados en Europa sobrepasa con creces a los producidos por otros patógenos de transmisión alimentaria, y su incidencia sigue una tendencia al alza desde 2008 pese a los esfuerzos dirigidos a mejorar las medidas de control, lo cual conlleva serias implicaciones socioeconómicas. Además, en un porcentaje pequeño de casos (1%) se pueden producir complicaciones graves (artritis reactiva y síndrome de Guillain-Barré) que pueden causar la muerte. El agente causante de la campylobacteriosis es *Campylobacter* spp., principalmente las especies *C. jejuni* (*ca.* 85% casos) y *C. coli* (*ca.* 10%). *Campylobacter* coloniza asintomáticamente el tracto gastrointestinal de mamíferos y aves, siendo los animales de granja (aves de corral y los rumiantes domésticos) importantes reservorios y fuentes de infección humana. La vía de infección es fecal-oral y se asocia principalmente al consumo de carne de pollo contaminada poco cocinada. La transmisión de *Campylobacter* por el consumo de carne roja también puede ocurrir, aunque la forma más común de infectarse a partir de rumiantes domésticos es a través del consumo de agua contaminada por efluentes de granja y contacto con los animales infectados. Se han descrito también casos asociados al consumo de leche cruda sin pasteurizar o contaminada tras el tratamiento térmico. Se ha visto que los casos de campilobacteriosis relacionados con cepas de *Campylobacter* spp. procedentes de rumiantes domésticos son más comunes en medios rurales mientras que los casos urbanos se vinculan más con el consumo de carne de ave. Las medidas de control dirigidas a la disminución de la colonización en animales destinados a la producción de alimentos podrían contribuir significativamente a reducir el riesgo de infección humana. Por tanto, un buen conocimiento de los diferentes aspectos epidemiológicos de *Campylobacter* en los diferentes reservorios es crucial para diseñar intervenciones efectivas en el sector primario, la base de la cadena alimentaria.

En este sentido, el objetivo general de esta tesis doctoral es estudiar distintos aspectos de la infección por *Campylobacter* en dos importantes reservorios: pollos de engorde y rumiantes domésticos. Para ello, se han definido estos objetivos específicos: 1) estimar la prevalencia a nivel de rebaño de *C. jejuni* y *C. coli* en rumiantes domésticos sanos del País Vasco; 2) determinar los perfiles de resistencias a antimicrobianos (RAM) de los aislados de *C. jejuni* y *C. coli* obtenidos de rumiantes mediante la determinación fenotípica de los valores de Concentración Mínima Inhibitoria (CMI) y comparar estos perfiles con los obtenidos en un estudio similar realizado hace diez años en la región; 3) evaluar el efecto de dos aditivos suplementados en pienso - lactosuero seco en polvo (un prebiótico) y butirato de calcio (un ácido orgánico en forma de sal) - sobre la colonización y los niveles de excreción de *Campylobacter* a través de un estudio experimental *in vivo* en pollos de engorde (broilers Ross-308); 4) investigar los posibles efectos de los dos aditivos alimentarios en el rendimiento productivo de los pollos de engorde así como su integridad intestinal mediante la evaluación de los parámetros productivos y las medidas histomorfológicas de las vellosidades duodenales; 5) evaluar el efecto de los aditivos en la comunidad microbiana del ciego de los pollos de engorde utilizando la técnica de secuenciación masiva de productos amplificados del gen ribosómico 16S RNAr; 6) caracterizar el desarrollo temporal de la microbiota cecal en dos razas de pollos de engorde - Ross-308 (raza de crecimiento rápido utilizada para producción intensiva) y Sasso-T451A (raza de crecimiento lento criada en un sistema extensivo campero tradicional) - para comparar su perfil taxonómico e identificar los taxones comunes y específicos para las diferentes razas y/o grupos de edad; y, 7) evaluar la coexistencia de *Campylobacter* spp. y los otros miembros de la microbiota cecal en cada raza de pollo.

Para abordar los dos primeros objetivos se ha llevado a cabo un estudio transversal (Estudio I) en el que se muestrearon heces de animales sanos de 301 explotaciones de rumiantes en el País Vasco, incluyendo los tres sistemas productivos principales: vacuno de leche (82), vacuno de carne (104) y ovino de

