Assessment of biofilm formation by *Campylobacter* spp. strains mimicking slaughterhouse conditions

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"Nothing is too wonderful to be true, if it be consistent with the laws of nature."

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisboa (Lisbon, Portugal), during the period of September 2019 to December 2020, under the supervision of Dr. Maria João dos Ramos Fraqueza. The thesis was co-supervised at Instituto Superior Técnico by Prof. Cristina Viegas.

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Resumo

O objetivo deste trabalho foi avaliar a capacidade de formação de biofilme de estirpes de *C. jejuni* e *C. coli* provenientes de amostras de carcaças de frango obtidas no matadouro, de forma a explicar a sua sobrevivência ao longo do processamento até ao consumidor. A genotipagem de uma coleção de isolados (n=145) foi efetuada por polimorfismo de comprimento de fragmentos de DNA (RFLP) do gene *flaA*. Foi também realizada eletroforese em campo pulsado. Os perfis foram analisados pelo Bionumerics versão 6.6., e pela similaridade e agrupamento hierárquico, foram selecionadas 17 estirpes para os testes de suscetibilidade antimicrobiana e ensaios de biofilme pelo método cristal violeta. Os ensaios de biofilme mimetizaram condições semelhantes às encontradas no ambiente fabril associadas à temperatura, atmosfera e nível de contaminação. *C. jejuni* com perfis *flaA*-RLFP semelhantes surgiram em períodos de abate distanciados no tempo. Todas as estirpes revelaram ser multirresistentes. A formação de biofilme foi dependente da estirpe, da concentração de bactérias e da tolerância ao oxigénio. A temperatura de 10ºC afetou negativamente a formação de biofilme. *C. jejuni* 46E, 61C e *C. coli* 65B destacaram-se com níveis significativos de produção de biofilme quando em microaerofilia e aerobiose a 42ºC. As estirpes formaram agregados (11 em 17), mantendo-se viáveis após 72h, em aerobiose a 10ºC. A maioria das estirpes foram fracas produtoras de biofilme de acordo com o sistema de classificação de Stepanović *et al.* (2000). Outros fatores poderão contribuir para a permanência de *C. jejuni* e *C. coli* no ambiente fabril.

Palavras-chave: *Campylobacter, frango, biofilme, sobrevivência, resistência antimicrobiana, flaA-RFLP.*

Abstract

The aim of this research was to evaluate biofilm formation of *C. jejuni* and *C. coli* strains isolated from poultry samples, taken at slaughterhouse level, in order to explain their survival and persistence through slaughter processing steps until the final consumer. Genotyping of an isolate collection (n=145) was performed by means of restriction fragment length polymorphism (RFLP) of the gene *flaA*. Pulse filed gel electrophoresis (PFGE) was also performed. Profiles were analysed with Bionumerics version 6.6., and by similarity and hierarchical clustering, 17 strains were selected for antimicrobial susceptibility tests and biofilm formation assays by crystal violet staining method. Biofilm assays were performed mimicking slaughterhouse environmental conditions, namely temperature, atmosphere, and contamination levels. Results obtained indicated that *C. jejuni* strains with similar *flaA*-RFLP profiles were present at slaughterhouse on different processing days. All strains tested were multidrug resistant, except one. Biofilm formation ability was strain dependent, and it was affected by inoculum concentration and tolerance to oxygen levels. The temperature of 10ºC negatively affected adherence levels. However, under microaerophilic and aerobic atmospheres at 42ºC, three strains: *C. jejuni* 46E, 61C and *C. coli* 65B, stood out exhibiting significant levels of biofilm formation. Strains formed aggregates (11 out of 17), and maintained viable cell counts after 72h of incubation at 10°C under aerobiosis. Most strains were considered weak biofilm producers by Stepanović *et al.* (2000) classification system. Other factors besides the ones studied could contribute to the permanence of some *C. jejuni* and *C. coli* strains at slaughterhouse.

Key words: *Campylobacter, poultry, biofilm, environmental survival, antibiotic resistance, flaA-RFLP.*

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List of symbols and Acronyms

AMC - Amoxacillin + Clavulanic acid
AMP - Ampicillin
BHI - Brain Heart Infusion
CDC - Centers for Disease Control and Prevention
CDT - Cytolethal distending toxin
CFU – colony forming units
cgMLST - Core genome multi-locus sequence typing
CHL - chloramphenicol
CIP - Ciprofloxacin
comb. nov. - Combinatio nova
CPP – Critical control point
CR - Carvacrol
DNA - Deoxyribonucleic acid
e.g. - exempli gratia in Latin, “for example.”
EC – European commission
ECDC - European Centre for Disease Prevention and Control
EDTA - Ethylenediaminetetraacetic acid
EFSA - European Food Safety Authority
EG - Eugenol
EPS - extracellular polymeric substances
ERI - Erythromicin
ESBL - Extended-spectrum beta-lactamase
ETP – Ertapenem
EU – European Union
FBO - food business operator
FDA - Food and Drug Administration
FoodNet - Foodborne Diseases Active Surveillance Network
GBS - Guillain Barré syndrome
GC-content - guanine-cytosine content
GEN - Gentamicin
GHP - good hygienic practices
GI - gastrointestinal
GM1 – monosialotetrahexosylganglioside
GRAS - generally recognized as safe
HACCP - Hazard Analysis and Critical Control Point
HIV - human immunodeficiency viroses
INE - Instituto Nacional de Estatística
INSA - Instituto Nacional de Saúde Doutor Ricardo Jorge

ISO - International Organization for Standardization
LOS - Lipo-oligossacharides
MALDI-TOF-MS - Matrix-assisted laser desorption/ionization
mCCD - Modified charcoal cefoperazone deoxycholate
MLST - multi locus sequence typing
MRSA - Methicillin-resistant *Staphylococcus aureus*
NA - Nalidixic acid
NARMS - National Antimicrobial Resistance Monitoring System
No. - Number
O.D.c. - Cut-off O.D.
OD – Optical density
PCR - Polymerase chain reaction
PFGE - Pulse Field Gel electrophoresis
pH - potential of hydrogen
QACs - quaternary ammonium compounds
RFLP - Restriction fragment length polymorphism
ROS - radical oxygen species
sp. nov. - species nova
STEC - Shiga toxin-producing *Escherichia coli*
STX - Trimethoprim + Sulfamethoxazole
TBE - Tris-Borate-EDTA
TC - Trans-cinnamaldehyde
TE - Tris-EDTA
TET - Tetracycline
TFP - 1,3,5-triphenylformazan formazan
T6SS - type VI secretion system
TTC - 2,3,5 triphenyltetrazolium chloride
UK – United Kingdom
USA – United States of America
UV – Ultraviolet
VBNC - viable but nonculturable
VRE - vancomycin resistant Enterococci
WHO – World Health Organization

ºC – Celsius degrees
< - Less-than
> - Greater-than
≈ - Approximately
= - Equal
≈ - Approximately

µm – Micrometre

µl – microliter

µg – microgram

% - Percentage

O_2 - Oxygen

CO_2 - Carbon dioxide

ZnO - Zinc oxide

a_w - Water activity

mL - milliliter

Mbp - megabase pair

bp – basepairs

h – hours

H_2 – Dihydrogen

kg - Kilogram

g - gram

A – Absorbance

KDa - kilodalton

σ - sigma factor

Introduction

Campylobacter jejuni and *Campylobacter coli* are the most frequently reported causative agents of gastroenteritis in the EU since 2005 (EFSA and ECDC, 2019). *Campylobacter jejuni* species in particular, is accountable for the vast majority of cases, followed by *Campylobacter coli* (Vidal *et al.*, 2016). Furthermore, *C. jejuni* is strongly associated with Guillain-Barré syndrome, a neuropathy demonstrated to be caused by molecular mimicry between *C. jejuni* lipo-oligossacharides (LOS) and human gangliosides, triggering an auto immune response (Goodfellow & Willison, 2016). Poultry reservoir is generally accepted as the primary source of infection (Teh *et al.*, 2014). Therefore, it is of great importance *C. jejuni* and *C. coli* survival and persistence at slaughterhouse, the last stage in the poultry meat production chain, before selling at retail.

The effects of adverse environmental conditions on vital functions of bacterial cells, lead to stress responses associated with changes in gene expression. It has been demonstrated that bacteria can make a transition to a state where metabolic activity is preserved by a “dormant state”, in which cell division is suspended. This phenomenon is designated “non culturable form”, and it protects bacteria until favourable conditions are available. The presence of these cells should be taken into account, especially in food industry environments. Another form of resistance is the development of biofilms on the surface of materials and foods. Biofilm formation is a central key phenomenon to study. Bacterial communities living in a biofilm possess extracellular polymeric substances (EPS) forming a thick matrix that prevents diffusion of chemicals (e.g. antimicrobial agents) inside the biofilm. Consequently these bacteria are more tolerant to various stresses, not only chemical, but also physical and biological (Efimochkina *et al.*, 2017).

When compared to other food-borne pathogens, such as *Salmonella enterica*, and shiga toxin-producing *Escherichia coli* (STEC), *C. jejuni* is more fastidious, having more restrict growth requirements. It requires a reduced oxygen atmosphere (microaerophilic) and an optimal growth temperature of 42ºC (thermophilic). Additionally, it is susceptible to several environmental stresses found in the food industry, such as high temperature, low pH and osmotic stress (Teh *et al.*, 2014). From this perspective, *C. jejuni* theoretically should not be able to survive and persist along the poultry food chain. This paradox has been the focus of many researches suggesting that biofilm formation could be the underlying mechanism that allows the pathogen to survive (Teh *et al.*, 2014). In this context, characterizing isolates obtained from poultry samples taken at slaughterhouse, and testing for their biofilm formation capacity, was the main driving motivation for this dissertation.

The following thesis is divided into 3 major chapters: a literature review, experimental work, results obtained and discussion. Literature review aims to provide recent information about thermophilic *Campylobacter* spp. (*C. jejuni* and *C. coli*), and their importance. First, the characterization of this bacteria species is presented, followed by the characterization of the disease (campylobacteriosis) and its implications. The following topics are addressed: clinical features of the disease (symptoms description); epidemiologic data on human campylobacteriosis; reservoirs, transmission routes and risk factors, including a focus on the current data about pathogen persistence along the food chain; virulence factors; and multidrug resistance. The last part of literature review is related to *C.jejuni*/*C. coli* ability to

form biofilm, comprising: biofilm definition; steps for biofilm formation; genes related, and importance in food industry.

As for the experimental work section, materials and methods are detailed, and it is described how a representative collection of *Campylobacter* isolates from different poultry flocks at slaughterhouse level was gathered. The aims were: to characterize *Campylobacter* isolates from poultry by *flaA*-RFLP genotyping and pulse field gel electrophoresis; to assess antimicrobial resistance of selected isolates to different classes of antibiotics; and to evaluate strains with different profiles for their ability to form biofilms, mimicking slaughterhouse conditions.

At last, results obtained were analysed and discussed according to statements made by several authors that performed techniques and assays related with each of the aims defined.

Chapter I - Literature review

1. *Campylobacter jejuni* and *Campylobacter coli*: species description

The first observation of bacteria belonging to the genus *Campylobacter* may have occurred in 1886 by Theodor Escherich. The physician described a non-culturable spiral shaped bacteria, found in the colon of children with a diarrhoeal disease called “cholera infantum” (Butzler, 2004; Epps *et al.*, 2013).

Campylobacter fetus was reported as the first species of *Campylobacter* isolated from ovine aborted fetuses by McFadyean and Stockman in 1913. They observed large numbers of ‘S’ shaped bacteria in smears made from uncontaminated exudate of foetal stomach. Staining was successful with methylene blue or diluted carbol-fuchsin. The authors classified the organism as “vibrio-like”, and therefore referred to it only as *Vibrio*.

The genus *Campylobacter* was only proposed in 1963 by Sebald and Véron, concomitant to the renaming of the species *Vibrio fetus*. and *V. bubulus* as *Campylobacter fetus* sp. nov., comb. nov., and *C. bubulus* sp. nov., comb. nov., respectively (Sebald & Véron, 1963). Formerly, *Campylobacter* spp. were classified as *Vibrio,* due to the morphologic similarities with *Vibrio cholerae*. However, there are significant differences in biochemical characteristics, growth conditions, and DNA base nucleotide content (Rettig, 1979). Today the genus is part of the family *Campylobactereceae*, only proposed in 1991 (Vandamme & De Ley, 1991). The taxonomic hierarchy related with *Campylobacter* is presented in Table 1.

Table 1. Taxonomic hierarchy of thermophilic Campylobacter.

Phylum:	Proteobacteria
Class:	Epsilonproteobacteria
Order:	*Campylobacterales*
Family:	*Campylobacteraceae*
Genus:	*Campylobacter*

The genus *Campylobacter* comprises a total of 33 validly published species on List of Prokaryotic names with Standing in Nomenclature (LPSN), isolated from various sources such as stools from humans with enteric infection, human oral cavity and gastrointestinal (GI) tract, poultry, rabbits, marine mammals, black-headed gulls, etc. Recently, between 2015 and 2020, 8 new species have been validly published: *Campylobacter geochelonis*, isolated from western Hermann's tortoise (reptile) (Piccirillo *et al.*, 2016); *Campylobacter hepaticus*, isolated from livers of chickens with spotty liver disease in Australia (Van *et al*., 2016); *Campylobacter pinnipediorum* isolated from abscesses and internal organs of different seal species in Scotland (UK) and California (USA) (Gilbert *et al*., 2017); *Campylobacter ornithocola* a novel member of the *Campylobacter lari* group isolated from wild bird faecal samples from the city of Valdivia (southern Chile) (Cáceres *et al.*, 2017); *Campylobacter blaseri* discovered during a study to assess the faecal microbiome of common seals in a Dutch seal rehabilitation center (Gilbert *et al.*, 2018); *Campylobacter armoricus isolated* from surface water and stools from humans with enteric infection (Boukerb *et al.*, 2019); *Campylobacter novaezeelandiae* identified during studies of *Campylobacter* isolation from bird faeces and rivers in New Zealand

(Bloomfield *et al*., 2020); and *Campylobacter portucalensis* isolated from the preputial mucosa of bulls in Alentejo (Portugal) (Silva *et al.,* 2020).

Within the genus *Campylobacter*, the species *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis,* form a genetically close group known as 'thermophilic campylobacters'. The name is based on their optimal growth temperature, 42°C (Fitzgerald, 2015).

In early years, the challenge of being able to culture, isolate and characterize these agents, underestimated their importance as cause of disease until the 70's (1970) (Sheppard & Maiden, 2015). The improvements made in isolation procedures renewed the interest in *Campylobacter* research in the 1980's. Currently, *C. jejuni* and *C. coli* are considered the most important enteropathogens among *Campylobacter* spp. (Epps *et al.*, 2013). They are the main causative agents of campylobacteriosis, the most commonly reported gastrointestinal foodborne disease in the European Union since 2005, accounting for 70% of all zoonoses reported followed by salmonellosis (EFSA & ECDC, 2019). Other species such as *C. lari* and *C. upsaliensis* have also been isolated from patients with diarrhoeal disease, but they were unfrequently reported (WHO, 2020).Originally, *C. jejuni* was isolated from faeces of cattle with diarrhea, while *C. coli* was isolated from diarrheic faeces of pigs (Ngulukun, 2017).

In terms of their characteristics, *Campylobacter* are Gram-negative, with thin cells, spirally curved, 0.2 to 0.8 µm wide and 0.5 to 5.0 µm long. They can be S-shaped, curved or with gull wing shape when two cells form a short chain. These bacteria have a single flagellum at one or both poles and present a characteristic corkscrew like motility. Flagella can have two to three times the length of the cell (Vandamme *et al.*, 2010). The feature that distinguishes them from other pathogenic bacteria transmitted by food is their restrictive growth conditions (Chlebicz & Śliżewska, 2018). *Campylobacter* optimal atmosphere conditions for growth are strictly microaerophilic with 3-15% oxygen and 3-15% carbon dioxide (Vandamme *et al.*, 2010). Optimal growth temperature ranges between 37-42°C (Public Health England, 2018). All parameters regarding growth limits are presented in Table 2.

Table 2. Limits for Campylobacter spp. growth adapted from "Campylobacter species food safety authority Ireland." (Food Safety Authority of Ireland, 2011).

Parameter	Range	Optimum
Temperature (°C)	32 - 45	42
pH	4.9 – 9.0	6.5 – 7.5
NaCl (%)	0 – 1.5	0.5
Water activity (a_w)	>0.987	0.997
Atmosphere	Microaerophilic	5% O_2 and 10% CO_2

These bacteria do not form spores; however, a coccoid form may appear in old cultures or cells exposed to oxygen (Ngulukun, 2017). In fact, when confronted with unfavourable conditions, *Campylobacter* has the ability to turn into a state known has: "viable but nonculturable" (VBNC) (Chlebicz & Śliżewska, 2018).

In terms of nutrition, they are chemo-organotrophs; don't ferment nor oxidize carbohydrates and do not produce acid or neutral end-products. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates (Vandamme *et al.*, 2010).

