Antimicrobial resistance and molecular epidemiology of *Campylobacter* spp. isolated from broiler flocks in southern Greece

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Preface

The work presented in this PhD thesis was carried out from February 2014 to March 2021 at the Department of Poultry Diseases, Faculty of Veterinary Science, University of Thessaly, Karditsa, Greece. Isolation, detection, enumeration and PCR speciation of *Campylobacter spp.* in caeca and poultry carcasses were performed in the National Reference Laboratory of *Salmonella* and Antimicrobial Resistance of Chalkida, Greece. Genotyping and phylogenetic analysis of *Campylobacter* isolates was performed in the Microbiological Department of the Biopathological Laboratory of Aeginition Hospital, Athens, Greece.

This PhD forms part of the research project CampyRisk (‘‘*Campylobacter* spp. in the broiler food chain: Measuring and monitoring the risk for public health’’) funded by the ‘‘General Secretariat of Research and Technology’’ - Ministry of Education and Religious Affairs - under the ‘‘Bilateral Cooperation R & T Program between Greece and France’’.

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Abstract

A cross-sectional study was conducted to estimate the prevalence of and risk factors for *Campylobacter* spp. colonization of broiler flocks and broiler carcasses contamination in Greek slaughterhouses. Moreover, the antimicrobial susceptibility of *Campylobacter* isolates derived from both caecal and neck skin samples were tested to 7 antimicrobials, including nalidixic acid, ciprofloxacin, erythromycin, tetracycline, gentamycin, streptomycin and ampicillin. Finally, flaA sequencing was performed for the subtyping of 62 *C. jejuni* and 58 *C. coli* strains and phylogenetic trees were constructed using the neighbor-joining method to study their evolutionary relationships.

Over a 14-month period, a pool of 10 caeca and 5 neck skin samples from chicken carcasses were collected from each of 142 batches of broiler flocks slaughtered in 3 different slaughterhouses. Information on potential risk factors for *Campylobacter* infection in broilers was collected by an on-farm interview and linked according to the *Campylobacter* contamination status of broiler flocks and differences in farm characteristics and management practices identified from questionnaires.

Campylobacter spp. was isolated from 73.94% of caeca (95% CI 65.92-80.94) and 70.42% of carcasses (95% CI 62.19-77.78), respectively. A significant correlation ($p < 0.001$) between the presence of *Campylobacter* spp. in broiler caeca and contamination of carcasses was found, suggesting the spread of the microorganism on the skin of carcasses during the slaughtering procedure. Two different species of *Campylobacter* (*C. jejuni*, *C. coli*) were recovered, while *C. coli* was found to be the predominant species identified both in caecal and neck skin samples.

A multiple logistic regression showed the disinfection of the poultry house being conducted by unskilled personnel (odds ratio [OR] 1⁄4 = 3.983) as a significant risk factor ($p < 0.05$) and the use of straw litter as bedding material (OR 1⁄4 = 0.170) and closure of windows during the intervals of production cycles (OR 1⁄4 = 0.396) as significant protective factors ($p < 0.05$) for broiler flock contamination.

A total of 98.5% of the strains were resistant to at least one antimicrobial agent. In terms of multidrug resistance, 11.7% of strains were resistant to three or more groups of antimicrobials. Extremely high resistance to fluoroquinolones (89%), very high resistance to tetracycline (69%) and low resistance to macrolides (7%) was detected.

No prevalence of a specific flaA type was observed indicating the genetic diversity of the isolates, while some flaA types were found to share similar antimicrobial resistance patterns. Seven clusters of the *C. jejuni* phylogenetic tree and three clusters of the *C. coli* tree were considered significant with bootstrap values > 75%. Some isolates clustered together were originated from the same or adjacent farms, indicating transmission via personnel or shared equipment.

These results are important and help further the understanding of the molecular epidemiology and antimicrobial resistance of *Campylobacter* spp. derived from poultry in Greece.

Περίληψη

Μια διατμηματική μελέτη πραγματοποιήθηκε για να υπολογιστεί ο επιπολασμός και οι παράγοντες επικινδυνότητας της μόλυνσης των σμηνών κρεοπαραγωγής και των σφαγίων τους με *Campylobacter* spp. στα πτηνοσφαγεία της Ελλάδας. Επιπλέον, ελέγχθηκε η αντιμικροβιακή αντοχή των απομονωθέντων στελεχών *Campylobacter* από το περιεχόμενο των τυφλών εντέρων και του δέρματος του λαιμού σε 7 αντιμικροβιακούς παράγοντες (ναλιδιξικό οξύ, σιπροφλοξασίνη, ερυθρομυκίνη, τετρακυκλίνη, γενταμυκίνη, στρεπτομυκίνη και αμπικιλλίνη). Τέλος, πραγματοποιήθηκε αλληλούχιση του γονιδίου flaA για την υποτυποποίηση 62 στελεχών *C. jejuni* και 58 στελεχών *C. coli* και κατασκευάστηκαν φυλογενετικά δέντρα χρησιμοποιώντας τη μέθοδο σύνδεσης γειτόνων για να μελετηθούν οι εξελικτικές τους σχέσεις.

Για μια περίοδο 14 μηνών, πραγματοποιήθηκε σειρά δειγματοληψιών 10 τυφλών εντέρων και 5 δερμάτων λαιμού από σφάγια ορνιθίων κρεοπαραγωγής για κάθε μία από τις 142 παρτίδες που σφάχτηκαν σε 3 διαφορετικά σφαγεία. Ακολούθησε συλλογή πληροφοριών σχετικά με τους πιθανούς παράγοντες επικινδυνότητας για τη μόλυνση των ορνιθίων κρεοπαραγωγής με *Campylobacter* ύστερα από συνέντευξη που έλαβε χώρα στις πτηνοτροφικές μονάδες από τις οποίες προέρχονταν τα πουλερικά με τη χρήση ειδικά σχεδιασμένου ερωτηματολογίου, και αυτά τα δεδομένα συσχετίστηκαν με την παρουσία *Campylobacter* στα σμήνη κρεοπαραγωγής και τις διαφορές στα χαρακτηριστικά των εκτροφών και τις διαχειριστικές πρακτικές που ακολουθούνταν.

Απομονώθηκαν *Campylobacter* spp. από το 73.94% των τυφλών (95% ΔΕ 65.92-80.94) και το 70.42% των δερμάτων λαιμού (95% ΔΕ 62.19-77.78), αντίστοιχα. Βρέθηκε στατιστικώς σημαντική συσχέτιση ($p < 0.001$) μεταξύ της παρουσίας των *Campylobacter* spp. στα τυφλά και τη μόλυνση του δέρματος των σφαγίων, υποδεικνύοντας τη διάδοση του μικροοργανισμού στο δέρμα των σφαγίων κατά τη διαδικασία της σφαγής. Δύο διαφορετικά είδη *Campylobacter* (*C. jejuni, C. coli*) ανακτήθηκαν, ενώ το *C. coli* ήταν το επικρατέστερο είδος που εντοπίστηκε τόσο στα τυφλά όσο και στα δείγματα δέρματος λαιμού.

Μια πολλαπλή ανάλυση παλινδρόμησης έδειξε ότι η απολύμανση των υποστατικών από μη εξειδικευμένο προσωπικό (OR 1⁄4 = 3.983) αποτελεί στατιστικώς σημαντικό παράγοντα επικινδυνότητας ($p < 0.05$) , ενώ η χρήση αχύρου ως στρωμνή (OR 1⁄4 = 0.170) και το κλείσιμο των παραθύρων κατά τη διάρκεια του υγειονομικού κενού μεταξύ των παραγωγικών κύκλων (OR 1⁄4 = 0.396) αποτελούν στατιστικώς σημαντικούς προστατευτικούς παράγοντες ($p < 0.05$) για τη μόλυνση των σμηνών κρεοπαραγωγής με *Campylobacter*.

Συνολικά 98.5% των απομονωθέντων στελεχών βρέθηκαν ανθεκτικοί σε τουλάχιστον έναν αντιμικροβιακό παράγοντα. Όσον αφορά την πολυανθεκτικότητα, 11.7% των στελεχών βρέθηκαν ανθεκτικά σε τρεις ή περισσότερες ομάδες αντιμικροβιακών. Ανιχνεύθηκε εξαιρετικά υψηλή αντοχή στις φλουοροκινολόνες (89%), πολύ υψηλή αντοχή στην τετρακυκλίνη (69%) και χαμηλή αντοχή στα μακρολίδια (7%).

Δεν παρατηρήθηκε επικράτηση συγκεκριμένου τύπου flaA, γεγονός που υποδεικνύει τη γενετική ποικιλομορφία των απομονωθέντων στελεχών, ενώ ορισμένοι τύποι flaA βρέθηκαν να παρουσιάζουν παρόμοιο προφίλ αντιμικροβιακής αντοχής. Επτά ομάδες (clusters) στο φυλογενετικό δέντρο των *C. jejuni* και τρεις ομάδες στο δέντρο των *C. coli* θεωρήθηκαν στατιστικώς σημαντικές με τιμές bootstrap > 75%. Ορισμένα στελέχη που ομαδοποιήθηκαν μαζί βρέθηκε ότι προέρχονταν από την ίδια ή παρακείμενες εκτροφές, γεγονός που υποδεικνύει μετάδοση μέσω του εργατικού προσωπικού της εκτροφής ή του κοινόχρηστου εξοπλισμού.

Τα αποτελέσματα αυτά είναι σημαντικά και βοηθούν στην περαιτέρω κατανόηση της μοριακής επιδημιολογίας και της αντιμικροβιακής αντοχής των *Campylobacter* spp. που προέρχονται από την πτηνοτροφία στην Ελλάδα.

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Dedication

Αφιέρωση

Στην αγαπημένη μου κόρη

Declaration

I declare that apart from the advice and assistance acknowledged, the work reported in this thesis is my own and has not been submitted for consideration for any other degree of academic qualification.

Georgios G. Natsos
June 2021

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals I-IV. In addition, some unpublished results have also been included.

I. Natsos, G., Koutoulis, K.C., Sossidou, E., Chemaly, M., Mouttotou, N.K., 2016. *Campylobacter* spp. infection in humans and poultry. Journal of the Hellenic Veterinary Medical Society 67, 65-77.

II. Natsos, G., Mouttotou, N.K., Ahmad, S., Kamran, Z., Ioannidis, A., Koutoulis, K.C., 2019. The genus *Campylobacter*: detection and isolation methods, species identification & typing techniques. Journal of the Hellenic Veterinary Medical Society 70, 12.

III. Natsos, G., Mouttotou, N.K., Magiorkinis, E., Ioannidis, A., Rodi-Burriel, A., Chatzipanagiotou, S., Koutoulis, K.C., 2020. Prevalence of and risk factors for *Campylobacter* spp. colonization of broiler chicken flocks in Greece. Foodborne pathogens and disease 17, 679-686.

IV. Natsos, G., Mouttotou, N.K., Magiorkinis, E., Ioannidis, A., Magana, M., Chatzipanagiotou, S., Koutoulis, K.C., 2021. Antimicrobial resistance, flaA sequencing, and phylogenetic analysis of *Campylobacter* isolates from broiler chicken flocks in Greece. Veterinaty Sciences 8(5), 68.

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LIST OF SCIENTIFIC CONFERENCE ATTENDANCE WITH PROCEEDINGS

I. Natsos, G., Mouttotou, N.K., Sossidou, E., Chemaly M., Koutoulis, C.K. Campylobacteriosis in broiler flocks: associated risk factors. Proceedings of 3rd Pan-Hellenic Congress on Farm Animal Practice & Food Hygiene, pp. 98, 2-4 May 2014, Ioannina, Greece.

II. Natsos, G., Sossidou, E., Chemaly, M., Mouttotou, N.K., Rodi-Burriel A., Koutoulis. C.K. *Campylobacter* spp. as a foodborne pathogen. Proceedings of Pan-Hellenic Congress on “Meat and its products - From stable to table”, pp 118-127, 27/2-1/3/2015, Thessaloniki, Greece.

III. Νάτσος, Γ. Αποτελέσματα μικροβιοαντοχής απομονωθέντων στελεχών *Campylobacter* spp. από σμήνη ορνιθίων κρεοπαραγωγής. Ημερίδα: “Το *Campylobacter* spp. στην Τροφική Αλυσίδα των Ορνιθίων Κρεοπαραγωγής: Εκτίμηση και Διαχείριση των Κινδύνων για τη Δημόσια Υγεία”, 12 Νοεμβρίου 2015, Καρδίτσα, Ελλάδα.

IV. Νάτσος Γ. Αποτελέσματα επιδημιολογικής διερεύνησης του *Campylobacter* spp. σε σμήνη ορνιθίων κρεοπαραγωγής. Ημερίδα: “Το *Campylobacter* spp. στην Τροφική Αλυσίδα των Ορνιθίων Κρεοπαραγωγής: Εκτίμηση και Διαχείριση των Κινδύνων για τη Δημόσια Υγεία”, 12 Νοεμβρίου 2015, Καρδίτσα, Ελλάδα.

V. Natsos, G., Mouttotou, N.K., Sossidou, E., Rodi-Burriel A., Koutoulis, C.K. Prevalence, risk factors for *Campylobacter* contamination of broiler flocks in Greece and antimicrobic resistance of isolates. Proceedings of Pan-Hellenic Congress "Meat and its products - From stable to table", 3-5 May 2017, Thessaloniki, Greece.

VI. Natsos, G., Mouttotou, N.K., Sossidou, E., Rodi-Burriel A., Koutoulis, C.K. Investigation of prevalence, risk factors for *Campylobacter* spp. colonization of broiler chicken flocks and antibiotic resistance testing of isolates in Greece. Proceedings of 4th Panhellenic Congress on Farm Animal & Food Hygiene, Hellenic Veterinary Medical Society, 12-14 May 2017, Volos, Greece.

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LIST OF ABBREVIATIONS

AFLP	Amplified Lenth Polymorphism
AIDS	Acquired Immunodefficiency Syndrome
AMP	Ampicillin
a_w	Water activity
CDC	Centers for Disease Control and Protection
CFU	Colony forming unit
CGF	Comparative genomic finterprinting
CI	Confidence Interval
CIA	Critically Important Antimicrobials
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
CN	Gentamicin
DALY’s	Disability-adjusted life years
DNA	Deoxiribonucleic acid
E	Erythromycin
EC	European Commission
ECDC	European Centers for Disease Protection and Control
EEA	European Economic Area

EFSA	European Food Safety Authority
EIA	Enzyme Immunoassay
EU	European Union
FISH	Fluorescent in situ hybridization
FlaA	Flagellin gene A
GBS	Guilain-Barrè Syndrome
HCDCP	Hellenic Center for Disease Control & Protection
I	Intermediate
ISO	International Organization for Standardization
log	logarithm
Mccda	Modified Charcoal Cefoperazone Deoxycholate Agar
MDR	Multidrug resistance
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
MS	Member State
NA	Nalidixic acid
No.	Number
^oC	Degree Celsius
OIE	Office International des Epizooties (World Organization for Animal Health)
OR	Odds Ratio

PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
pH	Potential of Hydrogen
QC	Quality Control
R	Resistant
RAPD	Random Amplified Polymorphic DNA analysis
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RS	Reference Sequence
S	Streptomycin
S	Susceptible
spp.	species
ST	Sequence Type
T	Tetracycline
USA	United States of America
WHO	World Health Organization

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GENERAL INTRODUCTION

Campylobacter spp. are ubiquitous bacteria, able to colonize mucosal surfaces, usually the intestinal tract, of most mammalian and avian species tested (OIE, 2008). They are small, oxidase positive, microaerophilic, slender spirally curved Gram-negative rods exhibiting corkscrew motility. *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide. The incidence of human campylobacteriosis has been steadily rising worldwide since 1990's (WHO, 2011; EFSA and ECDC, 2019). In the European Union, campylobacteriosis has been the most commonly reported zoonosis since 2005 (EFSA, 2006; EFSA and ECDC, 2019), in the United States, it is indicated as the second most common infection (CDC, 2014), while human campylobacteriosis is hyperendemic in many developing areas of the world (Coker et al., 2002).

The eating and handling of improperly cooked or raw broiler meat contaminated with *Campylobacter* spp. has been shown to be one of the most important sources of human campylobacteriosis (EFSA and ECDC, 2018). Birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs. Broilers are considered *Campylobacter* free after hatching and become colonized by exposure to viable bacteria from the environment (Lee and Newell, 2006). Several risk factors can result in the introduction of *Campylobacter* into the flocks making it difficult to keep chicken flocks free of *Campylobacter* throughout the rearing period. Lack of biosecurity measures, season, age, partial depopulation practices, flock size, type of production system, presence of other animals on farm, water quality, presence of rodents and mechanical transmission via insects are considered to be some of the risk factors associated with horizontal transmission (Natsos et al., 2016). The control of *Campylobacter* in poultry seems crucial for the reduction of human campylobacteriosis cases.

C. jejuni has been found to be the predominant species isolated from poultry samples and yet responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by *C. lari* (Zhang and Sahin, 2013). Other *Campylobacter* species, such as *C. upsaliensis* and *C. fetus*, may also be associated with human diarrhea. Although the detection of non-*C. jejuni/coli* is uncommon in human cases in the industrialized world, it is more common in the developing world (Lastovica and Allos, 2008).

The infection may be subclinical or cause disease of variable severity. Common signs include nausea, vomiting, stomachache, malaise, profuse watery diarrhea, blood in feces and high fever (Blaser and Engberg, 2008). In most cases the illness is self-limiting, but it may be severe and life threatening in susceptible people such as young children, the elderly, or people with immunosuppressive diseases, such as AIDS and cancer (EFSA, 2011). Infection is sometimes complicated by the development of serious post infection complications, such as Guillain-Barré syndrome and even death (Zilbauer et al., 2008).

Most patients infected with *Campylobacter* spp. will recover without any specific treatment and antimicrobials are usually not required, although effective treatment may shorten the duration of illness (EFSA and ECDC, 2020). In cases where antimicrobial treatment is needed, macrolides (e.g., azithromycin) and fluoroquinolones (e.g., ciprofloxacin) are considered as the first- and second-choice of antimicrobials, respectively (Yang et al., 2019). Since a rapidly increasing proportion of *Campylobacter* strains worldwide have been found to be resistant to these antimicrobials, attention should be paid choosing the most appropriate antimicrobial treatment (EFSA and ECDC, 2020). Transmission of antimicrobial resistance from food animals to humans can occur via the food chain, therefore food animals are a significant reservoir of antimicrobial resistant zoonotic pathogens (Moore et al., 2006). Antimicrobials have been indiscriminately used in animal production for decades in order to control, prevent and treat infections, and enhance animal growth, which is speculated to have led to the emergence and spread of antimicrobial resistance among *Campylobacter* spp. (Silva et al., 2011). In particular, quinolones, 3rd and higher generation cephalosporins, macrolides and ketolides, glycopeptides and polymyxins are regarded as Critically Important Antimicrobials (CIA), hence their use in food animals is recommended to be diminished (WHO, 2019).   Accordingly, the estimation of antimicrobial susceptibility of *Campylobacter* strains derived from animal samples is crucial.

Due to the impact of *Campylobacter* on public health, epidemiological investigations analyzing the clonality of the isolated strains are very important, in order to trace the sources and routes of transmission, to follow up the temporal and geographic distribution of important phenotypic characteristics and to develop effective strategies for the control and prevention of the pathogen spread, especially inside the food chain (Sheppard et al., 2010; Ioannidou et al., 2013). Numerous phenotyping and genotyping methods have been developed for epidemiological surveillance of *Campylobacter* infections (Natsos et al., 2019). FlaA typing based on the restriction analysis of PCR-amplified fragments or sequencing of the flagellin-encoding gene, have been described for *Campylobacter* (Wassennar and Newell, 2000; Korczak et al., 2009) and is a quick and high discriminatory sub-typing technique.

In conclusion, campylobacteriosis has become the leading foodborne disease worldwide and therefore a lot of effort is being done to achieve early diagnosis of human cases, while epidemiological investigations of campylobacteriosis outbreaks using the innovative and constantly developing typing and subtyping systems available are increasingly conducted, providing information to recognize outbreaks of infection and match cases with potential vehicles of infection (Natsos et al., 2019). Since poultry is regarded as the main cause of human campylobacteriosis, the necessity to study the prevalence of *Campylobacter* in the poultry population and identify the risk factors associated with colonization of broiler flocks and broiler carcass contamination in Greece should be stressed (Natsos et al., 2016). The cross sectional study carried out in Greece, helped further the information on prevalence and antimicrobial resistance of *Campylobacter* in poultry production and lays the foundation in understanding the epidemiology of the microorganism countrywide.

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CHAPTER ONE

LITERATURE REVIEW

A. Campylobacter spp.: detection and isolation methods, species identification and typing techniques

1.1. The genus *Campylobacter*: a historical overview

The generic name *Campylobacter*, from the Greek kampylos (curved) and baktron (rod), was given by Sebald and Veron (1963) to the group of bacteria formerly known as the microaerophilic vibrios, due to their special characteristics (On, 2001; Moore et al., 2005). It is believed that *Campylobacter* species were first described in 1886 by Theodore Escherich who observed non-culturable spiral-shaped bacteria in the large-intestinal mucus of infants who had died of cholera infantum (Escherich, 1886; Vandamme, 2000; King and Adams, 2008; Vandamme et al., 2010). In 1906, the first isolation of these organisms was made by McFadyean and Stockman (1913) from the uterine exudate of aborting sheep (Butzler 2004, Skirrow 2006). Although *Campylobacter* spp. have been known to be the cause of animal disease since 1909, they have been generally recognized as a cause of human disease after the study of Butzler et al. (1973), which raised the interest in *Campylobacter* by noting their high incidence in human diarrhea (On, 2001). The first successful isolation of *Campylobacter* from human feaces had been accomplished a few years earlier with the use of a filtration technique (Dekeyser et al., 1972). Later, the isolation of *Campylobacter* became a routine in the field of clinical microbiology, since many selective media were developed, and *Campylobacter* spp. rapidly became recognized as a common cause of bacterial gastroenteritis (Fitzgerald et al., 2008a)

1.2. Classification

In the 1970s there was much confusion over campylobacter nomenclature (Skirrow 1990), but the classification of Veron and Chatelain (1973) forms the basis of currently approved nomenclature. Advances in DNA technology enabled the application of molecular methods, notably 16S rRNA sequence analysis and DNA-DNA hybridization (Vandamme et al., 1996), which clarified the systematics of campylobacters. This resulted in the extensive restructuring of the genus showing that the family of *Campylobacteraceae* represents a diverse but phylogenetically distinct group, rRNA superfamily VI, within the group of Gram-negative bacteria (Vandamme et al., 1991; Vandamme, 2000; On, 2001;), which Vandamme et al. (1991) regarded as a distinct phylum far removed from other eubacteria. Trust et al. (1994) make a case for placing the group in the epsilon subdivision of the class Proteobacteria, which comprises rRNA homology groups I (*Campylobacter* and *Bacteroides ureolyticus*), II (*Arcobacter*), and III (*Helicobacter* and *Wolinella succinogenes*). The family *Campylobacteraceae*, proposed by Vandamme and De Ley (1991), consists of two genera, *Campylobacter* and *Arcobacter* (Vandamme, 2000), while the genus of *Campylobacter* currently contains 34 species and 14 subspecies (LPSN). The species *C. jejuni* includes two subspecies (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*) that can be discriminated on the basis of several phenotypic tests (nitrate reduction, selenite reduction, sodium fluoride, and safranine) and growth at 42 °C, since *C. jejuni* subsp. *doylei* does not grow at 42 °C (Garrity, 2005). The taxonomy of the *Campylobacter* genus, which has been revised many times (Debruyne et al., 2008), is reviewed by On (2001).

1.3. Morphology

Members of the *Campylobacter* genus are slender spirally curved, non-spore forming, Gram-negative rods. The size of the cells is small and range from 0.2 to 0.9 μm in width and 0.5 to 5 μm in length (Fitzgerald et al., 2008a; Silva et al., 2011). Some species, such as *C. hominis* and *C. gracilis*, form straight rods (Fitzgerald et al., 2008a). The majority of the species are motile by means of a single polar unsheathed flagellum inserted at one or both poles of the cells (monotrichate or amphitrichate) (Vandenberg et al., 2005; Fitzgerald et al., 2008a; Silva et al., 2011). The only exceptions are *C. showae*, which has up to five unipolar flagella, and *C. gracilis*, which has none and is non-motile (Debruyne et al., 2005; Vandenberg et al., 2005; Fitzgerald et al., 2008a; Silva et al., 2011). Motility is rapid and darting, with the bacteria spinning around their long axes in a corkscrew fashion (Vandenberg et al., 2005). *Campylabacters* are able to pass through membrane filters (0.45 to 0.65 μm) with relative ease, because of their small size and motility, a property used for isolating *Campylobacter* spp. from clinical samples (Steele and McDermott, 1984; Bolton 2001; Fitzgerald et al., 2008b).

1.4. Growth and Survival Characteristics

Under ideal conditions, *Campylobacters* produce visible growth after 24 hours at 37 °C, but colonies are not well formed until 48 hours. However, it may take up to 72-96 hours of incubation to observe some slow-growing strains (Corry et al., 1995). Depending on the media used, colonies of *Campylobacter* spp. may appear differently. If the agar is moist, the colonies may appear gray, flat, irregular and thinly spreading, whereas round, convex or glistening colonies may be formed when plates are dry (Cory et al., 1995; Vandenberg et al., 2005). Since the pathogenic *Campylobacter* species grow at 37-42 °C, with an optimum growth temperature of 41.5 °C, they are used to referred to as thermophilic *Campylobacters,* although Levin (2007) suggested the term "thermotolerant" since they do not exhibit true thermophily (growth at 55 °C or above). *Campylobacters* are incapable of growth below 30 °C, since they lack cold shock protein genes which play a role in low-temperature adaptation (Silva et al., 2011).

These non-spore-forming and fastidious bacteria neither ferment nor oxidize carbohydrates, but instead they obtain energy from the degradation of amino acids, or tricarboxylic acid cycle intermediates (Vandamme, 2000; Kelly 2001) and are essentially microaerophilic and do not grow in air on primary isolation, yet oxygen (5-10%) is normally required for growth (Vandenberg et al. 2005). However, recent studies indicate that some *C. jejuni* strains can use L-fucose as a substrate for growth (Muraoka and Zhang, 2011; Stahl et al., 2011). Moreover, most *Campylobacter* species require CO_2 (1-10%) for growth (Bolton and Coates 1983). An atmosphere with low oxygen tension (5% O_2, 10% CO_2, and 85% N_2) is regarded as the most suitable for *Campylobacter*'s incubation (Vandenberg et al. 2005; Garénaux et al., 2008). An atmosphere containing increased hydrogen appears to be a growth requirement for some species (Nachamkin, 2003; Vandenberg et al., 2005; Fitzgerald et al. 2008a). Oxidase activity is present in all species except for *C. gracilis* (Silva et al., 2011).

Except of their fastidious growth requirements, *Campylobacter* spp. are considered to be very fragile and more susceptible than most bacteria to many environmental conditions, such as temperature and pH changes, low humidity, presence of oxygen and UV irradiation and to many chemical agents, such as disinfectants (Vandenberg et al., 2005; Alter and Scherer, 2006; Isohanni, 2011). *Campylobacter* spp. are easily inactivated by heat treatments with their D-value being less than 1 min (Silva et al., 2011), while freezing and thawing causes a 1-2 $\log_{10}$ fall in viable numbers, yet bacteria remain alive for many months at -20 °C (Vandenberg et al., 2005). Most species have a pH growth range of 5.5-8.0, growing optimally at pH 6.5-7.5 (Silva et al., 2011). At pH values below 5.0 or above 9.0 there is a progressive inactivation although some strains of *C. fetus* can grow slowly at pH 9.0 (Vandenberg et al., 2005). Growth does not occur in environments with water activity (a_w) lower than 0.987 (sensitive to concentrations of sodium chloride (NaCl) greater than 2% w/v), while optimal growth occurs at $a_w = 0.997$ (approximately 0.5% w/v NaCl) (Silva et al. 2011).

In some species, notably *C. jejuni* and *C. lari*, cultures that are postmature or exposed to atmospheric oxygen undergo coccal transformation (Vandenberg et al., 2005), which seems to be a degenerative process in response to toxic oxygen derivatives and low osmolality (Harvey and Leach 1998; Reezal et al. 1998). Except of the exposure to oxygen, other unfavorable conditions such as changes in temperature and pH, dehydration and low nutrient availability may cause this transformation (Rollins and Colwell 1986; Portner et al., 2007; Jackson et al., 2009; Oliver 2010; Kassem et al., 2013). Those viable, but non-cultivable cells (VBNC) have been shown to be unable to grow in subculture even though the possibility that they can revert to spiral forms after passing through the intestinal tract of chickens or humans remains unanswered (Cappelier, 1997; Vandenberg et al., 2005; Olivier 2010) and even their existence is contentious (Vandenberg et al., 2005; Silva et al., 2011).

1.5. Laboratory isolation and detection methods

In a clinical context the main role of the laboratory is to detect campylobacters in the feaces of patients with diarrhea. The same purpose also applies when it comes for samples derived from animal stool, environmental materials or processed food. There are two main categories regarding the detection method used: the conventional culture-based isolation methods and the culture-independent methods.

1.5.1. Culture-based isolation methods

The conventional method for isolating the common enteric *Campylobacter* species from feaces is primary plating on selective media and incubation at 42 °C in a microaerobic atmosphere (Vandenberg et al., 2006). Faecal samples often contain large numbers of viable *Campylobacter*, thus their detection is easily possible by direct plating on selective media (Fitzgerald et al., 2008b). Food products and environmental samples, however, may have only low numbers of stressed *Campylobacter* cells, thus an enrichment step in liquid medium before plating on solid agar plates is indicated (Cory et al., 1995; Jacobs-Reitsma et al. 2008). Inclusion of an enrichment step may also be beneficial in instances where low numbers of organisms are expected due to delayed transport to the laboratory, or after the acute stage of disease when the concentration of organisms may be low, such as in the investigation of GBS following acute *Campylobacter* infection (Fitzgerald and Nachamkin, 2006). However, enrichment culture may not always perform better than direct plating when culturing fecal samples (Musgrove et al., 2001). Several enrichment broths (e.g. Bolton broth, *Campylobacter* enrichment broth and Preston broth) that are available to be used before plating have been compared for their efficacy (Baylis et al., 2000). During the first stages of enrichment, in order to permit recovery of damaged cells, lower incubation temperature is often used (4 hours at 37 °C) and gradually increased to the final incubation temperature of 41.5 °C (Jacobs-Reitsma et al. 2008; Silva et al., 2011). This methodology is the basis for one of the ISO standard methods (ISO, 1995, 2017a) used for detection of *Campylobacter* species. When an enrichment step is used, it should be controlled for less than 24 hours because a prolonged incubation in enrichment broth may actually decrease the isolation rate (Zhang and Sahin, 2013).

The first selective culture medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow. Since then more than 40 solid and liquid selective culture media for culturing *Campylobacter* from clinical and food samples have been reported and evaluated (Habib et al., 2007; Potturi-Venkata et al., 2007; Kiess et al., 2010; Le Bars et al., 2011), and have been reviewed by Corry et al (1995). All the selective media contain a basal media, either blood or other agents such as charcoal, to quench oxygen toxicity (Fitzgerald et al., 2008), and a variety of combinations of antimicrobials to which thermophilic *Campylobacter* species are intrinsically resistant, such as polymyxin , vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin (Zhang and Sahin, 2013) that suppress the growth of many background microbial flora present in samples allowing the isolation of slow-growing *Campylobacter* spp. (Vandenberg et al., 2005; Fitzgerald et al., 2008a; Zhang and Sahin, 2011). No single medium will isolate all *Campylobacter* species (Fitzgerald et al., 2008a). Some of the less common *Campylobacter* species are inhibited by conventional selective agents and incubation at 42 °C. Therefore, where the presence of such organisms is likely, such as in patients with autoimmune deficiency syndrome (AIDS), immunocompromised patients, and deprived children in developing countries, appropriate cultivation conditions are needed to be applied, such as stool membrane filtration, special atmospheric and temperature conditions, prolonged incubation and subsequent plating on non-selective media (Lastovica and le Roux 2000; Bolton 2001; Debruyne et al., 2008).

The most recent standard method (ISO, 2017a) for detection and isolation, as well as the direct plating method for enumeration of *Campylobacter* spp. (ISO, 2017b), both use mCCDA as the selective agar. Bolton broth is used for the enrichment step and the suspension is incubated in a microaerophilic atmosphere at 37 °C for 4-6 hours, then in 41.5 °C for 40-48 hours and subsequently follows the plating on mCCDA and another agar medium based on a different principle. For confirmation, at least one colony considered to be typical or suspected as being *Campylobacter* is taken from each plate, streaked onto a Columbia blood agar plate in order to allow the development of well-isolated colonies after incubation in a microaerobic atmosphere at 41.5 °C for 24-48 hours. The pure cultures are examined for morphology and motility (characteristic), microaerobic growth at 25 °C (absent) and the presence of oxidase (positive). An exception is *Campylobacter gracilis*, which does not produce oxidase and thus gives a negative result to the latter test (Vandenberg et al., 2006; Fitzgerald et al., 2008).

Alternative enrichment and plating combinations for enumeration and detection of *Campylobacter* in chicken meat have been evaluated (Habib et al., 2011) and found to be able to provide significantly better results. Conventional methods for isolation of *Campylobacter* from food samples, involving enrichment in a selective broth for up to 72 hours, followed by subculture on selective agars and phenotypic identification require four days to produce a negative result, and 6-7 days to confirm a positive result (Corry et al., 1995). For the recovery of *Campylobacter* from stool specimens, current recommendations stipulate that cultures be held for a minimum of 72 hours prior to signing out a negative result (Garcia, 2007).

1.5.2. Direct detection methods

Microscopic observation of direct smear or wet preparation, in the case of liquid feaces, may reveal the presence of curved rods characteristic of campylobacters (Vandenberg et al., 2005). Dark-field microscopy may also reveal, besides the characteristic morphology, the darting motility of *Campylobacter* species (Fitzgerald et al., 2008a). Moreover, the direct Gram stain with carbol-fuchsin counterstain method, though underutilized, may provide a presumptive result within 30 minutes of receipt of a faecal sample in the laboratory with relatively high sensitivity, and at low cost (Wang and Murdoch, 2004).

There are also nonculture-based methods for the direct detection of campylobacters in human or animal feaces and processed food samples, which allow the identification of this fastidious organism without the specialized media and equipment that is needed for *Campylobacter* culture. Several enzyme immunoassays (EIA), which are based on antigen-antibody interaction, have been developed for this purpose in human feaces and are commercially available in a form of kits (Hindiyeh et al. 2000; Tolkin et al., 2000; Dediste et al., 2003; Kawatsu et al., 2008; Granato et al., 2010; Bessede et al., 2011 ; Fitzgerald et al., 2011). According to the preliminary data of the Centers for Disease Control and Prevention (CDC), a positive EIA test alone is not sufficient to consider a case “confirmed” and further confirmation of positive EIA results using culture methods is required (Fitzgerald et al., 2011), since these tests exhibit excellent specificity but poor sensitivity values, thus often giving false-positive results (Granato et al., 2010; Bessede et al., 2011; Myers et al., 2011; Giltner et al., 2013). In addition, the utility of these assays for detection of campylobacters in chicken feaces, which represent the main reservoir of pathogenic *Campylobacter* species, remains to be determined (Zhang and Sahin, 2013). Regarding the food samples, although commercial EIAs are available for culture-independent identification of *Campylobacter* spp., these assays have not been extensively validated (Oyarzabal and Battie, 2012) and are mainly applied to enriched cultures (Wilma et al., 1992; Lilja and Hanninen, 2001; Bohaychuck, 2005; Reiter et al., 2005; Bailey et al., 2008; Kawatsu et al., 2010; Chon et al., 2011). Commercial and/or published immunological methods used to identify *Campylobacter* spp. in food and stool samples have been reviewed by Oyarzabal and Battie (2012).

Many PCR-based assays have been described to directly detect campylobacters in human stools from clinical cases (Lawson et al., 1999; LaGier et al., 2004; Persson and Olsen, 2005; Al Amri et al., 2007; Lin et al., 2008; Zhang et al., 2013), feacal samples from bovine (Inglis and Kalischuk, 2004) and pigs (Jensen et al., 2005; Leblanc-Maridor et al., 2011), caecal and feacal samples from broilers (Bang et al., 2001; Lundi et al., 2003; Rudi et al., 2004; Al Amri et al., 2007; Rodgers et al., 2012), samples from poultry meat (Mateo et al., 2005; Debretsion et al., 2007; Hong et al., 2007; Josefsen et al., 2010; Shnider et al., 2010; Fontanot et al., 2014) and environmental specimens (Waage et al., 1999; Rothrock et al., 2009), although thus far they have been used only for research applications. Advantages of using a PCR approach instead of culture include same-day detection and identification of *Campylobacter* to the species level, along with the identification of the less-common *Campylobacter* species that are often missed by conventional culture (Kulkarni et al. 2002). However, PCR methods are more expensive and labor-intensive than culture and do not provide an isolate for further characterization, such as typing and sensitivity testing.

Finally, fluorescent in situ hybridization (FISH) with the application of highly specific oligonucleotide probes may serve for the detection and identification of thermotolerant *Campylobacter* spp. in feacal and liver samples and looks promising to become a future monitoring system in a logistic poultry slaughter concept (Schmid et al., 2005).

1.6. Species identification

Among the *Campylobacter* spp. growing at 42 °C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli*. However, low frequencies of other species have been described. The most widely adopted approach for further differentiation to species level is based on classical phenotypic characters, which include morphological appearances, growth temperatures, biochemical reactions, and tolerances (Vandenberg et al., 2006). Nevertheless, speciation is difficult because of the complex and rapidly evolving taxonomy, along with the biochemical inertness of these bacteria. These problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group. (Fitzgerald et al., 2008a)

Campylobacters are biochemically inactive compared with many other bacteria, thus few phenotypic tests are available to identify them to the species level. Generally, *C. jejuni* can be differentiated from other species on the basis of the hydrolysis of hippurate as this is the only *Campylobacter* species that expresses the N-benzoylglycine amidohydrolase (hippuricase) gene, giving hippurate-positive result. However, variability in the hippurate reaction has been observed in some strains of *C. jejuni*, resulting in hippurate-negative results (Morris et al., 1985; Totten et al., 1987; Dennis et al., 1999; Fermer and Engvall, 1999; Rautelin et al., 1999; Steinbrueckner et al., 1999; Steinhauserova et al., 2001; Jensen et al., 2005). Hippuricase-negative *C. jejuni* strains cannot be differentiated from *C. coli* by phenotypic testing. Nalidixic acid and cephalothin susceptibility testing have been used in species identification in the past (Barret et al., 1988). Both *C. jejuni* and *C. coli* grow at 42 °C and are resistant to cephalothin and cefoperazone, which are valuable agents for inclusion in selective media (Vandenberg et al., 2006). Instead, *C. upsaliensis* is sensible to cephalothin (ISO 2006a). Nowadays sensitivity to nalidixic acid may give difficulties in interpretation (OIE, 2008), since fluoroquinolone resistant and cross-resistant to nalidixic acid *Campylobacter* species have become increasingly common, with rates reported to be as high as 80% (Engberg et al., 2001). Therefore, antimicrobial susceptibility tests can no longer be relied upon for the phenotypic identification of *Campylobacter* isolates (Fitzgerald et al., 2008a). More biochemical tests may be applied for species identification, such as the detection of catalase, which is absent in *C. upsaliensis* and the detection of indoxyl acetate hydrolysis, which is negative in *C. lari* (ISO 2006a), whereas more extensive speciation schemes have been described in the literature (On 1996; Vandamme 2000). Speciation results should be confirmed using defined positive and negative controls.

Because of the difficulties and the unreliability of the phenotypic identification, several molecular methods may be used as supplementary to biochemical tests or even replace them. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for *Campylobacter* species (On 1996; Vandamme 2000). Detection of species-specific sequences via PCR can be helpful especially in cases where the differentiation between hippuricase-negative *C. jejuni* strains and *C. coli*, which are closely related species, is needed and the application of biochemical tests alone is inadequate (Dennis et al., 1999; Burnett et al., 2002; Best et al., 2003; Englen et al., 2003; On and Jordan 2003; Persson and Olsen, 2005). However, some published evaluations of PCR assays highlight the importance of validating their sensitivity and specificity (Burnett et al., 2003; On and Jordan, 2003). The complexity and the cost of these procedures preclude their use in most diagnostic laboratories. Therefore, a pragmatic approach to identification is often taken in diagnostic laboratories, where virtually all isolates are assumed to be *C. jejuni* or *C. coli* and their differentiation has little or no clinical significance (Vandenberg et al., 2006). Only if there are epidemiological or other special reasons for speciation or typing is there a need for cultures to be referred.

1.7. Typing and subtyping

Classification of bacterial strains at the species or subspecies level is generally known as bacterial typing or subtyping. The main purposes of bacterial subtyping are the evaluation of taxonomy, the definition of phylogenetic relationships, the examination of evolutionary mechanisms and the conduct of epidemiological investigations (van Belkum et al., 2001). Moreover, the use of typing methods provides the opportunity to apply more rapid, precise and efficient food-borne pathogen surveillance and prevention practices (Wiedmann, 2002). Due to the impact of *Campylobacter* on public health, epidemiological investigations analyzing the clonality of the isolated strains are very important, in order to trace the sources and routes of transmission, to follow up the temporal and geographic distribution of important phenotypic characteristics and to develop effective strategies for the control and prevention of the pathogen spread, especially inside the food chain (Ioannidou et al., 2013; Sheppard et al., 2010). The sub-typing of clinical, animal, and food isolates remains an important requirement for epidemiological studies in order to (i) trace sources and routes of transmission of human infections; (ii) identify and monitor temporally and geographically specific strains with important phenotypic characteristics; and (iii) develop strategies to control organisms within the food chain (Ioannidis et al., 2006).

The ability to discriminate or subtype campylobacters below the level of species has been successfully applied to aid the epidemiological investigation of outbreaks of campylobacteriosis (Wassenaar and Newell, 2000; Fitzgerald et al., 2001; Sails et al., 2003; Siemer et al., 2005; French et al., 2011). Subtyping provides information to recognize outbreaks of infection, match cases with potential vehicles of infection, and discriminate these from unrelated strains. In addition, these methods are of essential importance in epidemiological research projects to identify potential reservoirs of strains that cause disease in humans, identify routes of transmission, and improve our understanding of *Campylobacter* epidemiology (Fitzgerald and Nachamkin, 2006).

Typing of *Campylobacter* is a dynamic field with older methods continually being advanced and new methodologies constantly being developed (Ross, 2009). A multitude of typing systems have been developed over the last few years, however, no single technique has been declare as universally acceptable and applicable (Sails et al., 2003), since each one has both advantages and disadvantages. A number of criteria are used to evaluate subtyping methods, defining their efficacy and efficiency, which are two major properties that any typing system should possess in order to be adapted for further use (ECDC 2009). The efficacy of any typing technique can be assessed in terms of typeability, reproducibility, consistency and power of discrimination, while the efficiency reflects the expertise required, time consumed or rapidity of the technique, flexibility and suitability to carry out a certain investigation (Mohan, 2011). From all these performance criteria, the most important is the discriminatory power, namely the ability to differentiate among unrelated isolates, and typeability, namely the ability of the method to provide unambiguous results for typeable isolates (Nielsen et al., 2000). The subtyping methods available to subtype *Campylobacter jejuni* vary considerably using these criteria, depending on the method used. Since there is no definitive gold-standard method for subtyping *C. jejuni* at present, the subtyping method of choice is ultimately determined by consideration of the basic microbiology of the organism in question, the nature of the microbiological question being asked, and, essentially, the ability of the typing method to detect significant epidemiological differences (Fitzgerald et al., 2008a).

Typing systems are based on the idea that clonally related isolates share characteristics that can be tested to differentiate them from unrelated isolates (Eberle and Kiess, 2012). They are broadly classified into two major categories: phenotyping, which applies phenotypic methods that detect the presence or absence of biological or metabolic activities expressed by the bacteria and genotyping, which utilize genotypic methods that involve analysis of genetic elements based on the bacteria's DNA and RNA (Arbeit, 1995). Typing of infectious pathogens was initially done based on phenotypic characteristics such as growth, morphology, biochemical, serological and functional properties. Genotyping came into existence with the advent of restriction enzymes, electrophoretic techniques and DNA sequencing (Riley 2004). Numerous phenotyping and genotyping methods have been developed for epidemiological surveillance of *Campylobacter* infections (Wassenaar and Newell, 2000; Natsos et al., 2019). Serotyping, biotyping and phage typing are the most common phetotypic methods. The major disadvantage of these techniques is their low discriminatory ability, resulting in high numbers of strains that cannot be typed. Currently, genetic-based methods, with enhanced sensitivity and discriminatory potential, show the most promise for research purposes (Ioannidis et al., 2006).