leche (115). Se observó que *C. jejuni* y/o *C. coli* son altamente prevalentes en rebaños de rumiantes en el País Vasco (86,6% en vacuno de leche, 78,8% en vacuno de carne y 54,8% en ovino), siendo el ganado vacuno el reservorio principal de *C. jejuni* (81,2% en comparación con 45,2% en ovino, OR=5.22, $p<0.001$) y las ovejas de *C. coli* (19,1% en comparación con 11,3% en vacuno, OR=1.71, $p=0.128$). Estos resultados resaltan la importancia de los reservorios no avícolas de *Campylobacter* y su riesgo potencial para la infección humana. Para el estudio de las RAM, se determinó la susceptibilidad de los aislados a 6 antimicrobianos mediante microdilución en cultivo liquido aplicando los valores de corte epidemiológicos (ECOFF) establecidos por EUCAST (*European Committee for Antimicrobial Susceptibility Testing*). Las RAM fueron significativamente más frecuentes entre los aislados de rumiantes de *C. coli* que de *C. jejuni* (94,1% *vs.* 65,1%), con una mayor prevalencia en los obtenidos de ganado vacuno lechero que vacuno de carne u ovino. Una alta proporción de aislados fueron resistentes a quinolonas y tetraciclinas, particularmente en *C. coli* (64,7% y 76,5% respectivamente), mientras que la resistencia a los macrólidos fue baja y restringida a *C. coli* procedente de ganado vacuno lechero (8.8%). La comparación de los perfiles de RAM observados en este estudio (2014-2016) y los de otro estudio similar realizado 10 años antes (2003-2005) mostró un aumento general de la resistencia. En particular, hubo un aumento significativo en la proporción de resistencias a las fluoroquinolonas, específicamente asociadas a *C. jejuni* de vacuno de carne (32,0% a 61,9%). En cambio, la resistencia a las tetraciclinas en *C. jejuni* disminuyó debido a una reducción importante en el vacuno lechero (75,0% a 43,2%). La resistencia a los macrólidos se mantuvo estable a tasas bajas, lo cual es tranquilizador ya que son los antimicrobianos de elección para tratar infecciones graves por *Campylobacter* en humanos.

En un segundo estudio se llevó a cabo un ensayo experimental con pollos de crecimiento rápido (broilers Ross-308) criados en intensivo durante 42 días (Estudio II). Los 600 broilers incluidos en el ensayo se distribuyeron de manera

aleatoria (diseño de bloques aleatorios) en 4 grupos de tratamiento (5 réplicas de 30 pollos por tratamiento) a los que se administró un pienso convencional a base de maíz y soja al que se añadieron distintos suplementos: i) 0.1% butirato de calcio; ii) 6% lactosuero; iii) 0.1% Butirato + 6% lactosuero; y, iv) dieta control sin suplemento (5 réplicas de 30 pollos por tratamiento). A la edad de 15 días se infectó experimentalmente con *C. jejuni* a 6 pollos por recinto. Los resultados obtenidos demostraron que la dieta suplementada con lactosuero (6%) en combinación con butirato de calcio (0.1%) favorece el crecimiento de los broilers y mejora el índice de conversión, tiene un efecto beneficioso sobre la salud intestinal y reduce la mortalidad, siendo particularmente significativos dichos efectos durante el periodo de iniciación. Estos resultados sugieren que la dieta de pienso convencional a base de maíz y soja suplementada con lactosuero (6%) y butirato de calcio (0.1%) podría ser una estrategia interesante a considerar para mejorar el rendimiento productivo en pollos broiler.

En cuanto a la dinámica de infección a nivel de explotación, los resultados bacteriológicos y moleculares mostraron una efectiva colonización y una rápida diseminación de la infección por *Campylobacter*. Seis días post-inoculación, la infección por *Campylobacter* estaba ya ampliamente extendida por los recintos, y la mayoría de las aves estaban eliminando *C. jejuni* a niveles similares independientemente de si habían sido inoculados oralmente o se habían infectado horizontalmente a partir de los primeros, lo que demuestra la rapidez de la diseminación de la infección tras la primera entrada. Las dos primeras semanas tras la infección experimental, correspondientes a las edades de 21 y 28 días, registraron los mayores niveles de colonización y excreción (aproximadamente 9,0 $\log_{10}$ y 5,0 $\log_{10}$ equivalentes genómicos (EG) de *Campylobacter* por gramo respectivamente); las semanas posteriores los niveles disminuyeron, pero los animales permanecieron infectados y excretando la bacteria hasta la edad de sacrificio. Aunque no se pudo aislar *Campylobacter* a partir de las muestras ambientales, sí que se detectó su presencia por PCR a tiempo real en las muestras de los bebederos, polvo y aire del interior de la nave

durante todo el periodo en que los animales fueron positivos. Sin embargo, en las condiciones de este estudio, ninguno de los suplementos añadidos a las dietas proporcionó a los animales ninguna ventaja aparente respecto a la colonización y excreción de *Campylobacter*.

Para investigar el efecto de las dietas no solo sobre *Campylobacter* sino sobre la composición microbiana total (microbiota), así como el efecto de otros factores como la edad (distintos momentos del ciclo productivo) y la raza (pollo broiler y pollo campero), se han empleado técnicas de secuenciación masiva de nueva generación (NGS) para caracterizar el microbioma cecal de los pollos (Estudio III). Para ello, se llevó a cabo la secuenciación masiva de los productos de amplificación de la región hipervariable V4 del 16S RNAr, que permite la descripción taxonómica de la comunidad bacteriana. El estudio se realizó sobre los pollos broiler del ensayo de las dietas (80 broilers Ross-308, 5 réplicas por dieta a las edades de 3, 14, 29, y 42 días) y sobre pollos camperos (38 pollos Sasso-T451A de edades 4, 18, 39, 58 y 81 días).