These bacteria are both oxidase and catalase positive (Public Health England, 2018). In fact, *C. jejuni* and *C. coli* are phenotypically closely related. Biochemically, these species can only be differentiated by the ability to hydrolyse hippurate. *C. jejuni* can hydrolyse hippurate although some subspecies are hippurate negative, while *C. coli* is unable to hydrolase hippurate (Ngulukun, 2017).

Campylobacter colonies are translucent when cultured on blood agar. They appear round, convex, with a regular edge and sometimes slightly pink. On the selective medium Charcoal cefoperazone deoxycholate agar, colonies are grey/white or creamy grey, and moist in appearance (Public Health England, 2018). Serum or blood can enhance their growth (Vandamme *et al.*, 2010).

As for their genome, *C. jejuni* and *C. coli* carry a rather small genome, composed by a singular circular chromosome with 1.59 to 1.77 Mbp in size, and an average GC-content of 30.3-30.6%. Additionally, because they possess a high gene content of 94-94.3%, their genome is considered one of the densest bacterial genomes sequenced to date (Backert *et al.*, 2016).

2. Campylobacteriosis

2.1. Historical perspective

Elizabeth King was the first raising the possibility that bacteria designated “related vibrios” (*Campylobacter* spp.) were associated with enteric disease in 1957 (Walker *et al.*, 1986). King was the first microbiologist that studied in depth *Campylobacter* strains (Skirrow, 1977), isolated from blood samples of infected children with diarrhoea. Important observations allowed the distinction of the so called “related vibrios” from *Vibrio fetus*, for example the fact that “related vibrios” failed to grow at 25 °C and grew much better at 42°C (King, 1957).

Fundamental findings that further allowed the establishment of a causal relationship between *Campylobacter* spp. and enteritis were the ones made by Butzler *et al.* (1973). Over a period of six months the authors conducted a research for common enteropathogens and *Vibrio* species in stools of hospitalized patients presenting diarrhoea. Butzler *et al.* (1973) stated that it was relatively easy to isolate the “related vibrios” by means of a filtration technique used in veterinary microbiology for coproculture, along with the use of a culture medium containing antibiotics. This filtration technique was successful based on the fact that *Campylobacter* spp. were small enough to pass through a filter that would hold back other microorganisms (Skirrow, 1977). Ultimately, Skirrow in 1977 confirmed these findings, using selective culture for *Campylobacter* (specifically *C. jejuni* and *C. coli*), and isolated the bacteria in 57 out of 803 random patients with diarrhoea and severe abdominal pain. The highest incidence was found to occur in young children. *Campylobacter* was still a relatively unrecognised cause of acute enteritis, but Skirrow’s findings suggested that after all, they were probably a common cause.

2.2. Symptoms/ Clinical features

Predominantly, infection caused by *C. jejuni* and *C. coli* is characterised by an acute, self-limited gastrointestinal illness. Gastroenteritis caused by *C. jejuni* is clinically similar from the one caused by *C. coli* (Kaakoush *et al.*, 2015). Humans infected with *C. jejuni* or *C. coli* usually present symptoms of acute watery or bloody diarrhoea, fever, abdominal pain, and sometimes vomiting (Chlebicz & Śliżewska,

2018). Cramps can last on average 6 days. The peak of illness can last 24 to 48h, and abdominal pain may mimic a case of appendicitis (Kaakoush *et al.*, 2015).

A human experimental study comprising 111 adult volunteers conducted by Robert E. Black *et al.*, tested the infective dose of two strains of *C. jejuni* isolated during two different outbreaks, in Connecticut and Minnesota. The lowest infective dose challenged on healthy young adults, was $8x10^2$ CFU suspended in 150mL of milk, and it was demonstrated to be enough to cause illness. Also, the risk of infection was increased when higher doses of the inoculum were administrated (Black *et al.*, 1988). Furthermore, the ingestion of 500 bacteria in 180mL of pasteurized milk, was reported to be enough to cause mild diarrhoea with mucus and abdominal cramps after 4 day of ingestion in a case report described by the epidemiologist Robinson in 1981. The strain tested was a known *C. jejuni* strain originated from a milk-borne outbreak (Robinson, 1981). According to the study and report mentioned, it was possible to presume that infective dose for *C. jejuni* is probably low.

As for the incubation time of the disease, it usually ranges between 1 to 7 days, and it can be longer in the case of exposure to a low infective dose (Chlebicz & Śliżewska, 2018).

Although people of all ages can get infection with *C. jejuni* or *C. coli*, in developing countries *Campylobacter* infections are more frequent on children under the age of 2 years, sometimes resulting in death (WHO, 2020).

In terms of diagnosis, the assessment of human infection is usually based on stool cultures. In addition to culture methods, non-culture methods like polymerase chain reaction (PCR) are also used for confirmation. For species identification of isolates submitted to the national reference laboratory, biochemical tests or molecular methods are conducted (EFSA and ECDC, 2019).

The human infection with *Campylobacter* usually does not require antibiotic therapy, although it is necessary for cases of immunocompromised individuals (e.g. HIV-positive), as well as for situations of persistent fever and bloody diarrhoea (during more than 7 days). Currently, azithromycin and erythromycin are the first line drugs of choice when antimicrobial therapy is indicated (García-Fernández *et al.*, 2018).

In rare cases, infection caused by *C. jejuni* and *C. coli* may have other outcomes in addition to the typical pattern of self-limiting diarrhoeal disease. Pancreatitis, peritonitis, and massive gastrointestinal haemorrhage are examples of some gastrointestinal manifestations that have been associated (Allos, 2001). Additionally, serious chronic diseases such as inflammatory bowel diseases (Crohn's disease and ulcerative colitis); and irritable bowel syndrome, have also been correlated (Kaakoush *et al.*, 2015). Moreover, extraintestinal complications can occur, even though they are very rare. Those include meningitis, endocarditis, septic arthritis, and bacteraemia, which are most likely to occur in immunocompromised patients (Allos, 2001). Guillain Barré syndrome (GBS) is the most concerning post infectious complication. GBS was associated with *C. jejuni* for the first time in 1982, as a result of a reported case of enteritis caused by *C. jejuni* (Mishu & Blaser, 1993). It has been difficult to confirm this association because bacteria are often eliminated from the body before the onset of neurological symptoms. The syndrome is characterised by an acute demyelinating disease of the peripheral nervous system, and it affects about 1-2 people per 100.000 in the USA population each year. It is a neurologic condition, in which progressive symmetrical weakness in the limbs takes place,

with or without hyporeflexia, which in turn can affect muscles innervated by cranial nerves and respiratory muscles (Poropatich *et al.*, 2010). *C. jejuni* is considered to be a common trigger of the syndrome, probably preceding 30% of cases. The risk of developing GBS after going through campylobacteriosis is very small, and estimations indicate about 1 case per 1000 *C. jejuni* infections (Poropatich *et al.*, 2010). An interesting case related to this issue, were the decreased rates of GBS occurring concomitant to the decreased number of campylobacteriosis cases, after the implementation of food safety measures to reduce contamination on fresh poultry meat in New Zealand (Baker *et al.*, 2012). Symptoms may only show 1 to 3 weeks after diarrhoeal disease, for this reason humoral immunopathogenic mechanisms are probably involved. It is assumed that the syndrome is the result of a molecular mimicry between peripheral nerve glycolipids or myelin proteins, and structures on the lipopolysaccharides (LOS) of some *Campylobacter* strains. The nerve damage is a consequence of cross-reactivity between antibodies produced in response to *C. jejuni* LOS and human gangliosides, like ganglioside GM1 (Goodfellow & Willison, 2016).

2.3. Disease Epidemiology

The foodborne disease caused by *Campylobacter* is named campylobacteriosis. Currently, it is the most common human gastrointestinal infection worldwide (WHO, 2020; Mughal, 2018; Laughlin *et al.*, 2019). Globally, an estimated number of 400-500 million people were affected by this disease each year (Mughal, 2018).

In the EU, *Campylobacter* is the most commonly reported gastrointestinal bacterial pathogen since 2005 (EFSA and ECDC, 2019). A significant increasing trend occurred during the period of 2008 to 2013, that finally stabilized between 2014 and 2018. The number of reported cases of campylobacteriosis was 246,158 and 246,571, in 2017 and 2018 respectively (EFSA and ECDC, 2019). In terms of cost to public health systems, and lost productivity in the EU, it was estimated that campylobacteriosis was accountable for about 2.4 billion euros a year (EFSA, 2014).

Regarding residents in the United States, estimations made by the CDC indicate that *Campylobacter* infection affects 1.5 million individuals every year (CDC, 2019).

United Kingdom, reported that the number cases increased from 52.381 in 2016 to 56.729 in 2017 (increase of 4.348 cases). Data provided by Public Heath England also indicated that individuals with 50-59 years old accounted for the highest number of laboratory reports (Public Health England, 2017).

Also, in Australia, *Campylobacter* was the most notified pathogen responsible for foodborne gastroenteritis. Moreover, Australia and New Zealand are among the high-income countries with the highest notification rates of *Campylobacter* in the world. In recent years, notification rates in Australia stabilized, but incidence raised to 139.7/100000 habitants in 2015, with 10 cases estimated for every notified case within the community. *C. jejuni* and *C. coli* were estimated to account for approximately 95% of human cases (Varrone *et al.*, 2018).

As for the African continent, significant gaps exist in epidemiological data. However, it is clear that infection is particularly prevalent in children under 5 years old, with a variable reported prevalence of 2% in Sudan to 21% in South Africa (Asuming-Bediako *et al.*, 2019).

Regarding Asia and the Middle East, data on campylobacteriosis is also limited. A study based on the detection rates of *Campylobacter* in raw chicken and consumption trend in China from 2007 to 2010, predicted that 1.6% of the urban population and 0.37% of the rural population were affected by campylobacteriosis every year (Kaakoush *et al.*, 2015). In Singapore, from 1990 to 2015, a total of 5370 campylobacteriosis cases were reported. Of the reported cases from 2001 to 2014, *C. jejuni* was the most isolated specie (84-100%) followed by *C. coli* (0-4%). The occurrence was higher in infants (0-4 years old), children (5-14 years old), and adults over 55 years old. Additionally, based on 2007-2011 reports to the International Society of Travel Medicine and to the CDC, *Campylobacter* was found to be a frequent cause of diarrhoeal disease in travellers returning from Asia (Premarathne *et al.*, 2017).

Regarding Portugal, in 2017 the incidence rate for campylobacteriosis was 5.8 cases/100 thousand habitants, which was superior to the incidence rate in 2016 (3.5). It is currently inserted within the group of most frequent diseases of obligate declaration, accounting for a total of 597 cases of residents in 2017 (INE, 2018). Between the period of 2009-2012, 837 strains were analysed by INSA, 84.5% were *C. jejuni*, 14.8% were *C. coli*, 0.2% *C. upsaliensis*, and 0.1% *C. concisus*. The age group identified as the most at risk corresponded to the age group comprising 1-15 years old (Duarte *et al.*, 2013).

2.4. Reservoirs, Transmission routes and risk factors

Campylobacter spp. is considered to be part of the normal microflora of the gastrointestinal tract of many domestic animals and birds. Examples of reservoirs are: wild birds, cattle, sheep, swine, pets (cats and dogs), and most importantly, broilers (Workman *et al.*, 2005), along with commercial turkeys and ducks, which can also serve as reservoirs for both *C. jejuni* and *C. coli* (Kaakoush *et al.*, 2015). It is impossible to recognise by naked eye if commercial broilers are carriers, because most often, they are asymptomatic (Brown *et al.*, 2014).

The detection of *Campylobacter* in food and animals, is based on classical culture methods. The confirmation of genus and specie is performed by biochemical, molecular methods (PCR), and mass-spectrometry methods such as MALDI-TOF-MS (EFSA and ECDC, 2019).
Due to broad range of hosts and high diversity of *C. jejuni* and *C. coli* genotypes, tracing and attributing source of infection can be hard (Kittl *et al.*, 2013).

In 2010, the scientific opinion on quantification of the risk posed by broiler meat to human campylobacteriosis in the EU, indicated that broiler meat may be accountable for a total of 20%-30% of human campylobacteriosis cases, while 50%-80% may be attributed to chicken reservoirs as a whole, including broilers and laying hens (EFSA, 2010).

Additionally, a recent systematic review of studies linked to source attribution using MLST data determined that chicken was consistently the main source associated to human infection, in high income countries. The second most associated source apparently were ruminants (Cody *et al.*, 2019). Thépault *et al.* (2017) also conducted an advanced approach for source attribution. Based on genome wide analyses, source attribution was estimated through a reference pan-genome list with 1,810 genes, identified by ‘gene by gene’ comparison of 884 genomes of *C. jejuni* isolates from animals, humans and environment. Host-segregating markers were selected to attribute the sources of 281 United Kingdom

isolates and 42 French isolates. Analyses performed assigned 56.8% of British isolates from clinical cases to chicken, while French isolates, also from clinical cases, ended up with an even distribution between chicken and ruminant reservoirs. Nevertheless, the findings emphasized the importance of chicken as a reservoir.

2.4.1. Human transmission routes and risk factors

There are several important environments and contributors to human infection, and their specific roles in the complex epidemiology of campylobacteriosis is still not fully understood (García-Sánchez *et al*., 2018). Main transmission routes and sources of infection are schematized in Figure 1.

When it comes to human exposure, the main risk factor is ingestion of contaminated food, although there is also a risk of getting infected through direct contact with animals (Kaakoush *et al.*, 2015). Transmission occurs through the fecal-oral route (Facciolà *et al.*, 2017), by ingestion of undercooked meat, contaminated ready-to-eat food, unpasteurized milk, or untreated waters (García-Sánchez *et al.*, 2018). In professional and domestic kitchens particularly, there are important risk factors related to cross contamination due to inappropriate handling of raw meat, the major ones are: inadequate refrigeration of food; poor washing of hands and surfaces such as cutting boards and dishes; close contact of raw meats with other foods and cooking meat to inadequate temperatures (European Commission, 2017).

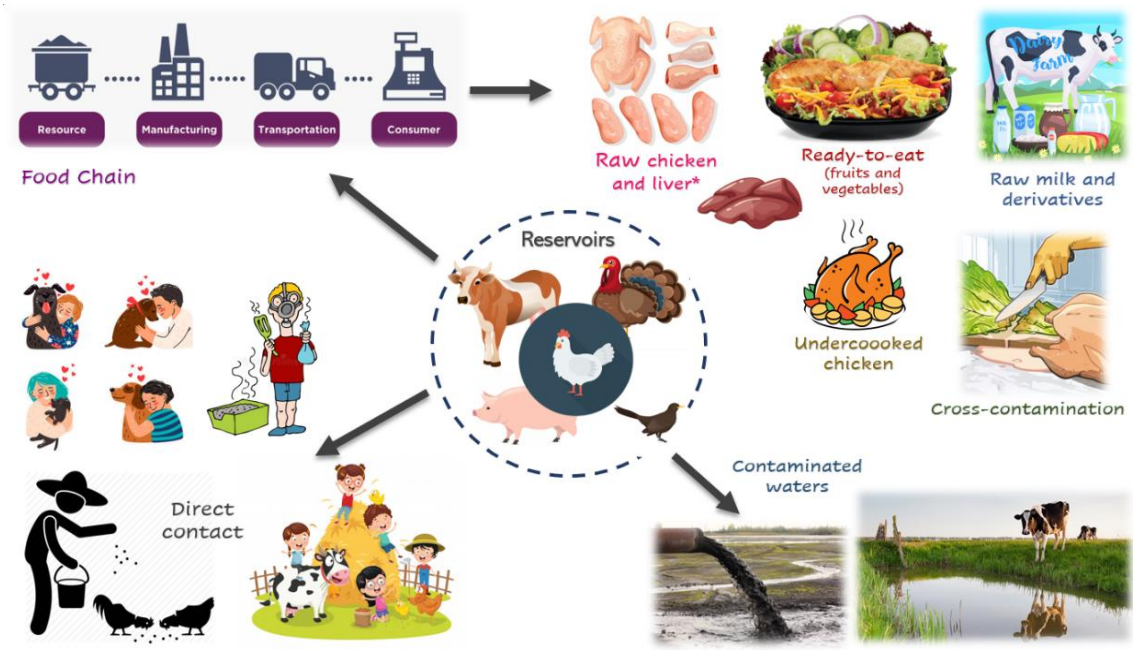


*Figure 1. Transmission routes, from reservoirs (in the centre) to sources of infection: poultry meat (raw or undercooked); raw milk; cross contamination; consumption of contaminated waters; direct contact. *Chicken and duck livers are important sources of Infection, implicated in outbreaks of campylobacteriosis.*

Another considerably important risk factor for humans is travelling. In the 2015 annual foodborne illness Surveillance Report from FoodNet, it was estimated that out of seven common foodborne bacterial pathogens (*Campylobacter*, *Listeria monocytogenes*, *Salmonella*, Shiga toxin-producing

Escherichia coli, *Shigella*, *Vibrio*, and *Yersinia*), *Campylobacter* was accountable for the highest number (686) of travel-associated reported infections (CDC, 2017). Unfortunately, in many of these cases, the link between the specific source and the human infection remains unidentified (García-Sánchez *et al*., 2018).