1.7.1. Phenotypic methods

The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, phage typing and multilocus enzyme electrophoresis. Even though most of these methods lack discriminatory power, they are still applied and are quite efficient in characterizing bacterial food-borne pathogens (Wiedmann, 2002).

Biotyping schemes based on the identification of bacterial isolates through the expression of metabolic activities, such as colonial morphology, environmental tolerances, and biochemical reactions (Eberle and Kiess, 2012) can group *C. jejuni*, *C. coli* and *C. lari* in broad categories (Vandenberg et al., 2006). The first biotyping scheme for *Campylobacter* spp. was developed by Bolton et al. (1984) utilising 12 biochemical tests, such as growth in 28 °C, hippurate analysis and antimicrobial resistance typing (resistotyping). Biotyping is useful as a first step for epidemiological investigation, since it is easy to perform, relatively inexpensive and can quickly identify bacterial isolates for further testing. Finer discrimination can be obtained by combining biochemical and resistance tests to give a numerical biotype code (Bolton et al. 1984). However, the reproducibility and stability of biotyping methods are poor and, moreover, have low discriminatory power and hence are often used with serotyping to make the scheme more useful (Sails et al. 2003).

Serologic typing, or **serotyping**, is based on the knowledge that different strains of bacteria differ in the antigens they carry on their cellular surfaces. In serotyping, antibodies and antisera are used to detect these surface antigens, thereby distinguishing strains by the differences in their surface structures (Arbeit, 1995; Wiedmann, 2002). There are two generally accepted, well-evaluated serotyping schemes that were developed in the 1980s for epidemiological characterization of *Campylobacter* isolates: the first one is based on the heat stable O antigens (LPS, LOS and CPS) using a passive hemagglutination technique and was described by Penner and Hennessy in 1980, and the other one developed by Lior et al. is based on heat labile antigens antigens using a bacterial agglutination method (Lior et al., 1982). Of these two schemes, the Penner typing scheme is the most frequently used technique in laboratories worldwide and has undergone further development with 66 different antisera being used for the typing of both *C. jejuni* and *C. coli* (McKay et al. 2001). Since the two schemes are complementary, they can give good discrimination when used together even with restricted panels of antisera (Vandenberg et al., 2005). The major drawback of all serotyping systems is their limited availability owing to the time and expense needed to maintain quality antisera (Vandenberg et al., 2005) and the lack of antisera standardization, preventing their routine application for strain characterization as new serotypes remain untyped (Wassenaar and Newell, 2000). Therefore, Penner serotyping is often used in conjunction with other typing methods (Mohan, 2011).

Phagetyping was initially performed to characterize *C. jejuni* and *C. coli* in 1985 by Grajewski et al., and is often used as an adjunct to serotyping. Concisely, the technique utilizes a set of virulent phages on a bacterial host irrespective of any receptors for attachment. In case that the phages are capable of attaching and infecting the bacterial hosts, they lyse the bacterial cells producing a characteristic lytic pattern on the cultured petri dishes, referred to as 'plaques' (Grajewski et al. 1985). Like serotyping, the usefulness of phagetyping is also limited by the occurrence of non-typeable isolates and problems with cross reactivity (Sails et al. 2003). This technique is labor intensive and expensive rendering it unsuitable for most clinical laboratories (Mohan, 2011).

In **multilocus enzyme electrophoresis (MLEE)**, bacterial isolates are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing conditions (Wiedmann, 2002). MLEE was first used by Aeschbacher and Piffaretti (1989) to characterize *Campylobacter* spp. in order to determine the relationships of *C. jejuni* and *C. coli* populations between strains from non-human and human sources. MLEE studies have been performed to determine the clonal framework of *C. jejuni* (Meinersmann et al. 2002). This technique has also been utilized to study the congruence between other typing schemes used for *C. jejuni*, such as multilocus sequence typing (MLST) and pulse field gel electrophoresis (PFGE) (Sails et al. 2003). Because of a number of limitations, MLEE has been rendered unsuitable for regular typing and has been superseded by a nucleotide-based technique, MLST, which essentially mimics the MLEE's multi loci principle (Mohan, 2011).

1.7.2. Genotyping methods

The limitations associated with phenotypic subtyping methods and the rapid growth of molecular biology techniques led to the development of a wide range of molecular subtyping methods (Fitzgerald et al., 2008a). While phenotypic traits form the basis of phenotyping, genes responsible for the production of those phenotypic characters form the foundation for genotyping (Mohan, 2011). Genotyping methods measure differences in parts of the genome that are relatively stable (Wassenaar and Newell 2000). Molecular methods have become widely applied to subtype *Campylobacter jejuni* since they provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeablility, and discriminatory power when compared with phenotypic typing methods (Wassenaar and Newell, 2000; Wiedmann, 2002, Fitzgerald et al., 2008a, Eberle and Kiess, 2012). They may be divided into two broad categories: macro-restriction mediated analyses, based on separation of restriction enzyme digested nucleotide sequences, and polymerase chain reaction (PCR) based assays (Mohan, 2011).

Pulse field gel electrophoresis (PFGE), also known as field alteration gel electrophoresis (FAGE) or macro-restriction profiling PFGE has emerged as one of the best molecular approaches to analyzing bacterial pathogens, including *Campylobacter* (Eberle and Kiess, 2012; Ahmed et al., 2012). In short, PFGE is a variation of agarose-gel electrophoresis; rare cutting restriction enzymes are employed to digest the chromosomal DNA resulting in five to fifteen DNA fragments (ranging from 1 to 1000 kb pairs) depending on the chromosome and restriction enzymes used (Wassenaar et al. 1998). The resultant digested DNA is electrophoresed in a pulse field within an agarose gel matrix and the fragments are separated depending on size. PFGE is considered the 'gold standard' for epidemiological investigations due to its enormous discriminatory power (Sails et al. 2003b). The Centers for Disease Control and Prevention in the United States of America introduced an initiative called PulseNet (www.cdc.gov/pulseNet) to overcome the shortcomings of PFGE such as protocol differences and interlaboratory profile comparisons, providing a universal nomenclature scheme (Gerner-Smidt et al., 2006). The reason rendering this technique unsuitable as a tool for routine use during outbreak investigations, is its sensitivity to small amounts of nucleotide variations which eventually results in more complex restriction patterns (Sails et al. 2003b, Wassenaar et al. 1998). As a result the true relationship between strains can become obscure (Sails et al., 2003b). Current evidence suggests that genomic rearrangements do play a role in strain diversity, which can make interpretation of PFGE data difficult (Fitzgerald et al., 2008a). However, PFGE has been extensively used in genetic and epidemiological investigations of *C. jejuni* and *C. coli* (Mohan, 2011; Ahmed et al., 2012)

The **polymerase chain reaction (PCR)** has certainly revolutionized molecular epidemiological studies with its versatility and ability to detect the presence or absence of an organism in any sample by detecting a specific gene unique to the particular organism of interest (Mohan, 2011). Several variations to the original PCR technique have been developed that are useful in detecting *Campylobacter* spp., including reverse-transcriptase PCR, multiplex PCR, and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Notably, multiplex PCR assays which are used for simultaneous differentiation of *Campylobacter* spp. have replaced monoplex PCR assays, which were widely used for detection and differential diagnosis of *Campylobacter* spp. in the past (Asakura et al. 2008, Yamazaki-Matsune et al. 2007). These techniques are easy to reproduce, highly discriminatory, and available in most laboratories. Although they may be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp. (Eberle and Kiess, 2012).

Apart from PCR being used as a diagnostic tool itself, most of the genotyping techniques are PCR based which are simple, rapid and cost effective (Asakura et al. 2008). **Random amplified polymorphic DNA analysis (RAPD)** and **amplified length polymorphism (AFLP)** are two PCR-based methods used for *Campylobacter* genotyping, which provide good discriminatory power, although due to certain limitations they are not successfully used as a routine genotyping tool (Mohan, 2011). **Ribotyping** is a ribosomal (r)RNA approach for the identification of bacterial isolates, which even though has a high level of typeablility for *Campylobacter* spp., its low number of ribosomal genes gives it poor discriminatory power (Eberle and Kiess, 2012). Moreover, this method is tedious, time-consuming and expensive, which makes it unsuitable for routine genotyping (Wassenaar and Newell, 2000). **Flagellin typing** using restriction fragment length polymorphism (RFLP) is another technique used for typing of *Campylobacter* species. Since the major flagellin gene, flaA, and the minor flagellin gene, flaB, which encode the flagella’s proteins, are different from one another and highly conserved, this flagellin gene locus is suitable for detection by RFLP from PCR products (Eberle and Kiess, 2012). Although flagellin gene typing is quick and can have high discriminatory power, it is suggested that this method should not be the sole technique used in epidemiological grouping of isolates and therefore it is often used in combination with other typing techniques mostly MLST (Dingle et al. 2005; Mohan, 2011; Eberle and Kiess, 2012). Polymorphic genes other than fla can be also used for **PCR-RFLP analysis**. Moreover, several polymorphic genes can be combined and analyzed by using a multiplex PCR in order to obtain increased discriminatory power (Wassenaar and Newell, 2000). Such a multiplex PCR was developed for *C. jejuni* by using the polymorphic genes gyrA and pflA, giving a level of discrimination similar to that of PFGE, which can be enhanced by including fla as a third gene target (Ragimbeau et al., 1998).

DNA sequencing of one or more selected bacterial genes represents another genetic subtyping method (Wiedmann, 2002), which is becoming increasingly automated and consequently is a reasonable alternative method for genotyping bacterial isolates (Wassenaar and Newell, 2000). **Multilocus sequence typing (MLST)** is a genotypic typing method that was first developed in 1991 based on the well-established principles of MLEE (Maiden et al., 1998). This technique differs from MLEE in that it assigns alleles directly by DNA sequencing of 7 to 11 housekeeping genes, rather than indirectly through the electrophoretic mobility of their gene products (Eberle and Kiess, 2012). A universal nomenclature scheme for storing and interpreting nucleotide sequence data is employed and each allele fragment is assigned a unique number in the order of discovery. For each locus, distinct allelic sequences are assigned with allelic numbers and each isolate is therefore designated with seven numbers constituting an allelic profile which, in turn, is given a sequence type (ST) or genotypic number (Mohan, 2011). The comparison of allelic profiles shows how closely related the isolates are to each other; the more sequence types the isolates have in common, the more they are related, and vice versa (Urwin and Maiden, 2003). The isolates that share at least four alleles in common are grouped under a common central genotype, referred to as the founder ST or the known central ancestor, the clonal complex genotype (Mohan, 2011). An important component of the MLST approach is the availability of databases (e.g. PubMLST) for use by public health and research communities, where the sequence data can be compared. In turn, researchers can submit the results of their findings to these databases (Maiden 2006).

Multilocus sequence typing is the leading molecular typing method for *Campylobacter* at this time (Ross, 2009). An MLST system specifically for the characterization *C. jejuni* strains was developed by Dingle et al. (2001) and is increasingly used in epidemiological studies and population structure analysis of *Campylobacter* spp. (Mohan, 2011). An extended MLST method that characterizes not only *C. jejuni* but also *C. coli*, *C. lari*, and *C. upsaliensis*, was designed by Miller et al. (2005). The advantages of using MLST include high discriminatory power, reproducibility, ease of interpretation, and transferability of information among laboratories (Wasenaar and Newell, 2000; Dingle et al., 2001). Another key advantage of MLST is that it can be used for population genetic studies as well as a typing tool for molecular epidemiological investigations (Maiden 2006); several reports are available on the use of MLST as an investigation tool (Sails et al. 2003, Urwin and Maiden 2003, McTavish et al. 2008). Additionally, multilocus sequence typing can detect mixed cultures, genetic exchange, and recombination between *Campylobacter* spp. (Miller et al., 2005). Even though MLST results are easy to reproduce, interpret and transfer, it is a complex and expensive technique to perform (Djordjevic ET AL., 2007; Lévesque et al., 2008; Ahmed et al., 2012). Recent work has shown that the seven loci used may be unsufficient to provide an accurate picture of gene content in all areas of the *C. jejuni* genome (Taboada et al., 2008). Moreover, MLST is unable to distinguish closely related strains in short-term outbreak investigations and additional methods, such as fla typing, may be required in order to obtain sufficient resolution (Sails et al., 2003) increasing the cost and time to acquire results adequate for detailed molecular epidemiological analysis.

Comparative genomics, namely the analysis of and comparison of two or more genomes, has not only served to uncover the large amount of within-species genomic diversity and the rapid pace of evolution of bacterial genomes, but it has also served to underscore some of the new challenges in bacterial genotyping and phylogenetic analysis (Ross, 2009). **Comparative genomic fingerprinting (CGF)** is a novel method of comparative genomics-based bacterial characterization developed in an attempt to circumvent the main problems associated with genome sequencing and microarray-based comparative genomics; i.e., cost, ease and throughput (Ross, 2009). CGF is based on the concept that differential carriage of these accessory genes can be used to generate unique genomic fingerprints for genotyping purposes. This technique assesses the conservation status of a small number of genes previously described as having high intraspecies variability based on comparative genomics studies (Ross, 2009). The more genes assessed using CGF, the higher is the resolution of the assay. Toboada et al. (2012) developed and validated a rapid and high-resolution 40-gene comparative genomic fingerprinting method for *C. jejuni* (CFG-40). The results obtained with this method suggest that it has a higher discriminatory power than MLST at both the level of clonal complex and sequence type, while it is also rapid, low cost, and easily deployable for routine epidemiologic surveillance and outbreak investigations (Clark et al., 2012; Tadoaba et al., 2012). It was shown that CGF and MLST are highly concordant, and that isolates with identical MLST profiles are comprised of isolates with distinct but highly similar CGF profiles. The high concordance with MLST coupled with the ability to discriminate between closely related isolates suggests that CFG40 is useful in differentiating highly prevalent sequence types, such as ST21 and ST45 (Taboada et al., 2012).

1.8.References

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B. *Campylobacter* spp. infection in humans and poultry

1.1. Campylobacteriosis and public health

1.1.1. Incidence, severity and costs

Since 1990's the incidence of human campylobacteriosis has been steadily rising worldwide (Baker et al., 2007; WHO, 2011). This is in accordance with the Community Zoonoses Reports of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). In the EU, campylobacteriosis has been the most commonly reported zoonosis since 2005, followed by salmonellosis (EFSA, 2006; EFSA and ECDC, 2019). Information submitted by 28 European Union Member States (EU MS) on the occurrence of zoonoses and food-borne outbreaks in 2018, showed that there were 246,571 confirmed human cases of campylobacteriosis (EFSA and ECDC, 2019). Thus, the overall notification rate of human campylobacteriosis was 64.1 per 100,000 population (range: 1.9-215.8 per 100,000 population). There was a wide variation in incidences between countries which probably reflects differences in the healthcare and reporting systems, and in microbiological methods for the detection of *Campylobacter* (Olson et al., 2008; Vally et al., 2009; EFSA and ECDC, 2019). Even though clinical cases of campylobacteriosis tended to be under-reported, "there may be not less than 2 million and possibly as high as 20 million cases of clinical campylobacteriosis per year in the 27 EU MS" (EFSA, 2010c).

There was a significantly increasing trend in the number of cases at EU/EEA level and at country level in half of the MS between 2009 and 2018. However, from 2014 to 2018 the EU notification rate did not change significantly as most MS reported stable trends (EFSA and ECDC, 2019). The increase in reported cases in some countries may not only reflect changes in exposure, but also improvements in surveillance systems, a better coverage of routine diagnostics across the country, requirement for medical laboratories to report positive test results and better knowledge and awareness among physicians. Moreover, *Campylobacter* was found to have a characteristic seasonality with a sharp increase of cases in the summer and early autumn (EFSA and ECDC, 2019). Considering the high number of human campylobacteriosis cases, the severity in terms of reported fatalities remains low (0.03%) (EFSA and ECDC, 2014).

According to Scallan et al (2011) *Campylobacter* is the third-leading cause of bacterial foodborne illness in the United States. Information provided by the Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Disease Control and Prevention (CDC), from 10 State Health Departments in the USA, indicated campylobacteriosis as the second most common infection (35%), following salmonellosis (40%). CDC also estimated that in 2012, the number of reported infections and incidence per 100,000 population by *Campylobacter* was 6,793 and 14.30, respectively (CDC, 2013). In the same report the estimated incidence of infection for *Campylobacter* showed a 14% increase in 2012, compared with 2006-2008. Also, in the USA, it is estimated that *Campylobacter* causes 2.5 million illnesses, 13,000 hospitalizations, and over 100 deaths each year (Patrick, 2007).

In Australia, *Campylobacter* is currently the most common cause of acute bacterial diarrhea among all the notified enteric pathogens with more than 15,000 cases each year (Stafford, 2010). The incidence of notified campylobacteriosis has steadily increased during the past 15 years from 67.0/100,000 population in 1991 to 121.4/100,000 in 2005 (Stafford, 2010). According to the same researcher, adjusting for under-reporting, there may be an estimation of 225,000 infections occurring each year in Australia, but most of which are sporadic in nature.

In many developing areas of the world, human campylobacteriosis is hyperendemic and the disease differs from campylobacteriosis in developed countries (Coker et al., 2002). In developing areas, campylobacteriosis is predominantly a pediatric problem affecting children under the age of five while adults are generally less prone to the disease (Oberhelman and Taylor, 2000; Coker et al., 2002). Generally, developing countries do not have national surveillance programs for campylobacteriosis; therefore, incidence values in terms of number of cases for a population do not exist (Coker et al., 2002). Most estimates of incidence came from laboratory-based surveillance of pathogens responsible for diarrhea. Oberhelman and Taylor (2000) estimated that *Campylobacter* isolation rates in developing countries ranged from 5 to 20%. In Asiatic countries like Thailand for example, the overall isolation rate of *Campylobacter* from diarrheal children under year 5 was 6.8% (Yang et al., 2008). This rate was 12.1% in Laos, with *C. jejuni* and *C. coli* occurring in 7.1% and 4% of enteric infection in children aged < 1 year and 1-5 years, respectively (Yamashiro et al., 1998).

There are no sufficient data on campylobacteriosis in Greece, because the disease is not under surveillance through Mandatory Notification System (EFSA and ECDC, 2018). According to Hellenic Center for Disease Control & Protection (HCDCP) factsheet of 2013, although there are few hospitals with laboratory ability of *Campylobacter* isolation, the number of positive cultures for this pathogen was high (623 positive cultures) in 2012, even greater than the frequency of salmonellosis (HCDCP, 2013). These data indicate the need of integration of campylobacteriosis on the Mandatory Notification System in order to achieve full illustration of the morbidity caused by the microorganism in question and the need of setting a specialized reference laboratory. Nevertheless, there have been several references about *Campylobacter* spp. and its contribution on acute gastroenteritis among patient in Greek hospitals, especially children (Kafetzis et al., 2001; Maltezou et al., 2001; Chatzipanagiotou et al., 2002; Chatzipanagiotou et al., 2003a; Maraki et al., 2003; Ioannidis et al., 2006; Papavasileiou et al., 2007; Ioannidis et al., 2009; Maragkoudakis et al., 2010; Mellou et al., 2010; Mammas et al., 2012; Maraki et al., 2012; Ioannidis et al., 2013). Moreover, the first diagnosed *C. jejuni*-associated Guillain-Barré Syndrome case from Greece in 2003 reported by Chatzipanagiotou et al. (2003b).

Common signs and symptoms of pathogenic *Campylobacter* infection include nausea, vomiting, abdominal pain, malaise, profuse watery diarrhea, blood in feces and high fever (Butzler, 2004; Blaser and Engberg, 2008; Igwaran and Okoh, 2019; Kaakoush et al., 2015). The infective dose of campylobacteriosis can be as low as few hundred cells (Black et al., 1988; Hara-Kudo and Takatori, 2011) and the incubation period lasts up to 3 days (Zilbauer et al., 2008). In most cases the illness is self-limiting, but it may be severe and life threatening in susceptible people such as young children, the elderly, or people with immunosuppressive diseases, such as AIDS and cancer (EFSA, 2011). In susceptible humans, *C. jejuni/coli* infection is associated with acute enteritis and abdominal pain lasting for up to seven days or longer (Allos, 2001). Infection is sometimes complicated by the development of serious post infection complications, such as bacteraemia, Guillain-Barré syndrome (GBS), reactive arthritis, inflammatory bowel disease, irritable bowel syndrome (Allos, 2001; Helms et al., 2003; Havelaar et al., 2005; Mangen et al., 2005; Smith and Bayles, 2007; Gradel et al., 2009; Haagsma et al., 2010; Zilbauer et al., 2008) and even death (Havelaar et al., 2005; Gradel et al., 2008; Zilbauer et al., 2008). GBS is an acute demyelinating disease of the peripheral nervous system resulting in temporary ascending flaccid paralysis (Allos, 2001). There are enough data on the incidence of GBS in Europe and North America (McGrogan et al., 2009; Sejvar et al., 2011). The disease has also been well studied in China, where it may implicate in outbreaks, and in Japan, whereas seasonal patterns of GBS have been described in Mexico, China, Argentina, Curacao, South Africa and other countries (Coker et al., 2002; WHO, 2013).

Most patients infected with *Campylobacter* spp. will recover without any specific treatment other than replacing lost fluids and electrolytes (Silva et al., 2011). Antimicrobial treatment is usually not required, but effective treatment may shorten the duration of illness (EFSA and ECDC, 2020). In cases where antimicrobial treatment is needed, macrolides (mostly erythromycin and azithromycin) and fluoroquinolones (e.g., ciprofloxacin) are considered as the first- and second-choice of antimicrobials, respectively (ECDC et al., 2009; Iovine, 2013; Yang et al., 2019). Since a rapidly increasing proportion of *Campylobacter* strains worldwide have been found to be resistant to these antimicrobials, attention should be paid choosing the most appropriate antimicrobial treatment (Allos, 2001; Humphrey et al., 2007; EFSA and ECDC, 2020). Antimicrobial resistant bacteria is a global problem associated with increased healthcare cost, prolonged infections with a greater risk of hospitalization and high mortality risk and rate (Founou et al., 2017). Infection with antimicrobial-resistant *Campylobacter* may lead to suboptimal outcomes of antimicrobial treatments or even treatment failure (Engberg et al., 2004). Therefore, other antimicrobials such as gentamicin, tetracycline, clindamycin, ampicillin, carbapenems and amoxicillin-clavulanic acid could be alternatively used for the treatment of systemic *Campylobacter* infections (Blaser et al., 2000; Dai et al., 2020). Transmission of antimicrobial resistance from food animals to humans can occur via the food chain, therefore food animals are a significant reservoir of antimicrobial resistant zoonotic pathogens (Moore et al., 2006). Antimicrobials have been indiscriminately used in animal production for decades in order to control, prevent and treat infections, and enhance animal growth, which is speculated to have led to the emergence and spread of antimicrobial resistance among *Campylobacter* spp. (Silva et al., 2011). Consequently, the estimation of antimicrobial susceptibility of *Campylobacter* strains derived from animal samples is crucial. World Health Organization, therefore, has published a list of critically important antimicrobials for human medicine emphasizing the importance of prudent use of antimicrobials both in human and veterinary medicine (WHO, 2019).

The socioeconomic costs of the disease in humans can be very high (Samuel et al., 2004) and this is expected, if one takes under consideration that there may be approximately nine million cases of human campylobacteriosis per year in the 27 EU MS (EFSA, 2011). The public health impact of campylobacteriosis and its sequelae is 0.35 million disability-adjusted life years (DALYs) per year and total annual costs are 2.4 billion euros (EFSA, 2011). These costs reflect to medical expenses, lost wages, product recalls, legal costs, and other indirect expenses (CAST, 1994). Havelaar et al. (2005), estimated that in the Netherlands (with approximately 80,000 cases of gastroenteritis per year), the costs of illness caused by campylobacteriosis are about 21 million euros/year.

1.1.2. Outbreaks of *Campylobacter* spp. - Sources and transmission of infection

Most campylobacteriosis cases are sporadic or small-scale family outbreaks (Olson et al. 2008). Even though outbreaks of *Campylobacter* infections are rarely reported, they might be more common than previously suspected (Gillespie et al., 2003; Miller et al., 2004; Fussing et al., 2007; Isohanni, 2013). Because the incubation period before the onset of symptoms can be long, it might be difficult to determine the source of infection. Numerous epidemiological studies have been conducted to identify potential sources for human campylobacteriosis. Risk factors for *Campylobacter* infections differ for outbreak cases and sporadic cases (Hue et al., 2011). Outbreaks are mainly due to untreated raw milk (Heuvelink et al., 2009) or contaminated water (Nygard et al., 2004; Abe et al., 2008; Karagiannis et al., 2010a; Karagiannis et al., 2010b), while consumption of raw or undercooked poultry meat or exposure to food cross-contaminated by contact with raw poultry constitutes the main risk factor for sporadic human infection (Tauxe et al., 1997; Studahl and Andersson, 2000; Corry and Atabay, 2001; Nadeau et al., 2002; Kapperud et al., 2003; Neimann et al., 2003; Friedman et al., 2004; Nielsen et al., 2006; Wingstrand et al., 2006; Humphrey et al., 2007; Stafford et al., 2007; Doorduyn et al., 2010; EFSA and ECDC, 2018). The consumption of chicken and chicken by-products has been increased due to their low price, special taste, and the short time required for preparation and consequently they have been implicated over the recent years in a large number of outbreaks of acute campylobacteriosis in human populations worldwide, in both industrialized and developing countries, and especially in children, the elderly and immuno-suppressed patients (Skirrow, 1998; Corry and Atabay, 2001). In particular, the handling, preparation and consumption of broiler meat accounted for 20% to 30% of campylobacteriosis cases, while 50% to 80% attributed to the chicken reservoir as a whole (EFSA, 2010c). Furthermore, broiler meat was the most commonly implicated food vehicle, accounting for 11of the 25 strong-evidence outbreaks (44.0%) (EFSA and ECDC, 2014).

Other possible sources of campylobacteriosis include other contaminated food, contaminated water, direct contact with farm animals, environmental sources and foreign travel. According to EFSA's report for 2012, among 19 EU MSs a total of 501 foodborne *Campylobacter* outbreaks were reported and this counted for 9.3 of the total reported foodborne outbreaks in the EU (EFSA and ECDC, 2014).

Besides broiler meat, contaminated livers constitute a notable source of human campylobacteriosis. Outbreaks of *Campylobacter* infections linked to chicken and duck liver pâté have been reported in the United Kingdom (O'Leary et al., 2009), Australia (Parry et al., 2012), Europe (EFSA and ECDC, 2013) and USA (Tompkins et al., 2013). In addition, since 2007, England and Wales have mentioned a significant increase in the proportion of *Campylobacter* outbreaks linked to the consumption of chicken livers used in pâté (Little et al., 2010). These outbreaks did not come as a surprise, given that previous studies had shown that 77% of retail chicken livers were contaminated with *Campylobacter* (Little et al., 2010).

Some researchers point out eggs as a possible route of transmission since fecal contamination of the shell may take place and the survival of *Campylobacter* on eggshell is being promoted by the shell's moisture (Cox et al., 2012). In a study conducted by Messelhäusser et al. (2011) viable bacteria of *Campylobacter* spp. were found in 4.1% of the eggshell samples, whereas Jones and Musgrove (2007) found 0.5% of the restricted shell eggs investigated positive for thermotolerant *Campylobacter* spp. In Japan, Sato and Sashihara (2010) found that between 27.9 and 36% of unpasteurized liquid egg samples were positive for *Campylobacter*. Therefore, a contaminated eggshell always creates the risk of cross-contaminating the egg yolk with pathogens and of initiating foodborne infections by producing ready-to-eat food with raw or undercooked egg content. The other possibility is cross-contamination from the eggshell to other ready-to-eat products which do not contain the egg content itself (Cox et al., 2012).

In addition to risks from food, contact with animals, either domestic pets or farm animals, presents another exposure pathway for human infection (Saeed et al., 1993; Schorr et al., 1994; Studahl and Andersson, 2000; Moore et al., 2005). Other foods (such as pork, beef and unpasteurized milk), or direct contact with these animals were mentioned in the literature as pathways to acquire *Campylobacter* infection (Moore et al., 2005; Jacobs-Reitsma et al., 2008). The digestive tract of healthy cattle can be a significant reservoir for a number of *Campylobacter* species, with a prevalence of the enteropathogen in cattle ranging from 0-80% (Atabay and Corry, 1998) whereas the prevalence of *Campylobacter* spp.in sheep was about 20% (Zweifel and Stephan, 2004). Pig carcasses have been shown to be more frequently contaminated than either beef or sheep (Nesbakken et al., 2003). This is most likely attributable to the fact that pig carcasses undergo a communal scalding stage early in the slaughtering process combined with the fact that the skin remains on the carcass following all of the dressing procedures (Moore et al., 2005).

Raw milk has also been identified as a vehicle of human gastroenteritis caused by *Campylobacter* spp. (Weltman et al., 2013; EFSA and ECDC, 2014). Especially, *C. jejuni* was found to be present in milk due to faecal cross-contamination during milking or as a result of udder infection (Orr et al., 1995).

Waterborne outbreaks of *Campylobacter* have been reported in many developed countries (Allos, 2001; Martin et al., 2006; Jakopanec et al., 2008; EFSA and ECDC, 2013).

In Greece, a waterborne *Campylobacter jejuni* outbreak occurred in Crete in 2009. Most cases originated from rural areas, served by a different water-supply system from that of the adjacent town and there was strong epidemiological evidence that tap water was the vehicle of the outbreak (Karagiannis et al., 2010a, Karagiannis et al., 2010b). Consumption of untreated water (Schorr et al., 1994) or rainwater (Eberhart-Phillips et al., 1997) was associated with campylobacteriosis in other studies. In an ecological study in Sweden, positive associations were found between the incidence of *Campylobacter* spp. and the average volume of water consumed per person. These observations suggested that drinking water and contamination from livestock might also be important factors in explaining at least a proportion of human sporadic campylobacteriosis cases (Nygard et al., 2004).

Contaminated shellfish have also been implicated as a vehicle in the dissemination of campylobacteriosis. Harvesting shellfish from *Campylobacter*-contaminated waters would appear to be the most likely cause of infection (Wilson and Moore, 1996).

Travel to a developing country is a risk factor for acquiring *Campylobacter*-associated diarrhea, which is more severe, and strains are more likely to be associated with antimicrobial resistance (Coker et al., 2002). Campylobacteriosis acquired abroad contributes to the number of cases reported in developed countries and, as a result, represents an important subset of all cases. In the USA, 13% of *Campylobacter* infections are associated with international travel, and *Campylobacter* is the most frequently reported travel-associated infection (Kendall et al., 2012). In Scandinavia, the proportion of travel-related cases is higher, and systematic reporting of such infections has provided proxy surveillance information for parts of the world where diagnostic testing or reporting of the infection is less frequent (Ekdahl and Andersson, 2004).

***1.2. Campylobacter* in broiler production**

1.2.1. Broiler farms

Broiler intestines are a particularly favorable environment for the proliferation of thermophilic *Campylobacters*, such as *C. jejuni* and *C. coli*. Birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs (Lee and Newell, 2006). Naturally acquired flock colonization has been found to be age dependent (Newell and Fearnley, 2003). Broilers are considered *Campylobacter* free after hatching, since most evidence suggest that vertical transmission plays a minor role, if any (Jacobs-Reitsma et al., 1995; Pearson et al., 1996; Petersen and Wedderkopp, 2001; Sahin et al., 2003; Callicott et al., 2006). Nevertheless, Cox et al. (2012) referred to trans-ovarian transmission since fecal bacteria, including *Campylobacter*, can contaminate the shell, shell membranes, and albumen of freshly laid eggs and the chick can become colonized after ingestion of the pathogen when it emerges from the egg.

In general, broiler flocks remain *Campylobacter* free for the first two weeks (the so-called lag phase) (Annan-Prah and Janc, 1988; Stern, 1992; Newell and Fearnley, 2003). This lag phase is likely to be an inherent property of the chick. An inhibitory effect produced by commensal organisms in the gut of young chicks (Schoeni and Doyle, 1992), the presence of maternal antibodies which may be protective and which decline by about 14 days of age (Sahin et al., 2001) and antimicrobial treatment contribute to the existence of the lag phase. After the first colonization (usually at two to three weeks of age), following exposure to viable bacteria from the environment, *Campylobacter* spread quickly within the flock. As chickens are coprophagic, fecal shedding is presumably an important factor in the dissemination of organisms around large broiler flocks once the first bird becomes colonized. Certainly, once flock colonization is detected, bird-to-bird transmission within flocks is extremely rapid, and the majority (up to 100%) of birds in a positive flock are colonized within only a few days (Shreeve et al., 2000; Newell and Fearnley, 2003). The presence of *Campylobacter* in the caeca can be at a detectable level few hours after the exposure (Bull et al, 2006), while birds remain highly colonized until slaughter (Berndtson et al. 1996a, van Gerwe et al. 2009), representing an important public health risk.

The prevalence of *Campylobacter* in broiler flocks varies among different countries. A harmonized baseline survey was conducted in the EU in 2008, generating representative data regarding national production, in order to estimate the prevalence of *Campylobacter* in broilers and on broiler meat (EFSA, 2010a). Approximately 71.2% of broiler batches were estimated to be colonized by *Campylobacter* at the slaughterhouse. The prevalence of *Campylobacter*-colonized broiler batches among the EU member states varied widely, ranging from as low as 2.0% up to 96.8% (EFSA 2010a). The results of the EU baseline survey were consistent with several other studies (Rasschaert et al., 2007; Allen et al., 2008; Kuana et al., 2008; Hue et al., 2010; Hue et al., 2011; Lawes et al., 2012; Powell et al., 2012). In 2012, the overall proportion of *Campylobacter*-positive broiler flocks was 33.56% (range: 0% - 83.6%) among the five MSs (e.g. Denmark, Germany, Hungary, Slovenia, Sweden) which reported flock-based data (EFSA and ECDC, 2014). Several other flock-based studies have showed a prevalence from 15% up to 76% (Barrios et al., 2006; Arseunault et al., 2007a; Guerin et al., 2007; McDowell et al., 2008; Sasaki et al., 2010; Ansari-Lari et al., 2010)

Campylobacter jejuni is the predominant species isolated from poultry samples, followed by *C. coli*, with other *Campylobacter* species such as *C. lari* being less detected. In the southern EU MSs the presence of *C. coli* was more abundant, whereas *C. jejuni* was the only species isolated in the northern countries (EFSA, 2010a). Climatic conditions, environmental reservoirs, broiler housing and age of slaughter that vary significantly from northern to southern Europe could partly explain the observed variation of the species distribution (EFSA, 2010a). In addition, *C. coli* is more frequently identified in older animals and particularly from organic systems (El-Shibiny et al., 2005). Some studies mention that *C. coli* is more commonly isolated from poultry in the developing world. Specifically, *C. coli* was the dominant *Campylobacter* species isolated from poultry in Nigeria and Thailand (Aboaba and Smith, 2005; Padungtod and Kaneene, 2005). Poultry flocks and individual chickens might be infected with different *Campylobacter* strains at the same time (Jacobs-Reitsma et al., 1995; Rivoal et al., 1999). Furthermore, mixed infections can result in new strains through the exchange of genetic material (Jacobs-Reitsma et al., 1995; De Boer et al., 2002; Hook et al., 2005).

There is a paucity of data about the prevalence and species distribution of *Campylobacter* in broiler flocks in our country since Greece did not participated in the European union-wide baseline survey carried out in 2008 (EFSA, 2010; Natsos et al., 2016) . The isolation, identification, and antimicrobial resistance of *Campylobacter* spp. from poultry farms and slaughter houses has been investigated and reported for the first time in Greece by Marinou et al (2013). The results of this study showed a low prevalence (16/830 (1.9%) fecal samples) of *Campylobacter* spp. in five poultry farms in a geographical region around Athens, with the predominance of *C. coli*. However, the need for a surveillance and monitoring system for the prevalence, risk factors and antimicrobial resistance of *Campylobacter* in poultry and other food animals is a requisite and more studies about this topic should be carried out.

The incidence and prevalence of *Campylobacter* in positive broiler flocks varies depending on geographical, farming and environmental conditions. Seasonality effects have been observed with a marked peak during summer months, much more noticeable in Northern Europe (Bouwknegt et al., 2004; Patrick et al., 2004; Hofshagen and Kruse, 2005; Hansson et al., 2007; van Asselt et al., 2008; Jore et al. 2010; Zoonosis Centre, 2012) than in Southern Europe (Nylen et al., 2002). In contrast, some studies in the United Kingdom, USA, and Canada have reported no seasonal influence on *Campylobacter* prevalence (Humphrey et al., 1993; Gregory et al., 1997; Nadeau et al., 2002). Seasonality effects could be explained by environmental factors, which require further investigation, such as humidity, temperature and sunlight (Wallace et al., 1997; Arsenault et al., 2007a; Guerin et al., 2008). For instance, a warmer mean temperature and the moister climate during summertime provide conditions favoring environmental *Campylobacter* survival, as well as increase the amount of insects, wild birds and rodents, which act as mechanical vectors for the pathogen, around the broiler house (Hald et al., 2004; Rushton et al., 2009; Jore et al., 2010). Except of the abundance of flies, the increased ventilation because of higher temperatures during the summer has also been related to the seasonal variation (Hald et al., 2008). It has been also claimed, that in the Nordic countries, the cold winters contribute to the decrease of the *Campylobacter* environmental load.

Remarkably, the increase in human cases can sometimes occur previous to infections in chickens, suggesting that there might be a common risk factor responsible for the increase in *Campylobacter* cases. Flies can transmit *Campylobacter* to chickens and humans and they could partly explain the seasonality of human cases (Hald et al., 2004; Nichols, 2005; Ekdahl et al., 2005; Nelson et al., 2006; Guerin et al., 2008; Hald et al., 2008; Nichols, 2010).

1.2.2. Broiler slaughterhouses - Carcasses

The intestinal colonization of broilers with *Campylobacter* during rearing is responsible for the contamination of the carcasses and equipment with *Campylobacter* during slaughtering (Rosenquist et al., 2006; Reich et al., 2008; Silva et al., 2011). Food processing areas that constitute critical control points in poultry processing plants are usually scalding, defeathering and evisceration, since the carcass contamination occurs there by leakage of the contaminated feaces from the cloaca and visceral rupture of the caeca carrying a high *Campylobacter* load (Berrang et al., 2001; Stern and Robach, 2003; Takahashi et al., 2006; Boysen and Rosenquist, 2009; Silva et al., 2011). Automated defeathering represents a high risk practice since cloacal contents can cause contamination of the carcasses (Berrang et al., 2001). *Campylobacter* spp. remain in a liquid film on the skin and become entrapped in its cervices and channels which provides a favourable environment for cross contamination (Chantarapanont et al., 2003). Cross-contamination of *Campylobacter* strains between slaughtered flocks may also occur via contacts with contaminated surfaces of the slaughter facilities, processing water and air (Peyrat et al., 2008; Perko-Mäkelä et al., 2009; Isohanni, 2013). Furthermore, the persistence and survival of *Campylobacter* spp. are fostered by a suitable microenvironment of the skin (Chantarapanont et al., 2003) and even under frozen conditions or storage at 4 °C, *Campylobacter* spp. are able to persist in the carcass (Maziero and de Oliveira, 2010). Previous studies reported that growth on skin stored at room temperature in a controlled atmosphere package is possible, increasing the risk for consumers if contaminated chicken is not adequately stored or handled (Lee et al., 1998; Scherer et al., 2006). It has been found that carcasses from batches with *Campylobacter*-positive caeca have significantly higher quantitative loads than those from batches with negative caeca, which is in accordance with other studies, indicates that reduction in intestinal contamination could be a possible way to reduce the amount of bacteria on carcasses (EFSA, 2010a; Hue et al. 2011).

Control of campylobacteriosis is commonly focused on reducing the occurrence of *Campylobacter* in broiler meats (Hue et al., 2010). Until recently, there were no regulations requiring that broiler meat be tested for *Campylobacter*, even though some countries like Iceland had an official policy of testing flocks prior to slaughter and requiring that meat derived from positive flocks be sold frozen (Stern and Robach, 2003). However, in 2017 the European Commission made an amendment of the standing Regulation as regards *Campylobacter* in broiler carcasses (2017/1495 EU), introducing the mandatory sampling of poultry carcasses for *Campylobacter* analysis at slaughterhouses on a regular basis.

The average prevalence of *Campylobacter* contamination on broiler carcasses worldwide is reported to be in the range of 60-80% (Suzuki and Yamamoto, 2009; Isohanni, 2013). According to EFSA (2010a), the prevalence in the EU of *Campylobacter*-contaminated broiler carcasses, in 2008, was reported as 75.8% and varied from 4.9% to 100.0% among the EU MSs. That prevalence is higher than the respective prevalence for broiler batches, which come into accordance with the results of other studies (Hue et al., 2011; Powell et al., 2012; Chokboonmongkol et al., 2013), assuming that cross-contamination from positive batches to negative batches does occur during the slaughtering process and associated carcass preparation (Jørgensen et al. 2002; Johannessen et al. 2007; EFSA, 2010a; Hue et al, 2011) through contamination of the slaughterhouse environment (Johnsen et al 2006). The counts of *Campylobacter* bacteria on broiler carcasses varied widely also between countries, which might be due to differences in slaughterhouse hygiene and processing practices (Habib et al., 2008; Sampers et al., 2008; EFSA, 2010a). In general there was a tendency for high counts in countries with high *Campylobacter* prevalence. Low *Campylobacter* numbers on broiler carcasses may reflect effective pre-harvest production procedures, good slaughter hygiene, low within-flock prevalence or low cross-contamination of carcasses of a *Campylobacter*-negative batch from a previous positive batch (Johannessen et al., 2007). The elevated levels of *Campylobacter* can be recovered from the broiler carcasses and transmitted in the food chain during further processing (EFSA 2010a).

The distribution of *Campylobacter* species isolated from broiler carcasses varies among different countries. *Campylobacter jejuni* proved to be the predominant species at EU level, with about two-thirds of the total isolates being identified as *C. jejuni*, while approximately one-third was *C. coli*. Other *Campylobacter* species are less frequently identified (EFSA, 2010a). Still, the reverse situation was observed in some MSs reporting dominance of *C. coli* isolates. Moreover, a high proportion of *C. coli* in poultry meat has been reported from some other parts of the world (Meeyam et al., 2004; Padungtod et al., 2005; van Nierop et al., 2005; Suzuki and Yamamoto, 2009). In Greece, no information is available, since there is no surveillance and monitoring system. According with the study performed by Marinou et al. (2013), no *Campylobacter* was isolated from the caecal samples of the chicken carcasses.

1.2.3. Retail broiler meat products

Broiler meat is considered to be the main foodborne source of human campylobacteriosis. According to EFSA (2014), a large share of retail broiler meat remains contaminated with *Campylobacter*. In 2012, approximately 30% of the samples of poultry meat in retail were found to be positive in the 9 EU MSs reporting data on testing of single broiler samples (range: 0% - 80.6%). The reported levels of *Campylobacter* in fresh broiler meat products at retail vary between log 1 to log 4 CFU/100g (or a fillet) of meat, depending on the different studies and methodologies used (Jacobs-Reitsma et al., 2008). Studies report that *C. jejuni* was usually the dominant *Campylobacter* species isolated from retail broiler meat products worldwide, but the ratio of *C. coli* to *C. jejuni* varied between countries (Suzuki and Yamamoto, 2009). Limited studies have been published on the prevalence of *Campylobacter* in broiler meat at the Greek retail level. The presence of *Campylobacter* spp. in poultry meat, along with isolation, identification at species level and determination of the antimicrobial resistance of the isolates has been investigated by Petridou and Zdragas (2009) in Northern Greece. The results of this study showed that 73% of the samples were *Campylobacter* positive, while *Campylobacter jejuni* seemed to be the predominant species. Moreover, the prevalence of *Campylobacter* spp. in raw broiler meat was investigated by Zisidis (2011) during the period from 2005 to 2010. The samples were collected from several slaughterhouses, poultry meat selling points and restaurants of Western Greece. The results showed that 28.7% of the samples were *Campylobacter* positive, with *C. jejuni* as predominant species and a remarkable decline of positive results was observed through the study from 50% in 2005 to 18.5% in 2010. However, there is still a need of more investigation in order to determine the true prevalence of *Campylobacter* spp. in our country.