La suplementación de la dieta de los broilers con butirato de calcio y lactosuero, a pesar de su efecto beneficioso sobre los parámetros productivos, no tuvo un efecto sobre la estructura global de la comunidad microbiana del ciego. La edad fue el factor determinante más importante para la composición de la microbiota del ciego, y en general, cada grupo de edad mostró un perfil propio, que se puede resumir en tres fases de desarrollo en broilers y cuatro en pollos camperos, con un periodo de transición intermedio, caracterizados por un aumento en la complejidad y biodiversidad de las comunidades con la edad. En ambas razas, Proteobaceria, Firmicutes y Bacteroidetes son los filos más abundantes durante toda la vida productiva, siendo los dos primeros los predominantes en animales jóvenes y el último en animales a edad de sacrificio. Sin embargo, a pesar de existir una comunidad microbiana común a ambas razas de pollos (*core* microbioma), existen diferencias sustanciales en la composición de la microbiota cecal de broilers y pollos camperos, siendo la microbiota más

rica y compleja en éste último tipo. Estas diferencias están probablemente asociadas a la mayor duración del periodo productivo y al contacto con el medioambiente de los pollos camperos. La mayor biodiversidad de la microbiota y la presencia de algunos grupos taxonómicos de manera exclusiva o más abundante en pollos camperos, sugiere que estos pollos camperos albergan una microbiota cecal más saludable.

En esta Tesis también se han identificado taxones asociados a la edad y géneros potencialmente correlacionados con las abundancias relativas de *Campylobacter* en ciego, información que puede ser útil como punto de partida para el desarrollo de futuros estudios de modulación de microbiota intestinal. Así, se ha observado que *Sutterella* y *Parabacteroides* están negativamente correlacionados con *Campylobacter* y por el contrario, géneros como *Faecalibacterium*, *Anaerotruncus* y *Blautia* mostraron una correlación positiva. Únicamente en broilers, *Lactobacillus* presentó correlación negativa con *Campylobacter*. Se necesitarán estudios adicionales para evaluar específicamente el efecto de la modulación de los niveles de abundancia de cada uno de estos miembros de la microbiota en el control de *Campylobacter*.

La metodología aplicada al estudio de las poblaciones microbianas nos ha permitido también identificar un grupo de animales con disbiosis, concretamente unos pollos camperos con signos de tiflitis en el lumen de su ciego. Estos animales mostraron una comunidad microbiana menos diversa y más similar entre ellos que a sus respectivos grupos de edad, confirmando la utilidad de estas técnicas para identificar situaciones de disbiosis.

8. Laburpena

Kanpilobakteriosia herrialde garatuetan intzidentzia gehien duen elikagaien bitartez transmitituriko enteritisa da, ondorio sozio-ekonomiko larriak sortzen dituena. Bereziki *Campylobacter* spp.-k sortutakoa da, batez ere *C. jejuni* (kasuen %85 inguru) eta *C. coli* (kasuen %10 inguru). *Campylobacter*ak hegaztien eta ugaztunen traktu gastrointestinala sintomarik gabe kolonizatzen du eta gizakienganako transmisioa gorotz-aho bidez gertatzen da. Gizakien infekzioa era adierazgarrian gutxitu liteke elikagai izango diren animalien konolizazioa gutxitu dezaketen neurriak hartuz. Gordailu desberdinetan *Campylobacter*ak dituen alderdi epidemiologiko desberdinen ezagutza ona izatea ezinbestekoa da elikagaien segurtasuna bermatzeko, era honetan baserrietan esku-hartze eraginkorrak egitea posible izango da. Dokrorego tesi honen helburu nagusia gizakien infekzioa sortzen duten 2 gordailu nagusien, oiloen eta etxe-hausnarkarien (abelgorrien eta ardien), alderdi desberdinetan jakinduria lortzea zen.

Tesi honen lehenengo ikerketaren (Study I) helburua Euskal Autonomia Erkidegoko (EAE) hausnarkari osasuntsuen *C. coli* eta *C. jejuni* talde prebalentzia ikertzea izan da, isolatu hauen antimikrobianoekiko erresistentzia (AMR) profilak aztertuz. Helburu hau bete ahal izateko, zeharkako ikerketa bat diseinatu zen, 301 hausnarkari talde osasuntsuren ondeste- eta gorotz-laginak jaso ziren (82 esne-behitalde, 104 haragi-behitalde eta 115 artaldetatik). *C. jejuni* esne-behitaldeen %85.4tan, haragi-behitaldeen %77.9tan eta artaldeen %45.2tan antzeman zen, aldiz *C. coli* esne-behitaldeen %17.1etan, haragi-behitaldeen %6.7tan eta artaldeen %19.1etan aurkitu zen. *C. jejuni* zabaltzearen arriskua abelgorrietan ardietan baina handiagoa da (%81.2 vs. %45.2; OR_{adj}=5.22, $p<0.001$), ostera *C. coli* zabaltzearen arriskua ardietan abelgorrietan baina handiagoa da (%19.1 vs. %11.3; OR_{adj}=1.71, $p=0.128$). “Broth microdilution” teknika erabiliz, EUCAST-en ebakipuntu epidemiologikoen balioekin (ECOFF) 6 antimikrobiorekiko sentikortasuna zehaztu zen. Antimikrobioekiko erresistentziak *C. coli*etan *C. jejuni* isolatuetan baino maiztasun handiagoarekin (era esanguratsuan, %94.7 *vs.* %65.1) antzeman ziren, artalde eta haragi