2.4.2. The poultry food chain

Giving the importance of poultry as a major source of infection, it is necessary to know the influence that each stage has on *Campylobacter* spp. "journey" from the farm to the retail store. Poultry food chain is comprised by: primary production at rearing farms; transportation to slaughterhouses; slaughter, and subsequent processing for production of chicken meat products; selling of products at retail, and finally handling and consumption in people's homes and restaurants. All the stages could be responsible for the transmission of *Campylobacter* to the final consumers. Production conditions between countries in the EU vary a lot, which consequently reflects on variation in the annual number of *Campylobacter* positive-flocks (Skarp *et al.*, 2016).

Fraqueza *et al.* (2014) evaluated *Campylobacter* prevalence at slaughterhouse level, and sampled chicken flocks from extensive, intensive and organic production system, representing in total 96,386 birds slaughtered. The frequency of *Campylobacter* on sampled intensive flock carcasses analysed was 100%, but after deboning the breasts from different flocks, 93% were positive for *C. jejuni* or *C. coli*. EFSA (2019) reported that few member states reported data on *Campylobacter* in foods. The highest occurrence was observed in fresh meat from broilers, with a frequency of 37.4%, followed by fresh meat from turkeys 31.5%.

2.4.3. Poultry colonization at farm

The entry and establishment of *Campylobacter* on farms always comes "from outside", meaning that it will be dependent on other animals, people, manure, water, etc. Young birds (chicks) are assumed to be "*Campylobacter* free", since no vertical transmission has ever been documented (European Commission, 2017). For this reason, in the farm environment, horizontal transmission is accepted as the usual route (García-Sánchez *et al.*, 2018). Colonisation generally begins after the first 2-3 weeks of age (European Commission, 2017). When *Campylobacter* finally gets established into a chicken flock, it spreads very rapidly, and as a result the intestinal tracts of the majority of chickens are colonized within 1 week (García-Sánchez *et al.*, 2018). Often, the prevalence can even reach 100% within a few days (European Commission, 2017). As time passes, broiler chickens remain colonized until slaughter and subsequent steps in the food chain, and eventually reach the consumer (García-Sánchez *et al.*, 2018).

Interactions occurring between farm animals, wild animals and humans are complex, because they are driven by many factors, such as: water flow, climatic conditions (and other ecological variables), presence of domestic animals, and defecation of wild birds on farm animals. Apart from these factors, there are also invertebrates such as flies, beetles and slugs, who have also been identified as carriers of *Campylobacter*, and could have a role as transmission vectors (García-Sánchez *et al.*, 2018). Indeed, Royden *et al.* (2016) actually indicated that dipteran flies were proven carriers of *Campylobacter*, and

through MLST (multi locus sequence typing) demonstrated that flies collected from four UK broiler farms were carrying broiler-associated sequence types responsible for human enteric disease.

The most important risk factors for *Campylobacter* colonization reported in the latest scientific opinion by EFSA panel on Biological Hazards are: slaughter age (higher prevalence and contamination levels at the end of producing cycle); seasonality, with higher risk in summer; thinning (partial depopulation of broilers for slaughter); contaminated drinking water; and previous *Campylobacter* positive flock in the aviary system, which leads to carry over (EFSA, 2020).

Presence of flocks of various ages in the same farm and farming of multiple species, use of non-potable water, presence of insects, inadequate cleaning of facilities, use of antibiotics, farming method used (higher prevalence in free range flocks linked to higher exposure to external environment), are all risk factors additionally mentioned in 2017 overview report of European Commission for mitigation measures on *Campylobacter* (European commission 2017).

There are other potential important risk factors such as the ones reported by Frosth *et al.* (2020) in a study performed on four Swedish broiler producers. Core genome multi-locus sequence typing (cgMLST) analysis concluded that drinking water pipes were found to be an important source of contamination. In fact, the same ST type (ST-257) was obtained from many different types of samples, for example: cattle faeces samples taken nearby; cecum samples of 26 batches during four different flock rotations; and water samples from drinking water pipes. Biofilms formed in the water pipes were suspected to be an important transmission route, since other studies had already reported *Campylobacter* isolation even after disinfection procedures. ST-257 wasn't detected again in any sample after thorough cleaning of water pipes by increasing and decreasing the pressure of water and air (Frosth *et al.*, 2020).

Summarized main risk factors for broiler *Campylobacter* colonization were outlined in Figure 2.

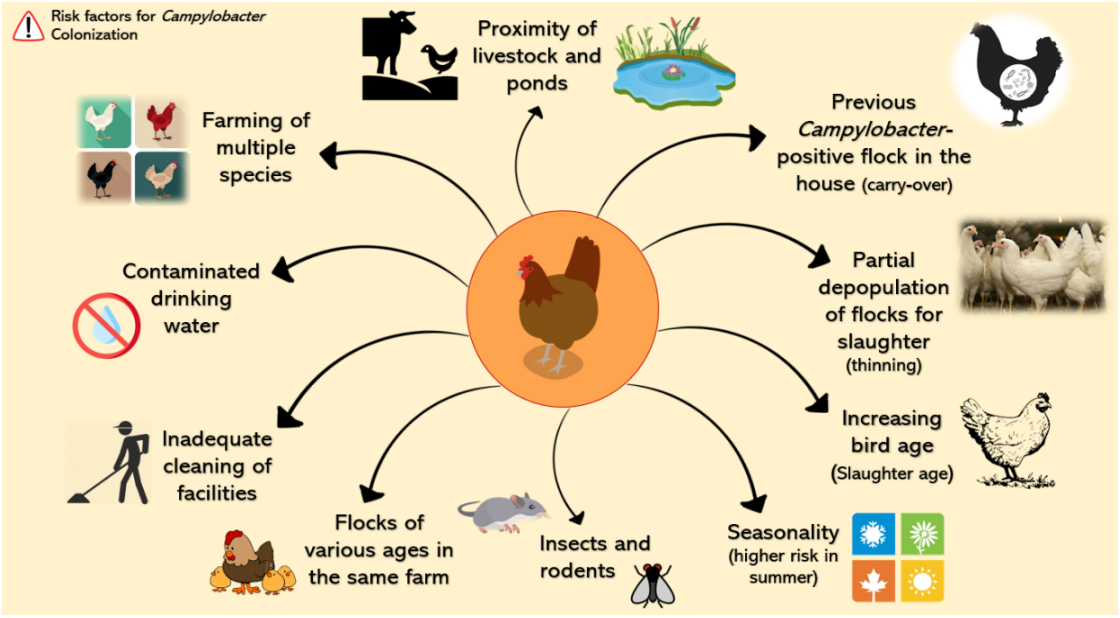


Figure 2. Major risk factors for Poultry colonization at primary production level.

2.4.4. From slaughterhouse to consumer: *Campylobacter* persistence

The world balance production of poultry meat had an estimated value of 124.6 million tonnes in 2018, and a forecast value of 130.5 million tonnes for 2019 (FAO, 2019). Additionally, in the U.S. the exports of red meat and poultry are expected to increase more than 4% in 2020 (USDA, 2019). These numbers were the highest when comparing to production estimations for bovine, pig and ovine meat (FAO, 2019). This reality emphasises the importance of poultry meat in the world, and the relevancy of food safety concerns in the poultry sector.

Many studies have demonstrated the persistence of specific *C. jejuni* genotypes isolated in the farm, along the rest of the poultry food chain (García-Sánchez *et al.*, 2018).

During processing at slaughterhouses, the risk of contamination is linked to positive flocks, working conditions, and practices followed by workers. Contamination of carcasses varies significantly among slaughterhouses due to the higher capability of some slaughterhouses to prevent and control contamination. Differences may be linked to the hygiene design of the equipment chosen, and the impact of specific process operations such as scalding, defeathering, evisceration, washing, and chilling (European Commission, 2017). Slaughter at processing plant, is critical in many ways. Transportation and holding time promote the spreading of *Campylobacter* present in the feathers and faeces, due to the proximity of chickens within the transportation crates. Several authors reported that reused or ineffectively cleaned/disinfected crates lead to cross contamination (Perez-Arnedo & Gonzalez-Fandos, 2019; Sibanda *et al.*, 2018; Slader *et al.*, 2002). Therefore, it is very important to minimize transportation and holding times before slaughter and guarantee efficient cleaning and disinfecting processes for crates, to achieve lower contamination levels. At slaughter line, broilers from a positive flock can carry a high load of *Campylobacter*, both on the outside (feathers and legs) and inside the GI tract. Contamination may increase significantly during the several operations that are usually carried out, and the slaughterhouse may play a major role in cross contamination between positive flocks and negative flocks. In fact, several studies have demonstrated that poultry meat from negative broiler flocks may be contaminated at the slaughterhouse, if the previous slaughtered flock was positive (García-Sánchez *et al.*, 2018). During slaughter, scalding with hot water can decrease carcass surface bacterial load. Still, poor conditions such as stagnant water, excessive excrements, and non-bactericidal temperatures, may turn the scald tank into a cross contamination system, in which pathogens are spread to all carcasses entering the tank. The next physical processes like defeathering and evisceration, are known to cause an increase in carcass contamination, and cross contamination between carcasses (Lu *et al.*, 2019).

The ineffectiveness of cleaning and disinfection procedures is also an important factor. Several studies have reported the presence of *Campylobacter* in equipment and surfaces, even after cleaning procedures. Prevalence of this pathogen at the processing plant is variable, but according to several authors it may be about 80% (García-Sánchez *et al.*, 2018).

At retail level, various types of poultry products are sold from fresh to frozen, and carcasses to portions, usually, the skinless portions like breast, are the pieces with the lowest levels of contamination (Skarp *et al.*, 2016). Contamination levels at poultry retail level depend a lot on mitigation strategies conducted at farm and slaughterhouse. Poultry meat storage conditions also affect final *Campylobacter*

counts. It is crucial to maintain low temperatures throughout the end stages of poultry meat production (processing and packaging) as well as in retail stores.

2.4.5. Management of risk factors in poultry food chain

2.4.5.1. Preventive measures at farm level

As for farm level control measures, mitigation of *Campylobacter* contamination levels can be achieved through strict biosecurity measures (European comission, 2017). Frosth *et al.* (2020) concluded that there are different sources and different transmission routes for broiler colonization among different breeders and their farms. The study highlighted the importance of 'custumized' measures for each producer, which can only be achieved if sources of contamination are investigated on each farm. Regardless, there are general control options, important for every poultry production farm, such as addition of disinfectants to drinking water (e.g. adding organic acids, chlorine-based biocides or hydrogen peroxide); employing few and well-trained staff (farm workers, maintenance personnel and catching crews disseminate the bacteria via contaminated boots and poor hygiene practices); hygienic anterooms (room between the outside door and the entry to the production area) at broiler house entrance: anteroom should be separated into a 'dirty' zone (area closest to the outside door) and a clean zone (area closest to the broiler production area) where the farmer changes footwear, puts on clean house specific clothes, and washes hands before entering to the production area; etc (EFSA, 2020).

EFSA ranked control options at primary production based on their probability of causing more than 10% risk reduction, feasibility, precision of concept, and proof of effectiveness. All control options considered are found in Table 3, divided according to their probability to have more or less than 10% effect on risk reduction (EFSA, 2020).

Table 3. EFSA ranking of control options at primary production (2020).

Control options with lower probability to have more than 10% effect in risk reduction	**Eight selected control options: identified as having a higher probability to have more than 10% effect in relative risk reduction**
- Effective rodent control - Downtime between flocks - Fly screens and keeping insects out of the broiler house - Clean litter and litter amendments - Stocking density and flock size - The number of broiler houses on site - Selective breeding - Feed structure	- Discontinued thinning - Employing few and well-trained staff - Vaccination - Feed and water additives - Avoiding drinkers that allow standing water - Addition of disinfectants to drinking water - Designated tools per broiler house - Hygienic anterooms at broiler house entrance * Four were excluded for the following reasons: - No farm animals in close proximity to the broiler houses: lack of feasibility of implementation; - Effective cleaning and disinfection of broiler house: definition of 'cleaning and disinfection' is not precise enough, (combines a group of activities and evidence of effectiveness is based on a variety of different activities); - Reduced slaughter age: current practices vary largely between EU countries; - Bacteriophages: lack of convincing evidence of effectiveness (shortage of field studies)

2.4.5.2. Preventive measures at slaughterhouse level

The current variation in prevalence and *Campylobacter* spp. levels of contamination between farms, emphasises the need and importance of further interventions at slaughterhouse level (European commission, 2017).

To begin with, food business operators should implement Hazard Analysis and Critical Control Point (HACCP), the food safety management system most widely used in the world (FAO, 2004). It is a scientific verifiable process to identify, control, reduce or eliminate any potential hazards to guarantee food safety. European legislation lays the responsibility for producing safe food on the food business operator (FBO), while the competent authority of the Member State is accountable for verifying correct implementation of the new rules. Regulation (EC) No. 852/2004 of the European Parliament and of the Council on the hygiene of foodstuffs applies directly to the food business operators (FBOs). Besides laying down general hygiene requirements to be respected by food businesses at all stages of the food chain, including primary production, it also indicates that all FBOs are required to put in place, implement and maintain a permanent procedure based on the Hazard Analysis and Critical Control Point (HACCP) principles (except for those involved in primary production).

However, there can be no successful implementation of a HACCP without the basic good hygienic practices (GHP). They comprise several prerequisite programs related to infrastructure and equipment, pest control, water quality, personal hygiene, plant layout (separation of clean and dirty areas), cleaning and sanitation procedures, waste disposal, etc. Nevertheless, these fundamental measures are not sufficient to deal with higher-level kind of risk that require more specific and targeted measures, that should ultimately be provided by a HACCP plan (FAO, 2004).

For HACCP implementation FBO need to respect and follow its basic 7 principles: a) hazard analysis; b) identification of critical control points (CCP's), (points in the production process where a loss of control could result in a biological, chemical or physical health hazard), Figure 3 indicates CCPs in a poultry slaughter line; c) establish critical limits: critical limits, or acceptable amount of deviation for each CCP; d) monitoring of critical limits; e) corrective actions (what steps should be taken if the process goes out of control); f) recordkeeping, which should include list of HACCP team members and their responsibilities; all products description and their intended use; flow diagrams with CCP's; list of all critical limits and preventive measures; monitoring and verification plans; plan of action when a critical limit deviation occurs, and person(s) responsible for corrective actions, etc. (Northcutt & Russell, 2010).

Measures taken to reduce *Campylobacter* risk at processing level were identified and gathered in a recent overview report on the outcome of a project carried out by Directorate-General for Health and Food Safety (European commission) between 2015 and 2016. Preventive measures observed started by the verification of the implementation of a process hygiene criterion by the food business operator (FBO) on the different stages of the poultry chain. Process hygiene criterions are an integral part of HACCP plan. Samples that reveal unsatisfactory results, must lead to corrective actions. Measures must be focused on hygiene conditions, optimization of the different process steps in slaughter line, and fine-tuning equipment (European commission, 2017).

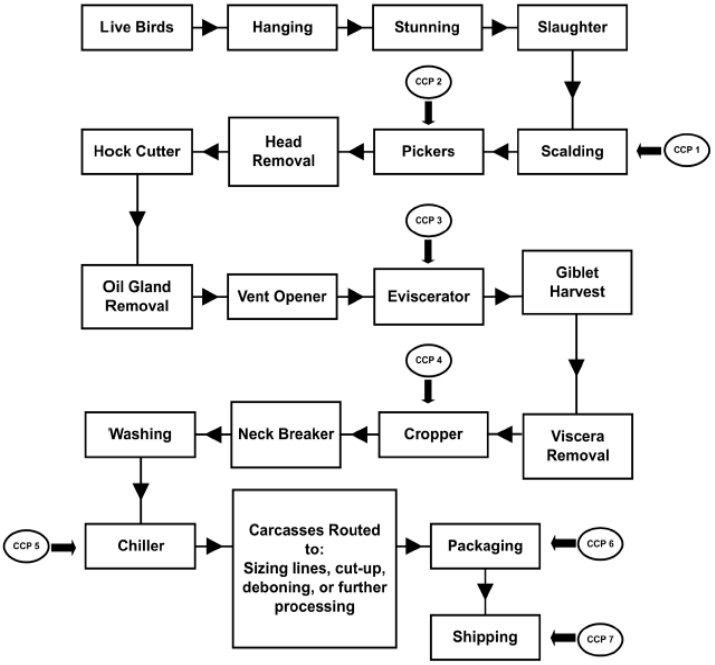


Figure 3. Example of Poultry Processing HACCP Flow Diagram. Source: Northcutt & Russel (2010)

FBO need to program own-check procedures aimed at preventing *Campylobacter* contamination. Before entering to the slaughterhouse, broilers are first submitted to transport and lairage conditions. The goal is to prevent re-contaminations and reduce "recycling" of strains from positive flocks to negative flocks. For this purpose, cleaning and disinfection procedures for crates and transport vehicles are essential and need to be effective. The effectiveness should be verified by periodical sampling procedures for microbiological testing. Appropriate storage of cleaned and disinfected crates is also very important, they should be kept in an area different from the reception area of poultry (European commission, 2017).