1.3. Risk factors associated with *Campylobacter* spp. colonization in broiler flocks and broiler carcasses contamination

Several risk factors can result in the introduction of *Campylobacter* into the flocks making it difficult to keep chicken flocks free of *Campylobacter* throughout the rearing period. Moreover, once introduced into the flock, *Campylobacter* is quickly spread to all birds, grows rapidly within each bird and large numbers are shed, heavily contaminating the broiler house environment and equipment (Battersby et al., 2016). The possible sources and transmission routes of *Campylobacter* for poultry flocks have been investigated extensively, focusing on different parts of the production processes and practices. Most epidemiological studies have focused on the outcome being the flock becoming infected, not considering the within flock prevalence nor the amount of *Campylobacter* in the infected chickens. The outside environment has been suggested as the ultimate source of colonization for broiler flocks. In addition, many factors - such as adjacent broiler units or other animals, farm workers, drinking water, rodents, wild birds, flies and other insects - may have a role in transmitting *Campylobacter* to broiler flocks (Hald et al., 2004; Bull et al., 2006; Rushton et al., 2009).

The most important risk factors associated with horizontal transmission of *Campylobacter* spp. to broiler flocks and broiler carcass contamination during the slaughtering process have been reviewed by Natsos et al. (2016) and are shown in Table 1.1 and Table 1.2 respectively.

Table 1.1. Risk factors with an increased association with *Campylobacter* spp. colonization in broiler flocks along with the corresponding references.

RISK FACTOR	REFERENCES
Season (summer months)	Bouwknegt et al., 2004; Barrios et al., 2006; Huneau-Salaün et al., 2007; Zweifel et al., 2008; McDowell et al., 2008; Ellis-Iversen et al., 2009; Jore et al., 2010; EFSA, 2010b; Lawes et al., 2012; Chowdhury et al., 2012a
Age of broilers	Berndtson et al., 1996b; Evans and Sayers, 2000; Bouwknegt et al., 2004; Barrios et al., 2006; McDowell et al., 2008; EFSA, 2010b; Ansari- Lari et al., 2011; Chowdhury et al., 2012a; Lawes et al., 2012; Sommer et al., 2013
Partial depopulation practices	Hald et al., 2000; Hald et al., 2001; Slader et al., 2002; Ellis-Iversen et al., 2009; Hannson et al., 2010; EFSA, 2010b; Lawes et al., 2012
Lack of biosecurity measures	Humphrey et al., 1993; Van de Giessen et al. 1996; Gibbens et al., 2001; Herman et al., 2003; Cardinale et al., 2004
Flock size	Berndtson et al., 1996b; Barrios et al., 2006; Guerin et al., 2007a; Nather et al., 2009
Human traffic and farm equipment	Berndtson et al., 1996b; Evans and Sayers, 2000; Hald et al., 2000; Cardinale et al., 2004; Ramabu et al., 2004; Hofshagen and Kruse, 2005

Other animals on the farm or very close to the farm	van de Giessen et al., 1996; Bouwknegt et al., 2004; Cardinale et al., 2004; Lyngstad et al., 2008; Ellis-Iversen et al., 2009; Hannson et al., 2010; Sommer et al., 2013
General farm hygiene	Hald et al., 2000; Evans and Sayers, 2000; McDowell et al., 2008; Hannson et al., 2010
Type of drinking system	Näther et al., 2009
Contaminated water	Pearson et al., 1993; Zimmer et al., 2003
Infected wild birds	Chuma et al., 2000; Craven et al., 2000
Contaminated air from adjacent poultry houses	Berndtson et al., 1996a
Mechanical transmission via insects	Berndtson et al., 1996a; Refregier-Petton et al., 2001
Health and welfare status	Bull et al., 2008
Presence of rodents	Gregory et al., 1997; Huneau-Salaün et al., 2007; McDowell et al., 2008; Sommer et al., 2013
Free-range & organic flocks	Näther et al., 2009

Table 1.2. Risk factors with an increased association with broiler carcass contamination along with the corresponding references.

RISK FACTOR	REFERENCES
Slaughter in summer months	EFSA, 2010b; Powell et al., 2012
Age of broilers	EFSA, 2010b
Previous thinning of the flock	Hue et al., 2010
Batch was not slaughtered first in the slaughter program	Hue et al., 2010
Temperature in evisceration room (ºC)	Hue et al., 2010
Presence of dirty marks on eviscerated carcasses	Hue et al., 2010
Time (hour) of sampling during day	EFSA, 2010b
Campylobacter-colonization in the broiler batch	Arsenault et al., 2007b; EFSA, 2010b
Batches with higher standard deviation of carcass weight	Mahler et al., 2011

1.4. Controlling of *Campylobacter spp.* infection through active surveillance

Burden of disease studies provide evidence that there is a need for control measures across all outcomes of campylobacteriosis while taking into consideration its underestimation (WHO, 2013). Nowadays, the implementation of effective controls to reduce the burden of disease in humans is considered a priority in many areas of the world. Consequently, the control of *Campylobacter* in poultry seems crucial for the reduction of human campylobacteriosis cases.

Nowadays risk assessment of *Campylobacter* in poultry plants is used as a tool for the prevention of human zoonotic diseases (Nauta et al., 2009). In order to assess the risks of acquiring an infection via poultry meat, it is essential to ascertain the degree of contamination of the raw poultry (Hue et al., 2011). It has been actually reported that reducing *Campylobacter* colonization in caecal contents of flocks by 2 $\log_{10}$ or 3 $\log_{10}$ CFU, would reduce human campylobacteriosis cases attributable to broiler meat by at least 76% or 90% respectively (Romero-Barrios et al., 2013). Therefore, the most common approach to *Campylobacter* control is the decrease of prevalence and bacterial load within the flock. Many efforts have been directed against finding appropriate intervention methods, which can be widely segregated into preharvest and postharvest intervention (Umaraw et al., 2017). *Campylobacter* control measures at farm level may include bio-security, vaccination, complete exclusion, bacteriophage therapy, food additives, probiotics or novel antibacterial treatment flocks (Newell et al., 2011; Zhang and Sahin, 2013), most of which are under development and not yet commercially available. Thus, the most effective intervention measure to control *Campylobacter* in broiler meat is to reduce *Campylobacter* levels on carcasses after evisceration, rather than reducing the prevalence of positive broiler flocks (Nauta et al., 2009; Hermans et al., 2011). In fact, it has been predicted that a 2 log reduction in the concentration of *C. jejuni* on broiler meat could result in a 30-fold decrease in the number of human campylobacteriosis cases related to broiler meat consumption (Rosenquist et al., 2003). Moreover, because cross-contamination may occur during slaughtering process, the knowledge of the contamination status of a flock constitutes an essential piece of information to help preserve its *Campylobacter*-free flocks (Hue et al., 2011).

European Food Safety Authority has emphasized the importance and recommended the establishment of an active surveillance of campylobacteriosis in all MS, including efforts to determine the uncertain and unreported campylobacteriosis cases. In addition, storage and genotyping of human and putative reservoirs of isolates in all MS have also been recommended (EFSA, 2011). Thereafter, it would be important to identify the *Campylobacter* properties of virulence, survival characteristics and ecology (EFSA, 2011).

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CHAPTER 2

MATERIALS AND METHODS

2.1. Experimental design

The unit for statistical analysis was the “slaughter batch” defined as a group of chickens from the same flock, delivered at the same time to the same slaughterhouse. The study dealt with 3 EC-approved Greek poultry slaughterhouses from Central Greece, each of which processes more than 5.000.000 chickens per year and in total 50.000.000 chickens per year, derived from 4 different Greek regional units (Arta, Attica, Boeotia and Euboea). All the data for this study were collected between February 24th 2014 and March 2nd 2015.

2.2. Sample size

For the purpose of the study, poultry samples were obtained from three different poultry slaughterhouses of Central Greece, conveniently selected according to location and throughput, derived from a sufficient number of broiler farms in the regional units of Arta, Attica, Boeotia and Euboea, which was based on the poultry population and density of each area. The sample size was set on the basis of an expected prevalence of 50% and a confidence interval of 95%. In total, 142 samples were collected, originating from 60 different poultry farms, of which 8 are situated in the regional unit of Arta, 9 in Attica, 20 in Boeotia and 23 in Euboea.

2.3. Sampling design

The sampling design was programmed so that a flock from each poultry enterprise to be examined, circumstances permitting, twice a year (summer period: March to August, winter season: September to February), in order to consider the effect of seasonality on the prevalence of the disease.

2.4. Sample collection

Caeca were randomly selected from 10 birds per batch during evisceration, and pooled into a sterile bag. Neck skin samples of 5 birds from the processing line after chilling were also taken using a clean pair of latex gloves and put into a sterile bag. After the sampling, the acquired samples were sent in an insulated box containing ice packs to maintain a low temperature within a few hours of the same day in the Veterinary Laboratory of Chalkida, where bacteriological analyses were performed. The referral for microbiological analysis accompanied each sample is shown in Appendix 1.

2.5. Sample analysis

Campylobacter spp. recovered from the caecal contents using the technique of direct isolation, in which 10 μl of each caecal sample, previously homogenized by adding Peptone Salt solution (Merck, Darmstadt, Germany), are plated on the selective medium modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid, Dardilly, France), followed by incubation for 44 ± 4 hours at 41.5 ± 1˚C under microaerobic conditions (5% O2, 10% CO2 and 85% N2). For each positive plate, if necessary, up to 5 typical *Campylobacter* colonies were then subcultured onto plates of Columbia Blood Agar (Oxoid, Dardilly, France) for further characterization, in accordance with standard procedure of International Organization for Standardization (ISO) 10272-1 (ISO, 2006a). The flock was considered *Campylobacter*-positive, when at least one confirmed *Campylobacter* isolated from a colony yielded a positive result by PCR procedure.

For the recovery of *Campylobacter* from the skin of carcasses, the procedure described in parts 1 and 2 of the ISO 10272 (ISO, 2006a; ISO, 2006b) was followed. For the detection of *Campylobacter*, 10 grams of neck skin placed in a sterile bag and diluted 1:10 with selective pre-enrichment Bolton Broth solution (Oxoid, Dardilly, France). The mix was then homogenized for 1 minute in a peristaltic homogenizer and the final suspension was incubated under microaerobic conditions for 4 hours at 37 ˚C and then for 44 ± 4 hours at 41.5 ± 1˚C. Subsequently, 10 μl of the suspension were plated onto mCCDA and Butzler (Oxoid, Dardilly, France) plates and followed by incubation for 44 ± 4 hours at 41.5 ± 1˚C. For each positive plate, up to 2 colonies typical of *Campylobacter* were subcultured onto Columbia Blood Agar plates for further characterization, according to standard method of ISO 10272-1:2006. *Campylobacter* spp. were enumerated by duplicate-plating 1 ml of the homogenate onto three plates of mCCDA. Ten-fold serial dilutions of the homogenate in tryptone salt broth were also prepared and plated onto one plate of mCCDA. All plates were incubated under microaerobic conditions for 44 ± 4 hours at 41.5 ± 1˚C. The colony-count technique and calculation method described in ISO10272-2:2006 was followed to estimate the bacterial load on carcasses.

2.6 Identification methods

For confirmation of *Campylobacter* species, the ISO 10272-1:2006 protocol was followed. Both biochemical and molecular identification methods were used for the speciation of *Campylobacter* isolates. For the biochemical identification of our isolates the rapid hippurate hydrolysis test was performed, which employs ninhydrin as an indicator that detects glycine, the second byproduct of hippurate hydrolysis. A positive test was indicated by the appearance of a deep blue/violet color within 30 minutes, while in case of a faint blue color or no color change the test was negative.

For the molecular identification of our isolates Polymerase Chain Reaction (PCR) was performed. DNA isolation was performed on pure cultures of *Campylobacter* on blood substrate. Bacterial suspensions were prepared with saline solution to be used for molecular typing and stored at -20 °C. Extraction of bacterial DNA was done using Instagene Matrix (BioRad Laboratories reagent, California USA) following the manufacturer's instructions. PCR was performed according to the protocol of the Community Reference Laboratory for Antimicrobial Resistance (DTU - Food) - PCR Protocol of *Campylobacter jejuni* and *C. coli* recommended by the EURL-AR (2013) - in a total volume of 25 μl using 12.5 μl of 2X Green PCR Master Mix, 9.5 μl of PCR H_2O and 0.5 μl of each primer shown in Table 2.1. Six primers were selected to identify the species *Campylobacter jejuni* and *Campylobacter coli* following the EURL-AR Protocol (PCR amplification of *Campylobacter jejuni* and *coli*; 1st version August 2013). Thermal cycler Biometra UNO II was used and the conditions of denaturation, hybridization of primers to the DNA ends and DNA amplification were: 10΄ at 95 °C, 30 cycles of 30΄΄ at 94 °C, 90΄΄ at 59 °C, 60΄΄ at 72 °C and 10΄ at 72 °C. PCR mixture loading was done onto 1.5% agarose gel in 1X TBE solution, which was stained with photosensitive dye GelRed Nucleic Acid Gel

Stain 10.000X (Biotium Inc.) followed by 45΄ of electrophoresis in 100 V. *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559 were used as control strains.

Table 2.1. Target-genes and their sequences used for molecular typing of *Campylobacter* species.

Target gene	Primer name	Sequence	Expected amplicon size
mapA$_{C.jejuni}$	MDmapA1	5’-CTA TTT TAT TTT TGA GTG CTT GTG-3΄	589bp
mapA$_{C.jejuni}$	MDmapA2	5’-GCT TTA TTT GCC ATT TGT TTT ATT A-3’	589bp
ceuE$_{C.coli}$	COL3	5’-AAT TGA AAA TTG CTC CAA CTA TG -3’	462bp
ceuE$_{C.coli}$	MDCOL2	5’-TGA TTT TAT TAT TTG TAG CAG CG-3’	462bp
16S	16S R primer 804RX	5'-GAC TAC CNG GGT ATC TAA TCC-3'	800bp
16S	16S F primer 10FX	5'-AGA GTT TGA TCC TGG CTN AG-3'	800bp

2.7 Antimicrobial resistance testing

For each *Campylobacter*-positive sample, antimicrobial susceptibility testing to ciprofloxacin, nalidixic acid, erythromycin, streptomycin, gentamicin and tetracycline was performed. Positive caecal samples were additionally tested for resistance in ampicillin. Antimicrobial disks for the disk diffusion method were obtained from Oxoid, Dardilly, France. Disk diffusion method in Miller-Hinton agar enriched with 5% defibrinated sheep’s blood was performed. Sterile cotton-tipped swabs were used inoculate broth culture diluted to match a 0.5 McFarland turbidity standard onto Mueller-Hinton blood agar plates to produce a confluent lawn of bacterial growth. After the inoculum on the plates was dried, antimicrobial disks were distributed over the inoculated plates using an Antimicrobial Susceptibility testing Disk Dispencer (Oxoid, Dardilly, France). These plates were then incubated at 42 °C for 24 hours under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). Isolates with insufficient growth after 24 hours incubation were reincubated immediately and inhibition zone read after a total of 40-48 hours incubation. *Campylobacter jejuni* ATCC 33560 was used as a quality-control (QC) strain and the acceptable ranges of Clinical and Laboratory Standards Institute (CLSI M45) were followed. Since there are no antimicrobial resistance breakpoints of disk diffusion method specific with respect to *Campylobacter* for nalidixic acid, gentamicin, streptomycin and ampicillin provided by CLSI M45, resistance breakpoints of enteric bacteria in the family *Enterobacteriaceae* were used to determine antimicrobial resistance of *Campylobacter* spp. (CLSI M100). The concentrations of antimicrobial agents tested in this study along with the zone diameter breakpoints are shown in Table 2.2. The record sheet where the results of antimicrobial resistance testing were kept is shown in Appendix 2.

Table 2.2. Breakpoints of the disk diffusion method used to determine antimicrobial susceptibility of *Campylobacter* isolates.

Antimicrobial Agent	**Disk Concentration (µg)**	**Zone diameter Breakpoint (mm)**[a]		
		S	**I**	**R**
Ciprofloxacin	5	≥ 24	21-23	≤ 20
Erythromycin	15	≥ 16	13-15	≤ 12
Tetracycline	30	≥ 26	23-25	≤ 22
Nalidixic acid	30	≥ 19	14-18	≤ 13
Gentamycin	10	≥ 15	13-14	≤ 12
Streptomycin	10	≥ 15	12-14	≤ 11
Ampicillin	30	≥ 17	14-16	≤ 13

[a] Zone diameter breakpoints of ciprofloxacin, erythromycin and tetracycline for *Campylobacter* spp. were recommended by the CLSI M45, whereas those of nalidixic acid, gentamycin, streptomycin and ampicillin for *Enterobacteriaceae* were recommended by the CLSI M100. S, susceptible; I, intermediate; R, resistant.

2.8. Statistical analysis

The prevalence of Campylobacter-positive batches of caeca and carcasses were calculated using Epi Info^{TM} 7 software (CDC, Atlanta, Georgia, USA). A batch was considered positive if *Campylobacter* was detected and/or enumerated. For enumeration purposes, bacterial counts were $\log_{10}$-transformed to obtain approximately normally distributed data.

An on-farm interview was performed to collect information on potential risk factors for *Campylobacter* infection in broilers, with questions concerning farm characteristics such as the environment around the farm and broiler houses, in-house environment (humidity, air quality, temperature), design of the broiler houses and on-farm management practices. Data concerned house surroundings, house characteristics, staff, sanitary practice, control of wild birds and rodents, dead bird management, feeding and watering practice, and various herd parameters were also tested for associations. Seasonal effect was taken into account since measurements and observations covered the four seasons of the year. Data concerning the structural and functional characteristics of slaughterhouses such as hygiene level, along with data derived from microbiology analysis were also statistically analyzed using the appropriate statistical methodology. The original questionnaire used is shown in Appendix 3.

Univariate statistical analysis was carried out using Epi Info^{TM} 7 software (CDC, Atlanta, Georgia, USA) to identify the main trend, variability and distribution of each individual variable. Variables with more than 20% of missing data and those for which there was no variability were excluded from the analysis. Bivariate analysis was performed using Epi Info^{TM} 7 software to study relationships between independent variables and *Campylobacter* contamination of caeca and neck skin samples. Finally, a multiple logistic regression including all the previously selected explanatory variables was performed. A downward selection, using Epi Info^{TM} 7 software, was performed, with variables introduced if $p < 0.20$ and excluded if $p > 0.05$.

2.9. FlaA sequencing

A PCR procedure was performed on the DNA extracts of 120 *Campylobacter* isolates. For each PCR 45 μl MasterMix and 5 μl DNA template were put in a 200 μl thin wall tube (Bio Rad Laboratories California USA) and followed the protocol described in Table 2.3. The primers used were composed by Eurofins Genomics, were in freeze-drying state and were selected based on a study of Meinersmann et al. (1997) as shown in Table 2.4. Electrophoresis of the PCR mixture on 100 ml of 1.5% agarose gel was followed applying 40 V for approximately 10 minutes and then 80 V for another two hours. For the sequencing of the flaA gene DNA STAR's Laser gene Evolution Suite software was used. All sequences were submitted to GenBank and issued accession numbers (MW713238 - MW713296 for *C. coli* sequences and MW713297 - MW713360 for *C. jejuni* sequences).

Table 2.3. The PCR protocol for flaA gene.

	Cycle	Time	Temperature
Initial Denaturation	1	2-5 min	95°C
Denaturation		20 sec	95°C
Annealing	35	15 sec	55°C
Extension		15-30 sec/kb	72°C
Final extension	1	1-5 min	72°C

Table 2.4. Primers used for PCR.

Target Gene	Primer	Primer sequence (5' to 3')	Tm (°C)	Molecular Weight	Volume for 100 pmol/ml
flaA	FLA4F	GGA TTT CGT ATT AAC ACA AAT GGT GC (26)	60.1	8009	185
	FLA630R	GCT CCA AGT CCT GTT CC (17)	55.2	5097	182

2.10. Phylogenetic trees

All available flaA sequences for *C. jejuni* and *C. coli* were downloaded from different geographic regions. Phylogenetic analysis was performed estimating the genetic distances between sequences using Tamura-Nei model (Tamura and Nei, 1993). Phylogenetic trees were constructed using the neighbor-joining method and the reliability of phylogenetic clusters was assessed using bootstrapping analysis of 1000 copies. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The alignment of all sequences was performed by Cluster W algorithm using the MEGA 5 software, while all positions containing gaps and missing data were manually edited.

2.11. References

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CHAPTER 3

RESULTS

3.1. Prevalence

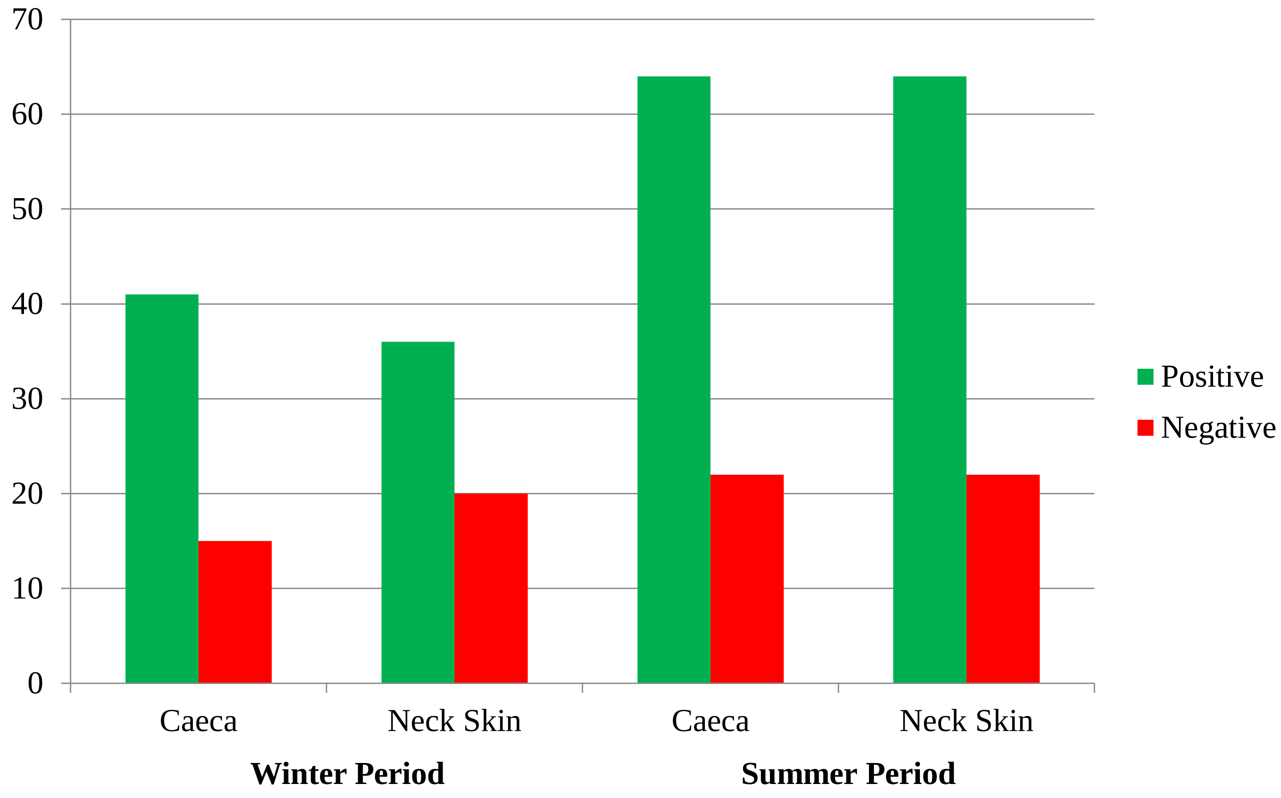
Overall prevalence of *Campylobacter*-positive batches based on caeca was 73.94% ($CI_{95\%}$ = [65.92; 80.94]). In the case of carcasses, 100 out of 142 (70.42%; $CI_{95\%}$ = [62.19; 77.78]) batches were positive for *Campylobacter* (Table 3.1). The presence of *Campylobacter* in broiler caeca was strongly correlated ($p < 0.001$) with contamination of carcasses of the same batch. In 20 batches (14.08) *Campylobacter* was detected in caeca but not on carcasses, while 17 batches (11.97) found to be *Campylobacter*-positive based on carcasses but negative based on caeca. Finally, in 20 batches (14.08%) *Campylobacter* spp. were detected neither in caeca nor in neck skin samples.

Table 3.1. Prevalence of *Campylobacter* in caeca and on carcasses (142 batches of broiler chickens, Greece, 2015).

Samples	Positive batches/ investigated batches	Prevalence (%)	± Standard deviation	95% Confidence interval
Caeca	105/142	73.94	44.05	[65.92; 80.94]
Carcasses	100/142	70.42	45.80	[62.19; 77.78]

Regarding the effect of seasonality on *Campylobacter* prevalence, out of the 112 samples collected during the winter period, 77 (68.75%) were found to be *Campylobacter*-positive, whereas 35 (31.25%) were negative. Respectively, 128 (74.42%) out of the total 172 samples collected during summer period found to be positive, while 44 (25.58%) were negative. The effect of seasonality on prevalence of *Campylobacter* in both caeca and carcasses is demonstrated in Figure 3.1.

Figure 3.1. Bar chart showing the effect of seasonality on *Campylobacter* prevalence in caecal and neck skin samples.



3.2. Quantification of *Campylobacter* on carcasses

The prevalence and load of *Campylobacter* on carcasses of positive batches of broiler chickens are reported in Table 3.2. Among the 101 enumerations performed on carcasses, 99 were countable for *Campylobacter* with a mean of positive values of 4.64 ± 0.11 $\log_{10}$ CFU/g. Contamination load of carcasses ranged from 3.22 $\log_{10}$ CFU/g to 5.96 $\log_{10}$ CFU/g and followed a normal distribution, with the statistical mean very close to the median.

Table 3.2. Numbers of *Campylobacter* on carcasses of positive batches of broiler chickens (142 batches of broiler chickens, Greece, 2015).

Positive batches/ investigated batches	Percentage of samples with positive results for enumeration purposes	Mean[a] (log_{10} CFU/g)	Median (log_{10} CFU/g)	± Standard deviation	95% Confidence interval
100/142 (70.42%)	99/100 (99%)	4.639	4.613	0.559	[4.528; 4.749]

Italic values represent prevalence of *Campylobacter* in the samples.

[a] includes only samples with positive results for enumeration purposes.

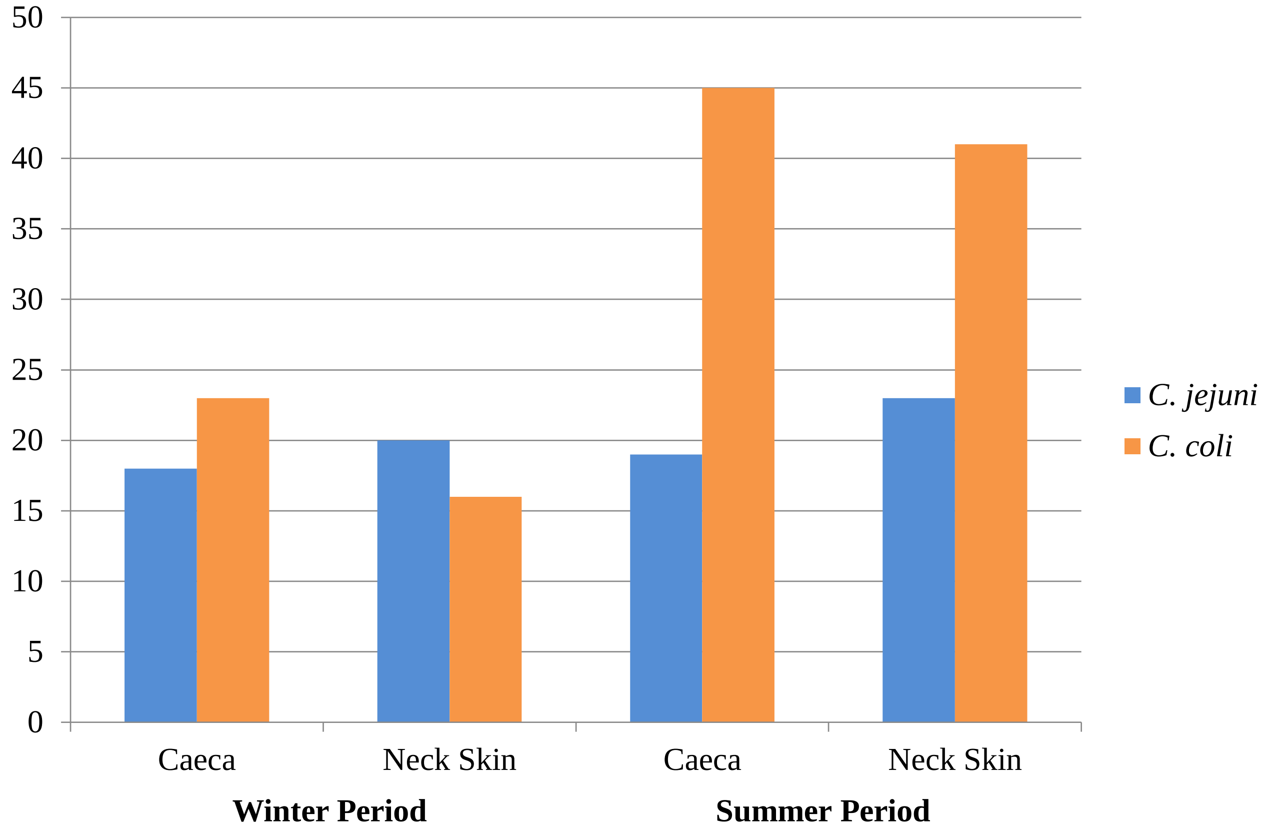
3.3. Identification of *Campylobacter*

Two different species of *Campylobacter* (*C. jejuni*, *C. coli*) were recovered from the 205 *Campylobacter*-positive caecal and neck skin samples tested using the molecular method of PCR. Considering one isolate per species per sample, both in caecal and neck skin samples *C. coli* was found to be the predominant species identified. More specifically, of the 205 samples tested, 80 (39.02%) were identified as *C. jejuni* and 125 (60.98%) as *C. coli*. Of the 105 positive caecal samples, 37 (35.24%) were identified as *C. jejuni* and 68 (64.76%) as *C. coli*, while of the 100 positive neck skin samples, 43 (43%) were identified as *C. jejuni* and 57 (57%) as *C.coli*.

Agreement concerning the *Campylobacter* species isolated between caecal and neck skin samples was observed in 63 (74.12%) of the 85 positive in both caeca and carcasses batches, while there was found to be difference between the isolated species in 22 (25.88%) slaughtered batches.

Regarding the effect of seasonality on the species of *Campylobacter*, out of the 77 *Campylobacter* positive samples collected during the winter period, 38 (49.35%) identified as *C. jejuni*, whereas 39 (50.65%) as *C. coli*. Respectively, 42 (32.81%) out of the total 128 *Campylobacter*-positive samples collected during summer period identified as *C. jejuni*, while 86 (67.19%) as *C. coli*. The effect of seasonality on speciation of *Campylobacter* in both caeca and carcasses is demonstrated in Figure 3.2.

Figure 3.2. Bar chart showing the effect of seasonality on *Campylobacter* speciation in caecal and neck skin samples.



3.4. Antimicrobial resistance

According to CLSI antimicrobial susceptibility breakpoints, 86.7% of *Campylobacter* isolates from caecal samples were classified as resistant to ciprofloxacin, 87.6% as resistant to nalidixic acid, 77.1% as resistant to tetracycline and 21% as resistant to ampicillin. On the other hand, very low resistance to erythromycin (7.6%) and streptomycin (11.4%) and no resistance to gentamicin was found. Similar results came out from antimicrobial resistance testing of neck skin samples (Table 3.3). The results of antimicrobial susceptibility in relation to the species of *Campylobacter* isolated are shown in Table 3.4.

Table 3.3. Antimicrobial susceptibility patterns of *Campylobacter* spp. identified by the disk diffusion method according to the sample tested[a].

Antimicrobial agent	Caecal Samples				Neck Skin Samples			
	No. of *Campylobacter* isolates[b]			% of Resistant isolates	No. of *Campylobacter* isolates[b]			% of Resistant isolates
	S	I	R		S	I	R	
Ciprofloxacin	14		91	86.7	8		92	92
Erythromycin	97		8	7.6	91	3	6	6
Tetracycline	22	2	81	77.1	39	6	61	53
Nalidixic acid	13		92	87.6	9	2	89	89
Gentamycin	105			0	100			0
Streptomycin	92	1	12	11.4	93		7	7
Ampicillin	71	12	22	21				

[a] The total number of *Campylobacter* isolates from caecal samples tested for antimicrobial resistance was 105 and from neck skin samples 100. Only isolates from caecal samples were tested for resistance in ampicillin.

[b] Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates identified by the disk diffusion method.

Table 3.4. Antimicrobial susceptibility patterns of *Campylobacter* isolates identified by the disk diffusion method according to the species[a].

Antimicrobial agent	***Campylobacter jejuni***				***Campylobacter coli***			
	No. of *Campylobacter* isolates[b]			**% of resistant isolates**	**No. of *Campylobacter* isolates[b]**			**% of resistant isolates**
	S	**I**	**R**		**S**	**I**	**R**	
Ciprofloxacin	7		95	93.1	15		88	85.4
Erythromycin	94		8	7.8	94	3	6	5.8
Tetracycline	29	1	72	70.6	32	1	70	68
Nalidixic acid	7	1	94	92.2	15	1	87	84.5
Gentamycin	102			0	103			0
Streptomycin	90	1	11	10.8	94	1	8	7.7
Ampicillin	36	2	9	19.1	35	10	13	22.4

[a] The total number of *Campylobacter jejuni* was 102 and *Campylobacter coli* 103. Only isolates from caecal samples were tested for resistance in ampicillin.

[b] Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates identified by the disk diffusion method.

Only three strains were susceptible to all antimicrobial agents. Additionally, 13 out of 205 (6.3%) *Campylobacter* isolates showed co-resistance to ciprofloxacin and erythromycin, whereas 24 out of 205 (11.7%) were resistant to three or more groups of antimicrobials (i.e. fluoroquinolones, macrolides, tetracyclines, aminoglycosides).

3.5. Statistical analysis

3.5.1. Selection of variables

The univariate analysis allowed the selection of 58 variables which were further considered for bivariate analysis. The variables related to similar practices of the slaughterhouses and to similar characteristics of chicken batches were eliminated (Table 3.5).

Table 3.5. Common characteristics and similar practices of poultry farms excluded for statistical analysis.

Presence of QualityAssurance system	0%
Presence of watercourses close to the farm	4%
Use of different boots for each house	6%
Farms with houses of different age	8%
Presence of house divider	8%
One disinfection per production cycle	8.5%
Presence of communal anteroom	9%
Disposal of droppings performed by special agency	91%
Presence of effluent collection system	96.5%

The bivariate analysis of explanatory variables allowed the selection of the variables most related with presence of *Campylobacter* on broiler caeca (Table 3.6). Fifteen variables significantly related to the presence of *Campylobacter* in caeca were selected for multivariate logistic regression. None of the parameters related to the slaughterhouse characteristics and slaughtering procedure e.g. type of chilling, time of batches' slaughter, temperature in evisceration room etc. was found to be statistically significant.

Table 3.6. Selected variables (threshold of 20%) entered in the multiple logistic model used to explain *Campylobacter*-positive batches (n = 142).

Variable[*]	**Variable modality**	**Size**	**% positive**	**P value (χ^2 test)**
Age of poultry house	< 15 years	18	55.56	0.082
	> 15 years	124	76.61	
Antimicrobial treatment during life	Yes	47	65.96	0.156
	No	95	77.89	
Bathroom	Presence	89	79.78	**0.049**
	Absence	53	64.15	
Bedding material	Straw	109	68.81	**0.012**
	Other[**]	33	90.91	
Closure of windows during the interruption of production cycles	Yes	60	81.67	0.084
	Maybe/ No	82	68.29	
Detergent	Presence	104	70.19	0.130
	Absence	38	84.21	
Disinfectant	Presence	60	80.00	0.179
	Absence	82	69.51	
Faucet	Presence	84	67.86	0.053
	Absence	58	82.76	

Hygiene level	Very good	66	68.18	0.180
	Poor	76	78.95	
Keeping of bedding material	In protected, clean room	82	82.93	**0.006**
	In unprotected room	60	61.67	
Number of disinfections	Only one	12	100	**0.036**
	Two	130	71.54	
Person who disinfects	Breeder/ personnel	129	77.52	**0.005**
	Special agency	13	38.46	
Responsible for thinning	Breeder/ personnel	105	70.48	0.131
	Specialists	37	83.78	
Sink	Presence	86	67.44	**0.032**
	Absence	56	83.93	
Watering System	Bell drinkers	98	79.59	**0.037**
	Nipple drinkers	44	61.36	

[*] All variables are significantly related to the presence of *Campylobacter* in caecal samples ($p < 0.20$).

[**] Sawdust, rice husk or mixed up with straw litter.

Values in bold are statistically significant ($p < 0.05$)

The multivariate logistic regression analysis produced a model weighted for seasonality, which showed 2 parameters as protective factors and one parameter as risk factor for contamination of broiler flocks (Table 3.7).

Table 3.7. Risk and protective factors for contamination of broiler flocks by *Campylobacter* spp. (n=142).

Variable	**Estimated parameters**	**Standard deviation**	**Odds Ratio**	**CI 95%**	***p* value**
Closure of windows during the interruption of production cycles					
•Yes	-0.925	0.444	0.396	0.166-0.947	0.067
•No	–	–	–		–
Person who disinfects					
•Breeder/ personnel	1.382	0.681	3.983	1.048-15.134	0.042
•Special agency	–	–	–		–
Bedding material					
•Straw	-1.772	0.660	0.170	0.047-0.619	0.007
•Other	–	–	–		–

3.5.2. Risk and protective factors

The risk of *Campylobacter* contamination was found to be decreased (OR ¼ = 0.396) when the windows were remained closed during the interruption of the production cycles. In 82 batches derived from farms that follow the closure of windows as a common practice the contamination rate was 60%, whereas it was 82% in the 60 batches derived from farms where the windows are kept open during the sanitary waiting period.

Batches derived from houses which had been disinfected by untrained farm staff seemed to have more chances to be *Campylobacter* positive (OR ¼ = 3.983) compared to those coming from farms where the disinfection was carried out be a special agency. Caeca from batches derived from farms where unskilled workers perform the disinfection had a relatively greater contamination rate (77.5%) than those coming from farms that hire skilled specialists to perform the programmed disinfections (38.4%).

The percentage of *Campylobacter* positive batches found to be lower (OR ¼ = 0.170) when straw was solely used as bedding material. Caeca derived from farms that use only straw as bedding material showed lower contamination rate (68.8%) compared to those derived from farms where sawdust or rice husk are used as bedding materials (90.9%).

3.6. flaA sequencing

From the processing of the sequences for both forward and reverse primers derived the flaA-types shown in Table 3.8. A high degree of genetic diversity was revealed, with a total of 38 different nucleotide types that correspond to 15 different peptide types. Peptide type 1 was the most predominant since it was recovered from 58 *Campylobacter* isolates. 92.6% (113 out of 122) of the isolates showed exact match with the already registered ones in the international database, whereas 7.4% (9 out of 122) displayed partial match, namely the isolates had a rate of homology though preserving different regions inside the sequences. Some isolates share the same nucleotide and peptide type in an exact match with the registered types in the international database, suggesting the occurrence of clonality. Moreover, some of these isolates share common antimicrobial profile (e.g. peptide type 1- DNA type 66).

Table 3.8. Results of antimicrobial resistance testing of *Campylobacter* isolates for ciprofloxacin (CIP), erythromycin (E), tetracycline (TE), gentamycin (CN), streptomycin (S) and ampicillin (AMP) along with fla-types derived from sequence typing.