abelgorrietan baina prebalentzia handiagoarekin esne-abelgorrietan lorturiko isolatuetan. Isolatuen proportzio handi bat ziprofloxazinoarekiko eta tetraziklinekiko erresistentea izan zen, *C. coli*ak bereziki (%63.2 eta %76.3 hurrenez hurren), aldiz makrolidoekiko erresistentziak baxuak eta esne-behiei mugatuak izan ziren (%13.2). 10 urte lehenago (2003-2005) burututako ikerketa antzeko batekin alderatuz, ikerketa honetan (2014-2016) antimikrobioekiko erresistentzien gorakada orokor bat erreparatzen da. Bereziki fluorokinolen aurkako erresistentzien proportzioaren areagotze esanguratsua azpimarra daiteke, haragi abelgorriei lotutako *C. jejuni* isolatuetan (%32.0tik %61.9ra). Esne behien *C. jejuni* isolatuetan ordea tetraziklinekiko erresistentzien beherapen esanguratsua gertatu da (%75.0tik %43.2ra). Makrolidoekiko erresistentziak indize baxuetan mantentzen dira, lasaigarria den datua, gizakien *Campylobacter* infekzio larrietan aukerako antibiotikoa baita. Emaitzek argi utzi dute hegazti ez diren *Campylobacter*aren gordailuek duten garrantzia eta hauek gizakien infekzioetan eta antibiotikoekiko erresistentzien zabaltzean izan dezaketen arriskua.

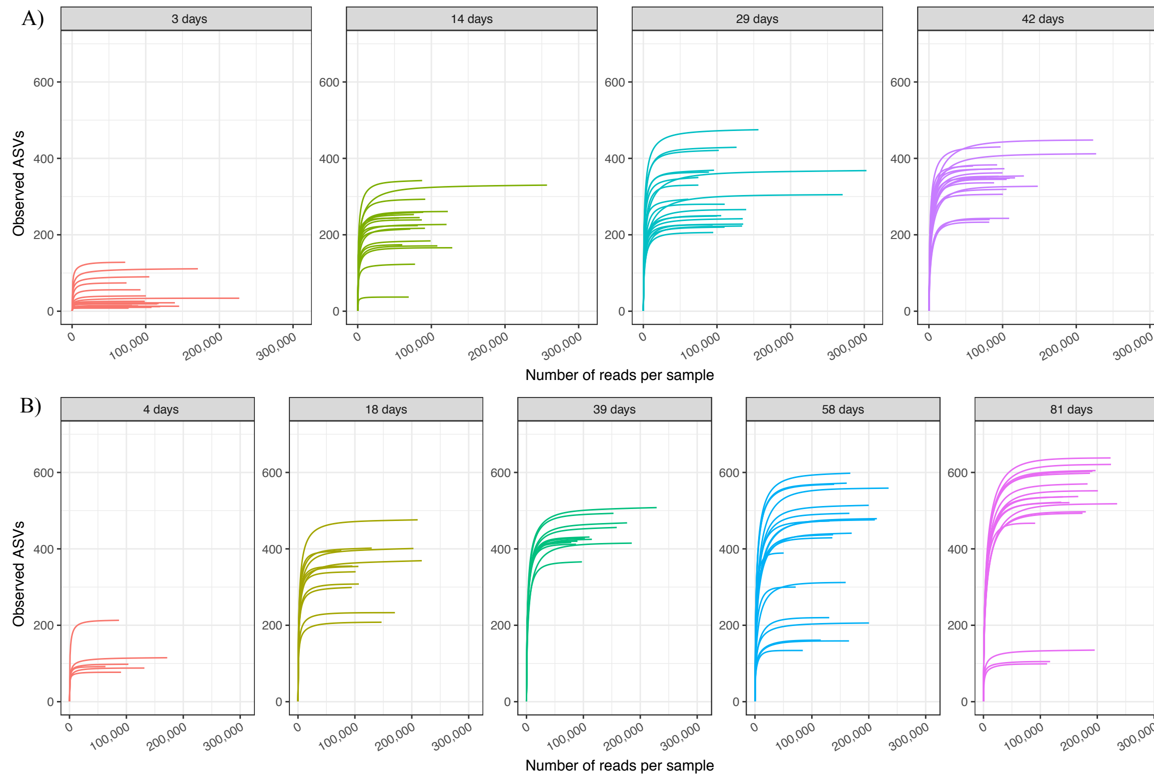
Bigarren ikerketa (Study II) batean oiloen dietan egindako esku hartze batzuen efektuak neurtu ziren, *Campylobacter*ak oiloak kolonizatzeko duen gaitasuna gutxitu eta/edo bakterioaren zabaltzea murrizteko kontrol estrategia bat izan daitekela uste da. Ondorioz hazkunde azkarreko oilo arraza (Ross-308 broilers) batekin ikerketa experimental bat egin zen, 42 egunez era intentsiboan haziak eta ohiko arto-soja pentsua gehigarri desberdinekin osatuz eman zitzaien: i) %0.1 kaltzio butiratoa; ii) %6 esne seruma; iii) %0.1 butiratoa + %6 esne seruma; eta iv) osagarririk gabeko kontrol pentsua (5 errepikapen egin ziren, elikadura desberdin bakoitzeko 30 oiloko taldetan). Talde bakoitzeko 6 oilo *C. jejuni*rekin esperimentalki inokulatzen ziren 15 eguneko adinarekin. Lortutako emaitzek erakutxi zutenez, hasierako denboraldian esne serumarekin (%6) eta kaltzio butiratoarekin (%0.1) osatutako pentsuaren elikadurak ondorio onuragarriak izan zituen alderdi produktiboari eta hesteetako osasunari dagokionez. Dena dela, pentsuari botatako osagarri desberdinetatik inork ez