Considering that it is impossible to eliminate *Campylobacter* spp. at farm, specific interventions at slaughterhouse level are being investigated, with focus on the most critical areas/steps that are most likely to have an impact on *Campylobacter* levels of contamination:

Scalding: usage of multiple scalding tanks offers a better cleaning process and reduce cross contamination between different batches. Water flow should be high and against the direction of incoming carcasses, aiming to prevent the built up of dry matter and bacteria. Additional positive effects can be achieved if water is replaced regularly, and temperature is maintained above 55 ºC.

Plucking (or defeathering): Machinery should be adjusted for bird size. Pressure cannot be too strong, because it could cause the release of faecal matter from the cloaca and consequently cross-contamination between carcasses. Plucking fingers need to be cleaned on a daily base and checked for ineffective cleaning or damaged ones that need replacement. Water flow rate should be ideal for washing out feathers. Aerosol control by use of specific ventilators is also a good recommendation both in the plucking and scalding areas, to avoid aerosols movement into cleaned areas.

Evisceration: the most important measure at this step is the observation of perforated intestines and faecal leakage. Equipment should be set and adjusted to bird size (it is important therefore, to group

birds of similar size). Visual monitoring and counting of the number of evisceration 'failures' (intestinal perforations) as a percentage for each batch, could indicate problems related to equipment.

Washing of carcasses: should be performed after evisceration with high pressure. Contaminations can be more likely with water immersion washing. Chilling step follows right after washing steps, (before carcasses dry out), cooling rapidly poultry carcasses. Fast drop in temperature is crucial for reducing *Campylobacter* adherence. Condensation and water pooling must be avoided.

Chilling: should be rapid and effective. Rapid drop on temperature is critical to reduce establishment and adherence of *Campylobacter* on carcasses. Condensation and water pooling need to be avoided. Use of sprays is unadvised.

Packaging: modified atmosphere or leak proof packaging for whole poultry carcases are widely used and recommended. Staff personnel that handles directly raw poultry should not touch packed products (it is recommended to have a team specific for packaging step).

FBOs also pointed out that the layout of the slaughter line cannot be designed in such way that scalded carcasses linger in scalding/plucking area. Delays can lead to cross contamination on the surface of carcasses. Equally important is airflow system, which should be installed in a way that prevents air flowing from contaminated areas, like scalding/plucking areas, to cleaned ones like evisceration area (European commission, 2017).

Innovative measure for carcass decontamination comprise: UV lighting; hot steam and ultrasound; rapid surface chilling (European commission, 2017) and ozonated water (Cano *et al.*, 2019).

Last but not the least, are the cleaning and disinfection plans, which should be established for all rooms and all equipment's. Nowadays many slaughter lines are automatic, and implicate a lot of machinery, which makes another problem because these complex equipment's are difficult to clean properly (García-Sánchez *et al.*, 2018). Cleaning is the action of removal of dirt and organic substances, from walls, floors, tools and equipment's. Nevertheless, many microorganisms stick very strongly to surfaces, in biofilms, which cannot be removed despite deep cleaning procedures (Rossi *et al*., 2017). If biofilms are already established, cleaning processes based on mechanical action are of most importance because friction is required to act on the matrix in order to cause disruption, and consequently expose the deeper layers. Disinfectants don't usually have the ability to penetrate into the biofilm matrix after an ineffective cleaning. For this reason, the cleaning step (which comes first) should never be neglected.

To inactivate microorganisms after an efficient removal of all food wastes, further treatments are required such as hot water, steam, or through the application of disinfectants (Rossi *et al*., 2017).

The most used group of chemicals are the chlorine-based sanitizers. unfortunately, some bacteria started to become resistant to them. An example is *Salmonella enterica*, a pathogen that demonstrated the ability of developing a cellulose production phenotype, correlated to the environmental stress conditions found in food processing plants (Rossi *et al*., 2017).

Another important group are the quaternary ammonium compounds (QACs). Chemically, QACs are positively charged cations that bind to the negatively charged surface of most microbes. Due to their positive charge, there is initial surface structure disruption, penetration to the cell membrane, and direct interaction with the phospholipids, leading to structure disruption, including leakage of cytoplasmic

components. To sanitize food contact surfaces, QACs can be applied at a range of 200 to 400 ppm. Although useful, some strains of bacteria demonstrated resistance against them, such as *Staphylococcus aureus* and *Listeria monocytogenes* (Chauret, 2014; McDonnell, 2009; Mereghetti *et al.*, 2000).

Examples of other useful disinfectants are: hydrogen peroxide, that generates free radicals thereby destroying biofilm structures without toxic side effects; and ozone, a toxic gas with potent oxidizing activity, also powerful enough to destroy biofilms (Galié *et al.*, 2018).

After disinfecting through the use of biocides approved by legislation, problems can still exist. Some research works demonstrated that even using recommended concentrations of sanitizing biocides, resistance of bacteria in biofilms can still occur (Rossi *et al*., 2017).

In the particular case of *C. jejuni*, concerns should also be raised, because these bacteria are also able to develop resistance. It has already been demonstrated with three common biocides after cultivation in increasing sub lethal concentrations: sodium hypochlorite, trisodium phosphate, and acetic acid. Another interesting finding on this pathogen, was the formation of biofilms with different structures after exposure to different biocides, which could indicate the secretion of different matrixes according to the chemical stresses in the environment (Galié *et al*., 2018).

All facts considered, it is important to always reinforce that every time a chemical agent is used to kill microbes, it will always exist the possibility of promoting resistance. The reason for this is because not all microorganisms present get killed. Reductions in contamination loads are only made with the goal of reaching a safe level. This issue reinforces the importance of assessing what organisms are present in the environment, in order to choose the right disinfectant, and proper strength.

2.5. Virulence factors: Mobility, chemotaxis, adhesion, invasion, and toxin production

To achieve colonization, *Campylobacter* cells need motility factors, toxin production, and capacity for adhesion and invasion. The process on itself (colonization) is quite effective, allowing *Campylobacter* to colonize poultry species cecum quite rapidly, as fast as 24h after ingestion (Bolton, 2015).

Concerning the motility system owned by *Campylobacter*, it is composed of one or two polar flagella, and a chemosensory system responsible for regulating flagellar movement. This system is ultimately essential for survival under different conditions in the gastrointestinal tract, and for colonization in the small intestine. Motility in *Campylobacter* is special, it is described as “corkscrew rotation” enabled by flagella and the bacteria’s helical shape (Bolton, 2015).

The flagella’s composition includes a hook-basal body and extracellular filament structural components. The extracellular filament contains the major flagellin protein FlaA (encoded by the gene *flaA*), a minor flagellin protein FlaB, and other multimers of the protein flagellin. The gene *flaA* is highly conserved among different *Campylobacter* isolates, and it is regulated by the σ28 promoter. The gene *flaB* on the other hand, is regulated by σ54, which also regulates other genes involved in the hook-basal body filament structure. A two-component regulatory system drives the transcription of σ54 dependent genes (Bolton, 2015).

Mutations on the genes that encode σ54 and σ28, respectively *rpoN* and *fliA*, result in an inhibition of colonization as well as changes in another gene that encodes a mobility accessory factor, *maf5* gene. For successful colonization, flagellin O-linked glycosylation seems to be critical, as it is linked to flagellin assembly and motility. Additionally, an experiment demonstrated that mutation in *flaA* leads to incapacity for colonization, making it evident that *flaA* is necessary for chick colonisation (Jones *et al.*, 2004).

C. jejuni is known to be a commensal in the avian gut, more specifically, in the mucus filled crypts of the ceca. To locate to these primary colonization sites, the bacteria makes use of chemotaxis, the mechanism that motile bacteria use to sense and move towards more favourable conditions. All evidences to date, point towards the assumption that chemotaxis in *Campylobacter*, is probably quite similar to the extensively studied chemotaxis of *E. coli*. It is provided by a single two component regulatory system, in which histidine protein kinase is dependent on a signal transduction pathway comprising six chemotaxis proteins, CheA,B,R,W,Y and Z, and methyl-accepting chemotaxis proteins. The major chemoattractants involved are: mucins and glycoproteins, main components of the mucus; alpha-ketoglutamate, L-aspartate, L-asparagine, L-cysteine and L-glutamate; electron donors including formate, L-malate, D-lactate and succinate; electron acceptors including fumarate, dimethyl sulfoxide, nitrite, nitrate and hydrogen peroxide pyruvate and L-serine. Bile salts, on the contrary, are extremely repellent for *C. jejuni* (Bolton, 2015).

When entering the human GI tract, an interaction with the mucus layer before binding to the epithelial cells of the intestine must occur. This stage of the attachment appears to be a necessary step for successive colonization and pathogenesis (Backert *et al.*, 2016)

For adhesion, the first step that needs to take place for further invasion and colonisation by *Campylobacter*, several adhesins on the bacterial surface are implicated as well as their correspondent host cell receptors. The adhesins described include: major outer membrane protein (MOMP); two outer membrane-embedded fibronectin binding proteins, *Campylobacter* adhesin to fibronectin (CadF) and fibronectin like protein A (FlpA); periplasmic binding protein (PEB1); *Campylobacter* autotransporter protein A (CapA); *Campylobacter* autotransporter protein A (JlpA) and p95 (Backert *et al.*, 2016). Likely, binding of the adhesins is crucial for efficient interaction between bacteria and host target cell (Backert *et al.*, 2016).

A well-known adhesin is CadF, a 37 KDa outer membrane protein that plays an important role in mediating adhesion to fibronectin, a glycoprotein present in the epithelial cells of the GI tract (Bolton, 2015). It is encoded by *cadF,* a highly conserved chromosomal gene. When binding occurs, a signalling process leading to the activation of GTPases Rac1 and Cdc42, induces *Campylobacter* cell internalisation. FlpA, a 46-KDa outer membrane-embedded fibronectin binding protein was proved to be equally important in the adhesion process. Talukdar *et al.* demonstrated by constructing a double deletion mutant (*C. jejuni ΔcadF ΔflpA*) in a binding assay with INT 407 cells, that neither *cadF* nor *flpA* complementation restored binding levels to those observed in the wild-type strain. Additionally, a fibronectin binding assay also confirmed that both genes were needed for maximal binding to epithelial cells (Talukdar *et al.*, 2020).

The protein CapA, encoded by *capA* gene was also potentially involved in the adhesion process (Bolton, 2015). It was initially thought to be an autotransporter lipoprotein, but further studies reported it as an adhesin, even though results have been divergent. A *capA* insertion mutant had a significantly reduced capacity for invasion of human Caco-2 cells (colorectal adenocarcinoma cells) and failed to colonize and persist in pathogen-free Rhode Island Red chickens (Ashgar *et al.*, 2007). In contrast, Flanagan *et al.*, despite obtaining a 47% reduction in binding to chicken LMH epithelial cells, demonstrated that *capA* mutant was capable to colonize chickens with the same efficiency as the wild type strain. Given the results, the authors concluded that CapA is indeed an adhesin, not essential for the colonization of chickens (Flanagan *et al.*, 2009).

JlpA is a 43-KDa protein encoded by *jlpA* gene, in which mutation leads to an 18-19.4% reduction in adherence of *C. jejuni* in cultured Hep-2cells. PEB1, a 28-KDa protein, was also linked to a decrease in adherence in cultured HeLa cells and prevented colonization in mice when *peb1A* was inactivated (Backert *et al.*, 2016).

There are two general strategies for most enteric pathogen to attack host target cells. One involves the binding of bacterial surface proteins to specific host cell receptors, resulting in internalization. This process, designated the “zipper” mechanism, has been described for example in *Yersinia* and *Listeria* species. The other, involves type III and type IV secretion systems that inject proteins that often mimic or hijack specific host cell factors in order to trigger the bacterial uptake (“trigger” mechanism), as reported in *Salmonella* and *Shigella* (O Cróinín & Backert, 2012). Regarding *Campylobacter*, in two *C. jejuni* strains isolated from chicken liver and gizzard, and in *C. jejuni* strain 81-176 (clinical strain), type IV secretion system was reported (Bacon *et al.*, 2000; Marasini *et al.*, 2020). Also reported, was the type VI secretion system (T6SS) in *C. jejuni* isolates obtained from humans and livestock (including chicken) (Liaw *et al.*, 2019; Siddiqui *et al.*, 2015). Various pathogens are known to possess this secretion system such as *Salmonella* spp., and *Escherichia coli*, serving as a tool to kill competing bacteria by a bacteriophage-like invasion and injection mechanism. In *C. jejuni*, T6SS has been found to be similar to the protein products encoded by the pathogenicity island in *Helicobacter hepaticus*, and it was demonstrated to mediate the lysis of red blood cells, likely contributing for the survival on retail meats where blood cells are abundant (Marasini *et al.*, 2020). Furthermore, in another study, it was demonstrated in biologically relevant models, that T6SS enhanced invasion and interactions with chicken primary intestinal cells, increasing the ability to colonize chickens (Liaw *et al.*, 2019).

Proof that *C. jejuni* is capable of invading human epithelial cells was reported in early 90’s (1993) by Oelschlaeger *et al.* whose work included transmission electron micrographs that showed *C. jejuni* in intercellular space and within endosomal vacuoles of human intestinal epithelial cells. Results supported that *C. jejuni* achieved internalization through a distinctive microtubule dependent endocytic process, in which the outcome was endosomal vacuole uptake. This finding contrasted with the well characterized, strictly microfilament dependent cell invasion systems, of many other bacteria such as enteroinvasive *E. coli*, *Legionella*, *Listeria*, *Salmonella*, *Shigella*, and *Yersinia* spp. (Oelschlaeger *et al.*, 1993). Today it is known that *C. jejuni* can enter cells through microtubule dependent and microtubule independent mechanisms (Backert *et al.*, 2016).

Even though *C. jejuni* doesn't encode the typical type III or type IV secretion system like *Salmonella*, *Shigella*, or *Bartonella* spp. do, it is known today that the bacteria uses its flagellum in a similar manner during the invasion step. Not only it is necessary for maximal cell invasion, the flagellar apparatus can also serve as a type 3 Secretion system (T3SS) for the secretion of different proteins into the extracellular milieu or even injection of some of them into the host cell, to support pathogenicity-associated processes (Neddermann & Backert, 2019). *C. jejuni* secreted factors comprise the flagellar co-expressed determinants (FedA-D) and *Campylobacter* invasion antigens (CiaA-H) (Backert *et al.*, 2016). The first secreted factor identified was CiaB (Kovács *et al.*, 2020). Presently, it is known that CiaB is involved in the invasion process, but it is still not clear if it is essential for invasion process itself. Novik *et al.* (2010) called into question its role when reporting that *ciaB*-deficient mutant of the strain *C. jejuni* 81-176 revealed no significant reduction in invasion of T84 cells (human colon carcinoma cell line).

FlaC is reported as another protein secreted through the flagellar apparatus, and it appears to also be important in invasion process. Song *et al.* demonstrated that *flaC* null mutant, although still motile, had 14% invasion level reduction in an assay with HEp-2 cells (Song *et al.*, 2004).

In addition to the above mentioned, serine protease HtrA (high temperature requirement A), is another virulence factor important for adherence, invasion and transmigration. This virulence factor (HtrA) is actively secreted into the extracellular space where it can encounter host cell proteins. Moreover, its proteolytic activity can split cell to cell junctions in epithelial cells, by cleaving E-cadherin and occludin, a calcium-dependent cell adhesion molecule and a correspondently tight junction protein (Backert *et al.*, 2016). All in all, invasion and transmigration processes in *C. jejuni* are highly complex and request further studies continued investigation.

An hiphotetical model of *C. jejuni* mechanism for cell internalization is presented in Figure 4 taken from Backert & Hofreuter (2013), along with first electron micrographs of of INT407 infected cells from Oelschlaeger *et al.* (1993).

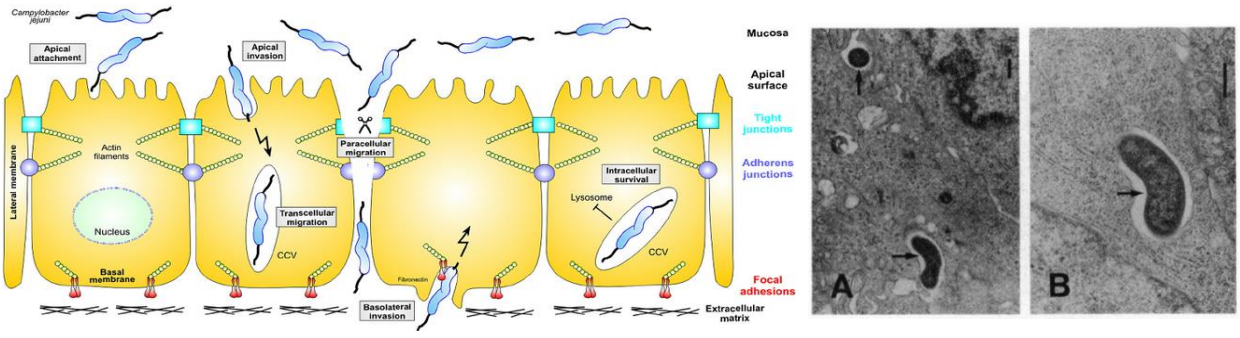


Figure 4. C. jejuni hyphotetical model. C. jejuni is able to interact, invade, and transmigrate across cells. Transmission electron micrographs of INT407 monolayers infected with C. jejuni 81-176 (A and B). Source: Backert & Hofreuter 2013; Oelschlaeger et al. (1993).