ISOLATE	**SPECIES**[a]	**SAMPLE**[b]	**MONTH**[c]	**SLAUGHTER HOUSE**[d]	**REGION**[e]	**FARM**	**CIP**[f]	**E**[f]	**T**[f]	**NA**[f]	**CN**[f]	**S**[f]	**AMP**[f]	**fla-type DNA**	**fla-type peptide**	**MATCH**
01FLA	1	1	2	1	2	11	3	1	3	3	1	1	3	14	11	exact
02FLA	1	1	2	1	1	1	3	1	2	3	1	1	1	18	20	exact
03FLA	1	1	2	3	3	31	3	1	3	3	1	1	1	14	11	exact
04FLA	1	1	2	3	2	12	3	1	1	3	1	1	2	49	1	exact
05FLA	1	1	3	3	1	2	3	3	1	3	1	2	3	265	1	exact
06FLA	1	1	3	3	1	3	3	1	3	3	1	1	1	18	20	exact
07FLA	1	1	3	1	3	33	3	1	3	3	1	1	1	12	14	exact
08FLA	1	1	3	1	3	34	1	1	3	1	1	1	1	325	1	exact

09FLA	1	1	4	1	3	41	3	1	1	3	1	1	1	67	8	exact
10FLA	1	1	5	1	1	8	3	1	3	3	1	1	1	34	1	exact
11FLA	1	1	5	1	2	24	3	1	3	3	1	1	1	14	11	exact
12FLA	1	1	5	1	3	30	1	1	3	3	1	1	1	14	11	exact
13FLA	1	1	5	2	3	43	3	1	3	3	1	1	1	14	11	exact
14FLA	1	1	5	2	4	52	3	1	1	3	1	1	1	350	2	exact
15FLA	1	1	5	2	3	43	3	1	3	3	1	1	1	49	1	exact
16FLA	1	1	6	2	4	53	1	1	1	1	1	1	1	121	10	exact
17FLA	1	1	6	2	4	54	3	1	3	3	1	1	3	34	1	exact
18FLA	1	1	6	2	3	45	3	1	3	3	1	1	1	14	11	exact
19FLA	1	1	6	2	3	45	3	1	3	3	1	3	3	222	33	exact
20FLA	1	1	8	1	2	29	3	1	3	3	1	1	1	325	1	exact

21FLA	1	1	8	3	3	38	3	1	3	3	1	1	1	66	1	exact
22FLA	1	1	8	3	3	39	3	1	3	3	1	1	1	14	11	exact
23FLA	1	1	9	2	4	52	3	1	3	3	1	3	1	117	8	exact
24FLA	1	1	9	2	2	25	3	3	1	3	1	1	1	99	-	partial
25FLA	1	1	10	2	4	54	3	1	1	3	1	1	3	49	1	exact
26FLA	1	1	10	2	4	53	3	1	3	3	1	3	1	49	1	exact
27FLA	1	1	10	2	3	43	3	1	3	3	1	1	1	49	1	exact
28FLA	1	1	10	2	3	43	3	1	3	3	1	1	1	49	1	exact
29FLA	1	1	10	2	3	46	3	1	3	3	1	1	1	287	8	exact
30FLA	1	1	10	3	3	17	3	1	3	3	1	3	1	34	1	exact
31FLA	1	1	10	3	2	16	3	1	3	3	1	1	1	245	49	exact
32FLA	1	1	10	2	4	57	3	1	3	3	1	1	3	11	11	exact

33FLA	1	1	11	3	3	31	3	1	3	3	1	1	3	30	11	exact
34FLA	1	1	1	1	2	15	3	1	3	3	1	1	1	45	1	exact
37FLA	1	1	11	3	1	2	3	1	1	3	1	1	1	34	1	exact
38FLA	1	1	11	3	2	20	3	1	3	3	1	1	1	10	66	exact
39FLA	1	1	1	1	3	50	3	1	3	3	1	1	3	66	1	exact
40FLA	1	1	1	1	1	5	3	3	3	3	1	1	2	66	1	exact
41FLA	1	1	2	1	2	27	3	1	3	3	1	1	3	17	11	exact
42FLA	1	1	2	1	2	28	3	1	3	3	1	3	1	325	1	exact
43FLA	1	1	2	1	2	29	3	1	3	3	1	1	1	325	1	exact
44FLA	1	1	2	1	1	9	3	3	3	3	1	1	1	902	1	exact
45FLA	1	1	2	1	2	23	3	1	3	3	1	1	1	16	12	exact
46FLA	1	1	2	1	3	41	3	1	3	3	1	1	1	140	4	exact

47FLA	1	1	3	1	2	19	3	1	3	3	1	1	1	140	4	exact
48FLA	1	1	3	3	3	7	3	1	1	3	1	1	1	14	11	exact
49FLA	1	1	3	3	2	21	3	1	1	3	1	1	1	66	1	exact
50FLA	1	2	2	1	1	1	3	1	3	3	1	1	-	12	245	exact
52FLA	1	2	3	3	1	2	3	1	3	3	1	1	-	245	49	exact
54FLA	1	2	3	1	3	32	3	1	3	3	1	1	-	49	1	exact
55FLA	1	2	3	1	2	13	3	1	1	1	1	1	-	34	1	exact
56FLA	1	2	3	1	3	34	3	1	3	3	1	1	-	28	10	exact
57FLA	1	2	3	1	1	5	3	3	3	3	1	1	-	66	1	exact
58FLA	1	2	3	1	1	6	3	3	3	3	1	1	-	66	1	exact
59FLA	1	2	3	1	2	14	1	1	1	1	1	3	-	255	1	exact
60FLA	1	2	3	1	2	15	3	1	3	3	1	1	-	815	10	exact

61FLA	1	2	4	3	2	16	3	1	1	3	1	1	-	16	12	exact
62FLA	1	2	4	3	3	37	1	3	1	3	1	1	-	66	1	exact
63FLA	1	2	4	3	3	38	3	1	3	3	1	1	-	66	1	exact
64FLA	1	2	4	3	3	39	3	1	3	3	1	1	-	66	1	exact
65FLA	1	2	4	3	2	18	1	1	3	1	1	1	-	16	12	exact
66FLA	1	2	4	1	3	40	3	1	1	3	1	1	-	14	11	exact
71FLA	1	2	4	3	2	20	3	1	3	3	1	1	-	301	12	exact
72FLA	1	2	4	3	2	21	3	1	1	3	1	1	-	301	12	exact
C3	2	1	2	1	3	30	3	1	3	3	1	1	1	23	1	exact
C8	2	1	3	1	3	32	3	1	1	3	1	1	1	-	10	exact
C9	2	1	3	1	2	13	1	1	1	1	1	1	1	82	-	partial
C12	2	1	3	1	3	35	3	1	3	3	1	1	2	-	1	exact

C13	2	1	3	1	3	36	1	1	1	1	1	3	1	255	1	exact
C14	2	1	3	1	1	4	3	1	2	3	1	1	2	40	-	partial
C15	2	1	3	1	1	5	3	1	3	3	1	1	1	-	10	exact
C16	2	1	3	1	1	6	3	3	3	3	1	1	3	66	1	exact
C19	2	1	4	3	2	16	3	1	1	3	1	1	1	90	-	partial
C20	2	1	4	3	3	37	3	1	3	3	1	1	1	66	1	exact
C22	2	1	4	3	3	39	3	1	3	3	1	1	2	66	1	exact
C23	2	1	4	3	2	18	1	1	3	1	1	1	1	16	12	exact
C24	2	1	4	1	3	40	3	1	3	3	1	3	1	16	12	exact
C25	2	1	4	1	3	34	1	1	3	1	1	1	2	325	1	exact
C27	2	1	4	1	3	36	1	1	1	1	1	3	1	255	1	exact
C30	2	1	4	3	2	20	3	1	1	3	1	1	1	51	-	partial

C31	2	1	4	3	2	21	1	1	3	1	1	1	1	62	-	partial
C32	2	1	4	3	1	3	3	1	1	3	1	1	1	1117	15	exact
C33	2	1	5	1	2	22	3	1	3	3	1	1	1	66	1	exact
C39	2	1	5	2	2	25	3	1	3	3	1	1	1	66	1	exact
C43	2	1	6	2	2	26	3	1	1	3	1	1	1	902	1	exact
C44	2	1	6	2	2	26	3	1	3	3	1	1	3	325	1	exact
C45	2	1	6	2	2	26	1	1	3	1	1	1	3	902	1	exact
C47	2	1	6	2	3	44	3	1	1	3	1	1	1	66	1	exact
C48	2	1	6	2	2	26	3	1	3	3	1	3	1	902	1	exact
C52	2	1	6	2	3	46	3	1	3	3	1	1	1	66	1	exact
C53	2	1	6	2	3	46	1	1	3	1	1	1	3	325	1	exact
C55	2	1	6	2	4	55	3	1	1	3	1	1	3	28	10	exact

C56	2	1	6	2	3	47	1	1	3	1	1	3	1	16	12	exact
C58	2	1	6	2	3	48	3	1	3	3	1	1	2	320	5	exact
C60	2	1	7	1	3	49	3	1	3	3	1	1	2	51	-	partial
C61	2	1	7	1	3	41	1	1	3	1	1	1	2	325	1	exact
C62	2	1	7	1	3	50	3	1	3	3	1	1	3	51	-	partial
C63	2	1	7	1	3	51	3	1	3	3	1	1	3	301	12	exact
C71	2	1	7	1	3	33	3	1	3	3	1	1	1	28	10	exact
C72	2	1	7	1	2	24	3	1	3	3	1	1	1	28	10	exact
C73	2	1	7	1	2	22	3	3	3	3	1	1	2	255	1	exact
C74	2	1	7	1	2	27	3	1	1	3	1	1	2	-	4	exact
C75	2	1	8	1	3	30	3	1	3	3	1	1	1	100	33	exact
C79	2	1	8	1	1	9	3	1	3	3	1	1	1	30	11	exact

C82	2	1	8	2	3	46	3	1	3	3	1	1	1	-	1	exact
C83	2	1	8	2	3	46	3	1	3	3	1	1	3	-	1	exact
C87	2	1	8	3	1	10	3	1	3	3	1	1	3	66	1	exact
C91	2	1	8	2	3	48	3	1	3	3	1	1	1	28	10	exact
C96	2	1	9	2	3	47	3	3	3	3	1	1	3	66	1	exact
C97	2	1	9	3	2	12	3	3	3	3	1	1	1	902	1	exact
C99	2	1	10	2	3	44	1	1	3	1	1	1	2	325	1	exact
C100	2	1	10	2	2	26	3	1	3	3	1	1	1	28	10	exact
C102	2	2	10	2	2	26	3	1	3	3	1	2	1	-	1	exact
C103	2	1	10	2	2	26	3	1	3	3	1	1	1	-	15	exact
C107	2	1	10	2	3	46	3	1	3	3	1	1	1	320	5	exact
C111	2	1	10	2	3	46	3	1	3	3	1	1	3	-	1	exact

C112	2	1	10	2	3	46	3	1	3	3	1	1	3	-	1	exact
C116	2	1	10	2	4	56	3	1	3	3	1	1	3	-	20	exact
C123	2	1	11	1	3	39	3	1	3	3	1	1	1	301	12	exact
C126	2	1	1	1	3	49	3	1	3	3	1	3	1	43	-	partial
C127	2	1	1	3	1	1	3	1	3	3	1	1	1	28	10	exact
C147	2	1	3	3	3	42	3	1	1	3	1	1	1	66	1	exact

[a] 1: *C. jejuni*, 2: *C. coli*

[b] 1: caeca, 2: neck skin

[c] 1: January, 2: February, 3: March, 4: April, 5: May, 6: June, 7: July, 8: August, 9: September, 10: October, 11: November

[d] 1: BIOKOT, 2: HQF, 3: KELAIDITIS

[d] 1: Attica, 2: Boeotia, 3: Euboea, 4: Arta

[e] 1: sensitive, 2: intermediate, 3: resistant

3.7. Phylogenetic trees

The phylogenetic trees of *C. jejuni* and *C. coli* isolates are shown in Figure 3.3 and Figure 3.4. Whereas most of the sequences found to be scattered inside the trees, seven clusters of the *C. jejuni* phylogenetic tree (Figure 3.3.b) and three clusters of the *C. coli* tree (Figure 3.4.b) were considered significant with bootstrap values > 75%. All clusters are shown in Appendix 4.

Among the 13 *C. jejuni* isolates of the first cluster, 8 share the same DNA and peptide fla-type, while two isolates (03FLA-33FLA) originated from the same poultry farm have the same antimicrobial profile. All 8 isolates from the second cluster share the same DNA and peptide fla-type, while there are two pairs (21FLA-63FLA and 40FLA-57FLA) that originate from the same farms and have similar antimicrobial profile. Likewise, in the third cluster there are two isolates (20FLA-43FLA) originated from the same farm and share both identical DNA and peptide fla-type and antimicrobial profile. All 5 isolates from the forth cluster have the same DNA and peptide fla-type and quite similar antimicrobial resistance. In the fifth cluster there are three isolates (15FLA-27FLA-28FLA) that originate from two adjacent houses of the same farm and share the same DNA and peptide fla-type and antimicrobial profile. All seven clusters include reference sequences isolated from different regions (mainly USA, Europe, Tanzania and Australia), however no clear connection between them and the isolates of the current study could be made.

In the first cluster of *C. coli* phylogenetic tree, 3 of 8 isolates (C60-C62-C63) originate from neighbor farms located in the same region and exhibit similar antimicrobial resistance patterns. The second cluster includes only 4 isolates (C82-C83-C111-C112), all of which originate from the same poultry farm and share similar antimicrobial profiles. Almost all reference sequences in the first and third cluster originate from USA with the exception of one sequence originated from Japan.

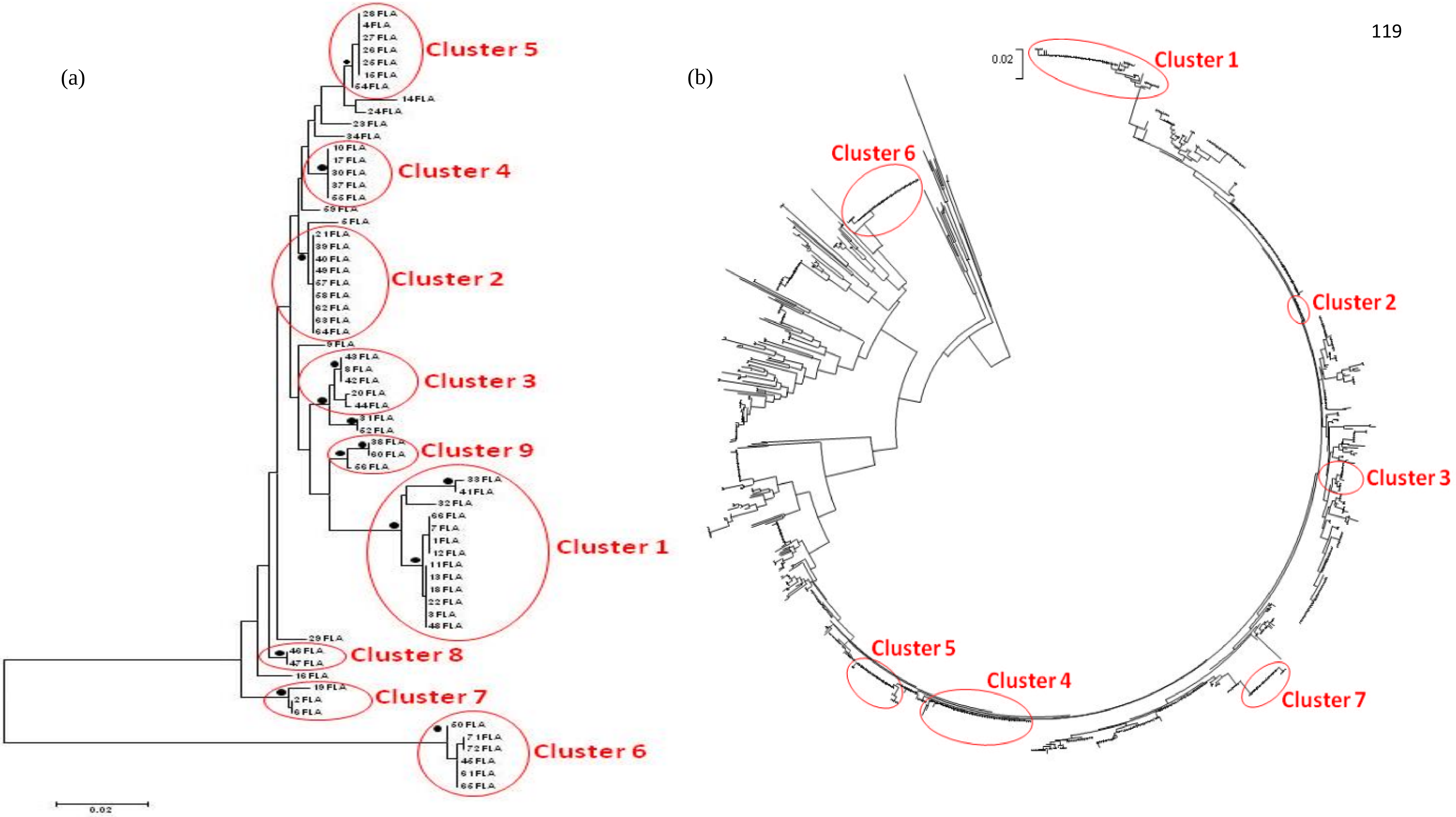


Figure 3.3. *Campylobacter jejuni* phylogenetic trees. Bullets represent clades which had bootstrap values > 75% of permuted trees. (**a**) The analysis involved 64 sequences. Most of the sequences were organized in nine significant clusters supported with high bootstrap values. (**b**) The optimal tree with the sum of branch length = 4.34019783 is shown. The analysis involved 64 sequences (isolates of our study) plus 960 *C. jejuni* flaA reference sequences (RS) downloaded from the GenBank database. Seven significant clusters containing our sequences along with RS were considered significant with high bootstrap values.

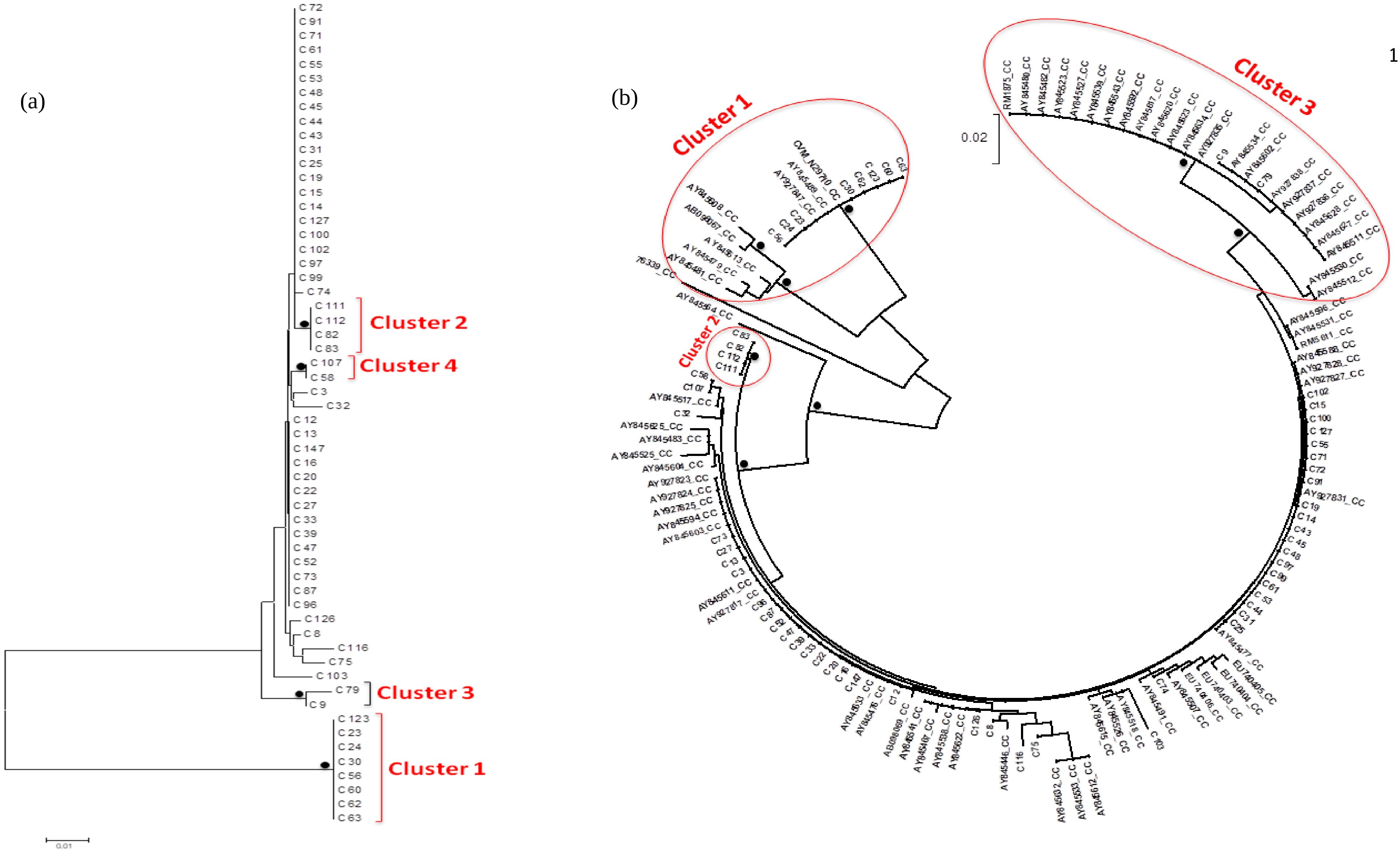


Figure 3.4. *Campylobacter coli* phylogenetic trees. Bullets represent clades which had bootstrap values > 75% of permuted trees. (**a**) The optimal tree with the sum of branch length=0.21165541 is shown. The analysis involved 58 sequences. Most of the sequences were dispersed within the tree whereas four significant sequence clusters were noticed. (**b**) The optimal tree with the sum of branch length=0.54 is shown. The analysis involved 58 sequences (isolates of our study) plus 74 *C. coli* flaA reference sequences downloaded from the GenBank database. Most of the sequences were dispersed within the tree whereas three significant sequence clusters were noticed.

CHAPTER 4

DISCUSSION

4.1. Prevalence and species identification

The current cross-sectional study carried out in Greece generated representative data on broiler caeca (73.94%) and carcass skin samples (70.42%), indicating the high prevalence of *Campylobacter* at the national level. These results are in agreement with several studies both for caeca and carcasses (Allen et al., 2008a; Hue et al., 2010, 2011; Lawes et al., 2012) and the EFSA scientific report for *Campylobacter*-positive batches (71.2%) and *Campylobacter*-contaminated carcasses (75.8%) in the EU member states (EFSA, 2010a).

A significant proportion of carcasses (12%) were positive for *Campylobacter* contamination, while caeca of the same batches were found to be *Campylobacter* negative. Rosenquist et al. (2006) and Figueroa et al. (2009) demonstrated that during the evisceration step, cross-contamination might be possible. Rupture of viscera from infected chickens may release high numbers of *Campylobacter* isolates that contaminate the surfaces of the slaughterhouse, explaining these results. A cross-contamination may also occur between batches from different flocks during the slaughterhouse process (Rivoal et al., 1999; Johannessen et al., 2007), and the level of contamination of noninfected chicken batches can be influenced by several factors such as the *Campylobacter* status of previously slaughtered batches, the amount of cross-contamination taking place, and the position of carcasses in subsequent negative batches (Hue et al., 2011). Therefore, the use of a logistic slaughtering schedule could help preserve *Campylobacter*-free batches, considering that the later in the day the batch is slaughtered, the higher the probability that it will be contaminated (Hue et al., 2010).

In recent years, quantitative risk assessment modeling is supported by a growing demand for quantitative data to describe the occurrence and dynamics of *Campylobacter* in the broiler meat chain (Uyttendaele et al., 2006; Nauta et al., 2009; Prachantasena et al., 2016). Researchers previously concluded that for enumeration of thermotolerant *Campylobacter* in chicken meat, direct spread plating on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) is an acceptable protocol and a reliable alternative to the most probable number method (Scherer et al., 2006; Rosenquist et al., 2007). Currently, mCCDA is the recommended medium by the ISO for enumeration of thermophilic Campylobacter in foods (ISO, 2006b), although alternative enrichment and plating combinations have been evaluated (Habib et al., 2011). In our study, the average concentration of *Campylobacter* recovered from carcasses was 4.639 ± 0.11 $\log_{10}$ CFU/g, while the normal distribution of positive values leads to an average close to the median, clearly separating values into two halves. The result of our study has shown a much higher colonization rate of *Campylobacter* than the respective results of previously published data (Scherer et al., 2006; Hue et al., 2011). This finding could be attributed to a high degree of caecal contamination, to visceral rupture and subsequent release of large numbers of *Campylobacter* isolates on the carcass skin, or even to short processing times or inadequate slaughterhouse hygiene and cleaning conditions, which possibly promote the survival and spread of *Campylobacter* spp. during the slaughtering process.

Of the 206 identifications performed, two different species of *Campylobacter* were identified (*C. jejuni* and *C. coli*) and *C. coli* was found to be the predominant species. This result is in line with a previous study from Greece (Marinou et al., 2012), but contradictory to other studies showing *C. jejuni* as being much more frequently associated with poultry meat than *C. coli* (Pepe et al., 2009; Hue et al., 2011). However, according to the results of a baseline survey conducted by EFSA in 2008, seven EU member states reported *C. coli* as the predominant species isolated from caeca and carcasses (EFSA, 2010a). Moreover, the same survey showed that in southern member states, *C. coli* was more abundant, whereas *C. jejuni* was the only species identified in northern member states. Climatic conditions, environmental reservoirs, housing systems of broiler chickens, and age of slaughter differ significantly between northern and southern Europe and could partially explain the observed variance of species distribution (EFSA, 2010b).

The contamination of slaughtered batches by these species fluctuated according to the sample, with *C. jejuni* being more frequently identified on carcasses than in caeca (43% and 35.24%, respectively). Therefore, it is possible that *C. jejuni* is more resistant than *C. coli* to stress encountered during slaughtering (Hue et al., 2011). It has been shown that *C. jejuni* adheres more to inert surfaces than *C. coli* (Sulaeman et al., 2010), which may allow *C. jejuni* to have better biofilm formation capacity, especially under stressful environmental conditions (Reuter et al., 2010; Teh et al., 2014). Swelling of the skin during slaughter and processing allows the survival of *Campylobacter* on poultry carcasses (Chantarapanont et al., 2003).

Moreover, since only one well-isolated colony from a pure culture underwent PCR for species identification, any contamination with both *Campylobacter* species could not be detected. The simultaneous presence of the two species both in caeca and on carcass skin is common (Hue et al., 2011) and could explain the observed disagreement in 22 batches between the identified species in caecal content and neck skin samples. Biofilm formation might also be attributed to a short processing time or inadequate cleaning procedures in the slaughterhouse and should be further investigated.

4.2. Antimicrobial resistance

In order to estimate the antimicrobial resistance of *Campylobacter*, the disk diffusion method was used. Although the agar dilution method is considered the standard antimicrobial susceptibility testing method for thermophilic *Campylobacter* species (McDermott et al., 2004), it is a labor-intensive, time-consuming and costly test (McDermott et al., 2005). On the other hand, the disk diffusion method is simple, inexpensive and can provide reproducible results if it is conducted carefully with appropriate standardization and quality controls (Gaudreau et al., 2008; Potz et al., 2004). The latter method has been standardized by the CLSI, but according to those standards, it should be used only as a screening method for resistance to erythromycin and ciprofloxacin; a disk diffusion zone of 6 mm indicates resistance, while any inhibition zone would require an MIC determination of susceptibility (CLSI M45). Several comparisons of agreement between the disk diffusion method and other susceptibility testing methods for *Campylobacter* have been conducted over the years (Gaudreau et al., 2008; Lehtopolku et al., 2012; Luangtongkum et al., 2007; McGill et al., 2009; van der Beek et al., 2010), some of which have concluded that disk diffusion method could be used as reliable alternative method for the testing of susceptibility of *Campylobacter* spp. to ciprofloxacin and erythromycin (Gaudreau et al., 2008; Luangtongkum et al., 2007). On the other hand, the results of other studies are different and indicate the unreliability of this method and the need of further standardization (Lehtopolku et al., 2012; van der Beek et al., 2010).

The selection of antimicrobials was done according to the published data concerning the widely used antimicrobial agents both in poultry production and in the treatment of human campylobacteriosis, and followed the panel of antimicrobials from the EU protocol for harmonized monitoring of antimicrobial resistance in human *Salmonella* and *Campylobacter* isolates (ECDC, 2016). *Campylobacter* isolates from each positive sample were tested for resistance to ciprofloxacin, nalidixic acid, erythromycin, streptomycin, gentamicin and tetracycline, as in the recent EU summary report (EFSA and ECDC, 2016), while ampicillin was also included in the susceptibility testing of isolates from caecal content.

The results of our study regarding the antimicrobial resistance are consistent with other studies (EFSA and ECDC, 2020; Fraqueza et al., 2014). More specifically, high resistance to ciprofloxacin and nalidixic acid was observed, on the order of 90%. Similar results were submitted on the view of the obligatory monitoring and report of antimicrobial resistance by Greece in 2014, while the overall resistance to quinolones at EU level was slightly lower (EFSA and ECDC, 2016). Spontaneous mutation is a major mechanism for acquisition of fluoroquinolone-resistance. In an environment where resistance confers a selective advantage, clonal reproduction among resistant lineages will lead to local expansion (Sproston et al., 2018). Resistance to fluoroquinolones in *Campylobacter* spp. was firstly reported in the late 80s and since then, there is a continuous increase of resistance to fluoroquinolones (Sproston et al., 2018). It has been observed that resistance appeared simultaneously with the introduction of these agents in animal production and veterinary medicine (Engberg et al., 2004; Silva et al., 2011). Nowadays, worldwide fluoroquinolone resistance is common (Yang et al., 2019). Since campylobacteriosis is considered to be a zoonosis, the presence of resistant strains in the food chain also has an influence on human infections (Silva et al., 2011). Moreover, it has been noted that the proportion of ciprofloxacin resistant members of the genus *Campylobacter* in poultry meat is often strikingly similar to the proportion observed in human clinical cases (EFSA and ECDC, 2016). However, the transmission of fluoroquinolone resistant bacteria from agricultural animals to humans is difficult to prove and a recent global report on surveillance of antimicrobial resistance emphasized the need to collect more data of the effects of antimicrobial resistance in foodborne bacteria and human health (Thanner et al., 2016; WHO, 2014). Some studies that tried to establish a direct and consistent link between agriculture and clinical isolates found either no relation (Silva et al., 2016) or some correlation that was neither direct nor concrete and remains controversial (Wieczorek et al., 2018). Besides their excessive use in agriculture, the use of fluoroquinolones for infections other than gastroenteritis, as well as "self-medication" are often causes of the observed resistance in developing countries (Coker et al., 2002). Therefore, traveling to developing countries has been implied to be a risk factor for gaining an infection caused by a resistant *Campylobacter* strain. In the developed world, one reason behind the fluoroquinolone resistance might also be their inadequate clinical use in the treatment of human infections. Patients treated with fluoroquinolones were later found to carry bacteria resistant to these antimicrobial agents (Bacon et al., 2000).

A low percentage (6.8%) of *Campylobacter* among the strains recovered from caeca and neck skin samples was resistant to erythromycin. This result agrees with the respective ones of EU survey (EFSA and ECDC, 2020). However, the majority of these isolates revealed multi-antimicrobial resistance properties, a finding demonstrated in other studies as well (Wieczorek et al., 2013). Target modifications caused by a point mutation in the 23S ribosomal RNA gene (23S rRNA) and post-translational changes in the ribosomal proteins L4 and L22 and active efflux are the main causes of macrolide resistance (Gibreel and Taylor, 2006). Resistance to erythromycin, as a rule, corresponds to cross resistance to other macrolides (for example, azithromycin and clarithromycin), as well as to related drugs of the group of lincosamides (in particular, to clindamycin) and streptogramins (Efimochkina et al., 2020). Resistance of *Campylobacter* spp. to macrolides has remained in low and stable levels for a long time. However, there is also evidence from some parts of the world that resistance rates to erythromycin and other macrolides in *Campylobacter* species are slowly increasing (Bae et al., 2005; Vlieghe et al., 2008). Since fluoroquinolone resistance is common, the macrolides have become important in the treatment of campylobacteriosis, resulting to the development of macrolide resistance (EFSA and ECDC, 2016). Use of macrolides in animal production as therapeutic or growth-promoting agents has been considered to be a significant factor in the selection of erythromycin-resistant *Campylobacter* strains (Ladely et al., 2007). However, acquisition of erythromycin resistance in *Campylobacter* species is a stepwise process and requires prolonged exposure in contrast to the rapidly evolving fluoroquinolone resistance (Lin et al., 2007). Moreover, Hao et al. (2009) have shown that erythromycin-resistant *Campylobacter* strains display a fitness disadvantage when compared with susceptible *Campylobacter* strains, which may lead to a low frequency of macrolide resistance in clinical isolates.

Regarding the remaining antimicrobial agents, resistance of *Campylobacter* isolates to tetracycline was found to be remarkable high, especially in strains derived from caecal content. Similarly high resistance rates were observed in the recent report of EFSA and ECDC (2020). Tetracyclines can be used in the treatment of campylobacteriosis, except in children under nine years of age (Moore et al., 2005). However, tetracycline resistance has emerged also among *Campylobacter* species (EFSA and ECDC, 2016). In *Campylobacter* spp. the most common tetracycline resistance mechanism is a plasmid-mediated ribosomal protecting protein Tet(O) encoded by the *tet*(O) gene (Gibreel et al., 2004). Ribosomal protecting proteins and efflux pumps can also work synergistically and cause high-level tetracycline resistance (Gibreel et al., 2007). No resistance to gentamycin and low resistance to streptomycin was found. Quite similar results have been observed in most EU members states (EFSA and ECDC, 2020). Guyard-Nicodème et al. (2015) tested the susceptibility of *C. jejuni* strains derived from broiler meat products collected in retail outlets and found similar results with our study for tetracycline and gentamycin. The main mechanism of aminoglycoside resistance in Campylobacter spp. is via aminoglycoside modifying enzymes, which are usually plasmid-borne (Iovine, 2013). Finally, a slightly high level of resistance to ampicillin was found in *Campylobacter* strains isolated from caeca. Resistance level in ampicillin tends to be high among *Campylobacter* and clinical isolates (Gallay et al., 2007). Three main mechanisms mediate β-lactam resistance in *Campylobacter*: (1) enzymatic inactivation by chromosomally-encoded β-lactamases, (2) reduced uptake due to alterations in outer membrane porins and (3) efflux (Iovine, 2013).

Only 3 *Campylobacter* isolates showed complete susceptibility to all antimicrobial agents tested. Similar results were submitted by Greece in frames of the EU survey (EFSA and ECDC, 2016). On the other hand, 7.6% of *C. jejuni* and 5% of *C. coli* were co-resistant to ciprofloxacin and erythromycin. This fact is worrying since these antimicrobial classes constitute the cornerstone in treatment of severe human campylobacteriosis. Moreover, 13.3% of *C. jejuni* and 10% of *C. coli* strains showed multidrug resistance (MDR), defined as resistance or no-susceptibility to at least three antimicrobial classes - fluoroquinolones, macrolides, tetracyclines or aminoglycosides (Magiorakos et al., 2012). The increase of multidrug resistant *Campylobacter* strains has increased (Chen et al., 2010; Qin et al., 2011) posing a serious risk of treatment failures, since there are very few treatment alternatives of campylobacteriosis caused by multidrug resistant strains (Yang et al., 2019). This increase may reflect the overuse of different antimicrobial agents in veterinary medicine and, especially, in poultry production (Silva et al., 2011; Thanner et al., 2016), as well as in human medicine especially when administered without medical prescription (Coker et al., 2002).

4.3. Risk and protective factors

In line with previous studies, the current results suggest that *Campylobacter* infection is a multifactorial problem and is caused by several potential sources. The closure of windows between production cycles seemed to decrease the chance of the poultry batch being infected by *Campylobacter*. This result could be probably attributed to prevention of the access of flies or other vectors into the house (Hald et al., 2008; Choo et al., 2011). Royden et al. (2016) demonstrated that flies may play a role in the transmission of *Campylobacter* to broilers, and due to the large number of flies around broiler house ventilation inlets, the risk of transmission is high. It seems that keeping windows firmly closed during the downtime prevents the introduction of *Campylobacter* into the farm by not letting the potential vectors enter the farm.

The results of our study suggest that disinfection of the house plays an important role on the *Campylobacter* status of the poultry batch as when it was performed by unskilled personnel the chances for the batch to be positive were substantially higher compared with when it was undertaken by a special agency. These findings suggest that insufficient disinfection of the farm leads to increased contamination rates. It is clear that effective cleaning and disinfection of broiler houses and their surroundings can decrease the risk of *Campylobacter* transmission between subsequent flocks (Battersby et al., 2017). Overall, the absence of sanitizing procedures can be considered an important risk factor for *Campylobacter* spp. contamination (Bouwknegt et al., 2004; McDowell et al., 2008; Newell et al., 2011), and even with the use of most efficient biosecurity programs, this pathogen may enter the facilities and colonize the birds (van de Giessen et al., 1998). Several disinfection programs have been tested and evaluated for their effects on the environmental *Campylobacter* contamination (Battersby et al., 2017; Castro Burbarelli et al., 2017) and it is suggested that these cleaning practices should be routinely tested on all broiler farms to determine their effectiveness in reducing exposure of poultry and humans to the pathogen.

The material used as bedding material seemed to affect the contamination status of the flock. In particular, the sole use of straw as a bedding material reduced *Campylobacter* contamination, compared with the use of other materials, such as sawdust, rice husk, or mixtures of these bedding materials. Different bedding materials (straw and wood shavings) have been compared on how they affect the total aerobic bacterial counts and it was found that less contamination was detected in wood shavings than straw (Fries et al., 2005). Wood can exhibit strong antibacterial characteristics due to a combination of hygroscopic properties of wood and the effects of wood extracts (Milling et al., 2005). Other studies have found that such essential oils have antioxidant activity by scavenging free radicals and have shown antimicrobial activity against a range of foodborne organisms (Zeng et al., 2012), including *Campylobacter* (Kurekci et al., 2013). However, the findings of our study suggest that straw litter protects from *Campylobacter* contamination and maybe this is due to the fact that wheat straw contains less moisture than rice husk and wood shavings (Monira et al., 2003). The higher water content in the bedding material may protect *Campylobacter* from the effects of desiccation, thus enhancing its survival (Smith et al., 2016). Although, environmental challenges linked to the disposal of bedding material impose the litter reuse in some countries, a practice that may have an impact on key food safety pathogens such as *Campylobacter*, a survey conducted by Chinivasagam et al. (2016) found no direct influence between reuse of litter and either the timing of emergence or the levels of *Campylobacter* concentration across sequential farming cycles.

4.4. Phylogenetic analysis

Phylogenetic analysis of our strains using reference sequences highlighted seven clusters of *C. jejuni* isolates and three clusters of *C. coli* isolates in our study population. Almost all significant clusters included both sequences of the current cross sectional study and reference sequences. No clear connection between our *C. jejuni* isolates and the reference sequences was found, even though most of the reference sequences originated from USA. However, almost all reference sequences in the first and third cluster of *C. coli* originate from two surveys conducted in USA. The first one dealt with isolates from retail chicken products and humans with gastroenteritis in central Michigan (Fitch et al., 2005), while the second one with isolates from European CampyNet collection and National Antimicrobial Resistance Monitoring System, derived mostly from humans, chicken, cattle and swine (Meinersmann et al., 2005). No safe conclusion could be drawn though.

Some of the strains grouped in the same cluster and shared similar antimicrobial profile and fla-types were isolated from the same farms in different sampling time or from adjacent houses of the same farm. This finding indicates persistence of the infective strains in the house during turnaround time and further contamination of subsequent batches and/or infection of equipment and working clothes leading to the spread of these strains from one house to another. Indeed, *Campylobacter* can be carried via boots and clothes of farm personnel and shared equipment between broiler houses of the same farm (Ellis-Iversen et al., 2012; Newell et al., 2011; Sibanda et al., 2018). Moreover, the presence of colonized flocks has been found to be linked to the turnaround time in a broiler house. Periods of over 14 days can decrease the possibility of residual bacterial contamination (Newell et al., 2011), while the rapid flock turnover contributes to *Campylobacter* carry over with increased risk being reported if houses are restocked within 9 days of depopulation (Battersby et al., 2016). In any case, the biosecurity and hygiene level should be maintained optimal during the empty time, as it is well-known that an external reservoir can host multiple *Campylobacter* strains, during the empty period, which will allow colonization of the new flock (Ellis-Iversen et al., 2012).

The presence of isolates with same fla-types and shared antimicrobial resistance patterns collected from different farms within a close distance in the same region could be attributed to vehicles that visit different farms in the same day without applying adequate disinfection, such as feed delivery trucks, vehicles for collection of litter and dead birds or transport from the hatchery and to processing plants, which act as mechanical vectors and allow the transmission of these strains from each farm to another. Farm personnel and equipment (e.g., feed trucks) can carry *Campylobacter* between broiler houses and onto subsequent or neighboring farms (Newell et al., 2011). Although feed is not seen as a high-risk *Campylobacter* contaminant within the broiler house, since the low water activity of the dry feed does not permit *Campylobacter* survival (Sibanda et al., 2018), it can be a vehicle for horizontal transmission into the broiler house (Silva et al., 2011). Hald et al. (2000) showed that the incidence of *Campylobacter* was lower in farms that feed home grown wheat compared to farms that are depended of external supplies. Moreover, a Danish study showed that the absence of infected neighbors in 2 km radius of susceptible farm, in the same month, has a significant protective effect in comparison with presence of infected neighbors in the same distance and time (Chowdhury et al., 2012). Likewise, Jonsson et al. (2012) found that livestock and broiler farms with flocks positive for *Campylobacter* spp. within a few kilometers distance constitute significant risks for colonization in broiler flocks. Furthermore, live bird crates being contaminated with *Campylobacter* from previous (or other) flocks are reintroduced on the farm during catching, and quite often these crates undergo inadequate washing at the slaughterhouse (Newell et al., 2011). Research has shown that *Campylobacter* can survive on crates post-sanitization (Allen et al., 2008b; Hansson et al., 2005). Crates can carry identical genotypes of microorganisms which originate from broiler flock and abattoirs, which suggests that transport crates are responsible for contamination during transport to slaughter or they could contribute to the *Campylobacter* colonization of broiler houses (Hastings et al., 2011).

4.5. Conclusion

In conclusion, the cross sectional study carried out in Greece produced valuable results concerning the prevalence, antimicrobial resistance and the molecular epidemiology of *Campylobacter* spp. in poultry production countrywide. A high prevalence of *Campylobacter* spp. in broiler flocks and on carcasses was found, along with a remarkably high load on broiler chicken carcasses, while the predominance of *C. coli* was noted both in caeca and on carcasses. High resistance to fluoroquinolones and tetracycline and low resistance to macrolides and aminoglycosides was found. The analysis of potential risk factors proposed that closure of windows during the downtime and the use of straw as the bedding material act as protective factors, whereas disinfection of the poultry house performed by unskilled personnel acts as a risk factor for contamination of the flock with *Campylobacter*. A high genetic diversity was found, while some specific flaA types found to share similar antimicrobial resistance patterns. Phylogenetic analysis of the isolates revealed seven clusters of *C. jejuni* and three clusters of *C. coli*. Some isolates clustered together were originated from the same or adjacent farms, indicating transmission via personnel or shared equipment. No clear connection between the reference sequences used and the isolates of the current study was found. These results help in understanding the molecular epidemiology and susceptibility patterns of *Campylobacter* spp. derived from poultry in Greece.

4.6. Future Work

The results of the cross sectional study conducted in Greece were valuable, since the prevalence, antimicrobial resistance and molecular epidemiology of *Campylobacter* spp. in poultry production countrywide had never been investigated before. Future studies that will involve larger sample size derived from all over Greece, including northern and southern regions, should be conducted in order to produce more representative results. Moreover, comprehensive data from every level of the food chain, including farming, slaughtering, retailing, handling in the kitchen and consumption patterns, should be collected and be used to build a risk assesment model. Possible scenarios for preventive bio-security measures and interventions at farm level, during transportation and at slaughterhouses should also be examined. Finally, the study of antimicrobial resistence genes of the *Campylobacter* isolates could provide useful information concering the multi-drug resistance and help explain the similar antimicrobial resistence patterns found to be shared among *Cammpylobacter* isolates with same flaA types.

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APPENDICES

APPENDIX 1: Referral for microbiological analysis (Παραπεμπτικό εργαστηριακής εξέτασης για *Campylobacter* spp.)

APPENDIX 2: Record sheet of antimicrobial resistance testing results (Φύλλο καταγραφής αποτελεσμάτων ποιοτικού ελέγχου μεθόδου προσδιορισμού μικροβιακής αντοχής “CampyRisk”)

APPENDIX 3: Original questionnaire (Ερωτηματολόγιο “CampyRisk”)

APPENDIX 4: Clusters of *C. jejuni* and *C. coli* phylogenetic trees

APPENDIX 1

Παραπεμπτικό εργαστηριακής εξέτασης για *Campylobacter* spp.

Κλινική Παθολογίας Πτηνών **Τμήμα Κτηνιατρικής Καρδίτσας** **Πανεπιστήμιο Θεσσαλίας** University of Thessaly 	**Προς: Κτηνιατρικό Εργαστήριο Χαλκίδας** **Πει Δοκού, Χαλκίδα** **Τηλ.: 22210 42521** **Αρ. πρωτ/ Ημερ. Παραλαβής Εργαστηρίου:**

Αποστέλλουμε δείγμα τυφλών/λαιμών από ορνίθια κρεοπαραγωγής (*Gallus-gallus*) **για εξέταση στα πλαίσια του ερευνητικού προγράμματος CampyRisk.**

Κωδικός αριθμός δείγματος	Κωδ.αρ. εκμετ.	Αρ. θαλάμου	Ημερ. Εκκόλαψης	Ημερ.σφαγής
Σφαγείο				
Είδος δείγματος Αριθμός δειγμάτων	Τυφλά		Δέρμα λαιμού	
Ημερομηνία/ Ώρα δειγματοληψίας				
Κατηγορία σμήνους:	συμβατικό ☐ βιολογικό ☐ ελεύθερης βοσκής ☐ περιορισμένης βοσκής ☐ απεριόριστης βοσκής ☐			
Χορήγηση αντιβιοτικών τις δύο τελευταίες εβδομάδες (ναι/όχι)	Ναι Όχι			
Χορηγούμενη ουσία-Σκεύασμα				
Διάρκεια χορήγησης της θεραπείας				
Αιτία χορήγησης της φαρμακευτικής αγωγής				
Προηγούμενες επίσημες δειγματοληψίες στο σμήνος για *Salmonella* spp.	Ναι Όχι		Ημερ/νία τελευταίας δειγματοληψίας:	
Άλλες παρατηρήσεις				
Όνομα υπευθύνου δειγματοληψίας	**Σφραγίδα-Υπογραφή**			

APPENDIX 2

ΦΥΛΛΟ ΚΑΤΑΓΡΑΦΗΣ ΑΠΟΤΕΛΕΣΜΑΤΩΝ ΠΟΙΟΤΙΚΟΥ ΕΛΕΓΧΟΥ ΜΕΘΟΔΟΥ ΠΡΟΣΔΙΟΡΙΣΜΟΥ ΜΙΚΡΟΒΙΑΚΗΣ ΑΝΤΟΧΗΣ “CampyRisk”

Οίκος και αριθμός παρτίδας *C. jejuni* ATCC® 33560	
Εξεταζόμενα στελέχη	
Ημερομηνία παρασκευής Mueller Hinton Agar	
Ημερομηνία παρασκευής πρότυπου διαλύματος 0,5 McFarland	
Παρατηρήσεις	

	Αντιμικροβιακός παράγοντας			**Αποδεκτά όρια *C. jejuni* ATCC® 33560**	**Αποτελέσματα *C. jejuni* ATCC® 33560**
1	Ciprofloxacin	**CIP**	5μg	32-45	
2	Erythromycin	**E**	15μg	26-38	
3	Gentamicin	**CN**	10μg		
4	Nalidixic acid	**NA**	30μg	25-34	
5	Streptomycin	**S**	15μg		
6	Tetracycline	**TE**	30μg		
7	Ampicillin	**AMP**	30μg		

Ημερομηνία έναρξης ανάλυσης:

Ημερομηνία λήξης ανάλυσης:

Ονοματεπώνυμο & υπογραφή αναλυτή:

ΕΡΩΤΗΜΑΤΟΛΟΓΙΟ CampyRisk
Πτηνοτροφικές Μονάδες

I. ΓΕΝΙΚΕΣ ΠΛΗΡΟΦΟΡΙΕΣ	
1. Ημερομηνία επίσκεψης	……/……/…………...
2. Όνομα αξιολογητή	Όνομα: ……………… Επίθετο: ……………..
3. Διεύθυνση εκτροφής	…………………………
4. Κωδικός & όνομα εκτροφής	Κωδικός εκτροφής: …………….. Όνομα εκτροφής: ………………
5. Όνομα συνεντευξιαζόμενου	Όνομα: ……………… Επίθετο: ……………..
6. Σύστημα ποιοτικού ελέγχου εκτροφής	☐ Ναι ☐ Όχι
7. Όνομα σφαγείου	....
8. Όνομα εκκολαπτηρίου	....
9. Ημερομηνία τοποθέτησης νεοσσών	......../........./...................
10. Αριθμός νεοσσών κατά την τοποθέτηση ανά θάλαμο	.... πτηνά
11. Γενότυπος	☐ Ross ☐ Hubbard ☐ Cobb ☐ Hybro
12. Αριθμός πτηνών ανά θάλαμο τη στιγμή της επίσκεψης	.... πτηνά
13. Μέσο βάρος πτηνών τη στιγμή της επίσκεψης	.... kg
14. Υπάρχουν διαχωριστικά στο θάλαμο; *(αν όχι, προχωρήστε στην ερώτηση 16)*	☐ Ναι ☐ Όχι
15. Αν ναι, σε πόσα διαμερίσματα χωρίζουν το θάλαμο;	.... διαμερίσματα

16. Τύπος διαχωριστικού	☐ Σύρμα ☐ Τοίχος
17. Μέγιστη χωρητικότητα θαλάμου	....
18. Τύπος συστήματος εκτροφής	☐ Εντατική ☐ Ελευθέρας βοσκής ☐ Βιολογική ☐ Άλλο
II. ΠΕΡΙΓΡΑΦΗ ΤΗΣ ΜΟΝΑΔΑΣ	
1. Αριθμός θαλάμων στην εκτροφή	.... θάλαμοι
2. Μέγιστη χωρητικότητα κάθε θαλάμου	Θάλαμος 1 πτηνά Θάλαμος 2 πτηνά Θάλαμος 3 πτηνά
3. Υπάρχουν θάλαμοι διαφορετικής ηλικίας κατασκευής στην εκμετάλλευση; *(αν όχι, προχωρήστε στην ερώτηση 6)*	☐ Ναι ☐ Όχι
4. Πριν πόσα χρόνια κατασκευάστηκε ο παλαιότερος θάλαμος;	☐ ≤ 1 έτος ☐ 2-5 έτη ☐ 6-10 έτη ☐ 10-15 έτη ☐ ≥ 15 έτη
5. Πριν πόσα χρόνια κατασκευάστηκε ο νεότερος θάλαμος;	☐ ≤ 1 έτος ☐ 2-5 έτη ☐ 6-10 έτη ☐ 10-15 έτη ☐ ≥ 15 έτη
6. Μέσος αριθμός παραγωγικών κύκλων ανά θάλαμο ανά έτος	☐ 4 παραγωγικοί κύκλοι ☐ 5 παραγωγικοί κύκλοι ☐ 6 παραγωγικοί κύκλοι ☐ 7 παραγωγικοί κύκλοι
7. Μέσος αριθμός πτηνών που σφάζονται ετησίως	.... πτηνά
8. Πυκνότητα εκτροφής	.... πτηνά/μ^2

9. Εκτροφή (ή παρουσία) άλλων ειδών πτηνών στην εκμετάλλευση	☐ Ναι ☐ Όχι
10. Αν ναι, διευκρινίστε:	☐ Όρνιθες ☐ Πάπιες ☐ Χήνες ☐ Γαλοπούλες ☐ Ορτύκια ☐ Άλλο, διευκρινίστε: ………….....
11. Εκτροφή (ή παρουσία) άλλων ζώων στην εκμετάλλευση	☐ Ναι ☐ Όχι
12. Αν ναι, διευκρινίστε:	☐ Αγελάδες γαλακτοπαραγωγής ☐ Μόσχοι ☐ Χοίροι ☐ Πρόβατα ☐ Αίγες ☐ Άλογα ☐ Κόνικλοι ☐ Σκύλοι ☐ Γάτες ☐ Άλλο, διευκρινίστε: …...………. ☐
13. Υπάρχουν άλλα ζώα δίπλα ή κοντά στον χώρο της εκμετάλλευσης;	☐ Ναι ☐ Όχι
14. Αν ναι, διευκρινίστε:	☐ Βοοειδή ☐ Χόιροι ☐ Πρόβατα ☐ Αίγες ☐ Άλογα ☐ Όρνιθες ωοπαραγωγής ☐ Ορνίθια κρεοπαραγωγής ☐ Οικόσιτες πάπιες ☐ Οικόσιτες χήνες ☐ Γαλοπούλες ☐ Σκύλοι ☐ Γάτες ☐ Άλλο, διευκρινίστε: …...……….