zuen *Campylobacter*aren kolonizazio eta eskrezio mailaren murrizketa nabarmenik erakutsi. Emaitzen arabera, alderdi produktiboen onerako arto eta sojan oinarritutako ohiko pentsuari esne seruma (%6) eta kaltzio butiratoa (%0.1) gehitzea interesgarria izango litzateke baina ez luke *Campylobacter*aren kontrol estrategian eraginik izango.

Beste ikerketa batean (Study III) mikrobiota osatzen duten mikrobio guztien inguruan, ez *Campylobacter*aren inguruan bakarrik, dietaren eta beste faktore batzuen eragina ikertzeko, hala nola adinarena (produkzio bizitzaren puntu desberdinetan) eta arrazarena (broilerrak eta landa oiloak). Oiloen gorozkien mikrobiomaren osaketa taxonomikoa bereizteko 16S rRNA-ren V4 eskualde oso aldakorra sekuentziatzen duen errendimendu handiko amplikoia erabili zen. Ikerketa Study II-ko broiler (80 Ross-308 broilerretan, 5 errepikapen pentsu mota bakoitzeko 3, 14, 29 eta 42 egunetan) eta landa oiloekin (38 Sasso-T451A oiloekin 4,18,39,58 eta 81 egunetan) egin zen. Esne seruma eta kaltzio butiratoa gehigarri zuten pentsuek ez zuten inolako efekturik erakutsi broilerren gorozkien mikrobio komunitatean, nahiz eta parametro produktiboak hobetu. Broiler eta landa oiloetan faktore garrantzitsuena adina izan zen, zahartzen zihoazen bitartean mikrobiotaren biodibertsitatea ere handitzen zihoan. Hala ere, mikrobiotaren egituran eta osaketan desberdintasunak aurkitu ziren arrazen artean, landa oiloetan broilerretan baino mikrobioma aberatsagoa eta komplexuagoa aurkitu zen. Hau landa oiloen bizialdi luzeagoaren eta landara ateratzeko aukeraren ondorio izan daiteke. Ikerketa honek etapa desberdinetan oiloen gorozkietako mikrobiomaren garapena eta *Campylobacter*ak oiloen heste itsuan, mikrobiota komunitate komplexu batean bere lekua nola elkarbanatzen duen sakonki deskribatzen du. Bi oilo arrazetan korrelazio positiboa aurkitu zen *Campylobacter* eta *Faecalibacterium*en, *Anaerotruncus*en eta *Blautia*ren artean eta aldiz korrelazio negatiboa *Sutterella* eta *Parabacteroides*ekin. *Lactobacillus*ak ere korrelazio negatiboa izan zuen *Campylobacter*arekin, baina broilerretan bakarrik. Ikerketa gehiago beharrezkoak izango dira mikrobiotaren zati diren bakterio hauen eta *Campylobacter* ugaritasun mailaren arteko efektuak zehatz-

mehatz neurtzeko. Gainera mikrobio komunitateak aztertzeko erabilitako teknikaren bidez disbiosia zuten animali talde batzuk identifikatzea lortu zen, batez ere heste itsuaren lumenean tiflitis zantzuak zituztenetan. Animalia hauen mikrobio komunitateak dibertsitate txikiagoa erakutsi zuen eta antzekotasun handiagoa zuten beraien artean, adin berdineko taldekideen artean baino. Teknika honek disbiosia identifikatzeko duen erabilgarritasuna agerikoa da ikerketan.