Regarding *C. jejuni* and *C.coli* toxins, the cytolethal distending toxin (CDT) induces cellular distention, nuclear enlargement and DNA damage. CdtB subunit is the active unit, nevertheless CdtA and CdtC are needed for the process of binding to target cells, and for the delivery of CdtB subunit. When inside the cell, CdtB subunit supports type I DNase activity responsible for DNA damage, consequently triggering DNA damage response. When infected cells fail to repair damages, they

undergo apoptosis (Bolton, 2015). To conclude, the most important virulence factors detected so far are resumed in Table 4.

Table 4. Campylobacter virulence factors, adapted from Bolton et al. (2015)

Virulence factors	Proteins associated
Motility factors	FlaA, major Flagellin Protein; FlaB, major Flagellin Protein; FliF, hookebasal body Protein; FliM and FliY, flagellar motor proteins; FlgI, P-ring in the peptidoglycan; FlgH, L ring in the outer membrane; FlgE and FliK, minor hook components; RpoN sigma factor known to be involved in flagella biosynthesis and bacterial motility; Proteins involved in flagellin O-linked glycosylation.
Chemotaxis factors	Chemotaxis proteins: Che A, B, R, V, W, & Z; Methyl-accepting chemotaxis proteins (MCPs) also called transducer-like proteins; CheY, response regulator controlling flagellar rotation; *Campylobacter* energy taxis system proteins CetA (Tlp9) and CetB (Aer2); AI-2 biosynthesis enzyme encoded by *luxS*; AfcB, methyl-accepting chemotaxis protein required for persistence in the cecum. RpoN sigma factor, associated with resistance to osmotic and acidic pH stresses.
Adhesion	CadF, outer membrane protein; CapA, *Campylobacter* adhesion protein; Phospholipase A; Peb1, periplasmic binding protein; Peb3, Peb4, chaperone playing an important role in exporting proteins to the outer memberane; FlpA, fibronectin-like protein A; Type IV secretion system possibly involved in adhesion; JlpA, 42-kDa lipoprotein involved in adhesion to Human epithelial type 2 cells (Hep-2 cells); WaaF, heptosyltransferase II enzyme involved in the biosynthesis of the LOS; Pgp1, peptidoglycan peptidase 1, required for helical shape.
Invasion	FlhA, FlhB, FliO, FliP, FliQ & FliR, components of the flagellar T3SS; FlaC protein secreted into the host cells and essential for colonisation and invasion; CiaB, 73-kDa protein involved in adhesion; CiaC, protein required for full invasion of INT-407 cells; CiaI, with reported role in intracellular survival; IamA, invasion associated protein; HtrA, chaperone involved in the proper folding of adhesins; VirK protein, may have a role in protection against antimicrobial proteins; FspA, protein with a role in apoptosis; Type VI secretion system.
Toxins	Cytolethal distending toxin (CDT) subunits; 1,3 galactosyltransferases involved in lipopolysaccharide production.

2.6. Multidrug resistance

Campylobacter has been exposed for many years to antimicrobials used in food animal production. Having to deal with antimicrobial selection, *Campylobacter* developed various mechanism of resistance, described in recent years. The most concerning classes of antibiotics for which *Campylobacter* has developed resistance mechanisms, are fluoroquinolones and macrolides, the drugs of choice to treat campylobacteriosis (Shen *et al.*, 2017).

As for fluoroquinolones, the main resistance mechanism used by *Campylobacter* is supported by point mutations in quinolone resistance-determining region of GyrA, a subunit of DNA gyrase (Shen *et al*., 2017). Veterinary use of fluoroquinoles in poultry production may have led to the selection of *Campylobacter* resistant strains that could easily enter the food supply chain. For this reason, FDA in

2000 made a proposition to withdraw the approval of fluoroquinolone use in US poultry. However, the effective retreat of the antimicrobial only occurred in September 2005. Since the intervention, fluoroquinolone resistance has not decreased nor in human nor in poultry isolates. National Antimicrobial Resistance Monitoring Systems (NARMS) reported in 2015 that ciprofloxacin resistance in *C. jejuni* isolated from humans was 25%, similar to the prevalence in 2005 (22%) (Whitehouse *et al.*, 2018). Moreover, surveillance data from the period 2015-2017 indicate an increase of resistant isolates from 25% in 2015 to 28% in 2017 (Dall, 2019).

For macrolide resistance, *Campylobacter* modifies the ribosomal target by enzyme-mediated methylation or possesses point-mutations in the 23S rRNA and/or in ribosomal proteins L4 and L22. Furthermore, *ermB*, a gene that confers high resistance to macrolides, was also recently identified in both *C. jejuni* and *C. coli* (Whitehouse *et al.*, 2018). According to NARMS surveillance data the proportion of macrolide-resistant *C. jejuni* isolates from humans and chickens remained low in 2016-2017, being less than 3%. Among *C. coli* isolates from humans, it declined from 13% to 7% (Dall, 2019). In Europe, combined microbiological, as well as clinical, resistance to both ciprofloxacin and erythromycin was generally low (microbiological resistance 1.1%, clinical resistance 1.0%) in *C. jejuni* and moderate (11.0% for both) in *C. coli* for 2018. Two countries, Poland and Portugal, reported higher levels of combined resistance in *C. jejuni* from humans (8.0% and 5.0%, respectively), four countries, Estonia, Finland, Italy and Spain, reported high levels (> 20%) of combined resistance in *C. coli* and Portugal reported very high levels (> 50%) (EFSA 2018).

In Portugal, the Department of Infectious Diseases of the National Institute of Health Dr Ricardo Jorge analysed antibiotic resistance in 125 strains (78 *C. jejuni* and 47 *C. coli*) from a collection of clinical strains analysed between the period of 2009-2012. Overall, there was a high rate of resistant strains, being *C. coli* more resistant than *C. jejuni*. 87.2% of strains were multiresistant (resistant to at least 3 different classes of antibiotics) (Duarte *et al.*, 2013).

As for the antibiotics that were mostly used to treat severe *Campylobacter* infection, 20% of strains were Erithromycin (macrolide) resistant, and 92,8% were ciprofloxacin (fluoroquinolone) resistant. Comparing the period of 2009-12 (n=125) with 1984-89 (n=108), there was a considerable raise in antibiotic resistance rates (Duarte *et al.*, 2013). Fraqueza *et al.* (2014) reached similar conclusions when analysing 167 *Campylobacter* isolates from chicken cecum, carcass, and breast meat samples in Portugal. Strains were tested for susceptibility to 11 antimicrobial agents by the disk diffusion method. Highest antimicrobial resistance ocurrence on *C. jejuni* and *C. coli* was noticed for quinolones class: nalidixidic acid (92 and 98%), ciprofloxacin (90 and 96%), norfloxacin (80 and 95%; $P < 0.05$), and ofloxacin (81 and 91%). Ampicillin resistance was high for *C. jejuni* and *C. coli* as well (67 and 82%). In contrast, penicillin combination of amoxacillin + clavulanic acid resulted in decreased occurrence of antimicrobial resistance (16 and 33% for *C. jejuni* and *C. coli*, respectively). High prevalence of fluoroquinolones resistance emphasized the need for reducing the use of antimicrobials in poultry sector.

3. *Campylobacter* ability to form biofilm

3.1. Biofilms definition

The term ‘biofilm’ was first used in technical and environmental microbiology already in 1935. However, the first two medical reports using the word ‘biofilm’ were only published in 1981 by dentists from the University of Lund, Sweden. In the same year (1981), Costerton used the term ‘biofilm’ in a technical microbiology report. The most appreciated definition of Biofilm was done by Donlan and Costerton (2002): “a microbiallly derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.” (Donlan & Costerton, 2002). These concentrated populations of microorganisms typically surrounded by EPS matrix are present in the natural, industrial and clinical environments (Costerton & Stewart, 2001).

The work made by Costerton and colleagues increased the importance of adherent bacteria in nature and disease in the scientific community (Mclean *et al.*, 2012). Costerton officially introduced the term ‘biofilm growth’ in medical microbiology in 1985 (Høiby, 2017). The author demonstrated the increased antimicrobial resistance of cells living in a biofilm compared to planktonic growing bacteria, and subsequently pioneered the work on physiology, and biochemistry of biofilm producing bacteria (Mclean *et al.*, 2012).

3.2. Steps for biofilm formation

The formation of what can be considered a biofilm, is divided in five main stages as demonstrated in Figure 5: (1) Cells attachment in a reversible manner; (2) irreversible cell attachment (starts secretion of extracellular polymeric substance); (3) microcolonies formation; (4) biofilm maturation (formation of a three dimensional structure) and (5) cells detach and dispersion occurs (Renner & Weibel, 2011).

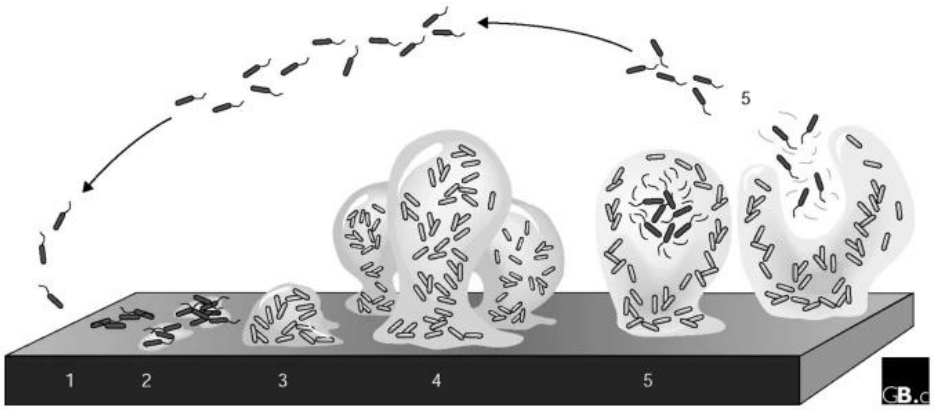


Figure 5. A model of the stages of bacterial biofilm development. Source: Sauer (2003)

In the first step, bacteria make use of extracellular organelles and proteins to “sense” and attach to surfaces, these include flagella, fimbriae, and outer membrane proteins (Renner & Weibel, 2011). Flagella in particular, is an organelle that helps overcoming hydrodynamic and repulsive forces involved

in adhesion, which gives motile bacteria (that possess flagella) an advantage. This has already been demonstrated in several pathogens: *Pseudomonas aeruginosa*, *Vibrio cholera*, *Listeria monocytogenes* and *Escherichia coli* (Kostakioti *et al.*, 2013).

Cells get attached to substrates that are immersed in, or in contact with, fluids incorporating electrolytes and macromolecules such as DNA, proteins, and humic acids. Soluble components end up having a very important influence on intrinsic chemical and physical properties of materials (Renner & Weibel, 2011). This is important for adhesion step, where planktonic bacteria get close to material surfaces. Chemical forces involved in the cohesion between microbial cells, and physical forces like attractive Van der Walls and repulsive electrostatic forces, are the ones mainly involved. Furthermore, environmental factors also have an important role. The most relevant are pH, temperature, and the material hydrophobicity. It is important to reinforce that bacteria attachment in its earlier stages, is still reversible (Achinas *et al.*, 2019).

In the second stage, extracellular polymeric substances (EPS), mainly composed by polysaccharides, proteins and DNA (Flemming & Wingender, 2010), are secreted. EPS promotes adhesion between cells and the surface. Then, in the third step, cells retained (adsorbed) on the surface, replicate and start forming microcolonies that continuously secrete EPS, which eventually forms a layer that becomes a physical barrier between the bacterial community and the external environment (Renner & Weibel, 2011). At this stage, chemical communication between cells plays an important role. It has been well described in a phenomenon called “Quorum sensing”, and it is thought to be very important for biofilm formation, modulating cellular functions in the community, namely: secondary metabolite production, motility, conjugation, nutrient acquisition and pathogenesis. When reaching the fourth step, the community grows and reaches maturation, at this stage cells “stick” together due to the EPS accumulation, and the structure formed is able to resist mechanical stresses. In the last step (5), cells can detach from the biofilm, and dispersion occurs. From here, released cells can get adsorbed in another place, and start the process all over again, by creating a new environmental “niche”. This last step allows propagation and renovation of the community (Renner & Weibel, 2011).

In terms of advantages, the formation of biofilm provides a lifestyle that is completely different from the planktonic state, which in some situations can be favourable for bacteria. The bacterial population growth in a biofilm is restricted; the bacteria energy is used primarily for production of EPS, which will in turn, confer protection while bacteria remain in a dormant state (Kokare *et al.*, 2009). The community formed gains protection against environmental stresses like dehydration, and also becomes more resistant to antimicrobials, disinfectants and UV light. Additionally, presence of extracellular DNA in the matrix constitutes an evolutionary advantage because it allows horizontal gene transfer by transformation in competent bacteria (Santos *et al.*, 2018).

3.3. Biofilm formation by *C. jejuni* and *C. coli* and related genes

Biofilms are assumed to be the ultimate form of protection and resistance for bacteria, therefore many studies have been conducted to assess the ability of *Campylobacter* to produce biofilms. In 1998, there was still no information available about the capacity of *Campylobacter* to produce biofilms (Buswell *et al.*, 1998).

C. jejuni is considered to be a fastidious bacterium, hard to culture and maintain in laboratory conditions (Solomon & Hoover, 1999). It has microaerophilic growth requirements, and unlike many other bacteria, it lacks the ability to utilize many carbohydrates as a carbon source, because it doesn't possess proper transporters for the intake of sugars like glucose or galactose, and several enzymes that play a role in the glycolytic pathway (Stahl *et al.*, 2012). Given all these reasons, it doesn't make sense how *C. jejuni* and *C. coli* are responsible for most cases of gastroenteritis in the world (WHO, 2020). Facts are, that they can adapt and survive somehow in the environment, exhibiting aerotolerance and resistance to starvation (García-Sánchez *et al.*, 2018). Another interesting concept that reinforced the idea of *Campylobacter* being able to form biofilms, is the formation of a microaerophilic environment (inside the biofilm), that allows them to survive until they can colonize a new appropriate host (Lamas *et al.*, 2018).

In 2006, according to Reeser *et al.* (2007), biofilm formation on abiotic surfaces by *C. jejuni* wasn't yet properly demonstrated. Therefore, a biofilm assay was conducted to assess *C. jejuni* biofilm formation on different materials: 24-well polystyrene plates; 4-cm coupons of acrylonitrile butadiene styrene plastic (ABS); polyvinyl chloride plastic (PVC); polystyrene and copper. Different temperatures (25ºC or 37ºC), culture media (Mueller-Hinton broth, Brucella, or Bolton) and levels of oxygen tension (10% CO2 atmosphere or aerobic conditions) were also tested. Additionally, biofilm formation of *flaAB* double mutant and *luxS* mutant (gene disruption was obtained by allelic exchange), was also demonstrated, to assess the importance of flagella and quorum sensing in this process. Significant biofilm formation only occurred when strains were incubated in MHB, which was the least nutritious medium compared to Brucella or Bolton broth. In terms of atmosphere and temperature conditions, biofilm production was enhanced in microaerophilic atmosphere at 42º, conditions already known to be favourable for *Campylobacter* growth. Of all the materials tested, better attachment and biofilm formation occurred on the hydrophobic materials polystyrene, polypropylene, polycarbonate, ABS, and PVC. On the contrary, there was a reduction in biofilm formation in the hydrophilic material copper. This was presumed to be a consequence of copper toxicity. Lastly, flagella and *quorum sensing* were demonstrated to have an important role for biofilm formation, since both mutants had a reduction in biofilm formation (Reeser *et al.*, 2007). Reeser's findings provided what may be considered some of the first relevant results on the matter 'biofilm formation by *C. jejuni* on abiotic surfaces'.

Other crucial works were released in the same year, like the one carried out by Joshua *et al.* (2006), where the authors stated that it was an "attractive hypothesis" that *C. jejuni* formed a biofilm, in order to survive under unfavourable conditions. In the experiments conducted, the pathogen developed three forms of monospecies biofilm formed in glass tubes with liquid culture (Brucella broth), incubated under microaerophilic conditions at 37°C: 1) attachment to glass surface; 2) unattached aggregate (designated floc); and 3) pellicle at liquid-gas interface. All three forms had the same appearance when visualized by scanning electron microscopy. Moreover, several mutant strains were tested. Results obtained indicated that attachment was mediated by flagella, since aflagellate *fliS* and *maf5* mutants were not able to attach. Also, non-motile strains failed to form pellicles. Results obtained proved the ability of *C. jejuni* to form a biofilm in monoculture, including an unattached aggregate with increased resistance to environmental stresses (Joshua *et al.*, 2006).