15. Εφόσον υπάρχουν γάτες στην εκτροφή, έχουν πρόσβαση στους θαλάμους της εκτροφής:

- ☐ Πάντα
- ☐ Όταν είναι κενοί, αλλά μόνο πριν τον καθαρισμό και την απολύμανση
- ☐ Όταν είναι κενοί, πριν και μετά τον καθαρισμό και την απολύμανση
- ☐ Ποτέ

16. Εφόσον υπάρχουν σκύλοι στην εκτροφή, έχουν πρόσβαση στους θαλάμους της εκτροφής:

- ☐ Πάντα
- ☐ Όταν είναι κενά, αλλά μόνο πριν τον καθαρισμό και την απολύμανση
- ☐ Όταν είναι κενοί, πριν και μετά τον καθαρισμό και την απολύμανση
- ☐ Ποτέ

17. Πως θα περιγράφατε τη δομική/ τεχνική κατάσταση της μονάδας;

- ☐ Νέα μονάδα (< 10 ετών), καλές συνθήκες
- ☐ Μονάδα > 10 ετών, ανακαινισμένη, καλές συνθήκες
- ☐ Νέα μονάδα, μέτριες συνθήκες
- ☐ Νέα μονάδα, κακές συνθήκες
- ☐ Παλιά μονάδα, κακές συνθήκες

III. ΠΕΡΙΒΑΛΛΟΝ ΧΩΡΟΣ

1. Περιγράψτε τον περιβάλλοντα χώρο του θαλάμου:

	ΧΩΡΟΣ ΠΡΟΣΒΑΣΗΣ	**ΠΕΡΙΜΕΤΡΙΚΑ ΤΟΥ ΘΑΛΑΜΟΥ**
Γρασίδι/βλάστηση		
Αποθηκευτικός χώρος		
Χώμα		
Χαλίκια, πέτρες		
Σκυρόδεμα		
Άλλο (διευκρινίστε)		

2. Υπάρχουν υδατορέματα (ρέμα, ποτάμι, λίμνη) στην περιοχή της φάρμας και σε ακτίνα 20 μέτρων από αυτή;

- ☐ Ναι
- ☐ Όχι

IV. ΒΙΟΑΣΦΑΛΕΙΑ ΚΑΙ ΔΙΑΧΕΙΡΙΣΗ

1. Υπάρχει προθάλαμος, χώρος προετοιμασίας ή τεχνητός φραγμός (π.χ. πόρτα ή χαμηλός τοίχος) στην είσοδο κάθε θαλάμου;

- ☐ Προθάλαμος/χώρος προετοιμασίας σε κάθε θάλαμο
- ☐ Προθάλαμος/χώρος προετοιμασίας σε κάποιους θαλάμους
- ☐ Υλικό φράγμα σε όλους τους θαλάμους
- ☐ Υλικό φράγμα σε κάποιους θαλάμους
- ☐ Ούτε προθάλαμος, ούτε υλικό φράγμα

2. Υπάρχει κοινός προθάλαμος ή χώρος προετοιμασίας μεταξύ ορισμένων θαλάμων;

- ☐ Ναι
- ☐ Όχι
- ☐ Σε ορισμένους θαλάμους

3. Σημειώστε την ύπαρξη ή όχι των ακόλουθων μέτρων βιοασφάλειας, καθώς επίσης και το επίπεδο υγιεινής τους:

	ΝΑΙ	ΟΧΙ	ΕΠΙΠΕΔΟ ΥΓΕΙΙΝΗΣ
Περίφραξη			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Λουτρό οχημάτων			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Αποδυτήρια			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Προθάλαμος			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Ποδόλουτρο			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Ποδόμακτρα			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό

4. Σημειώστε την ύπαρξη ή όχι των ακόλουθων ειδών εξοπλισμού υγιεινής του προσωπικού, καθώς επίσης και το επίπεδο υγιεινής τους:

	ΝΑΙ	ΟΧΙ	ΕΠΙΠΕΔΟ ΥΓΕΙΙΝΗΣ
Μπάνιο			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Βρύση			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Νερό			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Νιπτήρας			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Απορρυπαντικό			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Απολυμαντικό			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό
Πετσέτα			☐Άριστο ☐Καλό ☐Μέτριο ☐Κακό

5. Περιγράψτε το ρουχισμό του προσωπικού:

- ☐ Ρούχα και υποδήματα για τον κτηνοτρόφο και τον επισκέπτη, σωστή αλλαγή

☐ Ρούχα και υποδήματα για τον κτηνοτρόφο αλλά όχι για τον επισκέπτη, σωστή αλλαγή ☐ Ρούχα και υποδήματα για τον κτηνοτρόφο, ανεπαρκής αλλαγή ☐ Κοινόχρηστα βρώμικα ρούχα και υποδήματα ☐ Απουσία ρούχων και υποδημάτων, καμία αλλαγή	
6. Χρησιμοποιούνται ειδικές μπότες για την είσοδο σε κάθε θάλαμο;	☐ Ναι ☐ Όχι
7. Υπάρχουν ποδόλουτρα ή ποδόμακτρα στην είσοδο κάθε θαλάμου;	☐ Ναι ☐ Όχι
8. Υπάρχει εξοπλισμός που χρησιμοποιείται από κοινού σε διαφορετικούς θαλάμους	☐ Ναι ☐ Όχι
9. Αν ναι, διευκρινίστε	....
10. Κατά προσέγγιση πόσοι άνθρωποι εισέρχονται στο θάλαμο κατά τη διάρκεια ενός παραγωγικού κύκλου:	.... άνθρωποι
11. Τήρηση υγειονομικού κενού μεταξύ όλων των κύκλων εκτροφής;	☐ Ναι ☐ Όχι
12. Αν ναι, ποιος ο μέσος όρος του υγειονομικού κενού;	.... μέρες
13. Υπάρχει κάποιο πρόγραμμα καθαρισμού και απολύμανσης:	☐ Ναι ☐ Όχι
14. Απολύμανση θαλάμων μετά από κάθε παραγωγικό κύκλο;	☐ Ναι ☐ Όχι
15. Αν ναι, ποιος πραγματοποιεί την απολύμανση	☐ Κτηνοτρόφος/προσωπικό ☐ Εξειδικευμένη εταιρία ☐ Κτηνοτρόφος και εξειδικευμένη εταιρία
16. Αριθμός απολυμάνσεων ανά παραγωγικό κύκλο	☐ 1 απολύμανση ☐ 2 απολυμάνσεις ☐ Περισσότερες από 2, διευκρινίστε: ....
17. Μορφή της πρώτης απολύμανσης	☐ Νερό υπό πίεση (κρύο, θερμό) ☐ Θερμονεφελοποίηση (fogger) ☐ Υποκαπνισμός ☐ Άλλο, διευκρινίστε: ....

18. Ύπαρξη λάκκου συλλογής λυμάτων	☐ Ναι ☐ Όχι
19. Ημερομηνία πρώτης απολύμανσης μετά την απομάκρυνση της προηγούμενης παρτίδας	....../......../...................
20. Απολύμανση του εδάφους	☐ Ναι ☐ Όχι
21. Αν ναι, διευκρινίστε:	☐ Καυστική σόδα ☐ Φωτιά ☐ Άλλο, διευκρινίστε:……….
22. Περιγράψτε τη διαδικασία καθαρισμού κι απολύμανσης του θαλάμου: ☐ Πλήρης απομάκρυνση στρωμνής, στεγνό καθάρισμα, καθαρισμός με ζεστό νερό, απολύμανση με σπρέι, στέγνωμα ☐ Στεγνό καθάρισμα, καθαρισμός με κρύο νερό, απολύμανση, στέγνωμα ☐ Πρόχειρο στεγνό καθάρισμα, καθαρισμός με κρύο νερό, απολύμανση, στέγνωμα ☐ Πρόχειρο στεγνό καθάρισμα, καθαρισμός με κρύο νερό, απολύμανση μόνο στις υγρές περιοχές ☐ Ατελής απομάκρυνση στρωμνής, στεγνό καθάρισμα, απολύμανση μόνο στις υγρές περιοχές	
23. Υπάρχει κάποιο πρόγραμμα για τον έλεγχο των τρωκτικών;	☐ Ναι ☐ Όχι
24. Γίνεται εφαρμογή αγωγής εναντίον των εντόμων;	☐ Ναι ☐ Όχι
25. Γίνεται συντήρηση από εξειδικευμένη εταιρία καταπολέμησης εντόμων και τρωκτικών;	☐ Ναι ☐ Όχι
26. Αν ναι, με τι μεσοδιάστημα;	☐ Εβδομαδιαίως ☐ Μηνιαίως ☐ Κάθε 3 μήνες ☐ Κάθε 6 μήνες ☐ Ετησίως ☐ Μεταξύ των παραγωγικών κύκλων ☐ Άλλο, διευκρινίστε: .... ☐
27. Περιγράψτε το είδος της στρωμνής:	☐ Άχυρο ☐ Πριονίδι ☐ Ριζοφλοιός

28. Που γίνεται η φύλαξη της στρωμνής της εκτροφής:

☐ Σε κλειστό, προστατευμένο, καθαρό χώρο
☐ Σε κλειστό, προστατευμένο, αλλά ακάθαρτο χώρο
☐ Σε κλειστό, απροστάτευτο, ακάθαρτο χώρο
☐ Σε στεγασμένο χώρο που έχουν πρόσβαση τρωκτικά και πτηνά
☐ Σε μεγάλης έκτασης απροστάτευτη περιοχή, κοντά σε εξοπλισμό κι απορρίμματα

29. Που/πώς γίνεται η διάθεση της κοπριάς;

☐ Σε παρακείμενο βοσκότοπο
☐ Σε παρακείμενα καλλιεργήσιμα εδάφη
☐ Σε βοσκότοπο που δεν συνορεύει με την περιοχή
☐ Σε καλλιεργήσιμα εδάφη που δε συνορεύουν με την περιοχή
☐ Με αποτέφρωση
☐ Άλλο, διευκρινίστε: ....

30. Σε τι απόσταση από την εκτροφή γίνεται η διάθεση της κοπριάς;

☐ Σε απόσταση > 1 χλμ, οχυρωμένη
☐ Σε απόσταση 500-1000 μ
☐ Σε απόσταση 100-500 μ
☐ Σε απόσταση 20-100μ
☐ Ακριβώς δίπλα στο θάλαμο

31. Μέθοδος που χρησιμοποιείται για την απομόνωση των νεκρών πτηνών

☐ Θάψιμο σε λάκκο
☐ Κάψιμο
☐ Άλλο, διευκρινίστε: ....

32. Αν χρησιμοποιείται λάκκος, που βρίσκεται

☐ ≤ 50μ μακριά από τον θάλαμο
☐ ≥50μ από τον θάλαμο

V. ΣΥΣΤΗΜΑ ΣΥΛΛΟΓΗΣ ΠΤΗΝΩΝ ΓΙΑ ΣΦΑΓΗ

1. Περιγράψτε το σύστημα συλλογής:

☐ Οι συλλέκτες δεν έρχονται σε επαφή με άλλες πτηνοτροφικές μονάδες
☐ Οι συλλέκτες επισκέπτονται αποκλειστικά μια μονάδα ανά ημέρα
☐ Οι συλλέκτες επισκέπτονται > 1 μονάδες ανά ημέρα
☐ Οι συλλέκτες επισκέπτονται > 1 μονάδες ανά ημέρα, χρήση μηχανικής συλλογής πτηνών
☐ Οι συλλέκτες επισκέπτονται ≥ 2 μονάδες ανά ημέρα, όχι αλλαγή ρουχισμού, αναποτελεσματική απολύμανση

2. Τύπος συλλογής	☐ Χειρωνακτική ☐ Μηχανική
3. Εφαρμογή αραιώσεων	☐ Ναι ☐ Όχι
4. Συχνότητα αραίωσης	☐ Όχι εφαρμογή αραιώσεων ☐ 2 ημέρες αραιώσεων ☐ ≥3 ημέρες συλλογής
5. Πόση ώρα διαρκεί η απομάκρυνση των πτηνών από το θάλαμο κατά την πρώτη αραίωση;	.... ώρες
6. Κατά μέσο όρο, πόσες μέρες μεσολαβούν μεταξύ της πρώτης και της τελικής αραίωσης;	.... μέρες
7. Πόση ώρα διαρκεί η απομάκρυνση των πτηνών από το θάλαμο κατά την τελική αραίωση;	.... ώρες
8. Εφόσον έχει προηγηθεί αραίωση, προσδιορίστε: Αραίωση 1: αριθμός πτηνών ...... ημερομηνία ....../....../.......... Αραίωση 2: αριθμός πτηνών ...... ημερομηνία ....../....../.......... Αραίωση 3: αριθμός πτηνών ...... ημερομηνία ....../....../..........	
9. Εξοπλισμός που χρησιμοποιήθηκε στις προηγούμενες αραιώσεις ☐ Φορτηγά ☐ Εμπορευματοκιβώτια ☐ Φορτηγά και εμπορευματοκιβώτια ☐ Τίποτα από τα παραπάνω	

VI. ΑΕΡΙΣΜΟΣ

1. Όλοι οι θάλαμοι της εκτροφής έχουν τον ίδιο τύπο αερισμού;	☐ Ναι ☐ Όχι
2. Υποδείξτε τον τύπο αερισμού του θαλάμου	☐ Φυσικός ☐ Ίσης πίεσης ☐ Αρνητικής πίεσης ☐ Τούνελ
3. Παροχή εξαερισμού (εισερχόμενος αέρας) ☐ Ανεμιστήρες στην οροφή ☐ Ανεμιστήρες στους πλαϊνούς τοίχους ☐ Ανεμιστήρες στους τελικούς τοίχους	

☐ Είσοδοι αέρα χωρίς ανεμιστήρα στους πλαϊνούς τοίχους
☐ Είσοδοι αέρα χωρίς ανεμιστήρα στην οροφή
☐ Κουρτίνες στους πλαϊνούς τοίχους (για εξαερισμό-τούνελ)
☐ Κουρτίνες στους τελικούς τοίχους (για φυσικό εξαερισμό)
☐ Ανεμιστήρες (αναμικτήρες αέρα) μέσα στο θάλαμο

4. Έξοδος εξαερισμού (εξερχόμενος αέρας)

☐ Ανεμιστήρες στην οροφή
☐ Ανεμιστήρες στους πλαϊνούς τοίχους
☐ Ανεμιστήρες στους τελικούς τοίχους
☐ Αέρας εξέρχεται από την κορυφογραμμή

5. Τα σημεία εισόδου/εξόδου αέρα κλείνουν ερμητικά κατά το χρονικό διάστημα διακοπής του κύκλου παραγωγής;

☐ Ναι
☐ Όχι
☐ Δεν είναι γνωστό
☐ Δεν είναι εφικτό

6. Εφόσον υπάρχουν ανεμιστήρες, αντιστρέφονται κατά τους θερινούς μήνες;

☐ Ναι
☐ Όχι
☐ Δεν είναι γνωστό
☐ Δεν είναι εφικτό

VII. ΣΥΣΤΗΜΑ ΧΟΡΗΓΗΣΗΣ ΤΡΟΦΗΣ ΚΑΙ ΝΕΡΟΥ

1. Περιγράψτε το σύστημα χορήγησης τροφής του θαλάμου:

☐ Κλειστό σύστημα τροφοδοσίας, παράδοση τροφής, προστατευμένη περιοχή
☐ Κλειστό σύστημα τροφοδοσίας, παράδοση τροφής, απροστάτευτη περιοχή
☐ Σιλό, μεταφορά καλυμμένης τροφής
☐ Ανοιχτό δοχείο τροφοδοσίας, μεταφορά καλυμμένης τροφής
☐ Ανοικτό δοχείο τροφοδοσίας, μεταφορά τροφής

2. Χρήση προσθέτων στην τροφή

☐ Προβιοτικά
☐ Πρεβιοτικά
☐ Άλλο, διευκρινίστε: ……………

3. Παροχή νερού

☐ Νερό από κεντρικό αγωγό
☐ Νερό από ιδιόκτητη πηγή

☐ Υπόγεια ύδατα (γεώτρηση)
☐ Νερό από ιδιόκτητη πηγή, μολυσμένο με *E.coli/Enterococci*
☐ Υπόγεια ύδατα, μολυσμένα με *E.coli/Enterococci*

4. Ποια προϊόντα/μέθοδοι χρησιμοποιούνται για την επεξεργασία του νερού;

☐ Τίποτα
☐ Χλωρίωση
☐ Ιωδίωση
☐ Υποχλωριώδη
☐ Υπεροξείδια
☐ Οξειδωτικά απολυμαντικά
☐ Οξινοποιητές νερού
☐ Άλλο, διευκρινίστε: …………………………………..

5. Ποιος τύπος χορήγησης νερού χρησιμοποιείται στην εκμετάλλευση;	☐ Πιπίλες ☐ Πιπίλες με πιατάκια ☐ Καμπάνες

VIII. ΠΡΟΣΩΠΙΚΟ

1. Αριθμός ατόμων που εργάζονται στο θάλαμο	.... άτομα
2. Το ίδιο προσωπικό εργάζεται σε άλλους θαλάμους/κτήρια της μονάδας	☐ Ναι ☐ Όχι
3. Το ίδιο προσωπικό εργάζεται σε άλλη πτηνοτροφική μονάδα	☐ Ναι ☐ Όχι
4. Το προσωπικό της εκτροφής εργάζεται και σε άλλες πτηνοτροφικές επιχειρήσεις π.χ. σφαγείο;	☐ Ναι ☐ Όχι

5. Ποιος είναι υπεύθυνος για τις κύριες παρεμβάσεις σε επίπεδο εκτροφής

ΠΑΡΕΜΒΑΣΗ	ΚΤΗΝΟΤΡΟΦΟΣ	ΕΙΔΙΚΟΙ	ΑΛΛΟΙ
1η Τοποθέτηση			
Ενδιάμεση αραίωση			
Τελική αραίωση			

IX. ΜΕΤΡΑ ΠΡΟΛΗΨΗΣ

1. Έλαβαν τα κοτόπουλα αντιβιοτική αγωγή κατά την τοποθέτηση;	☐ Ναι, στο νερό ☐ Ναι, στην τροφή ☐ Καμία αγωγή
2. Αν ναι, αναφέρετε την εμπορική ονομασία του προϊόντος	....
3. Έλαβαν τα κοτόπουλα αντιβιοτική αγωγή κατά τη διάρκεια της ζωής τους;	☐ Ναι, στο νερό ☐ Ναι, στην τροφή ☐ Καμία αγωγή
4. Αν ναι, αναφέρετε την εμπορική ονομασία του προϊόντος	....
5. Έλαβαν τα πτηνά αντιβιοτική αγωγή στο νερό ή την τροφή;	☐ Καμία αγωγή ☐ Ναι μια φορά ☐ Ναι, δύο φορές ☐ Περισσότερες από δύο φορές

6. Αν ναι, προσδιορίστε τα ακόλουθα:

	ΑΣΘΕΝΕΙΑ	ΠΡΟΙΟΝ	ΗΛΙΚΙΑ	ΔΙΑΡΚΕΙΑ	ΤΡΟΦΗ	ΝΕΡΟ
1η Αγωγή						
2η Αγωγή						
3η Αγωγή						

7. Έλαβαν τα πτηνά προληπτική αντικοκκιδιακή αγωγή	☐ Ναι, στην τροφή ☐ Εμβολιασμός ☐ Καμία αγωγή
8. Αν ναι, αναφέρετε την εμπορική ονομασία του προϊόντος	....
9. Ποια είναι η θνησιμότητα μεταξύ της 0 και 10 ημέρας; *(βλ. Αρχεία εκτροφής)*	....

10. Σημειώστε το εμβολιακό πρόγραμμα των πτηνών:

ΑΣΘΕΝΕΙΑ	ΠΡΟΙΟΝ	ΗΜΕΡΑ	ΗΜΕΡΑ	ΝΕΡΟ	ΕΚΚΟΛΑΠΤΗΡΙΟ
IBV					
NDV					
IBD					
Marek					
Άλλο					

X. ΜΙΚΡΟΚΛΙΜΑ ΣΤΟ ΘΑΛΑΜΟ

Συμπληρώστε τον παρακάτω πίνακα με βάση τις μετρήσεις από τα ειδικά όργανα της εκτροφής, εφόσον υπάρχουν:

Θερμοκρασία °C	
Υγρασία %	
Ταχύτητα αέρα	
Συγκέντρωση αμμωνίας	
Ποιότητα αέρα (γενικό σχόλιο)	

XI. ΓΕΝΙΚΕΣ ΠΑΡΑΤΗΡΗΣΕΙΣ

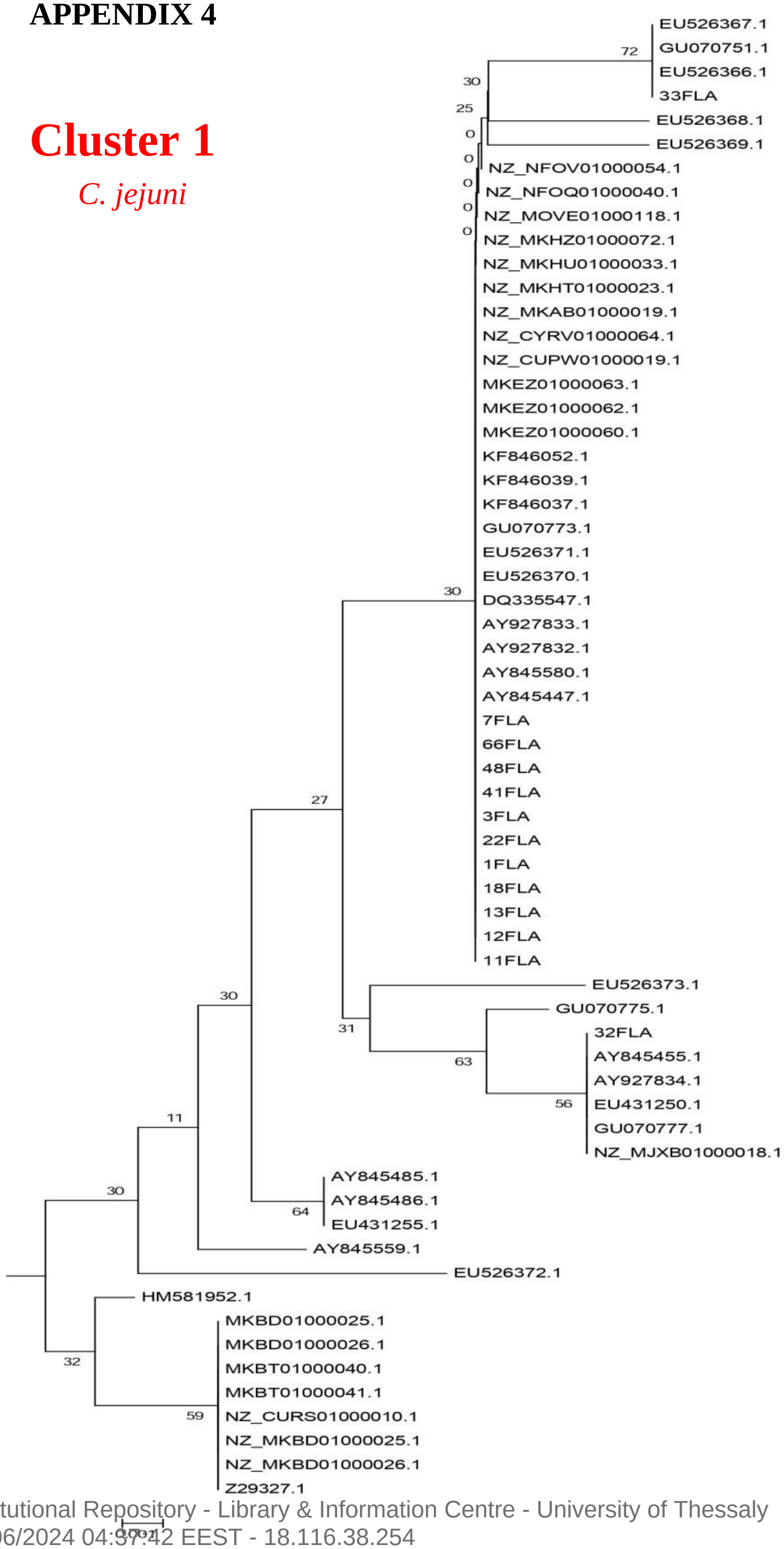
Σημειώστε τις γενικές παρατηρήσεις σας σε συνδυασμό με το επίπεδο υγιεινής της πτηνοτροφικής μονάδας

ΕΥΧΑΡΙΣΤΟΥΜΕ ΠΟΛΥ ΓΙΑ ΤΗ ΣΥΜΜΕΤΟΧΗ!!