9. Supplementary Material



Supplementary Figure S1. Rarefaction curves plotting the number of observed ASVs over the number of sequencing reads sampled per chicken from the broilers (A) and free-range chickens (B) datasets.

Supplementary Table S1. Number of reads that passed through each step of the pipeline in DADA2 for broilers and free-range chickens datasets.

Age	n[a]	Input		Filtered		Denoised		Merged		Tabled		Non-chimeric		Clean	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Broilers															
3 days	20	116868.3	42723.4	108785.6	39026.3	108785.6	39026.3	108750.6	39020.9	108750.4	39021.1	108373.4	39113.9	108367.9	39115.4
14 days	19	111009.8	45546.4	98606.4	43441.7	98606.4	43441.7	98493.7	43413.2	98412.8	43418.7	97869.6	43362.7	97865.3	43362.3
29 days	20	140027.8	64832.3	126423.3	60732.0	126423.3	60732.0	126221.5	60713.0	126219.4	60715.1	125747.3	60443.0	125735.8	60445.2
42 days	21	123647.4	46032.9	111836.6	42103.4	111836.6	42103.4	111592.0	42104.5	111592.0	42104.5	111334.0	42066.2	111312.8	42059.3
Free-range chickens															
4 days	6	119751.3	42305.2	108301.0	38593.1	108301.0	38593.1	108130.2	38572.1	108099.8	38544.7	107964.8	38443.3	107952.5	38446.0
18 days	12	153290.8	54570.6	139436.8	50398.4	139436.8	50398.4	139050.3	50267.9	139032.1	50256.5	138238.7	50263.9	138218.0	50262.6
39 days	12	145371.4	52813.1	133643.9	48039.7	133643.9	48039.7	133207.9	47940.1	133152.3	47896.6	132080.6	47477.9	131941.1	47454.9
58 days	20	168552.9	54017.5	153331.5	48464.3	153331.5	48464.3	152948.4	48398.6	152897.7	48370.9	152371.6	48226.5	152269.1	48206.1
81 days	20	180774.5	63566.0	160104.5	56730.2	160104.5	56730.2	159602.0	56616.2	159587.5	56609.4	159186.5	56513.9	172557.7[b]	40646.5

[a] n, number of samples. In the case of broilers and 4-day-old free-range chickens, the number of samples equals the number of animals analysed; in the case of free-range chickens of 18, 39, 58 and 81 days of age, where each of the two caeca of each animal were individually analysed, the number of samples doubles the number of animals analysed.

[b] Two samples (caeca from two different animals) were discarded due to low number of reads (<50,000) after the different filtering steps, so that final clean reads corresponded to 18 samples.

Supplementary Table S2. Proportions of taxonomic assignment of the amplicon sequence variants (ASVs) and number of unique taxa per taxonomic rank in both breeds.

Taxonomic level	% taxonomic assignment (n unique taxa)	
	Broilers (1163 ASVs)	Free-range chickens (2033 ASVs)
Kingdom	100.0 (1)	100.0 (2)
Phylum	97.5 (6)	95.7 (17)
Class	96.6 (14)	94.2 (28)
Order	96.2 (20)	93.5 (37)
Family	63.9 (32)	64.6 (55)
Genus	36.3 (43)	35.2 (77)
Species	5.8 (19)	5.9 (38)

Supplementary Table S3. Average relative abundance of taxa in broilers and free-range chickens for each age group, with ASVs collapsed to species rank. Relative abundance values >2% are highlighted in red.

This table is attached separately as an Excel file in electronic supplementary material, available at: https://www.nature.com/articles/s41598-019-39323-x#Sec18

Supplementary Table S4. Spearman's rank correlations between age (days) and relative abundance of genera in caecal microbiota of broilers and free-range chickens.