In the same year, Kalmokoff *et al.* (2006) conducted a protein expression analysis of stationary-phase planktonic cells and biofilm cells, grown on glass fibers, to assess differences in protein expression profiles between the two states. Incubation conditions were under reduced oxygen at 37ºC. Two-dimensional gel electrophoresis was performed for spot visualization and for protein identification. Overnight in-gel digests obtained with trypsin, were analysed by nano-liquid chromatography-tandem mass spectrometry (MS/MS). In summary, the proteins that revealed higher levels of expression were: proteins linked to the motility complex, namely flagellins FlaA and FlaB, filament cap FliD; basal body FlgG, FlgG2; chemotactic protein CheA; proteins involved in the general and oxidative stress response, GroEl,GroES and Tpx,Ahp; known adhesins Peb1 and FlaC; and lastly, proteins associated with biosynthesis, energy generation, and catabolic functions. All the findings ultimately indicated that flagellar motility complex has an essential role in attachment and biofilm formation (Kalmokoff *et al.*, 2006).

These studies marked the beginning of ongoing research regarding genes and their link to biofilm formation on *C. jejuni*. The major elements that are linked and influence biofilm formation are: motility and quorum sensing (pointed out above), chemotaxis, glycosylation, stress response regulators, metabolism and environment (Tram *et al.*, 2020).

As for chemotaxis, it is known to be crucial for *C. jejuni* ability to colonize and pathogenesis. For motile bacteria (possessing flagella) chemotaxis is the way to sense external stimulus in the environment and activate a signal transduction cascade that modulates flagella movement (Lübke *et al*., 2018). The mediators for this mechanism are the chemoreceptors, also designated transducer-like proteins (Tlps). Based on current knowledge, there are 13 Tlps and two aerotaxis (Aer) proteins that mediate chemotaxis in *C. jejuni*, and there could be potential interactions with biofilm state (Lübke et al., 2018). For example, an insertionally inactivated isogenic *tlp3* mutant displayed increased biofilm formation. Additionally, an assay to test the ability to colonize one day old chicks infected orally with a dose of 10^8 CFU *Campylobacter,* demonstrated that there were no differences between the mutant and the wild type strain, despite a defect in motility and reduced level of adherence and invasion in human intestinal Caco-2 cells (Rahman *et al.*, 2014).

Regarding protein glycosylation, one of the most common post-translational modifications in all life kingdoms, has been receiving more and more attention in the last 10 years because of its association with bacterial virulence. A substantial number of glycoproteins have been identified in pathogenic bacteria including *C. jejuni* (Lu *et al.*, 2015), known to produce four main types of glycosylated compounds: N-linked and O-linked glycosylated proteins, lipooligosaccharides (LOS), and capsular polysaccharides (Tram *et al.*, 2020). It was demonstrated that mutants lacking LOS outer core (Δ*waaF* and Δ*lgtF*) had enhanced biofilm formation. These mutants were not deficient in growth and survival, however displayed a colonization defect, likely due to *in vivo* phenomenon's inherent to the assay. Decreased membrane stability caused by loss of the outer core sugars may trigger biofilm formation, as a compensatory mechanism for cell stabilization (Naito *et al.*, 2010). On the other hand, Cain *et al.* (2020) demonstrated that glycosylation contributes to several phenotypes including electron transport, nutrient uptake and utilization, chemosensing, motility, cell stress and biofilm formation by creating a *pglB* defective mutant. The up regulation of biofilm production could have a compensatory mechanism

likewise to that of the strains lacking LOS outer core chains. As for capsule production there is no proof that it is related with biofilm formation to date (Tram *et al.*, 2020).

Several factors can have an influence in the shift between planktonic to biofilm state, such as energy production capacity, nutrient availability and metabolites, and oxygen saturation, act as signals to trigger the switch from planktonic to biofilm state (Tram *et al.*, 2020).

The capacity to regulate intracellular phosphate levels has been demonstrated to be important for biofilm formation. Mutants defective in *ppk1* and *ppk2* genes cannot synthesize inorganic polyphosphate, and interestingly display a hyper biofilm phenotype. However, the mutants had a defect in stress tolerance and ability to infect host. Mutant defective in *phosX*, an alkaline phosphatase and major contributor to inorganic phosphate pool in the cell, showed a decrease in infectivity and survival but again, an increase in biofilm production. All of these results indicate that when phosphate metabolism is disabled and energy production is affected, biofilm up regulation could be a way to protect cells (Tram *et al*., 2020).

Nutrient availability also causes great impact in biofilm formation. It has been reported that low nutrient media like Mueller Hinton broth is more favourable for biofilm formation than more nutritious culture media like nutrient broth nº2, Brucella broth and Bolton broth (Reeser *et al.*, 2007; Teh *et al.*, 2016). In contrary, osmotic stress (sucrose and NaCl) inhibit biofilm formation (Reeser *et al.*, 2007). Specific components can also influence the shift between planktonic and biofilm state.

A recent study called into question the principle that *C. jejuni* and *C. coli* are considered asaccharolytic bacteria that rely solely on carbon sources such as amino acids and intermediates from citric acid cycle for growth (Stahl *et al.*, 2012). *C. jejuni* NCTC 11168 was reported to have a *fuc* locus encoding a fucose permease, that allows fucose transport across the membrane (Dwivedi *et al.*, 2016). Dwivedi *et al.* (2016) shed light on the role of L-fucose in *C. jejuni* "lifestyle" and chemotaxis when inside human and chicken host, in which fucose can be found. More specifically, on epithelial cell surfaces, and also in human diet. *C. jejuni* binds to fucosylated structures. Such binding can be inhibited by fucose containing structures for example milk oligosshacarides. L-fucose is the only carbohydrate that is chemoattractant for *C. jejuni* and its usage in laboratory media improves *C. jejuni* NCTC11168 growth. By providing a competitive advantage in avian and animal colonization models, *C. jejuni* NCTC11168 was demonstrated to outcompete *fucP* deficient mutant at low infection doses in chicken fed with fucose rich diet. Dwivedi *et al.* (2016) demonstrated that biofilm formation by NCTC11168 is reduced when L-fucose is added to culture medium, but in contrast remains unchanged in *fucP* mutant. Authors proposed that there is an intracellular regulatory network that links external sensory response associated with biofilm formation in strains that conduct fucose uptake (Dwivedi *et al.*, 2016).

Besides nutrients available in the environment, meat juice residues on the abiotic surfaces can also influence biofilm formation. They can act as surface conditioners to support initial attachment and further biofilm formation. In meat processing environments, meat exudate can be found on machinery surfaces, and it has been demonstrated *in vitro* that chicken juice/chicken exudate enhances biofilm formation in *C. jejuni* (Brown *et al.*, 2014; Li *et al.*, 2017)

As for stress response regulators, CsrA was found to be potentially required for resistance to oxidative stress, activation of biofilm mode, motility, and adherence to host cells *in vitro*. Mutants in *csrA*

formed 50% less biofilm than wild type, and paradoxically had decreased invasion of human cells. Conclusions suggest that infection and biofilm states are distinct in *C. jejuni* life cycle, and the shifting of both states is regulated at least in part, by *csrA* (Fields & Thompson, 2008).
In opposition Joshua *et al.* (2006) speculated that biofilm formation could occur within the host (poultry, humans, livestock), and in that way provide a survival advantage in ambient environment (outside host) before transmission to the next.

Reuter *et al.* (2010) tested biofilm formation by *C. jejuni* under microaerophilic and aerobic conditions, at 37ºC in order to evaluate how *C. jejuni* responds when confronted with aerobic atmosphere. It was demonstrated that levels of biofilms formation were increased under aerobic conditions; flagella dependent motility facilitates biofilm formation; and biofilms constitute a reservoir of viable cells.

Aerobic atmosphere is a particularly important parameter to be tested, since oxygen is considered a stress factor for *C. jejuni* (Lamas *et al.,* 2018). Also, *C. jejuni* is evidently exposed to stressful levels of oxygen when it is transferred between host, and on food chain environment (Reuter *et al.*, 2010). Even though Reuter *et al.* (2010) demonstrated higher levels of biofilm formation under aerobic conditions, Brucella broth was utilized in their study for biofilm assay, which could lead to unreliable results. Brucella broth helps to create favourable conditions for *Campylobacter*, because it contains a reducing agent, sodium bisulfite, which is an oxygen scavenger that lowers the redox potential of medium, and in turn helps the bacteria against oxidative stress (Becton, Dickinson Company, 2015). Teh *et al.* (2017) confirmed that using this kind of medium can end up misrepresenting true aerobic conditions. Biofilm formation in *C. jejuni* was investigated under microaerophilic and aerobic conditions and dissolved oxygen was measured in three different types of culture media (Muller Hinton broth, Brucella, and Bolton broth) under different atmosphere conditions. The different broths had different dissolved oxygen contents, and this factor possibly affects biofilm formation ability. For this reason, the research group stated: “our results suggest that reports on the ability of *C. jejuni* to form biofilm under “aerobic” conditions in some of the literature may be overstated” (due to the use of broths containing reducing agents) (Teh *et al.*, 2017).

Oxidative stress regulators AhpC (alkyl hydroperoxide reductase) and *Campylobacter* oxidative stress regulator CosR defective mutants displayed increased biofilm formation (Oh & Jeon, 2014). The accumulation of radical oxygen species (ROS) may be a trigger to explain the biofilm phenotype in response to oxidative stress. In turn, maybe biofilms are the preferred state in aerobic conditions, outside the host (Tram *et al.*, 2020).

It is interesting to note that in most studies conducted on this subject (genes related to biofilm formation) the mainly utilized wild type strains are *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 (Joshua *et al*., 2006; Mahdavi *et al.*, 2014; Moe *et al.*, 2010; Oh & Jeon, 2014). Both these strains produce a biofilm, although in different mode. Maximum height, biomass volume, and ultrastructure was shown to be significantly different in both. *C. jejuni* NCTC11168 produces a biofilm that is thin but compact, multi-layered, with no complex organization. *C. jejuni* 81-176 on the other hand, is capable of forming a thick biofilm, provided with an open ultrastructure comprising voids and channels known to allow better nutrient flow and drainage of metabolic waste. This kind of structure is a signature of mature biofilms

(Turonova *et al.*, 2015). Additionally, they have genome sequence differences, for example, strain *C. jejuni* 81-176 naturally lacks the *fuc* locus, and has natural mutation in *tlp3* homologue making it devoided of a functional Tlp3. Both genes influence biofilm formation, making it clear again that both strains likely have different factors that trigger biofilm lifestyle (Dwivedi *et al.*, 2016; Rahman *et al.*, 2014). To conclude, it is important to emphasize that biofilm formation in *C. jejuni* and *C. coli* requires continued investigation. It is still questionable whether biofilm is a virulence associated trait, or simply a mean to survive harsh, starvation, aerobic stress induced conditions, until an appropriate host is encountered.

Another hypothesis that has been proposed to explain *C. jejuni* persistence, is that other bacteria, and pre-existing biofilms, may aid biofilm formation and survival by *C. jejuni*. Many works were conducted on this subject. A particularly interesting work was the one conducted by Ica *et al.* (2011) who utilized a unique different approach for the biofilm assay, that also investigated mixed species biofilms. Results provided a lot of information on *C. jejuni* monospecies biofilm and mixed biofilm with *Pseudomonas aeruginosa*. Briefly, a custom-built flat-plate reactor placed on top of an inverted microscope was designed for the quantification of biofilm, and imaging. The apparatus also included a custom-made microelectrode in its structure, for the measurement of dissolved oxygen levels in biofilms. The reactor allowed continuous recycling of nutrient solution by means of a mixing chamber, which was also aerated to incorporate oxygen in the medium. In summary, the main results were: a) *C. jejuni* mono species biofilm only occurred under limited flow conditions after five days; b) *C. jejuni* cells from monoculture biofilm were not culturable, probably due to oxygen exposure; c) dissolved oxygen levels indicated that monoculture biofilm didn't consume oxygen, which is compatible with the physiology of *C. jejuni* and the assumption that oxygen triggers the cells to enter into a VBNC state; d) mixed culture of *C. jejuni* and P. aeruginosa developed large cell clusters; *C. jejuni* and *P. aeruginosa* were able to form biofilms simultaneously; e) dissolved oxygen concentration in mixed culture biofilm was close to 0 mg/liter by the end of day five, which led to the conclusion that oxygen in these terms was consumed by *P.aeruginosa*, generating in turn a favourable environment for *C. jejuni* to survive.

3.4. Importance for food industry and new alternatives for biofilm elimination

Food industries operate under strict and regular hygiene standards; however, the detection and elimination of bacterial biofilms is still a challenge. Biofilms are a very common life form for bacteria, and many studies have even stated that "biofilms are ubiquitous" (Kerksiek, 2008; Chandki *et al.*, 2011; Costerton & Stewart, 2001). The main concern around this matter is supported by the role that biofilms may have in the transmission and survival of pathogens.

In recent time, evidence indicate that biofilms can ensure survival of *C. jejuni* throughout the poultry food chain. The formation of biofilm by this pathogen was already tested and demonstrated in relevant materials like stainless steel (Brown *et al.*, 2015). Even though it was proven that *C. jejuni* is able to form biofilm, it is arguable if strains from poultry sources/slaughter environment have the ability to form biofilm, since most of the conducted studies have used reference strains (Lamas, *et al.*, 2018). In conclusion, although a reasonable number of studies is already available, there are few studies about *Campylobacter* biofilm capacity under conditions that mimic those found in the food industry. Many assays have been conducted both under microaerophilic and aerobic conditions, but very few have

combined conditions of temperature and atmosphere along with the use of diverse wild strains obtained from the food supply chain and/or poultry sources. The use of wild strains is particularly important, because using reference strains won't provide information that can be extrapolated to reality (Lamas, *et al.*, 2018). Another important factor besides strains, temperature and atmosphere conditions, is the culture medium used. Many studies were conducted using Brucella, or Bolton broth to investigate biofilm formation under aerobic conditions. Because both these broths contain reducing agents such as sodium bisulfite and Sodium metabisulphite respectively, they end up misrepresenting true aerobic conditions, and therefore compromise accurate and reliable results (Teh *et al.*, 2017).

It would be relevant to use culture media that also mimics reality, with actual nutrients available in the food chain (Lamas *et al.*, 2018). Birk *et al.* (2004) created a food-based model system suitable to study the survival of *C. jejuni*. The model created is based on minimal processed and sterilized chicken juice used as culture media, to create conditions that resemble raw poultry environment. Furthermore, a recent study investigated the effect of chicken juice on the attachment and biofilm formation of *C. jejuni* in different surfaces (Brown *et al.*, 2014). Results indicated that *C. jejuni* formed more biofilm with chicken juice medium, than with a conventional growth medium under aerobic conditions. Furthermore, it was observed through electron microscopy that *Campylobacter* cells were associated with particles of the chicken juice rather than directly to the abiotic surface (Brown, *et al.*, 2014). However, the referred group only tested the formation of biofilm at 37ºC, and only used reference strains, therefore results still don't provide enough information. All of the parameters mentioned should be taken into consideration in future assays, in order to obtain more reliable results (Lamas *et al.*, 2018). The most relevant and recent studies on this subject are organized in Table 5 in Annex I.

Given the resistance that biofilms potentially confer in *Campylobacter* to disinfectants and antibiotics, it is important to consider alternative strategies to prevent their formation and/or eliminate them. The following novel methods could potentially be used in the future to control biofilm formation in the food industry: through enzymatic disruption (using enzymes to remove biofilms); steel coatings with nanoparticles; biosurfactants; bacteriophages; bacteriocins; compounds targeting quorum sensing system; essential oils with anti-biofilm properties; high hydrostatic pressure; photocatalysis with nanoparticles and lastly, non-thermal plasma, a partially ionized gas with temperature generally close to room temperature possessing interesting antimicrobial properties. Some of these are still very unavailable, and still need a lot of research (Galié *et al.* 2018). On the other hand, some studies have demonstrated that some of these methods have the potential to be effective against *Campylobacter* biofilms at food processing level. For example, an interesting work on enzymatic disruption mechanism, demonstrated that cell free extracts of a *C. jejuni* strain with DNase activity, have the ability to degrade *C. jejuni* biofilms, even after a ten-minute heat treatment. Conclusions suggested that extracellular DNase enzymes of the tested strain (RM1221) are fairly heat stable, and have the potential to be an easily obtainable, cost effective, antibiofilm agent to use during food chain cleaning processes. Further studies need to be made to ensure that supernatant derived products are safe for use (Brown *et al.*, 2015).