APPENDIX 4

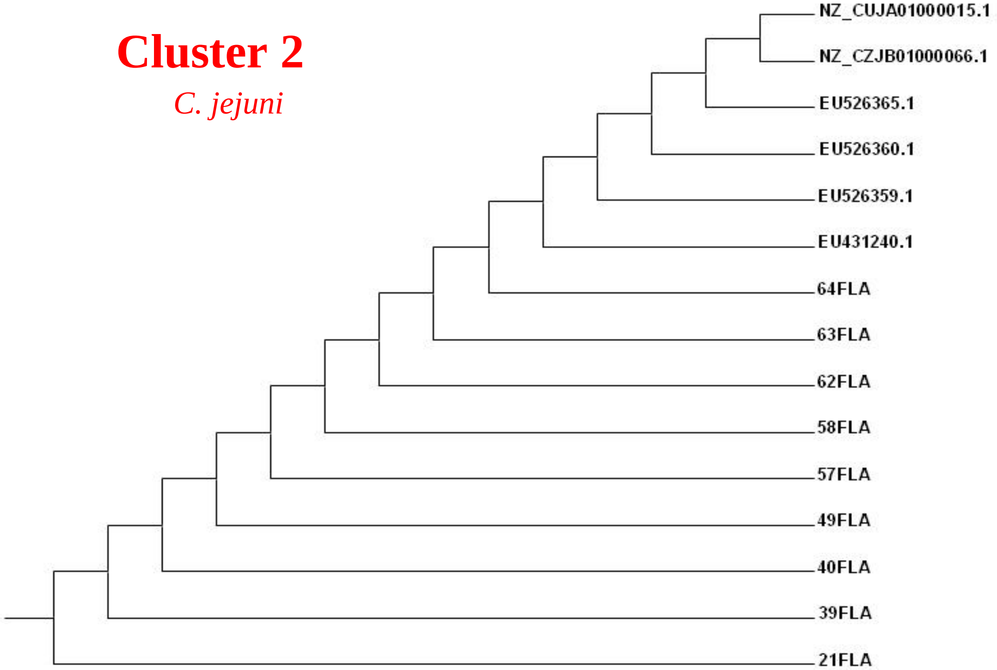
Cluster 1

C. jejuni



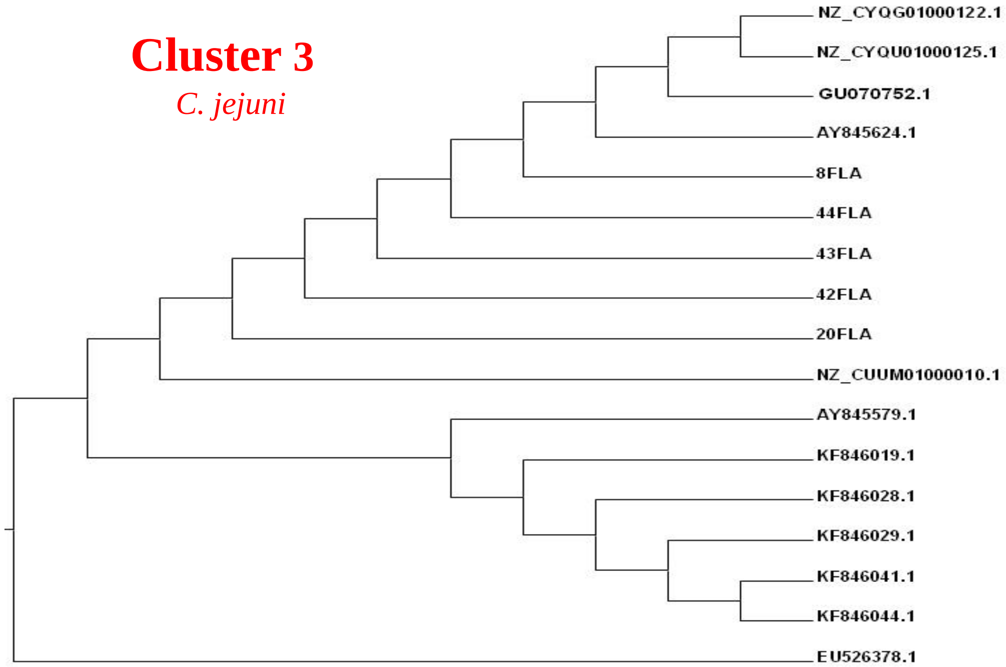
Cluster 2

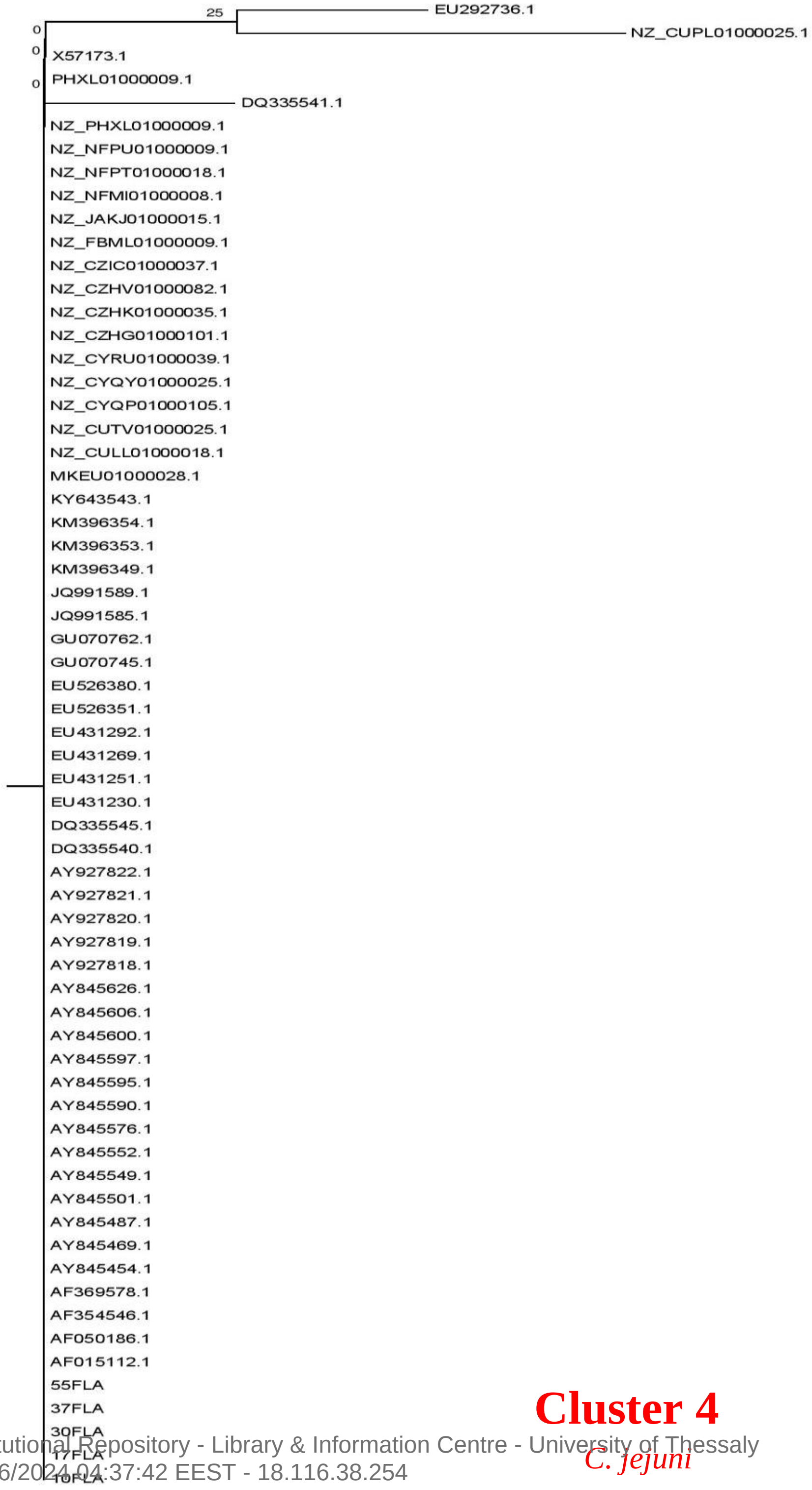
C. jejuni



Cluster 3

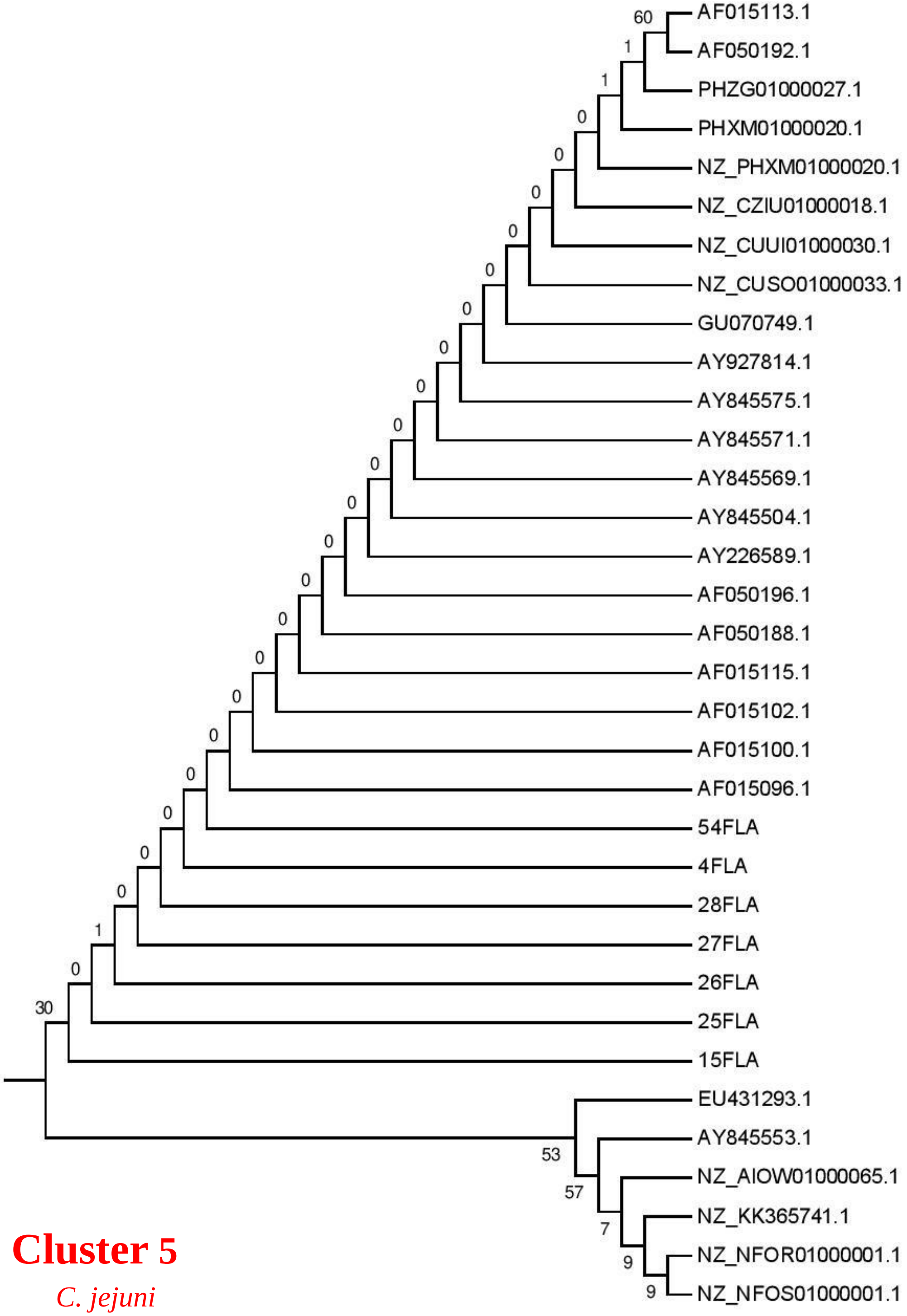
C. jejuni





Cluster 4

C. jejuni

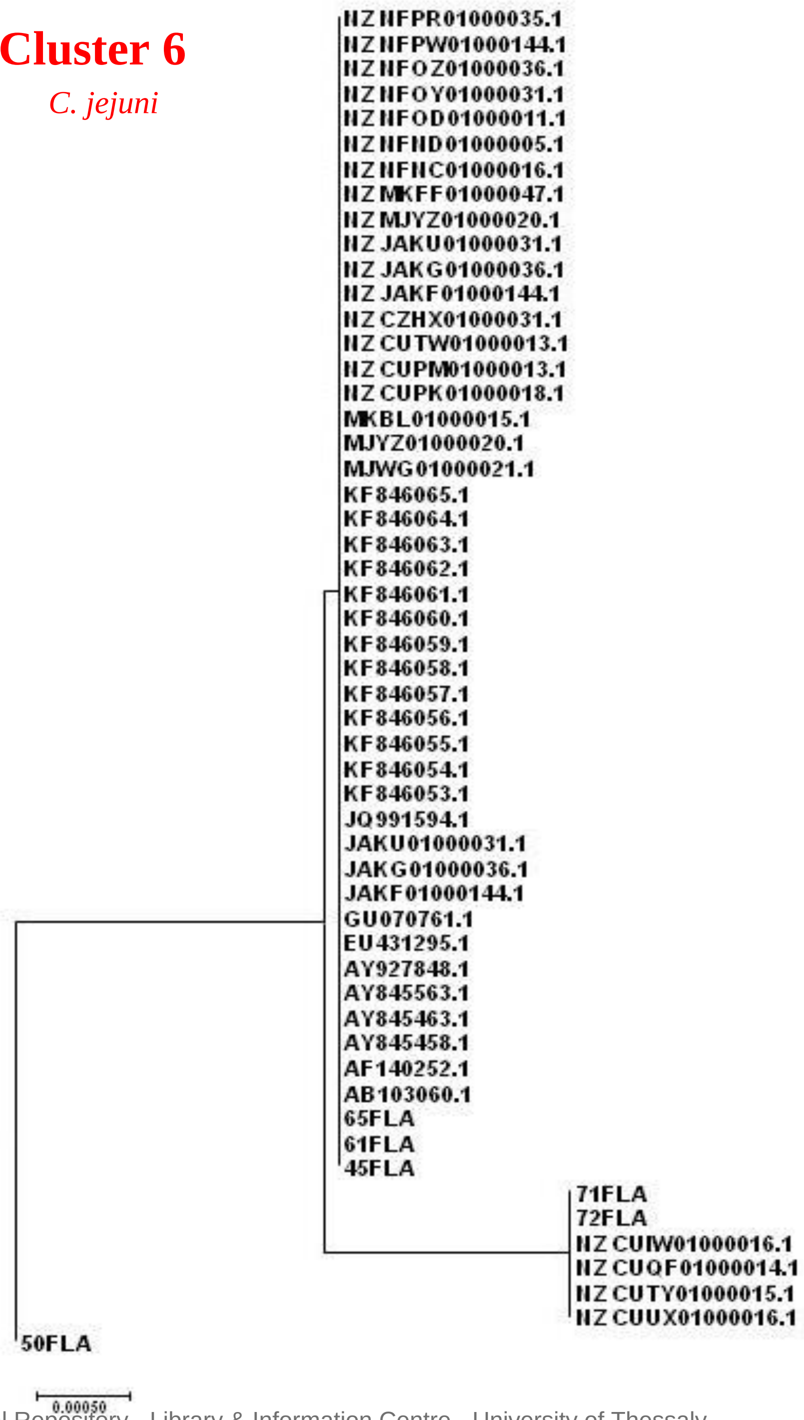


Cluster 5

C. jejuni

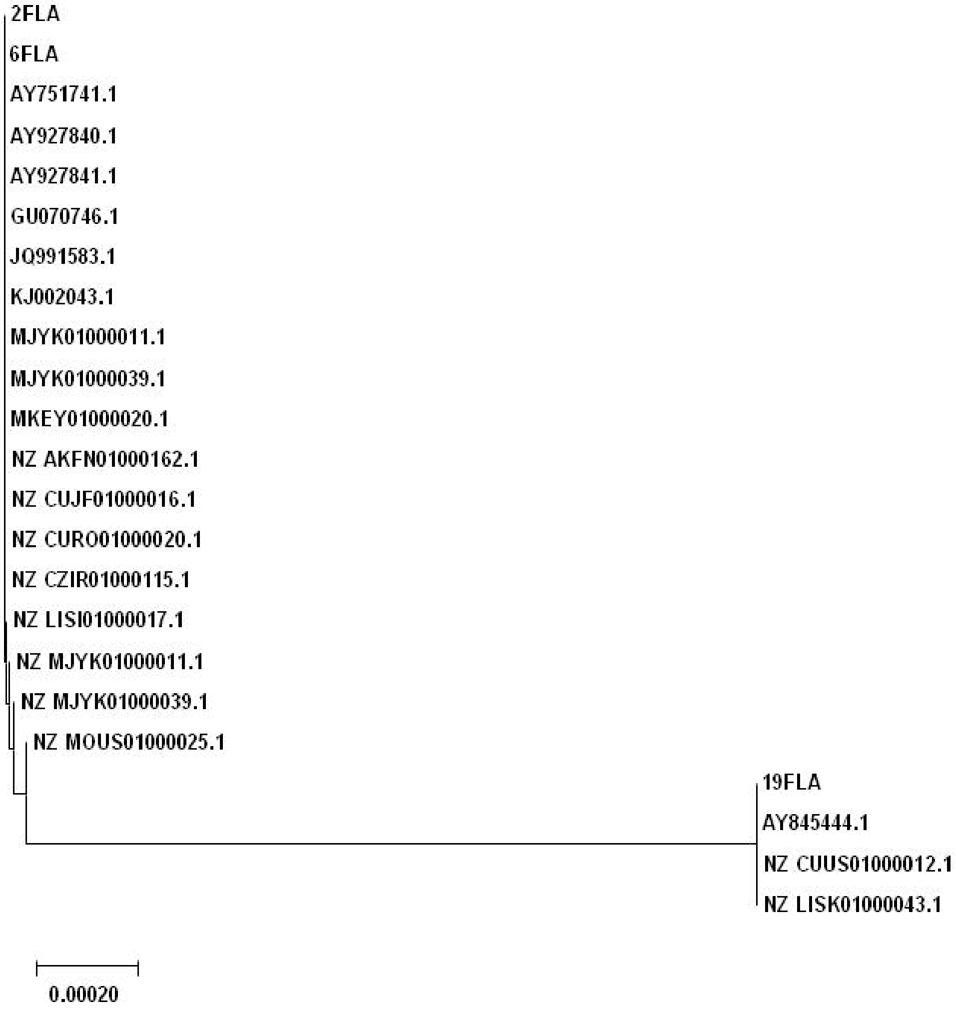
Cluster 6

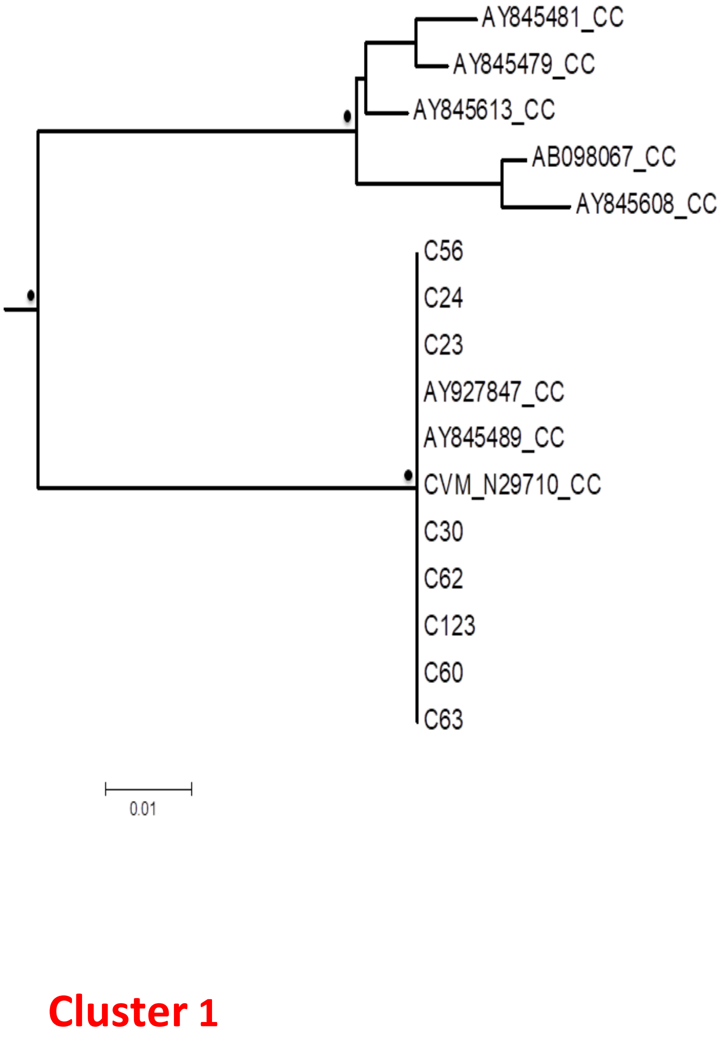
C. jejuni

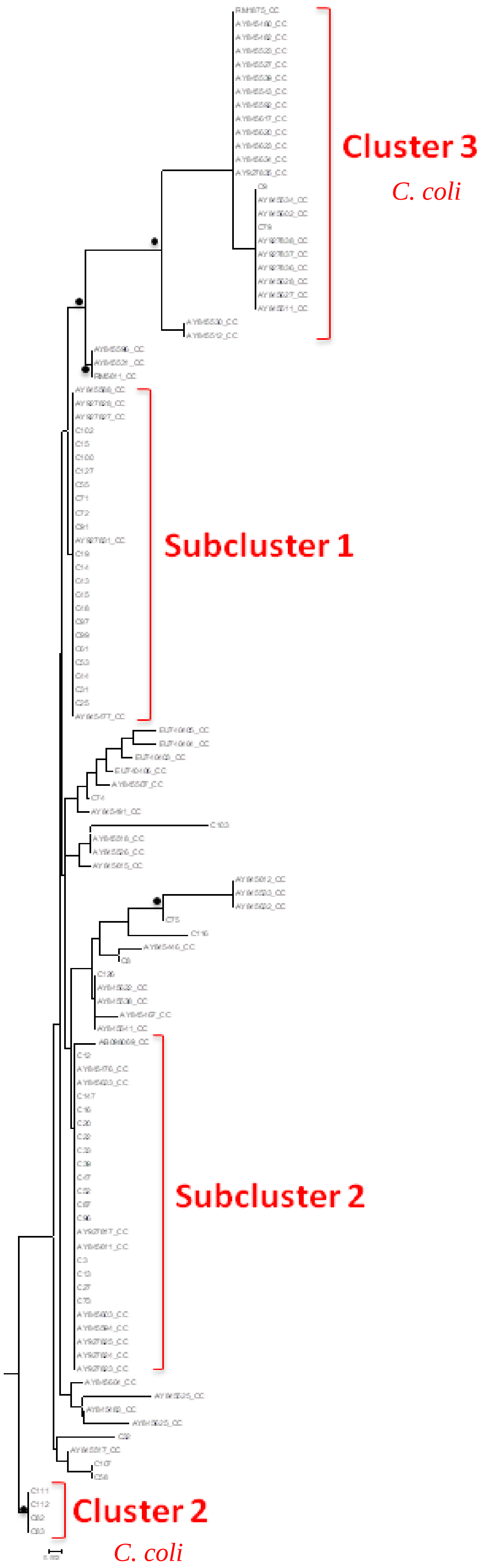


Cluster 7

C. jejuni




AY845481_CC
AY845479_CC
AY845613_CC
AB098067_CC
AY845608_CC
C56
C24
C23
AY927847_CC
AY845489_CC
CVM_N29710_CC
C30
C62
C123
C60
C63
0.01
Cluster 1
C. coli


Cluster 3
C. coli
Subcluster 1
Subcluster 2
C111
C112
Cluster 2
C. coli

ARTICLE I

Natsos G., Koutoulis K.C., Sossidou E., Chemaly M., Mouttotou N.K. (2016)

“*Campylobacter* spp. infection in humans and poultry.”

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Campylobacter spp. infection in humans and poultry

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Review article
Ανασκόπηση

Campylobacter spp. infection in humans and poultry

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Μόλυνση με Campylobacter spp. στους ανθρώπους και τα πτηνά

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ABSTRACT. Campylobacter is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide. The infection may be subclinical or cause disease of variable severity. The eating and handling of improperly cooked or raw broiler meat has been shown to be one of the most important sources of human campylobacteriosis. Birds carrying Campylobacter are asymptomatic colonizers without any clinical signs. Broilers are considered Campylobacter free after hatching and become colonized by exposure to viable bacteria from the environment. Several risk factors can result in the introduction of Campylobacter into the flocks making it difficult to keep chicken flocks free of Campylobacter throughout the rearing period. Lack of biosecurity measures, season, age, partial depopulation practices, flock size, type of production system, presence of other animals on farm, water quality, presence of rodents and mechanical transmission via insects are considered to be some of the risk factors associated with horizontal transmission. The control of

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Campylobacter in poultry seems crucial for the reduction of human campylobacteriosis cases. In Greece, there has been a dearth of information on prevalence and risk factors of Campylobacter in broiler flocks. Therefore, it is essential to initially investigate the prevalence of Campylobacter infection on farms and in poultry carcasses and subsequently the risk factors at all production stages of broiler meat and plan intervention studies to help reducing the disease in humans. This paper review the most recent data reported worldwide on Campylobacter infection in humans and poultry in order to provide an overview of trends, risks, possible causes and mechanisms of transmission routes.

Keywords: *broilers, Campylobacter, campylobacteriosis, foodborne pathogens, Greece, poultry, prevalence, risk factors*

ΠΕΡΙΛΗΨΗ. Το Campylobacter είναι παγκοσμίως αναγνωρισμένο ως η κύρια αιτία της διαρροϊκής, βακτηριακής αιτιολογίας, τροφοδηλητηρίασης. Η μόλυνση μπορεί αν είναι υποκλινικής μορφής ή να προκαλεί ασθένεια διαφορετικής σοβαρότητας. Η κατανάλωση και ο χειρισμός του πλημμελώς μαγειρεμένου ή ωμού ορνίθιου κρέατος έχει αποδειχθεί ως μία από τις πιο σημαντικές πηγές της ανθρώπινης καμπυλοβακτηρίωσης. Τα πτηνά που μεταφέρουν το Campylobacter είναι ασυμπτωματικοί φορείς, χωρίς κλινικά συμπτώματα. Τα ορνίθια κρεοπαραγωγής θεωρούνται ελεύθερα από Campylobacter μετά την εκκόλαψη και μολύνονται με την έκθεση τους στα βακτήρια από το περιβάλλον. Αρκετοί παράγοντες κινδύνου μπορούν να οδηγήσουν στην μόλυνση των σμηνών από Campylobacter, γεγονός που καθιστά πολύ δύσκολο να μείνουν τα σμήνη των πτηνών απαλλαγμένα καθ ‘όλη τη διάρκεια της εκτροφής. Η έλλειψη μέτρων βιοασφάλειας, η εποχή, η ηλικία, πρακτικές αραίωσης του πληθυσμού, το μέγεθος του σμήνους, το είδος του συστήματος παραγωγής, η παρουσία άλλων ζώων στην εκμετάλλευση, η ποιότητα των υδάτων, η παρουσία τρωκτικών και η μηχανική μετάδοση μέσω εντόμων θεωρούνται μερικοί από τους παράγοντες κινδύνου που συνδέονται με την οριζόντια μετάδοση. Ο έλεγχος του Campylobacter στα πτηνά είναι πολύ σημαντικός για τη μείωση των περιστατικών της καμπυλοβακτηρίωσης στους ανθρώπους. Στην Ελλάδα, υπάρχει έλλειψη δεδομένων σχετικά με τον επιπολασμό και τους παράγοντες κινδύνου του Campylobacter στα σμήνη ορνιθίων κρεοπαραγωγής. Έτσι, είναι πολύ σημαντικό να διερευνηθεί αρχικά, ο επιπολασμός του Campylobacter στις πτηνοτροφικές εκμεταλλεύσεις και στα σφάγια πουλερικών και στη συνέχεια, οι παράγοντες κινδύνου σε όλα τα στάδια της παραγωγής του ορνίθιου κρέατος, έτσι ώστε να σχεδιαστούν μελέτες παρέμβασης που θα βοηθήσουν να μειωθούν τα περιστατικά της ασθένειας στον άνθρωπο. Η εργασία αυτή κάνει μια ανασκόπηση των πιο σύγχρονων δεδομένων που έχουν αναφερθεί παγκοσμίως για τη μόλυνση με Campylobacter στους ανθρώπους και τα πτηνά προκειμένου να προσφέρει μια επισκόπηση των τάσεων, των παραγόντων κινδύνου, τις πιθανές αιτίες και των μηχανισμών των οδών μετάδοσης.

Λέξεις ευρετηρίασης: *ορνίθια κρεοπαραγωγής, Campylobacter, καμπυλοβακτηρίωση, τροφιμογενή παθογόνα, Ελλάδα, πτηνά, επιπολασμός, παράγοντες κινδύνου*

INTRODUCTION

Human campylobacteriosis is considered an important public health problem and poultry has been identified as a significant source for human infections with *Campylobacter* species. Although thermophilic *Campylobacter* spp. are not significant pathogens for poultry, they are of importance to food safety and public health, with *C. jejuni* being responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by *C. lari* (Zhang and Sahin, 2013). Other *Campylobacter* species, such as *C. upsaliensis* and *C. fetus*, may also be associated with human diarrhea. Although the detection of non-*C. jejuni/coli* is uncommon in human cases in the industrialized world, it is more common in the developing world (Lastovica and Allos, 2008). This paper review the most recent data reported worldwide on *Campylobacter* infection in humans and poultry in order to provide an overview of trends, risks, possible causes and mechanisms of transmission routes.

CAMPYLOBACTERIOSIS AND PUBLIC HEALTH

Incidence, severity and costs

Since 1990's the incidence of human campylobacteriosis has been steadily rising worldwide (Baker et al., 2007; WHO, 2011; EFSA, 2014). This is in accordance with the Community Zoonoses Reports of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). In the EU, campylobacteriosis has been the most commonly reported zoonosis since 2005, followed by salmonellosis (EFSA, 2006; EFSA, 2014). Information submitted by 27 European Union Member States (EU MS) on the occurrence of zoonoses and food-borne outbreaks in 2012, showed that there were 214,268 confirmed human cases of campylobacteriosis (EFSA, 2014). Thus, the overall notification rate of human campylobacteriosis was 55.49 per 100,000 population (range: 0.39 - 174.08 per 100,000 population). There was a wide variation in incidences between countries which probably reflects differences in the healthcare and reporting systems, and in microbiological methods for the detection of *Campylobacter* (Olson et al., 2008; Vally et al., 2009; EFSA, 2014). Even though clinical cases of campylobacteriosis tended to be under-reported, "there may be not less than 2 million and possibly as high as 20 million cases of clinical campylobacteriosis per year in the 27 EU MS" (EFSA, 2010c). The number of confirmed cases of campylobacteriosis in the European Union has followed a statistically significant ($p < 0.001$) increasing trend in the last five years (2008-2012), along with a clear seasonal trend (summer months) (EFSA, 2014). Considering the high number of human campylobacteriosis cases, the severity in terms of reported fatalities was low (0.03%) (EFSA, 2014).

According to Scallan et al (2011) *Campylobacter* is the third-leading cause of bacterial foodborne illness in the United States. Information provided by the Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Disease Control and Prevention (CDC), from 10 State Health Departments in the USA, indicated campylobacteriosis as the second most common infection (35%), following salmonellosis (40%). CDC also estimated that in 2012, the number of reported infections and incidence per 100,000 population by *Campylobacter* was 6,793 and 14.30, respectively (CDC, 2013). In the same report the estimated incidence of infection for *Campylobacter* showed a 14% increase in 2012, compared with 2006–2008. Also, in the USA, it is estimated that *Campylobacter* causes 2.5 million illnesses, 13,000 hospitalizations, and over 100 deaths each year (Patrick, 2007).

In Australia, *Campylobacter* is currently the most common cause of acute bacterial diarrhea among all the notified enteric pathogens with more than 15,000 cases each year (Stafford, 2010). The incidence of notified campylobacteriosis has steadily increased during the past 15 years from 67.0/100,000 population in 1991 to 121.4/100,000 in 2005 (Stafford, 2010). According to the same researcher, adjusting for under-reporting, there may be an estimation of 225,000 infections occurring each year in Australia, but most of which are sporadic in nature.

In many developing areas of the world, human campylobacteriosis is hyperendemic and the disease differs from campylobacteriosis in developed countries (Coker et al., 2002). In developing areas, campylobacteriosis is predominantly a pediatric problem affecting children under the age of five while adults are generally less prone to the disease (Oberhelman and Taylor, 2000; Coker et al., 2002). Generally, developing countries do not have national surveillance programs for campylobacteriosis; therefore, incidence values in terms of number of cases for a population do not exist (Coker et al., 2002). Most estimates of incidence came from laboratory-based surveillance of pathogens responsible for diarrhea. Oberhelman & Taylor (2000) estimated that *Campylobacter* isolation rates in developing countries ranged from 5 to 20%. In Asiatic countries like Thailand for example, the overall isolation rate of *Campylobacter* from diarrheal children under year 5 was 6.8% (Yang et al., 2008). This rate was 12.1% in Laos, with *C. jejuni* and *C. coli* occurring in 7.1% and 4% of enteric infection in children aged < 1 year and 1–5 years, respectively (Yamashiro et al., 1998).

There are no sufficient data on campylobacteriosis in Greece, because the disease is not under surveillance through Mandatory Notification System. According to Hellenic Center for Disease Control & Protection (HCDCP) factsheet of 2013, although there are few hospitals with laboratory ability of *Campylobacter* isolation, the number of positive cultures for this pathogen was high (623 positive cultures) in 2012, even greater than the frequency of salmonellosis (HCDCP, 2013). These data indicate the need of integration of campylobacteriosis on the Mandatory Notification System in order to achieve full illustration of the morbidity caused by the microorganism in question and the need of setting a specialized reference laboratory. Nevertheless, there have been several references about *Campylobacter* spp. and its contribution on acute gastroenteritis among patient in Greek hospitals, especially children (Kafetzis et al., 2001; Maltezou et al., 2001; Chatzipanagiotou et al., 2002; Chatzipanagiotou et al., 2003a; Maraki et al., 2003; Ioannidis et al., 2006; Papavasileiou et al., 2007; Ioannidis et al., 2009; Maragkoudakis et al., 2010; Mellou et al., 2010; Mammas et al., 2012; Maraki et al., 2012; Ioannidis et al., 2013). Moreover, the first diagnosed *C. jejuni*-associated Guillain-Barré Syndrome case from Greece in 2003 reported by Chatzipanagiotou et al. (2003b).

Human infections with *Campylobacter* pathogenic strains are characterized by nausea, vomiting, stomachache, malaise, profuse watery diarrhea, blood in feces and high fever (Blaser et al., 2008). The infective dose of campylobacteriosis can be as low as few hundred cells (Black et al., 1988). In most cases the illness is self-limiting, but it may be severe and life threatening in susceptible people such as young children, the elderly, or people with immunosuppressive diseases, such as AIDS and cancer (EFSA, 2011). In cases where antibiotic treatment is needed, fluoroquinolones and erythromycin are considered the drugs of choice, but attention should be paid since a rapidly increasing proportion of *Campylobacter* strains all over the world have been found to be resistant to these antibiotics (Allos, 2001; EFSA 2013b). The incubation period is up to 10 days with typical symptoms related to enteritis, with diarrhea, cramps, abdominal pain and fever. In susceptible humans, *C. jejuni/coli* infection is associated with acute enteritis and abdominal pain lasting for up to seven days or longer (Allos, 2001). Infection is sometimes complicated by the development of serious post infection complications, such as bacteraemia, Guillain–Barré syndrome (GBS), reactive arthritis, inflammatory bowel disease, irritable bowel syndrome (Allos, 2001; Helms et al., 2003; Havelaar et al., 2005; Mangen et al., 2005; Smith and Bayles, 2007; Gradel et al., 2009; Haagsma et al., 2010) and even death (Havelaar et al., 2005; Gradel et al., 2008). GBS is an acute demyelinating disease of the peripheral nervous system resulting in temporary ascending flaccid paralysis (Allos, 2001). There are enough data on the incidence of GBS in Europe and North America (McGrogan et al., 2009; Sejvar et al., 2011). The disease has also been well studied in China, where it may implicate in outbreaks, and in Japan, whereas seasonal patterns of GBS have been described in Mexico, China, Argentina, Curacao, South Africa and other countries (Coker et al., 2002; WHO, 2013).

The socioeconomic costs of the disease in humans can be very high (Samuel et al., 2004) and this is expected, if one takes under consideration that there may be approximately nine million cases of human campylobacteriosis per year in the 27 EU MS (EFSA, 2011). The public health impact of campylobacteriosis and its sequelae is 0.35 million disability-adjusted life years (DALYs) per year and total annual costs are 2.4 billion euros (EFSA, 2011). These costs reflect to medical expenses, lost wages, product recalls, legal costs, and other indirect expenses (CAST, 1994). Havelaar et al. (2005), estimated that in the Netherlands (with approximately 80,000 cases of gastroenteritis per year), the costs of illness caused by campylobacteriosis are about 21 million euros / year.

Outbreaks of *Campylobacter* spp. - Sources and transmission of infection

Most campylobacteriosis cases are sporadic or small-scale family outbreaks (Olson et al. 2008). Even though outbreaks of *Campylobacter* infections are rarely reported, they might be more common than previously suspected (Gillespie et al., 2003; Miller et al., 2004; Fussing et al., 2007; Isohanni, 2013). Because the incubation period before the onset of symptoms can be long, it might be difficult to determine the source of infection. Numerous epidemiological studies have been conducted to identify potential sources for human campylobacteriosis. Most cases of outbreaks in the literature were associated with handling raw poultry, eating raw or undercooked poultry meat or cross-contamination of raw to cooked foods (Tauxe et al., 1997; Studahl and Andersson, 2000; Corry and Atabay, 2001; Nadeau et al., 2002; Kapperud et al., 2003; Neimann et al., 2003; Nielsen et al., 2006; Stafford et al., 2007; Doorduyn et al., 2010; EFSA, 2014). The consumption of chicken and chicken by-products has been increased due to their low price, special taste, and the short time required for preparation and consequently they have been implicated over the recent years in a large number of outbreaks of acute campylobacteriosis in human populations worldwide, in both industrialized and developing countries, and especially in children, the elderly and immuno-suppressed patients (Skirrow, 1998; Corry and Atabay,2001). In particular, the handling, preparation and consumption of broiler meat accounted for 20% to 30% of campylobacteriosis cases, while 50% to 80% attributed to the chicken reservoir as a whole (EFSA, 2010c). Furthermore, broiler meat was the most commonly implicated food vehicle, accounting for 11of the 25 strong-evidence outbreaks (44.0%) (EFSA, 2014).

Other possible sources of campylobacteriosis include other contaminated food, contaminated water, direct contact with farm animals, environmental sources and foreign travel. According to EFSA's report for 2012, among 19 EU MSs a total of 501 foodborne *Campylobacter* outbreaks were reported and this counted for 9.3 of the total reported foodborne outbreaks in the EU (EFSA, 2014).

Besides broiler meat, contaminated livers constitute a notable source of human campylobacteriosis. Outbreaks of *Campylobacter* infections linked to chicken and duck liver pâté have been reported in the United Kingdom (O'Leary et al., 2009), Australia (Parry et al., 2012), Europe (EFSA, 2013a) and USA (Tompkins et al., 2013). In addition, since 2007, England and Wales have mentioned a significant increase in the proportion of *Campylobacter* outbreaks linked to the consumption of chicken livers used in pâté (Little et al., 2010). These outbreaks did not come as a surprise, given that previous studies had shown that 77% of retail chicken livers were contaminated with *Campylobacter* (Little et al., 2010).

Some researchers point out eggs as a possible route of transmission since fecal contamination of the shell may take place and the survival of *Campylobacter* on eggshell is being promoted by the shell's moisture (Cox et al., 2012). In a study conducted by Messelhäusser et al. (2011) viable bacteria of *Campylobacter* spp. were found in 4.1% of the eggshell samples, whereas Jones and Musgrove (2007) found 0.5% of the restricted shell eggs investigated positive for thermotolerant *Campylobacter* spp. In Japan, Sato and Sashihara (2010) found that between 27.9 and 36% of unpasteurized liquid egg samples were positive for *Campylobacter*. Therefore, a contaminated eggshell always creates the risk of cross-contaminating the egg yolk with pathogens and of initiating food-borne infections by producing ready-to-eat food with raw or undercooked egg content. The other possibility is cross-contamination from the eggshell to other ready-to-eat products which do not contain the egg content itself (Cox et al., 2012).

In addition to risks from food, contact with animals, either domestic pets or farm animals, presents another exposure pathway for human infection (Saeed et al., 1993; Schorr et al., 1994; Studahl and Andersson, 2000; Moore et al., 2005). Other foods (such as pork, beef and unpasteurized milk), or direct contact with these animals were mentioned in the literature as pathways to acquire Campylobacter infection (Moore et al., 2005; Jacobs-Reitsma et al., 2008). The digestive tract of healthy cattle can be a significant reservoir for a number of Campylobacter species, with a prevalence of the enteropathogen in cattle ranging from 0–80% (Atabay and Corry, 1998) whereas the prevalence of Campylobacter spp.in sheep was about 20% (Zweifel and Stephan, 2004). Pig carcasses have been shown to be more frequently contaminated than either beef or sheep (Nesbakken et al., 2003). This is most likely attributable to the fact that pig carcasses undergo a communal scalding stage early in the slaughtering process combined with the fact that the skin remains on the carcass following all of the dressing procedures (Moore et al., 2005).

Raw milk has also been identified as a vehicle of human gastroenteritis caused by ***Campylobacter*** spp. (Weltman et al., 2013; EFSA, 2014). Especially, ***C. jejuni was found to be*** present in milk due to faecal cross-contamination during milking or as a result of udder infection (Orr et al., 1995).

Waterborne outbreaks of *Campylobacter* have been reported in many developed countries (Allos, 2001; Martin et al., 2006; Jakopanec et al., 2008; EFSA, 2013a).

In Greece, a waterborne Campylobacter jejuni outbreak occurred in Crete in 2009. Most cases originated from rural areas, served by a different water-supply system from that of the adjacent town and there was strong epidemiological evidence that tap water was the vehicle of the outbreak (Karagiannis et al., 2010a, Karagiannis et al., 2010b). Consumption of untreated water (Schorr et al., 1994) or rainwater (Eberhart-Phillips et al., 1997) was associated with campylobacteriosis in other studies. In an ecological study in Sweden, positive associations were found between the incidence of Campylobacter spp. and the average volume of water consumed per person. These observations suggested that drinking water and contamination from livestock might also be important factors in explaining at least a proportion of human sporadic campylobacteriosis cases (Nygard et al., 2004).

Contaminated shellfish have also been implicated as a vehicle in the dissemination of campylobacteriosis. Harvesting shellfish from *Campylobacter*-contaminated waters would appear to be the most likely cause of infection (Wilson & Moore, 1996).

Travel to a developing country is a risk factor for acquiring *Campylobacter*-associated diarrhea, which is more severe, and strains are more likely to be associated with antibiotic resistance (Coker et al., 2002). Campylobacteriosis acquired abroad contributes to the number of cases reported in developed countries and, as a result, represents an important subset of all cases. In the USA, 13% of *Campylobacter* infections are associated with international travel, and *Campylobacter* is the most frequently reported travel-associated infection (Kendall et al., 2012). In Scandinavia, the proportion of travel-related cases is higher, and systematic reporting of such infections has provided proxy surveillance information for parts of the world where diagnostic testing or reporting of the infection is less frequent (Ekdahl and Andersson, 2004).

CAMPYLOBACTER IN BROILER PRODUCTION

Broiler farms

Broiler intestines are a particularly favorable environment for the proliferation of thermophilic *Campylobacters*, such as *C. jejuni* and *C. coli*. Birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs (Lee & Newell, 2006). Broilers are considered *Campylobacter* free after hatching, since most evidence suggest that vertical transmission plays a minor role, if any (Jacobs-Reitsma et al., 1995; Pearson et al., 1996; Petersen & Wedderkopp, 2001; Sahin et al., 2003; Callicott et al., 2006) and in general, broiler flocks remain *Campylobacter* free for the first two weeks (Annan-Prah & Janc, 1988; Stern, 1992). Nevertheless, Cox et al. (2012) referred to trans-ovarian transmission since fecal bacteria, including *Campylobacter*, can contaminate the shell, shell membranes, and albumen of freshly laid eggs and the chick can become colonized after ingestion of the pathogen when it emerges from the egg. After the first colonization (usually at two to three weeks of age), following exposure to viable bacteria from the environment, *Campylobacter* spread quickly within the flock. The presence of *Campylobacter* in the caeca can be at a detectable level few hours after the exposure (Bull et al, 2006), while birds remain highly colonized until slaughter (Berndtson et al. 1996a, van Gerwe et al. 2009), representing an important public health risk.

The prevalence of *Campylobacter* in broiler flocks varies among different countries. A harmonized baseline survey was conducted in the EU in 2008, generating representative data regarding national production, in order to estimate the prevalence of *Campylobacter* in broilers and on broiler meat (EFSA, 2010a). Approximately 71.2% of broiler batches were estimated to be colonized by *Campylobacter* at the slaughterhouse. The prevalence of *Campylobacter*-colonized broiler batches among the EU member states varied widely, ranging from as low as 2.0% up to 96.8% (EFSA 2010a). The results of the EU baseline survey were consistent with several other studies (Rasschaert et al., 2007; Allen et al., 2008; Kuana et al., 2008; Hue et al., 2010; Hue et al., 2011; Lawes et al., 2012; Powell et al., 2012). In 2012, the overall proportion of *Campylobacter*-positive broiler flocks was 33.56 % (range: 0 % - 83.6 %) among the five MSs (e.g. Denmark, Germany, Hungary, Slovenia, Sweden) which reported flock-based data (EFSA, 2014). Several other flock-based studies have showed a prevalence from 15% up to 76% (Barrios et al., 2006; Arseunault et al., 2007a; Guerin et al., 2007; McDowell et al., 2008; Sasaki et al., 2010; Ansari-Lari et al., 2010)

Campylobacter jejuni is the predominant species isolated from poultry samples, followed by *C. coli*, with other *Campylobacter* species such as *C. lari* being less detected. In the southern EU MSs the presence of *C. coli* was more abundant, whereas *C. jejuni* was the only species isolated in the northern countries (EFSA, 2010a). Climatic conditions, environmental reservoirs, broiler housing and age of slaughter that vary significantly from northern to southern Europe could partly explain the observed variation of the species distribution (EFSA, 2010a). In addition, *C. coli* is more frequently identified in older animals and particularly from organic systems (El-Shibiny et al., 2005). Some studies mention that *C. coli* is more commonly isolated from poultry in the developing world. Specifically, *C. coli* was the dominant *Campylobacter* species isolated from poultry in Nigeria and Thailand (Aboaba and Smith, 2005; Padungtod and Kaneene, 2005). Poultry flocks and individual chickens might be infected with different *Campylobacter* strains at the same time (Jacobs-Reitsma et al., 1995; Rivoal et al., 1999). Furthermore, mixed infections can result in new strains through the exchange of genetic material (Jacobs-Reitsma et al., 1995; De Boer et al., 2002; Hook et al., 2005).

There is a paucity of data about the prevalence of *Campylobacter* spp. in broiler flocks in our country since Greece did not participated in the European union-wide baseline survey carried out in 2008. The isolation, identification, and antimicrobial resistance of *Campylobacter* spp. from poultry farms and slaughter houses has been investigated and reported for the first time in Greece by Marinou et al (2013). The results of this study showed a low prevalence (16/830 (1.9%) fecal samples) of *Campylobacter* spp. in five poultry farms in a geographical region around Athens, with the predominance of *C. coli*. However, the need for a surveillance and monitoring system for the prevalence, risk factors and antimicrobial resistance of *Campylobacter* in poultry and other food animals is a requisite and more studies about this topic should be carried out.

The incidence and prevalence of *Campylobacter* in positive broiler flocks varies depending on geographical, farming and environmental conditions. Seasonality effects have been observed with a marked peak during summer months, much more noticeable in Northern Europe (Bouwknegt et al., 2004; Patrick et al., 2004; Hofshagen and Kruse, 2005; Hansson et al., 2007; van Asselt et al., 2008; Jore et al. 2010; Zoonosis Centre, 2012;) than in Southern Europe (Nylen et al., 2002). In contrast, some studies in the United Kingdom, USA, and Canada have reported no seasonal influence on *Campylobacter* prevalence (Humphrey et al., 1993; Gregory et al., 1997; Nadeau et al., 2002). Seasonality effects could be explained by environmental factors, which require further investigation, such as humidity, temperature and sunlight (Wallace et al., 1997; Arsenault et al., 2007a; Guerin et al., 2008). For instance, a warmer mean temperature and the moister climate during summertime provide conditions favoring environmental *Campylobacter* survival, as well as increase the amount of insects, wild birds and rodents, which act as mechanical vectors for the pathogen, around the broiler house (Hald et al. 2004, Rushton et al. 2009, Jore et al. 2010). Except of the abundance of flies, the increased ventilation because of higher temperatures during the summer has also been related to the seasonal variation (Hald et al., 2008). It has been also claimed, that in the Nordic countries, the cold winters contribute to the decrease of the *Campylobacter* environmental load.

Remarkably, the increase in human cases can sometimes occur previous to infections in chickens, suggesting that there might be a common risk factor responsible for the increase in *Campylobacter* cases. Flies can transmit *Campylobacter* to chickens and humans and they could partly explain the seasonality of human cases (Hald et al., 2004; Nichols, 2005; Ekdahl et al., 2005; Nelson et al., 2006; Guerin et al., 2008; Hald et al., 2008; Nichols, 2010).

Broiler slaughterhouses - Carcasses

The intestinal colonization of broilers with *Campylobacter* during rearing is responsible for the contamination of the carcasses and equipment with *Campylobacter* during slaughtering (Rosenquist et al. 2006, Reich et al. 2008; Silva et al., 2011). Food processing areas that constitute critical control points in poultry processing plants are usually scalding, defeathering and evisceration, since the carcass contamination occurs there by leakage of the contaminated faeces from the cloaca and visceral rupture of the ceca carrying a high *Campylobacter* load (Berrang et al., 2001; Stern & Robach, 2003; Takahashi et al., 2006; Boysen & Rosenquist, 2009; Silva et al., 2011). Automated defeathering represents a high risk practice since cloacal contents can cause contamination of the carcasses (Berrang et al., 2001). *Campylobacter* spp. remain in a liquid film on the skin and become entrapped in its cervices and channels which provides a favourable environment for cross contamination (Chantarapanont et al., 2003). Cross-contamination of *Campylobacter* strains between slaughtered flocks may also occure via contacts with contaminated surfaces of the slaughter facilities, processing water and air (Peyrat et al. 2008, Perko-Mäkelä et al. 2009; Isohanni, 2013). Furthermore, the persistence and survival of *Campylobacter* spp. are fostered by a suitable microenvironment of the skin (Chantarapanont et al., 2003) and even under frozen conditions or storage at 4°C, *Campylobacter* spp. are able to persist in the carcass (Maziero and de Oliveira, 2010). Previous studies reported that growth on skin stored at room temperature in a controlled atmosphere package is possible, increasing the risk for consumers if contaminated chicken is not adequately stored or handled (Lee et al.,1998; Scherer et al.,2006). It has been found that carcasses from batches with *Campylobacter*-positive caeca have significantly higher quantitative loads than those from batches with negative ceca, which is in accordance with other studies, indicates that reduction in intestinal contamination could be a possible way to reduce the amount of bacteria on carcasses (EFSA, 2010a; Hue et al. 2011).

The average prevalence of *Campylobacter* contamination on broiler carcasses worldwide is reported to be in the range of 60-80% (Suzuki & Yamamoto, 2009; Isohanni, 2013). According to EFSA (2010a), the prevalence in the EU of *Campylobacter*-contaminated broiler carcasses, in 2008, was reported as 75.8% and varied from 4.9% to 100.0% among the EU MSs. That prevalence is higher than the respective prevalence for broiler batches, which come into accordance with the results of other studies (Hue et al., 2011; Powell et al., 2012; Chokboonmongkol et al., 2013), assuming that cross-contamination from positive batches to negative batches does occur during the slaughtering process and associated carcass preparation (Jørgensen et al. 2002; Johannessen et al. 2007; EFSA, 2010a; Hue O. et al, 2011) through contamination of the slaughterhouse environment (Johnsen et al 2006). The counts of *Campylobacter* bacteria on broiler carcasses varied widely also between countries, which might be due to differences in slaughterhouse hygiene and processing practices (Habib et al., 2008; Sampers et al., 2008; EFSA, 2010a). In general there was a tendency for high counts in countries with high *Campylobacter* prevalence. Low *Campylobacter* numbers on broiler carcasses may reflect effective pre-harvest production procedures, good slaughter hygiene, low within-flock prevalence or low cross-contamination of carcasses of a *Campylobacter*-negative batch from a previous positive batch (Johannessen et al. 2007). The elevated levels of *Campylobacter* can be recovered from the broiler carcasses and transmitted in the food chain during further processing (EFSA 2010a).

The distribution of *Campylobacter* species isolated from broiler carcasses varies among different countries. *Campylobacter jejuni* proved to be the predominant species at EU level, with about two-thirds of the total isolates being identified as *C. jejuni*, while approximately one-third was *C. coli*. Other *Campylobacter* species are less frequently identified (EFSA, 2010a). Still, the reverse situation was observed in some MSs reporting dominance of *C. coli* isolates. Moreover, a high proportion of *C. coli* in poultry meat has been reported from some other parts of the world (Meeyam et al., 2004; Padungtod et al., 2005; van Nierop et al., 2005; Suzuki & Yamamoto, 2009). In Greece, no information is available, since there is no surveillance and monitoring system. According with the study performed by Marinou et al. (2013), no *Campylobacter* was isolated from the cecal samples of the chicken carcasses.

Retail broiler meat products

Broiler meat is considered to be the main foodborne source of human campylobacteriosis. According to EFSA (2014), a large share of retail broiler meat remains contaminated with *Campylobacter*. In 2012, approximately 30% of the samples of poultry meat in retail were found to be positive in the 9 EU MSs reporting data on testing of single broiler samples, (range: 0 % - 80.6 %). The reported levels of *Campylobacter* in fresh broiler meat products at retail vary between log 1 to log 4 cfu/100 g (or a fillet) of meat, depending on the different studies and methodologies used (Jacobs-Reitsma et al., 2008). Studies report that *C. jejuni* was usually the dominant *Campylobacter* species isolated from retail broiler meat products worldwide, but the ratio of *C. coli* to *C. jejuni* varied between countries (Suzuki and Yamamoto, 2009). Limited studies have been published on the prevalence of *Campylobacter* in broiler meat at the Greek retail level. The presence of *Campylobacter* spp. in poultry meat, along with isolation, identification at species level and determination of the antibiotic resistance of the isolates has been investigated by Petridou and Zdragas (2009) in Northern Greece. The results of Petridou & Zdragas study showed that 73% of the samples were *Campylobacter* positive, while *Campylobacter jejuni* seemed to be the predominant species. Moreover, the prevalence of *Campylobacter* spp. in raw broiler meat was investigated by Zisidis (2011) during the period from 2005 to 2010. The samples were collected from several slaughterhouses, poultry meat selling points and restaurants of Western Greece. The results showed that 28.7% of the samples were *Campylobacter* positive, with *C. jejuni* as predominant species and a remarkable decline of positive results was observed through the study from 50% in 2005 to 18.5% in 2010. However, there is still a need of more investigation in order to determine the true prevalence of *Campylobacter* spp. in our country.

Risk factors associated with *Campylobacter* spp. colonization in broiler flocks and broiler carcasses contamination

Several risk factors can result in the introduction of *Campylobacter* into the flocks making it difficult to keep chicken flocks free of *Campylobacter* throughout the rearing period. The possible sources and transmission routes of *Campylobacter* for poultry flocks have been investigated extensively, focusing on different parts of the production processes and practices. Most epidemiological studies have focused on the outcome being the flock becoming infected, not considering the within flock prevalence nor the amount of *Campylobacter* in the infected chickens. The outside environment has been suggested as the ultimate source of colonization for broiler flocks. In addition, many factors - such as adjacent broiler units or other animals, farm workers, drinking water, rodents, wild birds, flies and other insects - may have a role in transmitting *Campylobacter* to broiler flocks (Hald et al. 2004, Bull et al. 2006, Rushton et al. 2009).

The most important risk factors associated with horizontal transmission of *Campylobacter* spp. to broiler flocks and broiler carcass contamination during the slaughtering process are shown in *Table 1* and *Table 2* respectively.

Controlling of *Campylobacter* spp. *infection through active surveillance*

Burden of disease studies provide evidence that there is a need for control measures across all outcomes of campylobacteriosis while taking into consideration its underestimation (WHO, 2013). Nowadays, the implementation of effective controls to reduce the burden of disease in humans is considered a priority in many areas of the world. Consequently, the control of *Campylobacter* in poultry seems crucial for the reduction of human campylobacteriosis cases.

European Food Safety Authority has emphasized the importance and recommended the establishment of an active surveillance of campylobacteriosis in all MS, including efforts to determine the uncertain and unreported campylobacteriosis cases. In addition, storage and genotyping of human and putative reservoirs of isolates in all MS have also been recommended (EFSA, 2011). Thereafter, it would be important to identify the *Campylobacter* properties of virulence, survival characteristics and ecology (EFSA, 2011).

CONCLUDING REMARKS

In conclusion, the necessity to study the prevalence of the disease in the poultry population and identify the risk factors associated with this in Greece should be stressed. The cross sectional study which is currently being carried out in Greece, will give important information on prevalence of *Campylobacter* infection in poultry production and will be the foundation in understanding the epidemiology of the microorganism countrywide.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

Table 1. Risk factors with an increased association with Campylobacter spp. colonization in broiler flocks along with the corresponding references.

RISK FACTOR	REFERENCES
Season (summer months)	Bouwknegt et al., 2004; Barrios et al., 2006; Huneau-Salaün et al., 2007; Zweifel et al., 2008; McDowell et al., 2008; Ellis-Iversen et al., 2009; Jore et al., 2010; EFSA, 2010b; Lawes et al., 2012; Chowdhury et al., 2012a
Age of broilers	Berndtson et al., 1996b; Evans & Sayers, 2000; Bouwknegt et al., 2004; Barrios et al., 2006; McDowell et al., 2008; EFSA, 2010b; Ansari- Lari et al., 2011; Chowdhury et al., 2012a; Lawes et al., 2012; Sommer et al., 2013
Partial depopulation practices	Hald et al., 2000; Hald et al., 2001; Slader et al., 2002; Ellis-Iversen et al., 2009; Hannson et al., 2010; EFSA, 2010b; Lawes et al., 2012
Lack of biosecurity measures	Humphrey et al., 1993; Van de Giessen et al. 1996; Gibbens et al., 2001; Herman et al., 2003; Cardinale et al., 2004
Flock size	Berndtson et al., 1996b; Barrios et al., 2006; Guerin et al., 2007a; Nather et al., 2009
Human traffic and farm equipment	Berndtson et al., 1996b; Evans & Sayers, 2000; Hald et al., 2000; Cardinale et al., 2004; Ramabu et al., 2004; Hofshagen & Kruse, 2005
Other animals on the farm or very close to the farm	van de Giessen et al., 1996; Bouwknegt et al., 2004; Cardinale et al., 2004; Lyngstad et al., 2008; Ellis-Iversen et al., 2009; Hannson et al., 2010; Sommer et al., 2013
General farm hygiene	Hald et al., 2000; Evans & Sayers, 2000; McDowell et al., 2008; Hannson et al., 2010
Type of drinking system	Näther et al., 2009
Contaminated water	Pearson et al., 1993; Zimmer et al., 2003
Contaminated air from adjacent poultry houses	Berndtson et al., 1996a
Mechanical transmission via insects	Berndtson et al., 1996a; Refregier-Petton et al., 2001
Infected wild birds	Chuma et al., 2000; Craven et al., 2000
Health and welfare status	Bull et al., 2008
Presence of rodents	Gregory et al., 1997; Huneau-Salaün et al., 2007; McDowell et al., 2008; Sommer et al., 2013
Free-range & organic flocks	Näther et al., 2009

Table 2. Risk factors with an increased association with broiler carcass contamination along with the corresponding references.