Genus relative abundance[a]	Age (days)			
	Broilers		Free-range chickens	
	rho (ρ)[b]	p_{adj}[c]	rho (ρ)[b]	p_{adj}[c]
Bacteroides	**0.88**	**0.000**	0.75	**0.000**
SMB53	0.81	**0.000**	0.30	**0.018**
Streptococcus	0.79	**0.000**	0.05	0.731
Dehalobacterium	**0.78**	**0.000**	0.22	0.083
Sutterella[d]	**0.78**	**0.000**	**0.85**	**0.000**
Parabacteroides[d]	**0.75**	**0.000**	**0.84**	**0.000**
Campylobacter	0.71	**0.000**	0.39	**0.002**
Dorea	0.69	**0.000**	**-0.46**	**0.000**
Anaerostipes	**0.67**	**0.000**	-0.30	**0.018**
Corynebacterium	**0.65**	**0.000**	0.13	0.338
Anaeroplasma	0.51	**0.000**	-0.53	**0.000**
Coprococcus	0.48	**0.000**	-0.64	**0.000**
cc_115	0.48	**0.000**	-0.48	**0.000**
Lactobacillus	0.47	**0.000**	**-0.76**	**0.000**
Blautia	0.46	**0.000**	-0.46	**0.000**
Roseburia	0.45	**0.000**	0.01	0.921
Anaerofustis	0.39	**0.001**	-0.05	0.734
Faecalibacterium	0.39	**0.001**	-0.48	**0.000**
Anaerotruncus	0.33	**0.005**	-0.48	**0.000**
Proteus	0.32	**0.006**	-0.37	**0.003**
Staphylococcus	0.28	**0.019**	-0.11	0.403
Oscillospira	0.24	0.051	-0.65	**0.000**
Lachnospira	0.22	0.062	-0.39	**0.002**
Butyricicoccus	0.15	0.213	-0.61	**0.000**
Succinatimonas	0.15	0.213	**0.72**	**0.000**
Akkermansia	0.15	0.213	**0.37**	**0.003**
Coprobacillus	0.07	0.554	**-0.65**	**0.000**
Escherichia	0.02	0.835	-0.67	**0.000**
Candidatus_Arthromitus	-0.09	0.465	-0.44	**0.000**
Ruminococcus (Lachnospiraceae)	-0.16	0.173	**-0.49**	**0.000**
Ruminococcus (Ruminococcaceae)	-0.16	0.176	-0.68	**0.000**
Enterococcus	-0.29	**0.013**	0.22	0.092
Pediococcus[d]	**-0.31**	**0.009**	**-0.35**	**0.005**
Epulopiscium[d]	**-0.35**	**0.003**	**-0.41**	**0.001**
Clostridium (Lachnospiraceae)[d]	**-0.39**	**0.000**	**-0.72**	**0.000**
Clostridium (Erysipelotrichaceae)	**-0.44**	**0.000**	-0.20	0.125

a) Genera are listed in descending order according to the Spearman´s correlation coefficient in broilers.
b) Spearman´s correlation coefficient. Positive values indicate positive association between age and genus relative abundance while negative values express negative relationships. Values in bold indicate those associations in which the increase/decrease trend over time was continuous.
c) *p*-value of Spearman's rank correlation between age (days) and genus level composition of caecal microbiota after FDR correction. Significant p_{adj}-values are in bold.
d) Genera in which the correlation resulted significant and the increase/decrease trend over time was continuous in both breeds.

Supplementary Table S5. Differential analysis results of differentially abundant taxa (DESeq2) and potential biomarkers of age groups (LEfSe) for broilers and for free-range chickens.

Tabs with “DESeq2” prefix:

Differentially abundant taxa as calculated by DESeq2 across age groups by chicken breed. Taxa were agglomerated to genus level for each breed. Values represented $\log_2$ fold change in average relative abundance of the latter group compared to the first group in comparison, and significant values ($p_{adj} < 0.05$) were formatted in red and bold font. Coloured bars represent positive (blue) and negative (red) values of the fold change.

Tabs with “LEfSe” prefix:

Important taxa of each age group at each taxonomic rank as identified by LEfSe in broiler and free-range chickens. Only those classified taxas with LDA score > 2 are shown. Taxa are listed in descending order according to their effect size within each age group. Adjusted *p*-value cutoff = 0.05.

This table is attached separately as an Excel file in electronic supplementary material, available at: https://www.nature.com/articles/s41598-019-39323-x#Sec18

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Academic and research activities of the author

EDUCATION

PhD (2015-present). Doctoral Thesis carried out at the Department of Animal Health of NEIKER - Basque Institute for Agricultural Research and Development, under the doctoral program of the Department of Immunology, Microbiology and Parasitology of the University of the Basque Country (UPV/EHU). Spain

MSc (2017-present). Master´s degree in Research Methodology in Health Sciences in Autonomous University of Barcelona (UAB). Spain.

Post-graduate Diploma (2013-2015). Post-graduate Diploma in Statistics and Design in the Health Sciences in Autonomous University of Barcelona (UAB). Spain

MSc (2011-2012). Master´s degree in Research in Veterinary Science and Food Technology in University of León. Spain.

DVM (2006-2011). Degree in Veterinary Medicine in University of León. Spain.

PUBLICATIONS

Ocejo, M., Oporto, B., Hurtado, A. *Submitted*. Occurrence of *Campylobacter jejuni* and *Campylobacter coli* in cattle and sheep in Northern Spain and changes in antimicrobial resistance in two studies 10-years apart.

Oporto, B., Ocejo, M., Alkorta, M., Marimón, JM., Montes, M., Hurtado, A. 2019. Zoonotic approach to Shiga toxin-producing *Escherichia coli*: integrated analysis of virulence and antimicrobial resistance in ruminants and humans. *Epidemiology and Infection* 147: e164.

Ocejo, M., Oporto, B., Hurtado, A. 2019. 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. *Scientific Reports* 9(1): 2506.