Regarding the potential of phytochemicals in biofilm inhibition, Wagle *et al.* (2019) conducted a study to test the antibiofilm potential of trans-cinnamaldehyde (TC), an aldehyde extracted from the bark

of cinnamon (*Cinnamomum zeylandicum*); eugenol (EG) and carvacrol (CR), the active components of clove oil (*Eugenia caryophyllus*) and oregano oil (*Origanum glandulosum*), respectively. Food and Drug Administration of the United States classifies these three phytochemicals as "generally recognized as safe". To assess conclusions, a type strain was used, *C. jejuni* NCTC 11168, and biofilms were formed on polystyrene plates and steel coupons. Also, the ability to destroy biofilms was tested in the presence of 5% chicken juice, which represents reality better. Carvacrol was the most effective in inhibiting biofilm formation on polystyrene, but overall, the three phytochemicals were effective in reducing *C. jejuni* biofilm and inactivating mature biofilms at 20°C and 37°C. The group also used scanning electron microscopy, which revealed disruption of biofilm architecture and loss of extracellular polymeric substances. Additionally, a proteomic analysis indicated that selected genes and proteins critical for biofilm formation were modulated by the phytochemicals. Even though results were positive, it's mandatory to conduct experiments on multispecies biofilms, since in the real slaughterhouse environment, chances are, that most biofilms formed include a wide variety of species (Wagle *et al.*, 2019).

Finally, also worth mentioning is the work conducted by Zhong *et al.*, which is the first study exploring the ZnO nanoparticles to control *C. jejuni* biofilm formation. A very important variable in this study relies on the fact that tests were conducted using two *C. jejuni* strains isolated from retail food samples in China, which makes results more consistent with what could happen in the real environment of food production. Furthermore, the group also compared differences in biofilm formation between *C. jejuni* in pure culture, and mixed culture with *E. coli* (facultative anaerobic bacteria) and *P. aeruginosa* (aerobic bacteria). About Zinc oxide, it is "generally recognized as safe" (GRAS) according to the U.S. Food and Drug Administration. Its mechanism of action is based in the generation of reactive oxygen species on the surface of biofilms that consequently degrade them. Their final results indicated that 0.5 mg/mL ZnO nanoparticles inhibited biofilm formation, and therefore, nanosized ZnO particles could be useful in the future for biofilm inhibition, in new developed antibacterial products (Zhong *et al.*, 2020).

4. Objectives of the dissertation

The main goals of the research conducted were as follows:

- Characterize a *C. jejuni* and *C. coli* collection of isolates (n=145). For that, phenotypic (colony morphology, gram staining, oxidase, catalase, and hippurate tests) and genotypic (*cdt* genes, *flaA*-typing and PFGE) characterization was performed.
- Selection of different strains (with different *flaA* profiles) for antimicrobial susceptibility tests (disk diffusion) and biofilm formation assays performed by crystal violet staining method.
- Execute biofilm assays mimicking slaughterhouse conditions taking in consideration the following factors: temperature, atmosphere, nutrients available, and levels of contamination (inoculum concentration).
- Assess biofilm formation under optimal growth temperature (42°C) and atmosphere (microaerophilic) to understand the impact of temperature and atmosphere on biofilm formation.
- Classify strains as for their ability to form biofilm in a qualitative manner and perform a statistical analysis for the assessment of significant differences found.

Chapter II - Experimental work: Assessment of *Campylobacter* isolates ability to form biofilm mimicking slaughterhouse conditions

5. Materials and Methods

5.1. Sampling and collection of *Campylobacter* isolates

A collection of *C. jejuni* (n=140) and *C. coli* (n=5) isolates was provided by Food Technology and Safety Laboratory in Faculty of Veterinary Medicine, Lisbon University. *Campylobacter* were isolated between 2018 November to 2019 from poultry neck skin and fecal content samples (*caecum*) collected at slaughterhouse and from different broiler flocks. Sampling was performed for broiler carcasses neck skin from different flocks on different working days, at slaughterhouse level according to recommendations described in regulation (EU) 2017/1495 of 23 August 2017. As for fecal samples, those were obtained from cecum extracted from broiler carcasses as described by Fraqueza *et al.* (2016). Fecal samples were streaked with a loop on mCCD agar (*Campylobacter* selective agar, Neogen, USA) supplemented with Cefoperazone and Amphotericin (Cefoperazone/Amphotericin selective supplement, Neogen, USA). All plates were incubated at 42°C during 48h under microaerophilic conditions: 6% oxygen; 7.1% CO_2; 7.1% H_2 (Anoxomat, Advanced Instruments, USA).

All the samples (fecal and neck skin samples) were collected throughout the period of November 2018 to June 2019, during different trials performed. Isolates were obtained after *Campylobacter* plate counts according to the method described in ISO 10272-2 (colony count technique) (2017).

5.2. Morphological and biochemical characterization for *Campylobacter* identification

Afterwards, five presumptive colonies of *Campylobacter* (Table 6) were streaked on Columbia 5% sheep blood agar plates (bioMérieux, France), which were incubated during 48h at 42ºC, under microaerophilic conditions. After incubation period, all colonies presenting typical morphology were submitted to Gram staining, oxidase, catalase and Hippurate test, as described in ISO 10272-1 (2017). Lastly, a loop full of culture from each *Campylobacter* isolate was stored in cryotubes with Brain Heart Infusion broth (Scharlau, Spain) and 15% glycerol at - 80 ºC. Concomitantly, another loop full of culture from each isolate was saved in a 2 mL Eppendorf with TE 1X buffer (10 $mmol^{-1}$ Tris-HCl, 1 $mmol^{-1}$ EDTA, pH=8) for DNA extraction procedure. The collected number of isolates from each pool sample type is summarized on Table 6.

Table 6. Number of isolates from each pool sample type.

Pool sample type	*C. jejuni*	*C. coli*	Total nº of isolates
Faeces	97	0	97
Neck skin	43	5	48

5.3. Reference strains

The reference strain *C. jejuni* NCTC11168 was kindly provided by Naoaki Misawa from the University of Miyazaki, Miyazaki, Japan. *C. coli* SVA was kindly provided by Eva Olsson Engvall from Sweden CRL/SVA collection, European Community Reference Laboratory (CRL) for *Campylobacter*/

National Veterinary Institute (SVA), Uppsala, Sweden. *Campylobacter* jejuni *ATCC* 33560 (CCM 6214) was kindly provided by Dr. Mónica Oleastro from INSA (National Institute of Health Dr. Ricardo Jorge).

5.4. DNA extraction

DNA extraction was carried out by the Chelex100 extraction method, based on boiling, lysis and chelation with Chelex100 (Merck, Germany) as described by Talon *et al.* 2007. Chelex is a chelating ion-exchange resin created by Bio-Rad, composed of styrene divinylbenzene copolymers. It binds to the polar components of the cells, leading to cell lysis (Bio-Rad, 2000). The main steps for DNA extraction include vortexing, centrifugation, pellet resuspension in 6% Chelex100 solution, boiling, cooling, and final centrifugation (12000 rpm for 5 minutes). To check the amount and purity of extracted DNA, NanoDrop device (Thermoscientific spectrophotometer, USA) was used (Matlock, 2015).

5.5. Specie identification by Multiplex PCR for *cdtABC* genes

One of the main virulence factors related to *Campylobacter* spp. in animals and humans is the cytolethal distending toxin (CDT), encoded by three adjacent genes (*cdtA*, *cdtB*, *cdtC*) (Samosornsuk *et al.*, 2007).

To identify isolates collected as *Campylobacter*, a multiplex PCR for detection of *cdtA*, *cdtB* and *cdtC* genes was performed, as described by Samosornsuk *et al.* (2007), using VWR Dopio (VRW, Belgium) termocycler. The size of each amplicon obtained, and primers used are indicated in Table 7.

C. jejuni NCTC 11168 and *C. coli* CRL/SVA extracted DNA was used for positive controls. Gel Red (nucleic acid staining solution, Biotium, Fremont, EUA) and bromophenol blue (Merck, Germany) were homogenized with PCR product, before loading in agarose gel. PCR products were run in a 1.5% agarose gel (SeaKem LE Agarose, Lonza, USA), with TBE (Tris-borate-EDTA) 1X buffer, for 45 min under 90V. DNA molecular marker was 100bp (NZYTEC ladder V). Bands were visualized and photographed under UV light with ChemiDoc™ XRS+ (Bio-Rad Laboratories).

Table 7. Amplicon size of cdtB, cdtC and cdtA genes, and primers sequence used for PCR.

Gene	Primers - sequence (5' - 3')	Amplicon size
cdtB	*Cj-CdtBU: ATCTTTTAACCTTGCTTTTGC*	*C. jejuni*: 714 bp; *C. coli*: 329bp
	Cj-CdtBR: GCAAGCATTAAAATCGCAGC	
	Cc-cdtBU: TTTAATGTATTATTTGCCGC	
	Cc-cdtBR: TCATTGCCTATGCGTATG	
cdtC	*Cj-cdtCU: TTTAGCCTTTGCAACTCCTA*	*C.jejuni*: 524 bp *C. coli*: 313 bp
	Cj-cdtCR: AAGGGGTAGCAGCTGTTAA	
	Cc-cdtCU: TAGGGATATGCACGCAAAAG	
	Cc-cdtCR: GCTTAATACAGTTACGATAG	
cdtA	*Cj-cdtAU: AGGACTTGAACCTACTTTTC*	*C. jejuni*: 631bp *C. coli*: 329bp
	Cj-cdtAR: AGGTGGAGTAGTTAAAAACC	
	Cc-cdtAU: ATTGCCAAGGCTAAAATCTC	
	Cc-cdtAR: GATAAAGTCTCCAAAACTGC	

5.6. Genotyping of *Campylobacter* isolates

5.6.1. PCR amplification of *flaA* gene

The flagellin gene locus of *C. jejuni* contains two flagellin genes (*flaA* and *flaB*), which are arranged in tandem, and separated by approximately 170 nucleotides. This gene locus is highly conserved and possess variable regions, making it suitable for restriction fragment length polymorphism (RFLP) analysis of a PCR product (Wassenaar & Newell, 2000). Polymerase chain reaction was performed to amplify 1713bp *flaA* gene, according to Wassenaar & Newell (2000). The primers for the reaction were *flaA* foward and *flaA* reversed (STAB VIDA, Portugal) and their sequence can be found in Table 8. Optimization of annealing temperature was previously done with a gradient of temperature PCR technique.

The cycling conditions submitted in termocycler VWR Dopio (VRW,Belgium) were as follows: initial denaturation step at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing 50°C for 1 minute, extension at 72°C for 1 minute; and final elongation step at 72°C for 5 minutes. For positive control DNA extracted from *C. jejuni* NCTC11168 and *C. coli* CRL/SVA was used. As negative control a "blank" tube was made, with no DNA extract and solely DNAse free water (Sigma life science, UK), for every PCR reaction performed. PCR products were revealed after electrophoresis. Gel Red (nucleic acid staining solution, Biotum) and Bromophenol blue (Merck, Germany) loading buffer were mixed with PCR product and homogenized. PCR products (1713bp) were run in a 1.5% agarose gel (SeaKem LE Agarose, Lonza, USA), with TBE (Tris-borate-EDTA) 1X buffer, for 45 min under 100V. DNA molecular marker ranged from 200-10000bp (NZYTEC ladder III, Portugal). Bands were visualized and photographed under UV light with ChemiDoc™ XRS+MP Imaging System (Bio-Rad Laboratories).

Table 8. flaA forward and flaA reversed primers sequence.

Primers	Sequence
flaA forward	ATG GGA TTT CGT ATT AAC AC
flaA reversed	CTG TAG TAA TCT TAA AAC ATT TTG

5.6.1.1. Restriction fragment length polymorphism (RFLP)

The *flaA* PCR products (1713bp) were digested with *DdeI* restriction enzyme (New England Biolabs, USA), for one hour at 37°C, taking in consideration manufacturer's instructions for master mix preparation. Band separation was performed on 2% agarose gel, with 1X TBE (Tris-Borate-EDTA) buffer. Molecular size marker was 100-1000 bp DNA ladder (ladder V, NZYtech, Portugal). Gel red was the nucleic acid stain used for bands visualization under UV light (ChemiDoc™ XRS+, Bio-Rad Laboratories). Photographs were taken and saved throughout the experiment for further analysis with Bionumerics version 6.6. software.

5.6.2. Pulse Field Gel electrophoresis

Pulse Field Gel electrophoresis (PFGE) works by changing the electric field into different directions, that way, it can separate DNA fragments of up to ~10Mb, by reorientating and moving them at different speeds through the gel pores (Nassonova, 2008). This technique is commonly used by CDC

Pulse network for *Campylobacter* strains typing, however, it arbours disadvantages such as: expensive equipment; complex protocols; no standard methods for interpretation and data sharing; and the genetic variation among *Campylobacter* strains that may not be typable by the common restriction enzymes used such as *SmaI* (Di Giannatale *et al.*, 2019). Overall, it still provides a good representation of a fingerprinting pattern correspondent to an entire bacterial genome, highly reproducible (Sharma-Kuinkel *et al.*, 2016). This technique was performed in order to complement the genotyping of the isolates, firstly done by *flaA* typing technique.

5.6.2.1. DNA preparation

DNA preparation for Pulse Field Gel Electrophoresis was performed according to CDC pulse net protocol (2017). First, *Campylobacter* spp. isolates were recovered from storage cryotubes and reactivated by inoculation on Colombia 5% sheep blood agar plates (bioMérieux, France) at 42°C for 48h under microaerophilic conditions. Cells were collected with a 10µl loop to make suspensions in 2mL of Pett IV buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]). All suspensions were adjusted to MacFarland = 6-7 (to assure all plugs had roughly the same amount of DNA). 500µl of cells suspension was mixed and homogenized with 500µl of pre-heated (42°C) chromosomal grade agarose 1.5% (Agarose Prep, GE Healthcare, Denmark) in Eppendorf tubes. Each mixture was pulled by a 1mL sterilized syringe. The syringes were placed in the cold chamber at 4°C until agarose solidified. Afterwards, the extremity of the serynge was cutted off, and with the help of a coverslip, disks were cutted manually with an approximate size of 2-2.5 mm. The disks were placed into Falcon tubes with 3mL of ES buffer (0.5 M EDTA, 1% Sarkosyl, Sigma Life Science, Germany), for each isolate. 150µl of Proteinase K (1mg/mL) (Nzytech, Portugal) was added to each tube. Tubes were incubated overnight at 56°C with shaking. Finally, the lysis buffer was removed and washing steps were performed six times with TE buffer 1x (10mM Tris,1mM EDTA) for 30 minutes at 37°C, in order to remove proteinase k, cellular debris, lytic enzymes, and proteases present in the lysis step, which could consequently interfere with DNA digestion. Plugs were stored in TE 1x buffer at 4°C, until usage for enzymatic digestion.

5.6.2.2. Restriction digestion

The agarose disks containing the bacterial genomic DNA were placed in an eppendorf tube for digestion with *SmaI* (New England Biolabs, EUA). In each eppendorf (each corresponding to an isolate) was added 50µl of digestion Mix prepared as follows: 5µl enzyme buffer, 5 µl of BSA (Albumine Bovine Fraction V, NZYtech, Portugal), 40µl ddH_2O and 20U *SmaI*/per sample. The mix covered the plugs, which were incubated overnight at 25°C. After incubation 3µl of bromophenol blue was added (Merck, Germany).

5.6.2.3. Pulsed Field gel electrophoresis

For the band separation of the digested DNA, an electrophoresis was performed in a 1% agarose gel (SeaKem Gold Agarose, Lonza, USA), with a lambda PFGE ladder standard (New England Biolabs, USA) in TBE 0.5 X buffer at 14ºC in a CHEF-Dr III System apparatus (Bio-Rad Laboratories,

Hercules, USA). The electrophoresis parameters set in were as follows: 5 s initial switch time; 40 s final switch time; variable speed pump at 80, mA=0, 6V; buffer temperature at 14ºC; total run time of 22.5h. After electrophoresis, the gel was stained in 500mL of Gel Red solution (nucleic acid staining solution, Biotium, Fremont, EUA) 2x for 25 min. DNA fragments were visualised under UV transilluminator (ChemiDoc™ XRS+, Bio-Rad Laboratories), and photos were captured to be used for further analysis.

5.7. Antibiotic resistance: disk diffusion test

The antibiotic resistance pattern of the 17 selected strains (*C. jejuni* =13, *C. coli* =4) was assessed by disc susceptibility test, performed according to Fraqueza *et al.* (2016) for 10 antibiotics, each representing a different antimicrobial category. Isolates were recovered from cryotubes stored at -80ºC and grown in Colombia 5% sheep blood agar plates (bioMérieux, France) for 48h at 42ºC. After incubation cells were removed to prepare suspensions for each isolate all adjusted to 0.5 MacFarland. Each suspension was inoculated in Mueller-Hinton agar supplemented with 5% sheep blood (bioMérieux, France). Plates were incubated at 42°C for 48 h in a microaerophilic atmosphere (5% O2, 10% CO2 and 85% N2). The tested antibiotics included: ampicilin (10 µg), amoxycillin + clavulanic acid (20+10 µg), tetracycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), trimethoprim/sulfamethoxazole (25 µg) and ertapenem (10 µg) (Oxoid, Spain). The diameter of the inhibition zones was measured with calipers and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) guidelines, CLSI guidelines for *Enterobacteriaceae* (CLSI, 2018) and Comité de l'antibiogramme de la Société Française de Microbiologie (SFM, 2020)

5.8. Assessment of biofilm formation, mimicking slaughterhouse conditions

The conception of the assay for the assessment of biofilm formation was developed with the aim of testing conditions similar to those found at slaughterhouse level. Biofilm formation was tested at 10°C, because in the industry environment, premises where carcasses are processed are maintained under this temperature. In terms of the surface materials, the most commonly found are plastic and stainless steel. Since polystyrene is a plastic commonly used in premises, equipment, food packaging etc. (Cassidy & Elyashiv-Barad, 2007) 96-well polystyrene plates were suited to test biofilm producing ability. The culture medium used was Nutrient broth no. 2 (Oxoid Ltd., UK) supplemented with 10% (vol/vol) chicken juice in order to provide similar nutrients available in processing equipment surfaces.
As for the inoculum, biofilm formation was investigated in two assays. In the first assay, a low inoculum concentration was used, correspondent to 10^3 cfu/mL. In the second assay, a higher concentration of inoculum, correspondent to 10^6 cfu/mL was tested. This way, it was possible to assess if inoculum concentration had influence on biofilm formation.