RISK FACTOR	REFERENCES
Slaughter in summer months	EFSA, 2010b; Powell et al., 2012
Age of broilers	EFSA, 2010b
Previous thinning of the flock	Hue et al., 2010
Batch was not slaughtered first in the slaughter program	Hue et al., 2010
Temperature in evisceration room (oC)	Hue et al., 2010
Presence of dirty marks on eviscerated carcasses	Hue et al., 2010
Time (hour) of sampling during day	EFSA, 2010b
Campylobacter-colonization in the broiler batch	Arsenault et al., 2007b; EFSA, 2010b
Batches with higher standard deviation of carcass weight	Mahler et al., 2011

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ARTICLE II

Natsos G., Mouttotou N.K., Ahmad S., Kamran Z., Ioannidis A., Koutoulis K.C. (2019)

“The genus *Campylobacter*: detection and isolation methods, species identification & typing techniques.”

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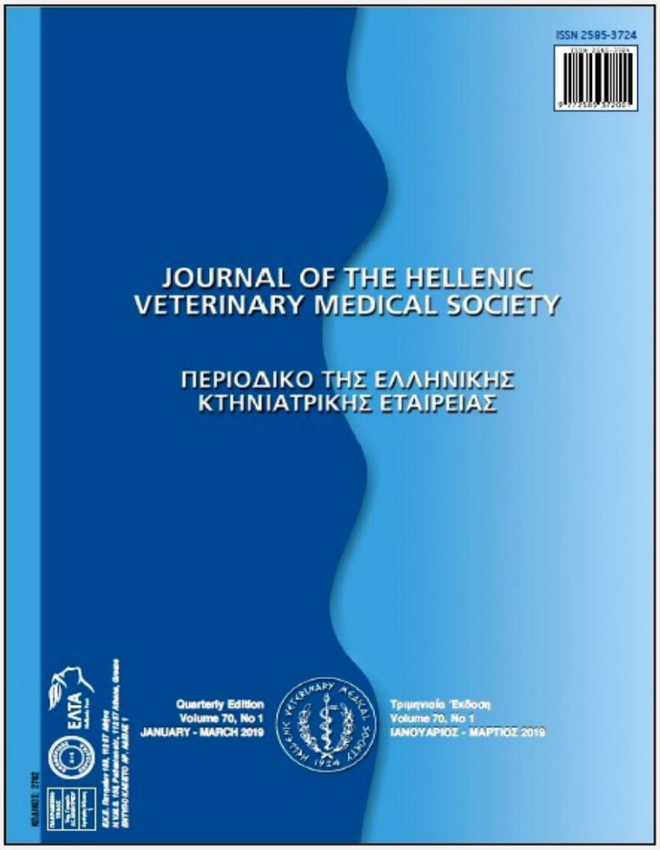

The genus Campylobacter: detection and isolation methods, species identification & typing techniques

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Review article
Ανασκόπηση

The genus *Campylobacter:* detection and isolation methods, species identification & typing techniques

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Το γένος *Campylobacter:* μέθοδοι ανίχνευσης και απομόνωσης, ταυτοποίηση είδους και τεχνικές τυποποίησης

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ABSTRACT. *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, poultry has been identified as a significant cause of campylobacter infection in humans. The *C. jejuni* has been found to be the predominant species isolated from poultry samples and, yet, responsible for the majority of human campylobacteriosis. *Campylobacter* spp. are small, oxidase positive, microaerophilic, curved gram-negative rods exhibiting corkscrew motility and colonize the intestinal tract of most mammalian and avian species. From its very first description in late 19th century by Theodor Escherich until nowadays, a lot of research has been carried out providing a wealth of information regarding its microbiological properties. Since novel technologies constantly emerge, increasingly advanced methods for detection, identification and typing of *Campylobacter* spp. are becoming available. The aim of this article is to review the recent bibliography on *Campylobacter* focusing, especially, on its survival and growth characteristics, the laboratory methods used for its detection and isolation from clinical, animal, environmental, and food samples, the reported methods applied for its speciation, as well as the typing systems developed for subtyping of *Campylobacter.*

Keywords: *Campylobacter* spp., detection, isolation, species identification, typing.

ΠΕΡΙΛΗΨΗ. Το *Campylobacter* είναι παγκοσμίως αναγνωρισμένο ως ο συχνότερος αιτιολογικός παράγοντας της βακτηριακής αιτιολογίας, διαρροϊκής τροφοδηλητηρίασης, ενώ τα πουλερικά έχουν αναγνωριστεί ως η κύρια αιτία μόλυνσης του ανθρώπου. Το *C. jejuni* είναι το είδος που απομονώνεται συχνότερα από δείγματα προερχόμενα από πουλερικά και συνεπώς ευθύνεται για τα περισσότερα περιστατικά ανθρώπινης καμπυλοβακτηρίωσης. Τα *Campylobacter* spp. είναι μικροί, θετικοί στη δοκιμή οξειδάσης, μικροαερόφιλοι,gram-αρνητικοί, κυρτοί βάκιλοι που παρουσιάζουν χαρακτηριστική ελικοειδή κίνηση και αποικούν τον εντερικό σωλήνα των περισσότερων θηλαστικών και πτηνών. Από την πρώτη περιγραφή τους στα τέλη του 19ου αιώνα από τον Theodor Escherich έως σήμερα, έχει διεξαχθεί σημαντική έρευνα που πρόσφερε πλούτο πληροφοριών σχετικά με τα μικροβιολογικά τους χαρακτηριστικά. Χάρη στη συνεχή εμφάνιση καινοτόμων τεχνολογιών, όλο και πιο προηγμένες μέθοδοι ανίχνευσης, ταυτοποίησης και γενοτύπησης γίνονται διαθέσιμες. Σκοπός αυτού του άρθρου είναι η ανασκόπηση της πρόσφατη βιβλιογραφία σχετικά με το *Campylobacter* εστιάζοντας κυρίως στα καλλιεργητικά του χαρακτηριστικά, τις εργαστηριακές μεθόδους που χρησιμοποιούνται για την ανίχνευση και την απομόνωσή του από κλινικά, ζωικά, περιβαλλοντικά και δείγματα τροφίμων, τις καταγεγραμμένες μεθόδους που χρησιμοποιούνται για τη ταυτοποίηση του είδους, καθώς και τα συστήματα γενοτύπησης που έχουν αναπτυχθεί για την υποτυποποίηση του *Campylobacter*.

Λέξεις ευρετηρίασης: Campylobacter spp., τροφιμογενή παθογόνα, Ελλάδα, πτηνά, επιπολασμός, παράγοντες κινδύνου.

INTRODUCTION

Campylobacters are ubiquitous bacteria, able to colonize mucosal surfaces, usually the intestinal tract of most mammalian and avian species tested (OIE, 2008). *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, the poultry has been identified as a significant source for *Campylobacter* infections in humans. The *C. jejuni* is the predominant species isolated from poultry samples, followed by *C. coli,* and other less-detected *Campylobacter* species such as *C. lari* (EFSA, 2010). The *C. jejuni* is considered responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by C. lari (Zhang and Sahin, 2013). The incidence of human campylobacteriosis has been steadily rising worldwide since 1990's (WHO, 2011). While in Greece there is a dearth of data (Natsos et al., 2016), in the European Union, campylobacteriosis has been the most commonly reported zoonosis since 2005 (EFSA, 2006; EFSA and ECDC, 2017), in the United States, the incidence of Campylobacter infections per 100,000 people was the highest along with Salmonella (CDC, 2018), in Australia *Campylobacter* has been found to be the most common cause of acute bacterial diarrhea among all the notified enteric pathogens (Stafford, 2010), while human campylobacteriosis is hyperendemic in many developing areas of the world (Coker et al., 2002).

THE GENUS Campylobacter: A HISTORICAL OVERVIEW

The generic name *Campylobacter*, from the Greek kampylos (curved) and baktron (rod), was given by Sebald and Véron (1963) to the group of bacteria formerly known as the microaerophilic vibrios, due to their special *characteristics* (Moore et al., 2005). It is believed that Campylobacter species were first described by Escherich (1885) who observed non-culturable spiral-shaped bacteria in the large-intestinal mucus of infants who had died of cholera infantum (Vandamme, 2000), while McFadyean and Stockman (1913) were the first to isolate these organisms from the uterine exudate of aborting sheep. A few years later, the study of Butzler et al. (1973) raised the interest in *Campylobacter* as a cause of human disease by noting their high incidence in cases of diarrhea. The first successful isolation of Campylobacter from human faeces had been accomplished one year before by using a filtration technique (Dekeyser et al., 1972). Later, the isolation of Campylobacter became a routine in the field of clinical microbiology and Campylobacter spp. rapidly became recognized as a common cause of bacterial gastroenteritis (Fitzgerald et al., 2008a).

CLASSIFICATION

In the 1970s, there was much confusion over *Campylobacter* nomenclature (Skirrow, 1994); however, the classification of Véron and Chatelain (1973) forms the basis of currently approved nomenclature. The family Campylobacteraceae, proposed by Vandamme and De Ley (1991), consists of two genera, *Campylobacter* and Arcobacter (Vandamme, 2000); while, the genus of *Campylobacter* currently contains 34 species and 14 subspecies (Parte, 2014). The taxonomy of the Campylobacter genus, which has been revised many times (Debruyne et al., 2008), is reviewed by On (2001).

MORPHOLOGY

Members of the *Campylobacter* genus are slender, spirally-curved, and non-sporeforming gram-negative rods. The size of the cells is small and ranges from 0.2 to 0.9 μm in width and 0.5 to 5 μm in length (Silva et al., 2011). Some species, such as *C. hominis* and *C. gracilis,* form straight rods (Fitzgerald et al., 2008a). Most species are motile by means of a single polar unsheathed flagellum inserted at one or both poles of the cells (monotrichate or amphitrichate) (Vandenberg et al., 2005). The only exceptions are *C. showae*, which has up to five unipolar flagella, and C. gracilis, which has none and is immotile (Debruyne et al., 2008). Motility is rapid and darting, with the bacteria spinning around their long axes in a corkscrew fashion (Vandenberg et al., 2005). Because of their small size and motility, *Campylobacter* spp. are able to pass through membrane filters (0.45 to 0.65 μm) with relative ease, a property used for isolating Campylobacter spp. from clinical samples (Bolton, 2000; Steele and McDermott, 1984).

GROWTH AND SURVIVAL CHARACTERISTICS

Under ideal conditions, Campylobacters produce visible growth after 24 h at 37 °C, but colonies are not well formed until 48 h; however, it may take up to 72-96 hours of incubation to observe some slow-growing strains (Corry et al., 1995). Depending on the media used, the appearance of *Campylobacter* colonies may vary. If the agar is moist, the colonies may appear gray, flat, irregular, and thinly spreading; whereas, round, convex, or glistening colonies may be formed when plates are dry (Corry et al., 1995; Vandenberg et al., 2005). Since the pathogenic *Campylobacter* species grow at 37-42 °C, with an optimum growth temperature of 41.5 °C, they are used to be referred as thermophilic Campylobacters: although Levin (2007) suggested the term "thermotolerant" since they do not exhibit true thermophily (growth at 55°C or above). Campylobacters are incapable of growth below 30°C, as they lack cold shock protein genes which play a role in low-temperature adaptation (Silva et al., 2011).

These non-spore-forming and fastidious bacteria neither ferment nor oxidize carbohydrates; instead, they obtain energy from the degradation of amino acids, or tricarboxylic acid cycle intermediates (Kelly, 2001; Vandamme, 2000). They are essentially microaerophilic, thus an atmosphere with low oxygen tension (5% O2, 10% CO2, and 85% N2) is regarded as the most suitable for *Campylobacter's* incubation (Garénaux et al., 2008). Oxidase activity is present in all species except for *C. gracilis* (Silva et al., 2011).

Except of their fastidious growth requirements, *Campylobacter* spp. are very fragile and more susceptible than most bacteria to many environmental conditions, such as temperature and pH changes, low humidity, presence of oxygen and UV irradiation, and to many chemical agents such as disinfectants (Isohanni, 2013). *Campylobacter* spp. are easily inactivated by heat treatments with their D-value being less than 1 min (Silva et al., 2011), while freezing and thawing causes a 1-2 log10 fall in viable numbers, yet bacteria remain alive for many months at -20 oC (Vandenberg et al., 2005). Most species have a pH growth range of 5.5-8.0, though optimal growth occurs at pH 6.5-7.5 and water activity (aw) equal to 0.997 (approximately 0.5% w/v NaCl), as mentioned by Silva et al. (2011).

In some species, notably *C. jejuni* and *C. lari,* cultures that are exposed to atmospheric oxygen (Vandenberg et al., 2005) or other unfavorable conditions, such as changes in temperature and pH, dehydration and low nutrient availability, may undergo coccal transformation (Jackson et al., 2009; Kassem et al., 2013; Oliver, 2010; Rollins and Colwell, 1986), which seems to be a degenarative process in response to these circumstances (Harvey and Leach, 1998; Reezal et al., 1998). Those viable but non-cultivable cells (VBNC) have been shown to be unable to grow in subculture; even though the possibility that they can revert to spiral forms after passing through the intestinal tract of chickens or humans remains unanswered (Oliver, 2010; Vandenberg et al., 2005) and even their existence is contentious (Silva et al., 2011).

LABORATORY ISOLATION AND DETECTION METHODS

In a clinical context, a laboratory is mainly asked to detect campylobacters in the faeces of patients with diarrhea. The same purpose also applies when it comes for samples derived from animal stool, environmental materials, or processed food. There are two main categories regarding the detection method used: the conventional culture-based isolation methods and the culture-independent methods.

Culture-based isolation methods

The conventional method for isolating the common enteric *Campylobacter* species from faecal samples is a primary plating on selective media followed by incubation at 42 oC in a microaerobic atmosphere (Vandenberg et al., 2006). Though faeces often contain large numbers of viable *Campylobacter* making their detection easily possible by direct plating on selective media (Fitzgerald et al., 2008b), food products and environmental samples tend to have fewer numbers of stressed *Campylobacter* cells, thus, an enrichment step in liquid medium before plating on solid agar plates is indicated (Corry et al., 1995). Several enrichment broths (e.g. Bolton broth, *Campylobacter* enrichment broth and Preston broth), that are available to be used before plating, have been compared for their efficacy (Baylis et al., 2000).

The first selective culture medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow. Since then more than 40 solid and liquid selective culture media for culturing *Campylobacter* from clinical and food samples have been reported and evaluated (Habib et al., 2008; Kiess et al., 2010; Potturi-Venkata et al., 2007). All the selective media contain a basal media, either blood or other agents such as charcoal, to quench oxygen toxicity (Fitzgerald et al., 2008a), and a variety of combinations of antibiotics to which thermophilic *Campylobacter* species are intrinsically resistant; such antibiotics (like polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin) suppress the growth of many background microbial flora present in samples allowing the isolation of slow-growing Campylobacter spp. (Vandenberg et al., 2005; Zhang and Sahin, 2013).

The most recent standard method (ISO, 2006a) for detection and isolation, as well as a direct plating method for enumeration of *Campylobacter* spp. (ISO, 2006b), use mCCDA as the selective agar, while Bolton broth is used for the enrichment step. Alternative enrichment and plating combinations for enumeration and detection of *Campylobacter* in chicken meat have been evaluated (Habib et al., 2011) and seem to provide significantly better results.

Direct detection methods

Microscopic observation of direct smear or wet preparation, in the case of liquid feaces, may reveal the presence of curved rods characteristic of *campylobacters* (Vandenberg et al., 2005). Dark-field microscopy may also reveal – besides the characteristic morphology – the darting motility of Campylobacter species (Fitzgerald et al., 2008a). Moreover, the direct Gram-stain with carbol-fuchsin counterstain method, though underutilized, may provide a presumptive result within 30 minutes of receipt of a feacal sample in the laboratory with relatively high sensitivity and at low cost (Wang and Murdoch, 2004).

There are also nonculture-based methods for the direct detection of campylobacters in human or animal faeces and processed food samples, which allow the identification of this fastidious organism without the specialized media and equipment needed for Campylobacter culture. Several enzyme immunoassays (EIA), which are based on antigen-antibody interaction, have been developed for this purpose in human faeces and are commercially available in a form of kits (Bessède et al., 2018; Dediste et al., 2003; Granato et al., 2010; Tolcin et al., 2000). While the culture-independent diagnostic tests (CIDTs) are convenient to use, the sensitivity, specificity, and positive predictive value of *Campylobacter* stool antigen tests have found to be highly variable (Bessède et al., 2011; Giltner et al., 2013; Granato et al., 2010) and therefore their use as standalone tests for direct detection of *Campylobacter* in stool is questioned. In addition, the utility of these assays for detection of campylobacters in chicken faeces, which represent the main reservoir of pathogenic *Campylobacter* species, remains to be determined (Zhang and Sahin, 2013). Regarding the food samples, although commercial EIAs are available for culture-independent identification of *Campylobacter* spp., these assays have not been extensively validated (Oyarzabal and Battie, 2012) and are mainly applied to enriched cultures (Bailey et al., 2008; Bohaychuk et al., 2005). Commercial and/or published immunological methods used to identify *Campylobacter* spp. in food and stool samples have been reviewed by Oyarzabal and Battie (2012).

Many PCR-based assays have been described to directly detect campylobacters in human stools from clinical cases (Al Amri et al., 2007;Lin et al., 2008; Zhang et al., 2013), feacal samples from bovine (Inglis and Kalischuk, 2004) and pigs (Jensen et al., 2005; Leblanc-Maridor et al., 2011), ceacal and feacal samples from broilers (Al Amri et al., 2007; Lund et al., 2003; Rodgers et al., 2012), samples from poultry meat (Debretsion et al., 2007; Fontanot et al., 2014; Hong et al., 2007; Josefsen et al., 2010; Schnider et al., 2010) and environmental specimens (Rothrock et al., 2009; Waage et al., 1999); although, so far these have been used only for research applications. Advantages of using a PCR approach instead of culture include same-day detection and identification of *Campylobacter* to the species level, along with the identification of the less-common Campylobacter species that are often missed by conventional culture (Kulkarni et al., 2002). However, PCR methods are more expensive and labor-intensive than culture and do not provide an isolate for further characterization such as typing and sensitivity testing.

Finally, fluorescent in situ hybridization (FISH), with the application of highly specific oligonucleotide probes, may serve for the detection and identification of thermotolerant Campylobacter spp. in feacal and liver samples, and looks promising to become a future monitoring system in a logistic poultry slaughter concept (Schmid et al., 2005).

SPECIES IDENTIFICATION

Among the Campylobacter spp. growing at 42 °C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli,* however, low frequencies of other species have also been reported. Speciation is difficult because of the complex and rapidly evolving taxonomy along with the biochemical inertness of *Campylobacter* spp., and these problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group (Fitzgerald et al., 2008a).

Campylobacters are biochemically inactive compared with many other bacteria, thus, few phenotypic tests are available to identify them to the species level. Generally, *C. jejuni* can be differentiated from other species based on the hydrolysis of hippurate as this is the only *Campylobacter* species that expresses the N-benzoylglycine amidohydrolase (hippuricase) gene, giving hippurate-positive result. However, variability in the hippurate reaction has been observed in some strains of *C. jejuni* resulting in hippurate-negative results (Denis et al., 1999; Jensen et al., 2005; Rautelin et al., 1999). Nalidixic acid and cephalothin susceptibility testing have been used in species identification in the past (Barrett et al., 1988). Both *C. jejuni* and *C. coli* grow at 42 °C and are resistant to cephalothin and cefoperazone, which are valuable agents for inclusion in selective media (Vandenberg et al., 2006). Instead, *C. upsaliensis* is sensible to cephalothin (ISO, 2006a). Nowadays sensitivity to nalidixic acid may give difficulties in interpretation (OIE, 2008) since fluoroquinolone resistant and cross-resistant to nalidixic acid *Campylobacter* species have become increasingly common with rates reported to be as high as 80% (Engberg et al., 2001), therefore, antimicrobial susceptibility tests can no longer be relied upon for the phenotypic identification of *Campylobacter* isolates (Fitzgerald et al., 2008a). More biochemical tests may be applied for species identification, such as the detection of catalase which is absent in *C. upsaliensis,* and the detection of indoxyl acetate hydrolysis which is negative in *C. lari* (ISO, 2006a); whereas, more extensive speciation schemes have been described in the literature (On, 1996; Vandamme, 2000).

Because of the difficulties and the unreliability of the phenotypic identification, several molecular methods may be used as supplementary to biochemical tests or even to replace them. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for *Campylobacter* species (On, 1996; Vandamme, 2000). Detection of species-specific sequences via PCR can be helpful, especially, in cases where the differentiation between hippuricase-negative *C. jejuni* strains and *C. coli* – which are closely related species – is needed, and the application of biochemical tests alone is inadequate (Denis et al., 1999; Persson and Olsen, 2005).

TYPING AND SUBTYPING

Classification of bacterial strains at the species or subspecies level is generally known as bacterial typing or subtyping. The main purposes of bacterial subtyping are the evaluation of taxonomy, the definition of phylogenetic relationships, the examination of evolutionary mechanisms, and the conduct of epidemiological investigations (Van Belkum et al., 2001). Moreover, the use of typing methods provides the opportunity to apply more rapid, precise, and efficient foodborne pathogen surveillance and prevention practices (Wiedmann, 2002). The ability to discriminate or subtype campylobacters below the level of species has successfully been applied to aid the epidemiological investigation of outbreaks of campylobacteriosis (French et al., 2011; Sails et al., 2003a; Wassenaar and Newell, 2000), providing information to recognize outbreaks of infection, to match cases with potential vehicles of infection and to discriminate these from unrelated strains.

Typing of *Campylobacter* is a dynamic field with older methods continually being advanced and new methodologies constantly being developed (Ross, 2009). A multitude of typing systems have been developed over the last few years, however, no single technique has been declared as universally acceptable and applicable (Sails et al., 2003a), since each one has both advantages and disadvantages. A number of criteria are used to evaluate subtyping methods to define their efficacy and efficiency: two major properties that any typing system should possess in order to be adapted for further use (ECDC, 2009). The efficacy of any typing technique can be assessed in terms of typeability, reproducibility, consistency, and power of discrimination; while, the efficiency reflects the expertise required, time consumed or rapidity of the technique, flexibility, and suitability to carry out a certain investigation (Mohan, 2011).

Typing systems are based on the idea that clonally related isolates share common characteristics which can be tested to differentiate them from unrelated isolates (Eberle and Kiess, 2012). They are broadly classified into two major categories: phenotyping – applies phenotypic methods that detect the presence or absence of biological or metabolic activities expressed by the bacteria, and genotyping – utilizes genotypic methods that involve analysis of genetic elements based on the bacteria's DNA and RNA (Arbeit, 1995).

Phenotypic methods

The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, phage typing, and multilocus enzyme electrophoresis. Even though most of these methods lack discriminatory power, they are still applied and are quite efficient in characterizing foodborne bacterial pathogens (Wiedmann, 2002).

Biotyping schemes based on the identification of bacterial isolates through the expression of metabolic activities, such as colonial morphology, environmental tolerances, and biochemical reactions, can group *C. jejuni, C. coli* and *C. lari* in broad categories (Eberle and Kiess, 2012; Vandenberg et al., 2006). Biotyping is useful as a first step for epidemiological investigation as it is easy to perform, relatively inexpensive, and can quickly identify bacterial isolates for further testing, however, due to its poor reproducibility and stability, and low discriminatory power it is often combined with serotyping to make the scheme more useful (Sails et al., 2003b).

Serologic typing, or serotyping, is based on the knowledge that different strains of bacteria differ in the antigens they carry on their cellular surfaces. In serotyping, antibodies and antisera are used to detect these surface antigens, thereby, distinguishing strains by the differences in their surface structure (Arbeit, 1995; Wiedmann, 2002). There are two generally accepted and well-evaluated serotyping schemes that were developed in the 1980s for epidemiological characterization of *Campylobacter* isolates: the first one is based on the heat stable O antigens (LPS, LOS and CPS) using a passive hemagglutination technique and was described by (Penner and Hennessy, 1980), and the other one, developed by Lior et al. (1982), is based on heat labile antigens using a bacterial agglutination method. Since the two schemes are complementary, they can give good discrimination when used together even with restricted panels of antisera (Vandenberg et al., 2005).

Phagetyping was initially performed to characterize *C. jejuni* and by (Grajewski et al., 1985) and is often used as an adjunct to serotyping. Concisely, the technique utilizes a set of virulent phages on a bacterial host irrespective of any receptors for attachment. If the phages are able to attach and infect the bacterial hosts, they lyse the bacterial cells producing a characteristic lytic pattern on the cultured petri dishes, referred to as 'plaques' (Grajewski et al., 1985). Like serotyping, the usefulness of phagetyping is also limited by the occurrence of non-typeable isolates and problems with cross reactivity (Sails et al., 2003b).

In multilocus enzyme electrophoresis (MLEE), bacterial isolates are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing conditions (Wiedmann, 2002). This technique has been utilized to study the congruence between other typing schemes used for *C. jejuni,* such as multilocus sequence typing (MLST) and pulse field gel electrophoresis (PFGE) (Sails et al., 2003b). Because of its limitations, MLEE has been rendered unsuitable for regular typing and has been superseded by a nucleotide-based technique, MLST, which essentially mimics the MLEE's multi loci principle (Mohan, 2011).

Genotyping methods

The limitations associated with phenotypic subtyping methods along with the rapid growth of molecular techniques have led to the development of a wide range of molecular subtyping methods (Fitzgerald et al., 2008a). While phenotypic traits form the basis of phenotyping, genes responsible for the production of those phenotypic characters form the foundation for genotyping (Mohan, 2011). Molecular methods have become widely applied to subtype *Campylobacter* jejuni since they provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeablility, and discriminatory power, when compared with phenotypic typing methods (Eberle and Kiess, 2012; Wassenaar and Newell, 2000; Wiedmann, 2002). These may be divided into two broad categories: macro-restriction mediated analyses based on separation of restriction enzyme digested nucleotide sequences, and polymerase chain reaction (PCR) based assays (Mohan, 2011).

Pulse field gel electrophoresis (PGFE), also known as field alteration gel electrophoresis (FAGE) or macro-restriction profiling PFGE, has emerged as one of the best molecular approaches to analyze bacterial pathogens, including *Campylobacter* (Ahmed et al., 2012; Eberle and Kiess, 2012). The PFGE is considered the 'gold standard' for epidemiological investigations due to its enormous discriminatory power (Sails et al., 2003a). Although the interpretation of PFGE data is difficult, rendering this technique unsuitable as a tool for routine use during outbreak investigation (Sails et al., 2003a), it has been extensively used in genetic and epidemiological investigations of *C. jejuni* and *C. coli* (Ahmed et al., 2012; Mohan, 2011).

The polymerase chain reaction (PCR) has certainly revolutionized molecular epidemiological studies thanks to its versatility and ability to detect the presence or absence of an organism in any sample by detecting a specific gene unique to the particular organism of interest (Mohan, 2011). Several variations of the original PCR technique have been developed and are applied for detecting *Campylobacter* spp., including reverse-transcriptase PCR, multiplex PCR, and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Notably, multiplex PCR assays, which are used for simultaneous differentiation of *Campylobacter* spp., have replaced monoplex PCR assays which were widely used for detection and differential diagnosis of *Campylobacter* spp. in the past (Asakura et al., 2008; Yamazaki-Matsune et al., 2007). These techniques are easy to reproduce, highly discriminatory, available in most laboratories and though may be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp. (Eberle and Kiess, 2012).

Apart from PCR being used as a diagnostic tool itself, most of the genotyping techniques are PCR based since it is simple, rapid, and cost effective (Asakura et al., 2008). Random amplified polymorphic DNA analysis (RAPD) and amplified length polymorphism (AFLP) are two PCR-based methods used for *Campylobacter* genotyping which provide good discriminatory power, although, due to certain limitations, these are not used successfully as a routine genotyping tool (Mohan, 2011). Ribotyping is an rRNA approach for the identification of bacterial isolates, which though has a high level of typeablility for *Campylobacter* spp., its low number of ribosomal genes gives it poor discriminatory power (Eberle and Kiess, 2012). Flagellin typing, using restriction fragment length polymorphism (RFLP), is another technique used for typing of *Campylobacter* species. Although flagellin gene typing is quick and can have high discriminatory power, it is recommended that it should not be the sole technique used in epidemiological grouping of isolates, and, therefore, it is often used in combination with other typing techniques mostly MLST (Dingle et al., 2005; Eberle and Kiess, 2012; Mohan, 2011).

DNA sequencing of one or more selected bacterial genes represents another genetic subtyping method (Wiedmann, 2002), which is becoming increasingly automated and, consequently, is a reasonable alternative method for genotyping bacterial isolates (Wassenaar and Newell, 2000). Multilocus sequence typing (MLST) is a genotypic typing method that was first developed in 1991 based on the well-established principles of MLEE (Maiden et al., 1998). This technique differs from MLEE in that it assigns alleles directly by DNA sequencing of 7 to 11 housekeeping genes rather than indirectly through the electrophoretic mobility of their gene product (Eberle and Kiess, 2012). An important component of the MLST approach is the availability of databases (e.g. PubMLST) for use by public health and research communities, where the sequence data can be compared. In turn, researchers can submit the results of their findings to these databases (Maiden, 2006).

MLST is currently the leading molecular typing method for *Campylobacter* (Ross, 2009). An increasingly used in epidemiological studies MLST system specific for the characterization *C. jejuni* strains was developed by Dingle et al. (2001), while an extended MLST method able to characterize not only *C. jejuni* but also *C. coli, C. lari,* and *C. upsaliensis,* was designed by Miller et al. (2005). The advantages of using MLST include high discriminatory power, reproducibility, ease of interpretation and transferability of information among laboratories (Dingle et al., 2001; Wassenaar and Newell, 2000), however, it is a complex and expensive technique to perform (Ahmed et al., 2012; Djordjevic et al., 2007; Lévesque et al., 2008). Moreover, recent work has shown that the seven loci used may be insufficient to provide an accurate picture of gene content in all areas of the *C. jejuni* genome (Taboada et al., 2008). MLST is also unable to distinguish closely related strains in short-term outbreak investigations, and additional methods like fla typing may be required in order to obtain sufficient resolution (Sails, et al., 2003b).

Comparative genomics, namely the analysis and comparison of two or more genomes, has also served to underscore some of the new challenges in bacterial genotyping and phylogenetic analysis (Ross, 2009). Comparative genomic fingerprinting (CGF) is a novel method of comparative genomics-based bacterial characterization which is based on the concept that differential carriage of accessory genes can be used to generate unique genomic fingerprints for genotyping purposes (Ross, 2009). Taboada et al. (2012) developed and validated a rapid and high-resolution 40-gene comparative genomic fingerprinting method for *C. jejuni* (CFG-40). The results obtained with this method suggest that it has a higher discriminatory power than MLST at both the level of clonal complex and sequence type; while, it is also rapid, low cost, and easily deployable for routine epidemiologic surveillance and outbreak investigations (Clark et al., 2012; Taboada et al., 2012). It was shown that CGF and MLST are highly concordant, and that isolates with identical MLST profiles are comprised of isolates with distinct but highly similar CGF profiles.

CONCLUSIONS

Campylobacteriosis has become the leading foodborne disease worldwide and therefore a lot of effort is being done to achieve early diagnosis of human cases using a wide variety of direct and indirect detection methods along with specific identification tests, while epidemiological investigations of campylobacteriosis outbreaks using the innovative and constantly developing typing and subtyping systems available are increasingly conducted, providing information to recognize outbreaks of infection and match cases with potential vehicles of infection. No sole technique is perfect, thus the development of a novel typing method that combines efficiency with efficacy, while overcomes the shortcomings of currently used methods, is considered crucial

CONFLICT OF INTEREST

The authors declare no conflict of interest. ■

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ARTICLE III

Natsos G., Mouttotou N.K., Magiorkinis E., Ioannidis A., Rodi-Burriel A., Chatzipanagiotou S., Koutoulis K.C. (2020)

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Prevalence of and Risk Factors for *Campylobacter* spp. Colonization of Broiler Chicken Flocks in Greece

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Abstract

The prevalence and risk factors for *Campylobacter* spp. colonization of broiler flocks and broiler carcass contamination in Greek slaughterhouses were investigated. Over a 14-month period, a pool of 10 ceca and 5 neck skin samples from chicken carcasses were collected from each of 142 batches of broiler flocks slaughtered in 3 different slaughterhouses. Information on potential risk factors for *Campylobacter* infection in broilers was collected by an on-farm interview and linked according to the *Campylobacter* contamination status of broiler flocks and differences in farm characteristics and management practices identified from questionnaires. *Campylobacter* spp. was isolated from 73.94% and 70.42% of ceca (95% CI 65.92–80.94) and carcasses (95% CI 62.19–77.78), respectively. A significant correlation ($p < 0.001$) between the presence of *Campylobacter* spp. in broiler ceca and contamination of carcasses was found, suggesting the spread of the microorganism on the skin of carcasses during the slaughtering procedure. A multiple logistic regression showed the disinfection of the poultry house being conducted by unskilled personnel (odds ratio [OR] ¼ =3.983) as a significant risk factor ($p < 0.05$) and the use of straw litter as bedding material (OR ¼ =0.170) and closure of windows during the intervals of production cycles (OR ¼ =0.396) as significant protective factors ($p < 0.05$) for broiler flock contamination. These results are important and help further the understanding of the epidemiology of *Campylobacter* spp. derived from poultry in Greece.

Keywords: *Campylobacter* spp., risk factors, prevalence, broiler, Greece

Introduction

Campylobacter spp. are of high importance to food safety and public health since they are identified as the most common cause of foodborne bacterial diarrhea in humans worldwide (Silva *et al.*, 2011).

The prevalence of human campylobacteriosis has been progressively rising worldwide since the 1990s (WHO, 2011). In the European Union (EU), campylobacteriosis has been reported as the most common cause of human foodborne zoonoses since 2005 (EFSA, 2006; EFSA and ECDC, 2018). *Campylobacter jejuni* is the predominant species isolated from poultry samples, followed by *Campylobacter coli*, with other *Campylobacter* species such as *Campylobacter lari* being less detected (EFSA, 2010a). Moreover, *C. jejuni* is considered responsible for the majority of human campylobacteriosis, while *C. coli* and *C. lari* are less frequently implicated in human infections (Zhang and Sahin, 2013). In Greece, there has been a lack of information on *Campylobacter* prevalence and species distribution in broiler flocks (Natsos *et al.*, 2016), as well as on human campylobacteriosis cases, and sufficient data on the epidemiology of the pathogen are not available since cases of campylobacteriosis are not monitored by the Mandatory Notification System in the European Union (EFSA and ECDC, 2018).

Different risk factors for *Campylobacter* infections are related to outbreak or sporadic cases (Hue *et al.*, 2011). Untreated raw milk (Heuvelink *et al.*, 2009) and contaminated water (Abe *et al.*, 2008; Karagiannis *et al.*, 2010) are mainly related to outbreaks, while the main risk factor for sporadic infection in humans is the consumption of poultry

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meat or exposure to food cross-contaminated by contact with raw poultry (Doorduyn *et al.*, 2010; EFSA and ECDC, 2018). Thus, reduction of *Campylobacter* in broiler meat is the main focus of controlling campylobacteriosis (Hue *et al.*, 2010). Until recently, broiler meat be tested for *Campylobacter* was not required by law; however, in 2017, the European Commission made an amendment to the standing regulation regarding *Campylobacter* in broiler carcasses (EU, 2017/1495), introducing the mandatory sampling of poultry carcasses for *Campylobacter* analysis at slaughterhouses on a regular basis.

Risk assessment of *Campylobacter* in poultry slaughterhouses is applied as a means to prevent human zoonotic diseases (Nauta *et al.*, 2009) and therefore it is essential to ascertain the degree of contamination of raw poultry (Hue *et al.*, 2011). Romero-Barrios *et al.* (2013) reported that a reduction of *Campylobacter* colonization in cecal contents of flocks by 2 $\log_{10}$ or 3 $\log_{10}$ CFU (colony-forming units) would decrease human campylobacteriosis cases attributable to broiler meat by at least 76% or 90%, respectively. Therefore, the most common approach to *Campylobacter* control is the decrease of prevalence and bacterial load within the flock and during the slaughterhouse process (Prachantasena *et al.*, 2016). Extensive research efforts have been made to look for appropriate intervention methods, which can be broadly segregated into preharvest and postharvest interventions (Umaraw *et al.*, 2017). *Campylobacter* control measures at the farm level may include biosecurity, vaccination, complete exclusion, bacteriophage therapy, food additives, probiotics, or novel antibacterial treatment of flocks (Newell *et al.*, 2011; Zhang and Sahin, 2013), most of which are under development and not yet commercially available. Thus, the reduction of *Campylobacter* levels on carcasses after evisceration is the most effective intervention measure to control *Campylobacter* in broiler meat, rather than reducing the prevalence of positive broiler flocks (Nauta *et al.*, 2009; Hermans *et al.*, 2011).

Several risk factors can lead to introduction of *Campylobacter* into flocks, and once introduced into the flock, *Campylobacter* quickly spreads to all birds and large numbers are shed, leading to heavy contamination of the broiler house environment and equipment (Battersby *et al.*, 2016). The possible sources and transmission routes of *Campylobacter* in poultry flocks have been well investigated, focusing on different parts of the production processes and practices. The main potential risk factors suggested include season (summer and/or autumn) (Ellis-Iversen *et al.*, 2009), higher age of broilers at slaughter (Ansari-Lari *et al.*, 2011), use of partial depopulation practice (Ellis-Iversen *et al.*, 2009; EFSA, 2010b; Hansson *et al.*, 2010; Lawes *et al.*, 2012), distribution of drinking water (Nather *et al.*, 2009) and its quality (Ellis-Iversen *et al.*, 2009), presence of other animals in the surrounding area of the farm (Hansson *et al.*, 2010) or in the same farm (Ellis-Iversen *et al.*, 2009), and presence of rodents (McDowell *et al.*, 2008; Sommer *et al.*, 2013) and flies (Royden *et al.*, 2016). In contrast, *Campylobacter* infection can be reduced by good hygiene practices by farmers or seldom or never thinning (Hansson *et al.*, 2010). The most frequently reported risk factors associated with horizontal transmission of *Campylobacter* spp. to broiler flocks and broiler carcass contamination during the slaughtering process have been reviewed by Natsos *et al.* (2016).

In this article, we describe the study undertaken to (i) assess *Campylobacter* prevalence in broiler flocks and on broiler carcasses, along with the level of contamination by *Campylobacter* on broiler carcasses collected during the slaughtering process; and (ii) identify risk factors associated with the presence of *Campylobacter* spp. in broiler flocks and on chicken carcasses.

Materials and Methods

Experimental design

The study was performed in 3 european commission (EC)-approved Greek poultry slaughterhouses located in central Greece, each of which processes more than 5,000,000 chickens per year. Flocks were derived from 4 different Greek regional units (Arta, Attica, Boeotia, and Euboea) producing in total 50,000,000 chickens per year (45% of the total Greek chicken production). In total, 142 samples were collected, originating from 60 different poultry farms, of which 8 are situated in the regional unit of Arta, 9 in Attica, 20 in Boeotia, and 23 in Euboea. The sample size was set on the basis of an expected prevalence of 50% and a confidence interval of 95%, and the unit for statistical analysis was the slaughter batch defined as a group of chickens from the same flock, delivered at the same time to the same slaughterhouse.

Ten birds per batch were randomly selected during evisceration and their ceca were pooled into a sterile bag. Neck skin samples of five birds from the processing line after chilling were also taken using a clean pair of latex gloves and put into a sterile bag. Samples were sent in an insulated box containing ice packs to maintain a low temperature within 2–4 h to the Veterinary Laboratory of Chalkida where bacteriological analyses were performed the same day of sampling.

Sample analysis

Campylobacter spp. were recovered from cecal contents by direct isolation following the procedure described by Hue *et al.* (2011). For each positive plate, if present, up to five typical *Campylobacter* colonies were then subcultured onto plates of Columbia Blood Agar (Oxoid, Dardilly, France) for further characterization in accordance with the standard procedure of International Organization for Standardization (ISO) 10272-1 (ISO, 2006a). Speciation of *Campylobacter* strains was carried out following the protocol for polymerase chain reaction (PCR) amplification of *C. jejuni* and *C. coli* recommended by the EURL-AR (2nd version, November 2013). The flock was considered *Campylobacter* positive when at least one colony typical for *Campylobacter* yielded a positive result by the PCR procedure.

For recovery and detection of *Campylobacter* from the skin of carcasses, the procedures described in parts 1 and 2 of ISO 10272 (ISO, 2006a, b) and by Hue *et al.* (2011) were followed. For each positive plate, up to two colonies typical of *Campylobacter* were subcultured onto Columbia Blood Agar plates for further characterization and enumeration, according to the standard method of ISO 10272-1:2006.

Statistical analysis

Epi Info™ 7 software (CDC, Atlanta, GA) was used to calculate the prevalence of *Campylobacter*-positive batches of ceca and carcasses. If *Campylobacter* was detected and/or

Table 1. Prevalence of *Campylobacter* in Ceca and on Carcasses of Broiler Flocks ($n=142$, Greece, 2015)

Samples	*Positive batches/investigated batches*	*Prevalence (%)*	*± Standard deviation*	*95% Confidence interval*
Ceca	105/142	73.94	44.05	65.92–80.94
Carcasses	100/142	70.42	45.80	62.19–77.78

enumerated, a batch was considered positive, and for enumeration purposes, bacterial counts were $\log_{10}$-transformed to obtain approximately normally distributed data.

Information on potential risk factors for *Campylobacter* infection in broilers was collected by an on-farm interview, with questions concerning the farm and house characteristics such as the environment around the farm and broiler houses, sanitary practice, control of wild birds and rodents, in-house environment (humidity, air quality, and temperature), design of the broiler houses, and on-farm management practices (Supplementary Data). Data concerning the structural and functional characteristics of slaughterhouses such as hygiene level along with data derived from the microbiology analysis were also statistically analyzed using the appropriate statistical methodology.

Univariate statistical analysis was carried out to identify the main trend, variability, and distribution of each individual variable, and bivariate analysis was performed to study relationships between independent variables and *Campylobacter* contamination of ceca and neck skin samples. Variables with more than 20% of missing data and those for which there was no variability were excluded from the analysis. Finally, a multiple logistic regression, including all the previously selected explanatory variables, was performed. A downward selection, using Epi Info 7 software, was performed, with variables introduced if $p<0.20$ and excluded if $p>0.05$.

Results

Prevalence

Overall prevalence of cecum-based *Campylobacter*-positive batches was 73.94% (number of infected/total examined samples). In the case of carcasses, 100 of 142 (70.42%) batches were positive for *Campylobacter* (Table 1). The presence of *Campylobacter* in broiler ceca was strongly correlated ($p<0.001$) with contamination of carcasses of the same batch. In 20 batches (14.08%), *Campylobacter* was detected in ceca, but not on carcasses, while 17 batches (11.97%) were found to be *Campylobacter* positive based on carcasses, but negative based on ceca. Finally in 20 batches (14.08%), *Campylobacter* spp. were not detected either in ceca or in neck skin samples. The number of *Campylobacter* isolates recovered from carcasses ranged from 3.22 up to 5.96 $\log_{10}$ CFU/g with a mean value of 4.639 ± 0.11 $\log_{10}$ CFU/g (Table 2).

Selection of variables

Fifty-eight variables were selected by univariate analysis, which were further processed by bivariate analysis. Variables related to common management practices and characteristics of poultry farms were eliminated from further analysis (Table 3).

Bivariate analysis of explanatory variables allowed the selection of variables most related to the presence of *Campylobacter* in broiler ceca (Table 4). Multivariate logistic regression was used to analyze further 15 variables significantly related to the presence of *Campylobacter* in ceca. None of the parameters related to slaughterhouse characteristics and slaughtering procedure, for example, type of chilling, time of slaughter, and temperature in the evisceration room, were found to be statistically significant.

The multivariate logistic regression analysis produced a model, which showed two parameters as protective factors and one parameter as a risk factor for contamination of broiler flocks (Table 5).

Risk and protective factors

The risk of *Campylobacter* contamination decreased (odds ratio [OR] ¼ =0.396) when windows were kept closed during the interruption of production cycles. In 82 batches derived from farms that follow closure of windows as a common practice, the contamination rate was 60%, whereas it was 82% in the 60 batches derived from farms where windows are kept open during the sanitary waiting period.

Batches derived from houses that had been disinfected by untrained farm staff seemed to have more chances to be positive for *Campylobacter* (OR ¼ =3.983) in comparison with those coming from farms where disinfection was carried out by a special agency. Ceca from batches derived from farms where unskilled workers perform the disinfection had a relatively greater contamination rate (77.5%) than those coming from farms that hire skilled specialists to perform the programmed disinfection (38.4%).