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CONTRIBUTION TO CONFERENCES

Ocejo, M., Oporto, B., Hurtado, A. (September 2017). Caecal microbiome modifications across age in a free-range poultry flock naturally-infected with *Campylobacter*. Oral communication presented at CHRO 2017 - 19th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, Nantes - France.

Ocejo, M., Oporto, B., Hurtado, A. (September 2017). Occurrence of zoonotic *Campylobacter* species in cattle and sheep farms. Poster presented at CHRO 2017 - 19th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, Nantes - France.

Oporto, B., Ocejo, M., Hurtado, A. (July 2017). Shiga toxin-producing *Escherichia coli* (STEC) prevalence and virulence genes in cattle and sheep. Poster presented at FEMS 2017; 7th Congress of European Microbiologists, Valencia - Spain.

Ocejo, M., Oporto, B., Lanzén, A., Juste, RA., Hurtado, A. (July 2017). Effects of feed supplementation with dry whey powder and calcium butyrate on *Campylobacter* colonization and caecal microbial community composition in broilers. Poster presented at FEMS 2017, 7th Congress of European Microbiologists, Valencia - Spain.

Ocejo, M., Oporto, B., Lanzén, A., Quince, C., Hurtado, A. (October 2016). 16S rRNA gene-based characterization of the chicken caecal microbiome across age. Poster presented at 4th World Congress on Targeting Microbiota 2016 (International Society of Microbiota), Paris - France.

Ocejo, M., Oporto, B., Hurtado, A. (October 2015). Dynamics of *Campylobacter jejuni* shedding in a broiler flock experimentally infected with two different strains. Poster presented at BIOMICROWORLD 2015; VI International Conference on Environmental, Industrial and Applied Microbiology, Barcelona - Spain.

Ocejo, M., Oporto, B., Hurtado, A. (October 2015). Prevalence of *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, verotoxigenic *E. coli* in healthy domestic ruminants in Northern Spain. Poster presented at BIOMICROWORLD 2015; VI International Conference on Environmental, Industrial and Applied Microbiology, Barcelona - Spain.

PARTICIPATION IN RESEARCH PROJECTS

2015-2018. Epidemiology of *Campylobacter* infection in meat chickens: variations in caecal microbiota with age, breed and feed, and its impact on infection as a basis for the design of sustainable control strategies.
Principal Investigator: Dr. Ana Hurtado.
Institution: NEIKER - Basque Institute for Agricultural Research and Development.
Funding body: Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria INIA.

2013-2016. Prevalence and characterization of bacterial pathogens at the primary production level - current situation and comparison with previous data.
Principal Investigator: Dr. Ana Hurtado.
Institution: NEIKER - Basque Institute for Agricultural Research and Development.
Funding body: Departamento de Desarrollo Económico e Infraestructuras of the Basque Government.

ATTENDED CONFERENCES

March 2018 - *Society for Veterinary Epidemiology and Preventive Medicine Conference*; SVEPM 2018 Annual Meeting, Tallinn - Estonia.

September 2017 - CHRO 2017; *19th International Workshop on* Campylobacter, Helicobacter *and Related Organisms*, Nantes - France.

July 2017 - FEMS 2017; *7th Congress of European Microbiologists*, Valencia - Spain.

October 2016 - *4th World Congress on Targeting Microbiota 2016* (*International Society of Microbiota*), Paris - France.

October 2015 - BIOMICROWORLD 2015; *VI International Conference on Environmental, Industrial and Applied Microbiology*, Barcelona - Spain.

September 2012 - *30th Joint Meeting of European Veterinary Pathologists, Annual Meeting of the European College of Veterinary Pathologists, 24th Annual Meeting of the Spanish Society of Veterinary Pathology*, León - Spain.

September 2011 - 46 AVEPA National Conference, *Southern European Veterinary Conference*, Barcelona - Spain.

RESEARCH EXPERIENCE

PhD Student (2015-present). Department of Animal Health of NEIKER - Basque Institute for Agricultural Research and Development, Spain.

Research assistant fellow (2013-2015). Department of Animal Health of NEIKER - Basque Institute for Agricultural Research and Development, Spain.

Master´s Thesis student (2011-2012). Department of Animal Health of University of León, Spain.

SCHOLARSHIPS

2017 **EMBO Short-Term Fellowship** (3 months). European Molecular Biology Organization.

2015 **Predoctoral grant** (4 years). Departamento de Desarrollo Económico e Infraestructuras of the Basque Government, Spain.

2013 **Young Researcher grant** (2 years). Departamento de Medio Ambiente, Planificación Territorial, Agricultura y Pesca of the Basque Government, Spain.

2011 **Scholarship for University Studies and Higher Education** (1 year). Departamento de Educacion, Universidades e Investigación of the Basque Government, Spain.

2010 **General and Mobility Purpose scholarship** (1 year). Departamento de Educacion, Universidades e Investigación of the Basque Government, Spain.