Table 2. Numbers of *Campylobacter* on Carcasses of Positive Batches of Broiler Flocks ($n=142$, Greece, 2015)

Positive batches/ investigated batches	*Percentage of samples with positive results for enumeration purposes*	*Mean*[a] *($\log_{10}$ CFU/g)*	*Median ($\log_{10}$ CFU/g)*	*± Standard deviation*	*95% Confidence interval*
100/142 (*70.42%*)	99/100 (*99%*)	4.639	4.613	0.559	4.528–4.749

Italic values represent the prevalence of *Campylobacter* in the samples.

[a] Includes only samples with positive results for enumeration purposes.

Table 3. Common Management Practices and Characteristics of Poultry Farms Excluded from Statistical Analysis

Presence of quality assurance system	0%
Presence of watercourses close to the farm	4%
Use of different boots for each house	6%
Farms with houses of different age	8%
Presence of house divider	8%
One disinfection per production cycle	8.5%
Presence of communal anteroom	9%
Disposal of droppings performed by special agency	91%
Presence of effluent collection system	96.5%

The percentage of *Campylobacter*-positive batches was found to be lower (OR ¼ =0.170) when straw was solely used as the bedding material. Ceca of broilers from farms that use only straw as the bedding material showed a lower contamination rate (68.8%) compared with those derived from farms where sawdust or rice husk was used as the bedding material (90.9%).

Discussion

The current cross-sectional study carried out in Greece generated representative data on broiler ceca (73.94%) and carcass skin samples (70.42%), indicating the high prevalence of *Campylobacter* at the national level. These results are in agreement with several studies both for ceca and carcasses (Allen *et al.*, 2008; Hue *et al.*, 2010, 2011; Lawes *et al.*, 2012) and the EFSA scientific report for *Campylobacter*-positive batches (71.2%) and *Campylobacter*-contaminated carcasses (75.8%) in the EU member states (EFSA, 2010a).

A significant proportion of carcasses (12%) were positive for *Campylobacter* contamination, while ceca of the same batches were found to be *Campylobacter* negative. Rosenquist *et al.* (2006) and Figueroa *et al.* (2009) demonstrated that during the evisceration step, cross-contamination might be possible. Rupture of viscera from infected chickens may release high numbers of *Campylobacter* isolates that contaminate the surfaces of the slaughterhouse, explaining these results. A cross-contamination may also occur between batches from different flocks during the slaughterhouse process (Rivoal *et al.*, 1999; Johannessen *et al.*, 2007), and the level of contamination of noninfected chicken batches can be influenced by several factors such as the *Campylobacter* status of previously slaughtered batches, the amount of cross-contamination taking place, and the position of carcasses in subsequent negative batches (Hue *et al.*, 2011). Therefore, the use of a logistic slaughtering schedule could help preserve *Campylobacter*-free batches, considering that the later in the day the batch is slaughtered, the higher the probability that it will be contaminated (Hue *et al.*, 2010).

In recent years, quantitative risk assessment modeling is supported by a growing demand for quantitative data to describe the occurrence and dynamics of *Campylobacter* in the broiler meat chain (Uyttendaele *et al.*, 2006; Nauta *et al.*, 2009; Prachantasena *et al.*, 2016). Researchers previously concluded that for enumeration of thermotolerant *Campylobacter* in chicken meat, direct spread plating on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) is an acceptable protocol and a reliable alternative to the most probable number method (Scherer *et al.*, 2006; Rosenquist *et al.*, 2007). Currently, mCCDA is the recommended medium by the ISO for enumeration of thermophilic *Campylobacter* in foods (ISO, 2006b), although alternative enrichment and plating combinations have been evaluated (Habib *et al.*, 2011). In our study, the average concentration of *Campylobacter* recovered from carcasses was 4.639 ± 0.11 $\log_{10}$ CFU/g, while the normal distribution of positive values leads to an average close to the median, clearly separating values into two halves. The result of our study has shown a much higher colonization rate of *Campylobacter* than the respective results of previously published data (Scherer *et al.*, 2006; Hue *et al.*, 2011). This finding could be attributed to a high degree of cecal contamination, to visceral rupture and subsequent release of large numbers of *Campylobacter* isolates on the carcass skin, or even to short processing times or inadequate slaughterhouse hygiene and cleaning conditions, which possibly promote the survival and spread of *Campylobacter* spp. during the slaughtering process.

Of the 206 identifications performed, two different species of *Campylobacter* were identified (*C. jejuni* and *C. coli*) and *C. coli* was found to be the predominant species. This result is in line with a previous study from Greece (Marinou *et al.*, 2012), but contradictory to other studies showing *C. jejuni* as being much more frequently associated with poultry meat than *C. coli* (Pepe *et al.*, 2009; Hue *et al.*, 2011). However, according to the results of a baseline survey conducted by EFSA in 2008, seven EU member states reported *C. coli* as the predominant species isolated from ceca and carcasses (EFSA, 2010a). Moreover, the same survey showed that in southern member states, *C. coli* was more abundant, whereas *C. jejuni* was the only species identified in northern member states. Climatic conditions, environmental reservoirs, housing systems of broiler chickens, and age of slaughter differ significantly between northern and southern Europe and could partially explain the observed variance of species distribution (EFSA, 2010b).

The contamination of slaughtered batches by these species fluctuated according to the sample, with *C. jejuni* being more frequently identified on carcasses than in ceca (43% and 35.24%, respectively). Therefore, it is possible that *C. jejuni* is more resistant than *C. coli* to stress encountered during slaughtering (Hue *et al.*, 2011). It has been shown that *C. jejuni* adheres more to inert surfaces than *C. coli* (Sulaeman *et al.*, 2010), which may allow *C. jejuni* to have better biofilm formation capacity, especially under stressful environmental conditions (Reuter *et al.*, 2010; Teh *et al.*, 2014). Swelling of the skin during slaughter and processing allows the survival of *Campylobacter* on poultry carcasses (Chantarapanont *et al.*, 2003).

Moreover, since only one well-isolated colony from a pure culture underwent PCR for species identification, any co-contamination with both *Campylobacter* species could not be detected. The simultaneous presence of the two species both in ceca and on carcass skin is common (Hue *et al.*, 2011) and could explain the observed disagreement in 22 batches between the identified species in cecal content and neck skin samples. Biofilm formation might also be attributed to a short processing time or inadequate cleaning procedures in the slaughterhouse and should be further investigated.

In line with previous studies, the current results suggest that *Campylobacter* infection is a multifactorial problem and is caused by several potential sources. The closure of windows between production cycles seemed to decrease the chance of the poultry batch being infected by *Campylobacter*.

TABLE 4. SELECTED VARIABLES (THRESHOLD OF 20%) ENTERED IN THE MULTIPLE LOGISTIC MODEL USED TO EXPLAIN *CAMPYLOBACTER* SPP.-POSITIVE BATCHES ($N = 142$)

Variable[a]	*Variable modality*	*Size*	*% Positive*	p *(χ^2 test)*
Age of poultry house	<15 years	18	55.56	0.082
	>15 years	124	76.61	
Antibiotic treatment during life	Yes	47	65.96	0.156
	No	95	77.89	
Bathroom	Presence	89	79.78	**0.049**
	Absence	53	64.15	
Bedding material	Straw	109	68.81	**0.012**
	Other[b]	33	90.91	
Closure of windows during the interruption of production cycles	Yes	60	81.67	0.084
	Maybe/no	82	68.29	
Detergent	Presence	104	70.19	0.130
	Absence	38	84.21	
Disinfectant	Presence	60	80.00	0.179
	Absence	82	69.51	
Faucet	Presence	84	67.86	0.053
	Absence	58	82.76	
Hygiene level	Very good	66	68.18	0.180
	Poor	76	78.95	
Keeping of bedding material	In a protected and clean room	82	82.93	**0.006**
	In an unprotected room	60	61.67	
Number of disinfection treatments	Only one	12	100	**0.036**
	Two	130	71.54	
Person who disinfects	Breeder/personnel	129	77.52	**0.005**
	Special agency	13	38.46	
Responsible for thinning	Breeder/personnel	105	70.48	0.131
	Specialists	37	83.78	
Sink	Presence	86	67.44	**0.032**
	Absence	56	83.93	
Watering System	Bell drinkers	98	79.59	**0.037**
	Nipple drinkers	44	61.36	

Values in bold are significantly different.

[a] All variables are significantly related to the presence of *Campylobacter* in cecal samples ($p < 0.20$).

[b] Sawdust, rice husk, or mixed up with straw litter.

This result could be probably attributed to prevention of the access of flies or other vectors into the house (Hald *et al.*, 2008; Choo *et al.*, 2011). Royden *et al.* (2016) demonstrated that flies may play a role in the transmission of *Campylobacter* to broilers, and due to the large number of flies around broiler house ventilation inlets, the risk of transmission is high. It seems that keeping windows firmly closed during the downtime prevents the introduction of *Campylobacter* into the farm by not letting the potential vectors enter the farm.

The results of our study suggest that disinfection of the house plays an important role on the *Campylobacter* status of the poultry batch as when it was performed by unskilled personnel the chances for the batch to be positive were substantially higher compared with when it was undertaken by a special agency. These findings suggest that insufficient disinfection of the farm leads to increased contamination rates. It is clear that effective cleaning and disinfection of broiler houses and their surroundings can decrease the risk of *Campylobacter* transmission between subsequent flocks (Battersby *et al.*, 2017). Overall, the absence of sanitizing procedures can be considered an important risk factor for *Campylobacter* spp. contamination (Bouwknegt *et al.*, 2004;

TABLE 5. RISK AND PROTECTIVE FACTORS FOR CONTAMINATION OF BROILER FLOCKS BY *CAMPYLOBACTER* SPP. ($N = 142$)

Variable	*Estimated parameters*	*Standard deviation*	*OR ¼*	*95% CI*	p
Closure of windows during the interruption of production cycles					
Yes	–0.925	0.444	0.396	0.166–0.947	0.067
No	—	—	—		—
Person who disinfects					
Breeder/personnel	1.382	0.681	3.983	1.048–15.134	0.042
Special agency	—	—	—		—
Bedding material					
Straw	–1.772	0.660	0.170	0.047–0.619	0.007
Other	—	—	—		—

OR, odds ratio.

McDowell *et al.*, 2008; Newell *et al.*, 2011), and even with the use of most efficient biosecurity programs, this pathogen may enter the facilities and colonize the birds (van de Giessen *et al.*, 1998). Several disinfection programs have been tested and evaluated for their effects on the environmental *Campylobacter* contamination (Battersby *et al.*, 2017; Castro Burbarelli *et al.*, 2017) and it is suggested that these cleaning practices should be routinely tested on all broiler farms to determine their effectiveness in reducing exposure of poultry and humans to the pathogen.

The material used as bedding material seemed to affect the contamination status of the flock. In particular, the sole use of straw as a bedding material reduced *Campylobacter* contamination, compared with the use of other materials, such as sawdust, rice husk, or mixtures of these bedding materials. Different bedding materials (straw and wood shavings) have been compared on how they affect the total aerobic bacterial counts and it was found that less contamination was detected in wood shavings than straw (Fries *et al.*, 2005). Wood can exhibit strong antibacterial characteristics due to a combination of hygroscopic properties of wood and the effects of wood extracts (Milling *et al.*, 2005). Other studies have found that such essential oils have antioxidant activity by scavenging free radicals and have shown antimicrobial activity against a range of foodborne organisms (Zeng *et al.*, 2012), including *Campylobacter* (Kurekci *et al.*, 2013). However, the findings of our study suggest that straw litter protects from *Campylobacter* contamination and maybe this is due to the fact that wheat straw contains less moisture than rice husk and wood shavings (Monira *et al.*, 2003). The higher water content in the bedding material may protect *Campylobacter* from the effects of desiccation, thus enhancing its survival (Smith *et al.*, 2016). Although, environmental challenges linked to the disposal of bedding material impose the litter reuse in some countries, a practice that may have an impact on key food safety pathogens such as *Campylobacter*, a survey conducted by Chinivasagam *et al.* (2016) found no direct influence between reuse of litter and either the timing of emergence or the levels of *Campylobacter* concentration across sequential farming cycles.

In conclusion, the cross-sectional study carried out in Greece produced valuable results concerning the prevalence of *Campylobacter* spp. in poultry production countrywide. A high prevalence of *Campylobacter* spp. in broiler flocks and on carcasses was found, along with a remarkably high load on broiler chicken carcasses, while the predominance of *C. coli* was noted both in ceca and on carcasses. The analysis of potential risk factors proposed that closure of windows during the downtime and the use of straw as the bedding material act as protective factors, whereas disinfection of the poultry house performed by unskilled personnel acts as a risk factor for contamination of the flock with *Campylobacter*. The results help in understanding the epidemiology of *Campylobacter* spp. derived from poultry in Greece and indicate the need for further investigation.

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Supplementary Material

Supplementary Data

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ARTICLE IV

Natsos G., Mouttotou N.K., Magiorkinis E., Ioannidis A., Magana M., Chatzipanagiotou S., Koutoulis K.C. (2021)

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Antimicrobial Resistance, FlaA Sequencing, and Phylogenetic Analysis of *Campylobacter* Isolates from Broiler Chicken Flocks in Greece

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Abstract: Human campylobacteriosis caused by thermophilic *Campylobacter* species is the most commonly reported foodborne zoonosis. Consumption of contaminated poultry meat is regarded as the main source of human infection. This study was undertaken to determine the antimicrobial susceptibility and the molecular epidemiology of 205 *Campylobacter* isolates derived from Greek flocks slaughtered in three different slaughterhouses over a 14-month period. A total of 98.5% of the isolates were resistant to at least one antimicrobial agent. In terms of multidrug resistance, 11.7% of isolates were resistant to three or more groups of antimicrobials. Extremely high resistance to fluoroquinolones (89%), very high resistance to tetracycline (69%), and low resistance to macrolides (7%) were detected. FlaA sequencing was performed for the subtyping of 64 *C. jejuni* and 58 *C. coli* isolates. No prevalence of a specific flaA type was observed, indicating the genetic diversity of the isolates, while some flaA types were found to share similar antimicrobial resistance patterns. Phylogenetic trees were constructed using the neighbor-joining method. Seven clusters of the *C. jejuni* phylogenetic tree and three clusters of the *C. coli* tree were considered significant with bootstrap values >75%. Some isolates clustered together were originated from the same or adjacent farms, indicating transmission via personnel or shared equipment. These results are important and help further the understanding of the molecular epidemiology and antimicrobial resistance of *Campylobacter* spp. derived from poultry in Greece.

Keywords: *Campylobacter* spp.; poultry; antimicrobial resistance; flaA typing; phylogenetic trees; Greece

1. Introduction

Campylobacter spp. are ubiquitous bacteria, able to colonize mucosal surfaces, usually the intestinal tract, of most mammalian and avian species [1,2]. Thermophilic *Campylobacter* spp. are essentially commensal in birds and insignificant for poultry health [3]. However, they are of high importance to food safety and public health, since they are recognized as the leading cause of bacterial foodborne diarrheal disease worldwide [1,4]. Birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs [5]. Broilers are considered *Campylobacter* free after hatching, since most evidence suggests that vertical transmission plays a minor role, if any [1], and, in general, broiler flocks remain

Campylobacter free for the first two weeks (the so-called lag phase) [6]. This lag phase is likely to be an inherent property of the chick. An inhibitory effect produced by commensal organisms in the gut of young chicks [7], the presence of maternal antibodies, which may be protective and which decline by about 14 days of age [8], and antimicrobial treatment contribute to the existence of the lag phase. As chickens are coprophagic, fecal shedding is presumably an important factor in the dissemination of organisms around large broiler flocks once the first bird becomes colonized. Certainly, once flock colonization is detected, bird-to-bird transmission within flocks is extremely rapid, and the majority (up to 100%) of birds in a positive flock are colonized within only a few days [6,9].

Consumption of poultry meat or ready-to-eat meat cross-contaminated by contact with raw poultry products constitutes the main risk factor for sporadic human infection [1,10,11]. Thus, control of campylobacteriosis is commonly focused on reducing the occurrence of *Campylobacter* in broiler meat [12]. *C. jejuni* is the predominant species isolated from poultry samples, followed by *C. coli*, with other *Campylobacter* species such as *C. lari* being less detected [13]. However, the predominance of *C. coli* has been reported in Greece [14,15] and other southern European countries [13], which could be attributed to the differences in climatic conditions, environmental reservoirs, housing systems of broiler chickens, and age of slaughter between northern and southern Europe [16]. *C. jejuni* is, as well, considered responsible for the majority of human campylobacteriosis, followed by *C. coli*, and, rarely, by other emerging *Campylobacter* species, including *C. concisus*, *C. ureolyticus*, *C. upsaliensis*, and *C. lari* [4].

In the European Union (EU), campylobacteriosis has been the most commonly reported cause of human foodborne zoonoses since 2005 [17,18]. Antimicrobial treatment is usually not required, but effective treatment may shorten the duration of illness [19]. In cases where antimicrobial treatment is needed, macrolides (mostly erythromycin and azithromycin) and fluoroquinolones (e.g., ciprofloxacin) are considered as the first and second choices of antimicrobials, respectively [20,21]. Since a rapidly increasing proportion of *Campylobacter* strains worldwide have been found to be resistant to these antimicrobials, attention should be paid to choosing the most appropriate antimicrobial treatment [19]. Infection with antimicrobial-resistant *Campylobacter* may lead to suboptimal outcomes of antimicrobial treatments or even treatment failure [22]. Therefore, other antimicrobials such as gentamicin, carbapenems, and amoxicillin-clavulanic acid could be alternatively used for the treatment of systemic *Campylobacter* infections [23]. Transmission of antimicrobial resistance from food animals to humans can occur via the food chain. Therefore, food animals are a significant reservoir of antimicrobial-resistant zoonotic pathogens [24]. Consequently, the estimation of antimicrobial susceptibility of *Campylobacter* strains derived from animal samples is crucial. The World Health Organization, therefore, has published a list of critically important antimicrobials for human medicine, emphasizing the importance of prudent use of antimicrobials both in human and veterinary medicine [25].

Due to the impact of *Campylobacter* on public health, epidemiological investigations analyzing the clonality of the isolated strains are very important, in order to trace the sources and routes of transmission, to follow up the temporal and geographic distribution of important phenotypic characteristics, and to develop effective strategies for the control and prevention of the pathogen spread, especially inside the food chain [26,27]. The subtyping of clinical, animal, and food isolates remains an important requirement for epidemiological studies in order to (1) trace sources and routes of transmission of human infections; (2) identify and monitor, temporally and geographically, specific strains with important phenotypic characteristics; and (3) develop strategies to control organisms within the food chain [28]. Classical pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), as well as flaA typing based on the restriction analysis of PCR-amplified fragments or sequencing of the flagellin-encoding gene, have been described for *Campylobacter* [29–31]. Although, multilocus sequence typing (MLST) has been described as the gold standard method in this field, MLST is still time consuming and expensive and, therefore, not feasible for routine testing [30,31].

The aims of the present study were multiple: (1) to determine the antimicrobial resistance of *Campylobacter* isolates derived from Greek flocks in relation to common antimicrobial substances used in poultry practice and for human medicine, (2) to subtype them using the flaA gene sequencing typing technique, and (3) to perform a phylogenetic analysis in order to study their molecular epidemiology.

2. Materials and Methods

2.1. Experimental Design

The experimental procedure was conducted in commercial flocks. Therefore, an ethical approval from the University's Animal Ethics Committee was not required. Samples were collected from 142 slaughter batches, originating from 60 different poultry farms between February 2014 and March 2015 [15]. Caeca were randomly selected from 10 birds per batch during evisceration and pooled into a sterile bag. Neck skin samples of five birds from the processing line after chilling were also taken, using a clean pair of latex gloves and put into a sterile bag. After the sampling, the acquired samples were sent, in an insulated box containing ice packs to maintain a low temperature, within a few hours of the same day to Veterinary Laboratory of Chalkida, where bacteriological analyses were performed.

2.2. Sample Analysis

Campylobacter spp. recovered from the caecal contents using the technique of direct isolation, in which 10 μL of each caecal sample, previously homogenized by adding Peptone Salt solution (Merck, Darmstadt, Germany), were plated on the selective medium, modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid, Dardilly, France), followed by incubation for 44 ± 4 h at 41.5 ± 1 °C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). For each positive plate, if necessary, up to five typical *Campylobacter* colonies were then subcultured onto plates of Columbia Blood Agar (Oxoid, Dardilly, France) for further characterization, in accordance with standard procedure of International Organization for Standardization (ISO) 10272-1 [32]. The flock was considered *Campylobacter*-positive, when at least one confirmed *Campylobacter* isolated from a colony yielded a positive result by PCR procedure.

For the recovery of *Campylobacter* from the skin of carcasses, the procedure described in ISO 10272 was followed. For the detection of *Campylobacter*, 10 g of neck skin was placed in a sterile bag and diluted 1:10 with selective pre-enrichment Bolton Broth solution (Oxoid, Dardilly, France). The mix was then homogenized for 1 min in a peristaltic homogenizer and the final suspension was incubated under microaerobic conditions for 4 h at 37 °C and then for 44 ± 4 h at 41.5 ± 1 °C. Subsequently, 10 μL of the suspension were plated onto mCCDA and Butzler (Oxoid, Dardilly, France) plates and followed by incubation for 44 ± 4 h at 41.5 ± 1 °C. For each positive plate, up to five colonies typical of *Campylobacter* were subcultured onto Columbia Blood Agar plates for further characterization, according to standard method of ISO 10272-1:2006.

2.3. Antimicrobial Susceptibility Testing

For each *Campylobacter*-positive sample, antimicrobial susceptibility testing to ciprofloxacin, nalidixic acid, erythromycin, streptomycin, gentamicin, and tetracycline was performed. Antimicrobial disks for the disk diffusion method were obtained from Oxoid, Dardilly, France. Disk diffusion method in Mueller–Hinton agar enriched with 5% defibrinated sheep's blood was performed. Sterile cotton-tipped swabs were used to inoculate broth culture diluted to match a 0.5 McFarland turbidity standard onto Mueller–Hinton blood agar plates to produce a confluent lawn of bacterial growth. After the inoculum on the plates was dried, antimicrobial disks were distributed over the inoculated plates using an Antimicrobial Susceptibility testing Disk Dispencer (Oxoid, Dardilly, France). These plates were then incubated at 42 °C for 24 h under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). Isolates with insufficient growth after 24 h of incubation were re-incubated immediately and inhibition zone was read after a total of 40–48 h of incubation. *Campylobacter jejuni* ATCC 33560 was used as a quality-control (QC) strain and the acceptable ranges of Clinical and Laboratory Standards Institute (CLSI M45) were followed. Since there were no antimicrobial susceptibility breakpoints for disk diffusion method specific with respect to *Campylobacter* for nalidixic acid, gentamicin, and streptomycin provided by CLSI M45, breakpoints of *Enterobacteriaceae* were used (CLSI M100). The concentrations of antimicrobial agents tested in this study along with the zone diameter breakpoints are shown in Table 1.

Table 1. Breakpoints of the disk diffusion method used to determine antimicrobial susceptibility of *Campylobacter* isolates.

Antimicrobial Agent	Disk Concentration (μg)	Zone Diameter Breakpoint (mm) [1]		
		S	I	R
Ciprofloxacin	5	≥24	21–23	≤20
Erythromycin	15	≥16	13–15	≤12
Tetracycline	30	≥26	23—25	≤22
Nalidixic acid	30	≥19	14–18	≤13
Gentamicin	10	≥15	13–14	≤12
Streptomycin	10	≥15	12–14	≤11

[1] Zone diameter breakpoints of ciprofloxacin, erythromycin, and tetracycline for *Campylobacter* spp. were recommended by the CLSI M45, whereas those of nalidixic acid, gentamicin, and streptomycin for Enterobacteriaceae were recommended by the CLSI M100. S, susceptible; I, intermediate; R, resistant.

2.4. FlaA Sequencing

A PCR procedure was performed on the DNA extracts of 122 *Campylobacter* isolates. The primers used (FLA4F and FLA630R) were composed by Eurofins Genomics, were in freeze-drying state, and were selected based on a study of Meinersmann et al. [33]. Sanger sequencing was performed in a 3130 Genetic Analyzer (Applied Biosystems Life Technologies Ltd., Paisley, UK). For the sequencing of the flaA gene, DNA STAR's Laser gene Evolution Suite software was used. All sequences were submitted to GenBank and issued accession numbers (MW713238–MW713296 for *C. coli* sequences and MW713297–MW713360 for *C. jejuni* sequences).

2.5. Phylogenetic Trees

All available flaA sequences for *C. jejuni* and *C. coli* were downloaded from different geographic regions. For *C. jejuni*, the analysis involved 64 sequences isolated in our study plus 960 flaA reference sequences (RS) downloaded from the GenBank database. For *C. coli,* the numbers were 58 sequences plus 74 flaA reference sequences, respectively. Phylogenetic analysis was performed, estimating the genetic distances between sequences using Tamura–Nei model [34]. Phylogenetic trees were constructed using the neighbor-joining method and the reliability of phylogenetic clusters was assessed using bootstrapping analysis of 1000 copies. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The alignment of all sequences was performed by Cluster W algorithm using the MEGA 5 version 5.0 software, while all positions containing gaps and missing data were manually edited.

3. Results

3.1. Antimicrobial Resistance

According to CLSI antimicrobial susceptibility breakpoints, 86.7% of *Campylobacter* isolates from caecal samples were classified as resistant to ciprofloxacin, 87.6% as resistant to nalidixic acid, and 77.1% as resistant to tetracycline. On the other hand, very low resistance to erythromycin (7.6%) and streptomycin (11.4%) and no resistance to gentamicin were found. Similar results came from antimicrobial resistance testing of neck skin samples (Table 2). The results of antimicrobial susceptibility in relation to the species of *Campylobacter* isolates are shown in Table 3.

Table 2. Antimicrobial susceptibility patterns of *Campylobacter* spp., identified by the disk diffusion method, according to the sample tested [1].

Antimicrobial Agent	**Caecal Samples**				**Neck Skin Samples**			
	No. of *Campylobacter* Isolates [2]			**% of Resistant Isolates**	**No. of *Campylobacter* Isolates [2]**			**% of Resistant Isolates**
	S	**I**	**R**		**S**	**I**	**R**	
Ciprofloxacin	14		91	86.7	8		92	92
Erythromycin	97		8	7.6	91	3	6	6
Tetracycline	22	2	81	77.1	39		61	61
Nalidixic acid	13		92	87.6	9	2	89	89
Gentamicin	105			0	100			0
Streptomycin	92	1	12	11.4	93		7	7

[1] The total number of *Campylobacter* isolates from caecal samples tested for antimicrobial resistance was 105 and from neck skin samples was 100. [2] Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates identified by the disk diffusion method.

Table 3. Antimicrobial susceptibility patterns of *Campylobacter* isolates, identified by the disk diffusion method, according to the species [1].

Antimicrobial Agent	***Campylobacter Jejuni***				***Campylobacter Coli***			
	No. of *Campylobacter* Isolates [2]			**% of Resistant Isolates**	**No. of *Campylobacter* Isolates [2]**			**% of Resistant Isolates**
	S	**I**	**R**		**S**	**I**	**R**	
Ciprofloxacin	7		95	93.1	15		88	85.4
Erythromycin	94		8	7.8	94	3	6	5.8
Tetracycline	29	1	72	70.6	32	1	70	68
Nalidixic acid	7	1	94	92.2	15	1	87	84.5
Gentamicin	102			0	103			0
Streptomycin	90	1	11	10.8	94	1	8	7.7

[1] The total number of *Campylobacter jejuni* was 102 and *Campylobacter coli* was 103. [2] Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates identified by the disk diffusion method.

Only three strains were susceptible to all antimicrobial agents. Additionally, 13 out of 205 (6.3%) *Campylobacter* isolates showed co-resistance to ciprofloxacin and erythromycin, whereas 24 out of 205 (11.7%) were resistant to three or more groups of antimicrobials (i.e., fluoroquinolones, macrolides, tetracyclines, aminoglycosides).

3.2. *FlaA Sequencing*

A high degree of genetic diversity was revealed, with a total of 38 different nucleotide types that corresponded to 15 different peptide types. Peptide type 1 was the most predominant since it was recovered from 58 *Campylobacter* isolates. Of the isolates, 92.6% (113 out of 122) showed exact match with the already registered ones in the international database, whereas 7.4% (9 out of 122) displayed partial match; namely, the isolates had a rate of homology though preserving different regions inside the sequences. Some isolates shared the same nucleotide and peptide type in an exact match with the registered types in the international database, suggesting the occurrence of clonality. Moreover, some of these isolates shared common antimicrobial profile (e.g., peptide type 1-DNA type 66).

3.3. *Phylogenetic Trees*

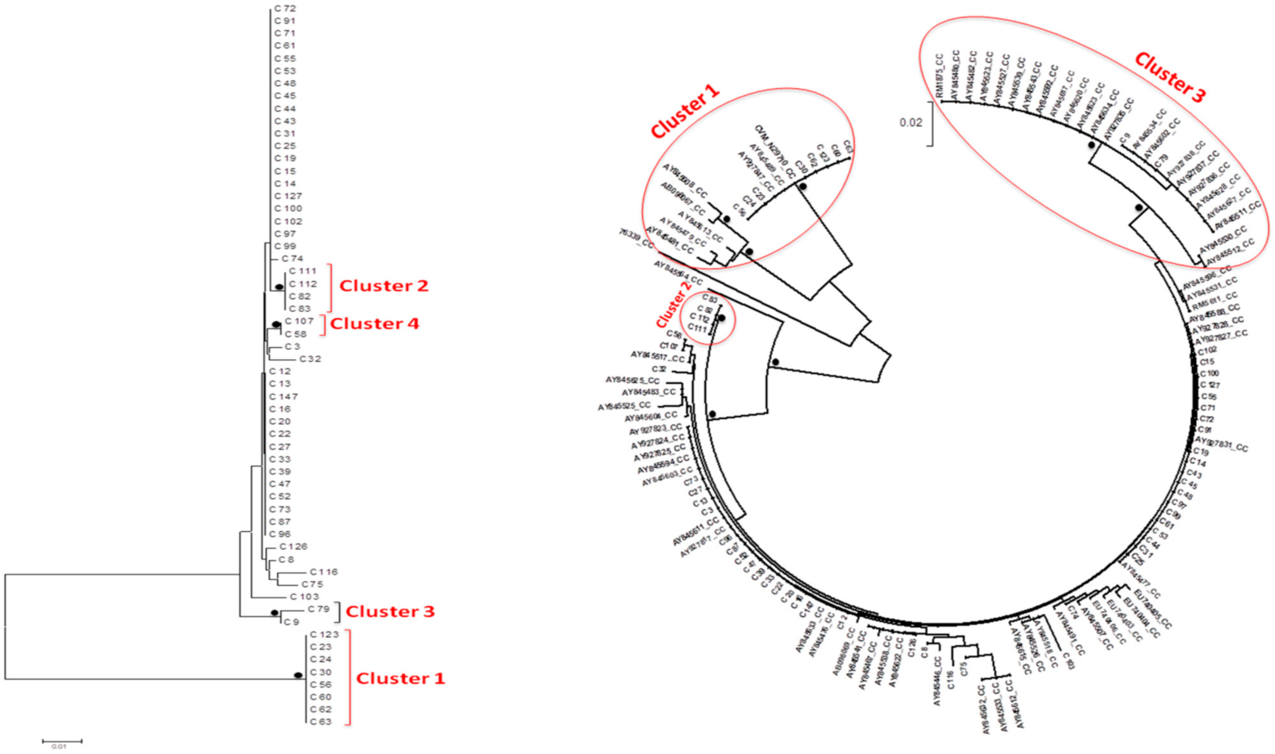
The phylogenetic trees of *C. jejuni* and *C. coli* isolates are shown in Figures 1a and 2a. Whereas most of the sequences found to be scattered inside the trees, seven clusters of the *C. jejuni* phylogenetic tree (Figure 1b) and three clusters of the *C. coli* tree (Figure 2b) were considered significant with bootstrap values >75%.

Among the 13 *C. jejuni* isolates of the first cluster, eight shared the same DNA and peptide fla type, while two isolates (03FLA-33FLA) originated from the same poultry farm had the same antimicrobial profile. All eight isolates from the second cluster shared the same DNA and peptide fla type, while there were two pairs (21FLA-63FLA and 40FLA-57FLA) that originated from the same farms and had similar antimicrobial profile. Likewise, in the third cluster, there were two isolates (20FLA-43FLA) that originated from the same farm and shared both identical DNA and peptide fla type and antimicrobial profile. All five isolates from the fourth cluster had the same DNA and peptide fla type and quite similar antimicrobial resistance. In the fifth cluster, there were three isolates (15FLA-27FLA-28FLA) that originated from two adjacent houses of the same farm and shared the same DNA and peptide fla type and antimicrobial profile. All seven clusters included reference sequences isolated from different regions (mainly USA, Europe, Tanzania, and Australia). However, no clear connection between them and the isolates of the current study could be made.

In the first cluster of *C. coli* phylogenetic tree, three of eight isolates (C60-C62-C63) originated from neighboring farms located in the same region and exhibited similar antimicrobial resistance patterns. The second cluster included only four isolates (C82-C83-C111-C112), all of which originated from the same poultry farm and shared similar antimicrobial profiles. Almost all reference sequences in the first and third clusters originated from the USA, with the exception of one sequence that originated from Japan.

(**a**) (**b**)

Figure 1. *Campylobacter jejuni* phylogenetic trees. Bullets represent clades, which had bootstrap values >75% of permuted trees. (**a**) The analysis involved 64 sequences. Most of the sequences were organized in nine significant clusters supported with high bootstrap values. (**b**) The optimal tree with the sum of branch length = 4.34019783 is shown.



(**a**) (**b**)

Figure 2. *Campylobacter coli* phylogenetic trees. Bullets represent clades, which had bootstrap values >75% of permuted trees. (**a**) The optimal tree with the sum of branch length = 0.21165541 is shown. The analysis involved 58 sequences. Most of the sequences were dispersed within the tree, whereas four significant sequence clusters were noticed. (**b**) The optimal tree with the sum of branch length = 0.54 is shown.

4. Discussion

The results of our study regarding the antimicrobial resistance are consistent with other studies [20,35]. More specifically, high resistance to ciprofloxacin (89.3%) and nalidixic acid (88.3%) was observed. Similar results were submitted on the view of the obligatory monitoring and report of antimicrobial resistance by Greece in 2014, while the overall resistance to quinolones at the EU level was slightly lower [36]. Resistance to fluoroquinolones in *Campylobacter* spp. was firstly reported in the late 1980s and, since then, there is a continuous increase of resistance to fluoroquinolones [37]. It has been observed that resistance appeared simultaneously with the introduction of these agents in animal production and veterinary medicine [11,22]. Since campylobacteriosis is considered to be a zoonosis, the presence of resistant strains in the food chain also has an influence on human infections [11]. Moreover, it has been noted that the proportion of ciprofloxacin-resistant members of the genus *Campylobacter* in poultry meat is often strikingly similar to the proportion observed in human clinical cases [36]. However, the transmission of fluoroquinolone-resistant bacteria from food-producing animals to humans is difficult to prove, and a recent global report on surveillance of antimicrobial resistance emphasized the need to collect more data of the effects of antimicrobial resistance in foodborne bacteria and human health [38,39]. Besides their excessive use in agriculture, the use of fluoroquinolones for infections other than gastroenteritis, as well as "self-medication", are often causes of the observed resistance in developing countries [40]. Therefore, traveling to developing countries has been implied to be a risk factor for gaining an infection caused by a resistant *Campylobacter* strain. In the developed world, one reason behind fluoroquinolone resistance might also be their inappropriate empirical use in the treatment of human infections. Patients treated with fluoroquinolones were later found to carry bacteria resistant to these antimicrobial agents [41].

A low percentage (6.8%) of *Campylobacter* among the strains recovered from caeca and neck skin samples was resistant to erythromycin. This result agrees with the respective ones of the EU survey [19]. However, the majority of these isolates revealed multi-antimicrobial-resistance properties, a finding demonstrated in other studies, as well [42]. Resistance to erythromycin, as a rule, corresponds to cross resistance to other macrolides (for example, azithromycin and clarithromycin), as well as to related drugs of the group of lincosamides (in particular, to clindamycin) and streptogramins [43]. Resistance of *Campylobacter* spp. to macrolides has remained in low and stable levels for a long time. However, there is also evidence from some parts of the world that resistance rates to erythromycin and other macrolides in *Campylobacter* species are slowly increasing [44,45]. Since fluoroquinolone resistance is common, the macrolides have become important in the treatment of campylobacteriosis, resulting in the development of macrolide resistance [36]. Use of macrolides in animal production as therapeutic or growth-promoting agents has been considered to be a significant factor in the selection of erythromycin-resistant *Campylobacter* strains [46]. However, acquisition of erythromycin resistance in *Campylobacter* species is a stepwise process and requires prolonged exposure, in contrast to the rapidly evolving fluoroquinolone resistance [47]. Moreover, Hao et al. have shown that erythromycin-resistant *Campylobacter* strains display a fitness disadvantage when compared with susceptible *Campylobacter* strains, which may lead to a low frequency of macrolide resistance in clinical isolates [48].

Regarding the remaining antimicrobial agents, resistance of *Campylobacter* isolates to tetracycline was found to be remarkably high, especially in strains derived from caecal content. Similarly high resistance rates were observed in the recent report of EFSA and ECDC [19]. Tetracyclines can be used in the treatment of campylobacteriosis, except for children under 9 years of age [49]. However, tetracycline resistance has emerged also among *Campylobacter* species [36]. In *Campylobacter* spp. the most common tetracycline resistance mechanism is a plasmid-mediated ribosomal protecting protein, Tet(O), encoded by the *tet*(O) gene [50]. No resistance to gentamicin and low resistance to streptomycin were found. Quite similar results have been observed in most EU members states [19]. Guyard-Nicodème et al. [51] tested the susceptibility of *C. jejuni* strains derived from broiler meat products collected in retail outlets and found similar results with our study for tetracycline and gentamicin. The main mechanism of aminoglycoside resistance in *Campylobacter* spp. is via aminoglycoside-modifying enzymes, which are usually plasmid-borne [20]. Only three *Campylobacter* isolates showed complete susceptibility to all antimicrobial agents tested. Similar results were submitted by Greece in the frame of the EU survey [36]. On the other hand, 7.6% of *C. jejuni* and 5% of *C. coli* were co-resistant to ciprofloxacin and erythromycin. This fact is worrying since these antimicrobial classes constitute the cornerstone in treatment of severe human campylobacteriosis. Moreover, 13.3% of *C. jejuni* and 10% of *C. coli* strains showed multidrug resistance (MDR), defined as resistance or no-susceptibility to at least three antimicrobial classes—fluoroquinolones, macrolides, tetracyclines, or aminoglycosides [52]. The increase of multidrug-resistant *Campylobacter* strains has increased [53,54], posing a serious risk of treatment failures, since there are very few treatment alternatives of campylobacteriosis caused by multidrug-resistant strains [21]. This increase may reflect the overuse of different antimicrobial agents in veterinary medicine and, especially, in poultry production [11,39], as well as in human medicine, especially when administered without medical prescription [40].

In order to determine the antimicrobial resistance of *Campylobacter*, the disk diffusion method was used. Although the agar dilution method used to determine the minimal inhibitory concentration (MIC) is considered the standard antimicrobial susceptibility testing method for thermophilic *Campylobacter* species [31], it is a labor-intensive, time-consuming, and costly test [55]. On the other hand, the disk diffusion method is simple, inexpensive, and can provide reproducible results if it is conducted carefully with appropriate standardization and quality controls [56,57]. The latter method has been standardized by the CLSI. However, according to those standards, it should be used only as a screening method for resistance to erythromycin and ciprofloxacin; a disk diffusion zone of 6 mm (growth up to the edge of a 6-mm disk) indicates resistance, while any inhibition zone would require an MIC determination of susceptibility (CLSI M45). Due to the lack of breakpoints for the rest of the antibiotics, it was decided to use breakpoints of *Enterobacteriacea* provided by CLSI M100 [58]. This study revealed a high-level correlation between the standardized agar dilution method and the agar disk diffusion method for aminoglycosides, quinolone/fluoroquinolones, erythromycin, and tetracycline in evaluating the resistance of *Campylobacter* spp. Several comparisons of agreement between the disk diffusion method and other susceptibility testing methods for *Campylobacter* have been conducted over the years [57–61], some of which have concluded that disk diffusion method could be used as a reliable alternative method for the testing of susceptibility of *Campylobacter* spp. to ciprofloxacin and erythromycin [57,58]. On the other hand, the results of other studies are different and indicate the unreliability of this method and the need of further standardization [60,61].

The selection of antimicrobials was done according to the published data concerning the widely used antimicrobial agents, both in poultry production and in the treatment of human campylobacteriosis, and followed the panel of antimicrobials from the EU protocol for harmonized monitoring of antimicrobial resistance in human *Salmonella* and *Campylobacter* isolates [62]. *Campylobacter* isolates from each positive sample were tested for resistance to ciprofloxacin, nalidixic acid, erythromycin, streptomycin, gentamicin, and tetracycline, as in the recent EU summary report [36].

Phylogenetic analysis of our strains using reference sequences highlighted seven clusters of *C. jejuni* isolates and three clusters of *C. coli* isolates in our study population. Almost all significant clusters included both sequences of the current cross-sectional study and reference sequences. No clear connection between our *C. jejuni* isolates and the reference sequences was found, even though most of the reference sequences originated from the USA. However, almost all reference sequences in the first and third clusters of *C. coli* originated from two surveys conducted in the USA. The first one dealt with isolates from retail chicken products and humans with gastroenteritis in central Michigan [63], while the second one dealt with isolates from the European CampyNet collection and National Antimicrobial Resistance Monitoring System, derived mostly from humans, chicken, cattle, and swine [64]. No safe conclusion could be drawn, though.

Some of the strains grouped in the same cluster and shared similar antimicrobial profile and fla types were derived from the same farms in different sampling time or from adjacent houses of the same farm. This finding indicates persistence of the infective strains in the house during turnaround time and further contamination of subsequent batches and/or infection of equipment and working clothes, leading to the spread of these strains from one house to another. Indeed, *Campylobacter* can be carried via boots and clothes of farm personnel and shared equipment between broiler houses of the same farm [65–67]. Moreover, the presence of colonized flocks has been found to be linked to the turnaround time in a broiler house. Periods of over 14 days can decrease the possibility of residual bacterial contamination [65], while the rapid flock turnover contributes to *Campylobacter* carryover with increased risk being reported if houses are restocked within nine days of depopulation [68]. In any case, the biosecurity and hygiene level should be maintained optimally during the empty time, as it is well known that an external reservoir can host multiple *Campylobacter* strains during the empty period, which will allow colonization of the new flock [66].

The presence of isolates with the same fla types and shared antimicrobial resistance patterns collected from different farms within a close distance in the same region could be attributed to vehicles that visit different farms in the same day without applying adequate disinfection, such as feed delivery trucks, vehicles for collection of litter and dead birds, or transport from the hatchery and to processing plants, which act as mechanical vectors and allow the transmission of these strains from each farm to another. Farm personnel and equipment (e.g., feed trucks) can carry *Campylobacter* between broiler houses and onto subsequent or neighboring farms [65]. Although feed is not seen as a high-risk *Campylobacter* contaminant within the broiler house, since the low water activity of the dry feed does not permit *Campylobacter* survival [67], it can be a vehicle for horizontal transmission into the broiler house [11]. Hald et al. [69] showed that the incidence of *Campylobacter* was lower in farms that feed homegrown wheat compared to farms that are dependent on external supplies. Jonsson et al. [70] found that livestock and broiler farms with flocks positive for *Campylobacter* spp. within a few kilometers' distance constitute significant risks for colonization in broiler flocks. Furthermore, live bird crates being contaminated with *Campylobacter* from previous (or other) flocks are reintroduced on the farm during catching, and quite often these crates undergo inadequate washing at the slaughterhouse [65]. Crates can carry identical genotypes of microorganisms that originated from broiler flock and abattoirs, which suggests that transport crates are responsible for contamination during transport to slaughter or they could contribute to the *Campylobacter* colonization of broiler houses [71].

5. Conclusions

In conclusion, the cross-sectional study carried out in Greece produced valuable results concerning the antimicrobial resistance and the molecular epidemiology of *Campylobacter* spp. in poultry production countrywide. High resistance to fluoroquinolones and tetracycline and low resistance to macrolides and aminoglycosides was found. A high genetic diversity was found, while some specific flaA types were found to share similar antimicrobial-resistance patterns. Phylogenetic analysis of the isolates revealed eight clusters of *C. jejuni* and three clusters of *C. coli*. Some isolates clustered together originated from the same or adjacent farms, indicating transmission via personnel or shared equipment. No clear connection between the reference sequences used and the isolates of the current study was found. These results are of high importance and constitute the foundation in understanding the molecular epidemiology and susceptibility patterns of *Campylobacter* spp. derived from poultry in Greece.

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