5.8.1. Isolates revivification

The isolates revivification was performed has described by Melo *et al.* (2017) with slight modifications. Briefly, the strains stored in cryotubes at −80°C in BHI (Brain heart infusion broth, Scharlau, Spain) with 15% glycerol were revivificated by subculturing, for 48 h in Bolton broth (Oxoid

Ltd. UK) supplemented with 5% sheep defibrinated blood (Thermoscientific, UK) at 42°C, in microaerophilic conditions (Anoxomat, Advanced Instruments, USA). After recovery process, a loop full (10µl) was taken from cultures in Bolton broth, and inoculated in COS agar plates (bioMerieux, France), incubated at 42°C during 48h, under microaerophilic conditions.

5.8.2. Chicken juice preparation

Whole fresh chicken was bought at the supermarket, with an approximate weight of ± 1,3 Kg. Chicken juice was prepared according to Pang and Yuk method (Pang & Yuk, 2018), with slight modifications. Steps were as follows: 1. Whole chicken was placed inside a sterile vacuum bag and 700 mL of sterile saline solution was added. 2.Chicken was vigorously massaged manually for 3 minutes. 3. Saline solution was removed to a sterile glass flask. 4. The solution was distributed in Falcon sterile tubes, and centrifuged at 12,000g for 15 minutes to remove solids. 5. Supernatant was sterilized with 0.22 µm pore-size syringe filters (Membrane solutions, USA). 6. Sterilized supernatant was stored at -20°C. When thawed, it was filtered again as described in step 5.

5.8.3. Qualitative Biofilm formation assay – crystal violet staining method

Crystal violet staining method for biofilm quantification was performed according to O'Toole (2011) in two major assays in this work:

1) Assay 1: the first assay in which the goal was to test biofilm formation ability with a low inoculum, using as a criteria the maximum limit allowed (10^3 cfu/g) in the Reg. (EU) 2017/1495. Culture medium used was Nutrient broth no. 2 (Oxoid, UK) with the addition 10% chicken juice.
2) Assay 2: in the second assay a higher inoculum concentration was tested (10^6 cfu/mL). Culture medium used was Nutrient broth no. 2 (Oxoid, UK) with the addition of 10% chicken juice. In this assay, two strains of *C. coli* (*C. coli* 62E and 62D) were added to the group of selected strains (n=15), to obtain more results on this specie.

Four different conditions were tested in both assays described above: a) 10°C aerobiosis; b) 10°C microaerophilic; c) 42°C aerobiosis and d) 42°C microaerophilic. These conditions were tested to assess the influence of slaughterhouse environment temperature (10-12°C) and atmosphere in biofilm formation.

Overnight cultures (volume = 20 mL) of *Campylobacter* isolates previously selected were made with Nutrient Broth No.2 (Oxoid, UK) supplemented with 10% chicken juice, using as inoculum, cultures from Columbia 5% sheep blood agar plates. The OD600nm of overnight cultures was determined and adjusted in order to reach the desired inoculum concentration. To achieve a suspension with 10^3 CFU/mL, OD600nm was set as approx. =0.400-.0.440 and two serial dilutions were made (10^{-1}; 10^{-2};). For 10^6 CFU/mL suspension, overnight cultures were adjusted to an OD600nm approx. = 0.500-0.555. The CFU/mL correspondent to the ODs were previously assessed by plate counts in CampyFood agar plates (bioMérieux, France). From the bacterial suspensions made, aliquots of 200 µl were placed in individual wells of 96-well polystyrene plates (Frilabo, Portugal). Each isolate was tested in triplicate (6x3). For positive biofilm control the strain *Campylobacter jejuni* NCTC 11168 was used. The negative control was performed with wells inoculated only with culture medium. A template of the 96-well plate is

shown in Figure 6. External wells weren't used because of edge effect caused by the phenomenon of evaporation (peripheral wells are more ventilated) (Shukla & Rao, 2017).

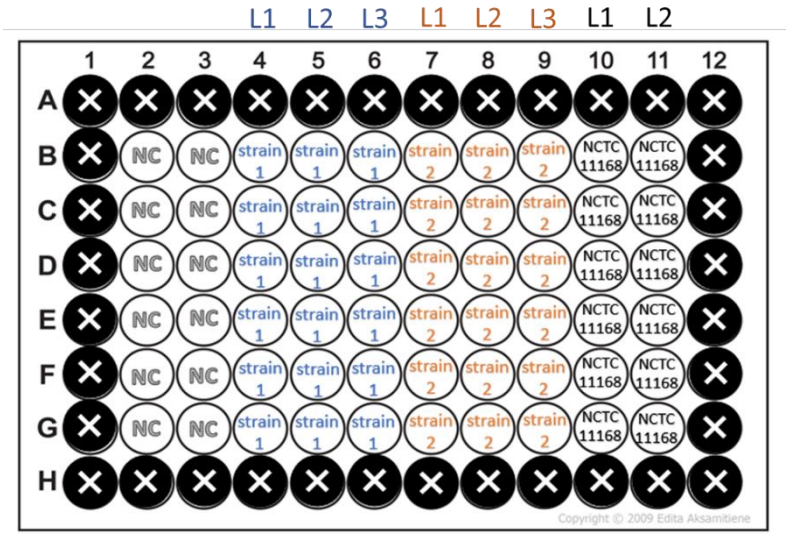


Figure 6. Experimental lay-Out of 96-well plate. NC-negative control (wells only filled with culture media). C. jejuni NCTC 11168 was the positive biofilm control strain used. Each strain was inoculated in 3 replicates (L1, L2, L3). Control strain (C. jejuni NCTC 11168) only had two replicates in every plate (L1, L2).

After incubation period (72h) crystal violet staining method was performed as described by O'Toole (2011). Briefly, media was removed, and wells were washed three times by pipetting and removing distilled water from the wells. Excess water was removed, and total biomass was measured by fixation with 1% crystal Violet (Sigma Life Science, India) solution, followed by 3 washing steps, 2 by pipetting distilled water, and a third by submerging the plate in a recipient with distilled water. Elution of crystal violet was performed with alcohol/acetone solution (80%/20%). Eluted dye was removed from each well and placed in a new 96-well microtiter plate for reading at OD_{580nm} (absorbance microplate reader, Tecan Sunrise, Switzerland).

ODs obtained from crystal violet staining method were corrected for all isolates by subtracting the mean of negative controls in each plate. Negative controls, despite always being necessary, in this assay were particularly important because it has been demonstrated that chicken juice components (particles) can bind to an abiotic surface and consequently increase crystal violet staining (Brown *et al.*, 2014).

To classify biofilms, Stepanović *et al.* (2000) criteria was used. The mean from negative control wells of each plate, was subtracted from each obtained OD value, for each strain tested. For each strain, in each assay, there were 3 replicates per plate (3x6). Then, standard deviation of negative controls was assessed. The cut-off O.D. (O.D.c. at 580nm) value was established as 3 standard deviations of the mean O.D. of negative controls. This way, biofilm formation ability was categorized into 4 levels: O.D. ≤ O.D.c = no biofilm producer, O.D.c < O.D. ≤ (2 x O.D.c) = weak biofilm producer, (2 x O.D.c) < O.D. ≤ (4 x O.D.c) = moderate biofilm producer and O.D. > (4 x O.D.c) = strong biofilm producer.

5.8.4. Aggregate formation

Aggregate formation was assessed according to Joshua. *et al.* (2006). Overnight cultures incubated under microaerophilic conditions, were placed in shaker, at 37°C, 50 r.p.m., for 3 days in aerobic atmosphere. Culture medium used was Nutrient broth no. 2 supplemented with 10% chicken juice. Aggregates were classified as: absent (-); small (+) (just visible); intermediate (++); and extensive (+++).

5.8.5. Qualitative assessment of *Campylobacter* viability

To assess the number of viable cells in the wells (planktonic cells) after the 3 incubation days, plate counts were performed on Campyfood agar (bioMérieux, France). For that purpose, before proceeding to crystal violet staining, an aliquot of each well, correspondent to each different strain, was plated (200µl). No serial dilutions were performed because counts were unpredictable. Only the condition at 10°C under aerobiosis was assessed after the incubation period (72h) for every strain.

5.9. Data Analysis (Bionumerics and SAS)

Bionumerics software version 6.6. (Applied maths) was used to analyse all *flaA*-RFLP profiles obtained for each isolate (n=145). Dice coefficient and UPGMA clustering method (unweighted pair group method with arithmetic mean) were used, with an optimization setting of 1.5% and a band position tolerance of 1.5%. All the obtained PFGE profiles were also analysed with Bionumerics.

For antimicrobial susceptibility data, Bionumerics was used to create a profile of resistance for each isolate. Additionally, *flaA*-RFLP profiles were linked to each profile, with same criteria used for *flaA*-RFLP clustering.

For biofilm assay data, Statistical Analysis Systems (SAS) software package, version 9.4 (SAS Institute, Cary, NC, USA) was used to make statistical analysis. The GLM procedure of SAS was used to perform analyses of variance, by comparing the results obtained for the main factors applied: strains, temperature, atmosphere, and inoculum concentration. The model also considered interactions between strains, temperature, atmosphere, and inoculum concentration.

Chapter III – Results and discussion: *Campylobacter* persistence throughout time, biofilm formation, and antibiotic resistance

6. Results and Discussion

6.1. Genotyping characterization of *Campylobacter* isolates: *flaA* typing and PFGE

Campylobacter spp. isolates were characterized and most of them were identified as *C. jejuni* (n=140). Only five of the isolates were *C. coli.* (n=5). All the isolates presented *cdtABC* genes, needed for the potential to produce cytolethal distending toxin (CDT).

The *flaA*-RFLP typing method was chosen to assess the genetic diversity of the *Campylobacter* isolates collection in this research (n=145). It discriminates *Campylobacter* isolates by generating different DNA band patterns, relying on the extensive internal variability observed in the sequence of the flagellin gene (El-Adawy *et al.*, 2013; Scarcelli *et al.*, 2009).

In total, 145 *flaA*-RFLP profiles were obtained by enzymatic digestion and submitted to Bionumerics for cluster analysis. The dendrogram created by Bionumerics is presented in Figure 7. Each isolate profile in the dendrogram was carefully analyzed within each cluster formed. Initially, twelve main clusters were defined, using as criteria 90% of similarity. There were three exceptions made for cluster 3, 5 and 8, in which more isolates were included due to very similar band patterns that could be visualized by naked eye, despite not presenting 90% similarity. The reason for this consideration was the fact that these profiles, due to variations in gel runs, weren't recognized by Bionumerics as 90% similar, even though they were noticeably similar by naked eye, with the same number of bands, with same approximate weight.

As for species discrimination, *flaA* typing technique could not provide species differentiation for all *C. coli* isolates. Three *C. coli* isolates had the same genotype obtained for *C. jejuni* isolates. The explanation probably relies on the lack of discriminatory power of *flaA* typing, attributable to the use of a single restriction enzyme (De Boer *et al*., 2000).

As a complement to the *flaA* typing technique, pulse field gel electrophoresis (PFGE) was also performed. PFGE technique is more complex and labor-intensive; however, it provides genetic profiles resultant of the enzymatic digestion of the whole bacterial genome, unlike *flaA*-typing, which is only based on one gene (*flaA*). PFGE is still considered the gold-standard for *Campylobacter* spp. genotyping, despite that, nowadays whole genome sequencing (WGS) is becoming the best method as it provides additional information on virulence, persistence, and clonal tracing, with an unprecedented detail compared with PFGE (EFSA, 2019).

For the execution of PFGE technique, isolates with different band patterns were selected from the dendrogram obtained for *flaA*-RFLP profiles (Figure 7). One strain was picked from each main cluster formed, along with 6 strains that had single profiles. In total, 18 isolates were selected and used to perform PFGE method: 105C, 46E, 22A, 20C, 62D, 105E, 63E, 19B, 104B, 62E, 25C, 106A, 105B, 106B, 65E, 65B, 36B, 19D. The different band patterns captured were submitted to Bionumerics for a cluster analysis. The dendrogram constructed can be observed in Figure 8.

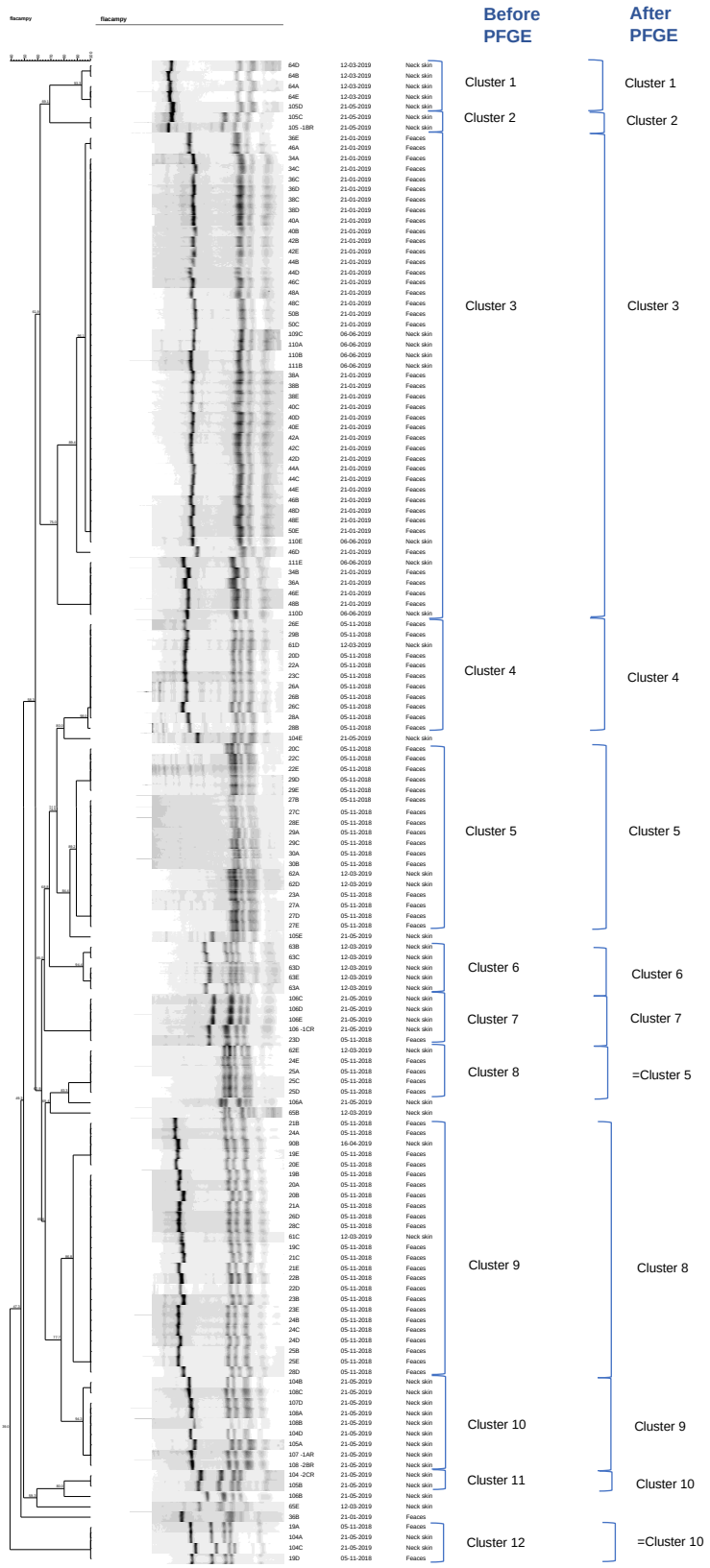


Figure 7. Campylobacter flaA-RFLP profiles of 145 isolates obtained from different flocks at slaughterhouse level and their cluster association according to profile similarity. A Cluster was considered a group comprising two or more isolates.

Eleven different PFGE profiles were obtained, with 8 to 13 bands, resultant from *SmaI* restriction (Figure 8). The bands obtained had a molecular size ranging from 727.5 Kb-48.5kb.

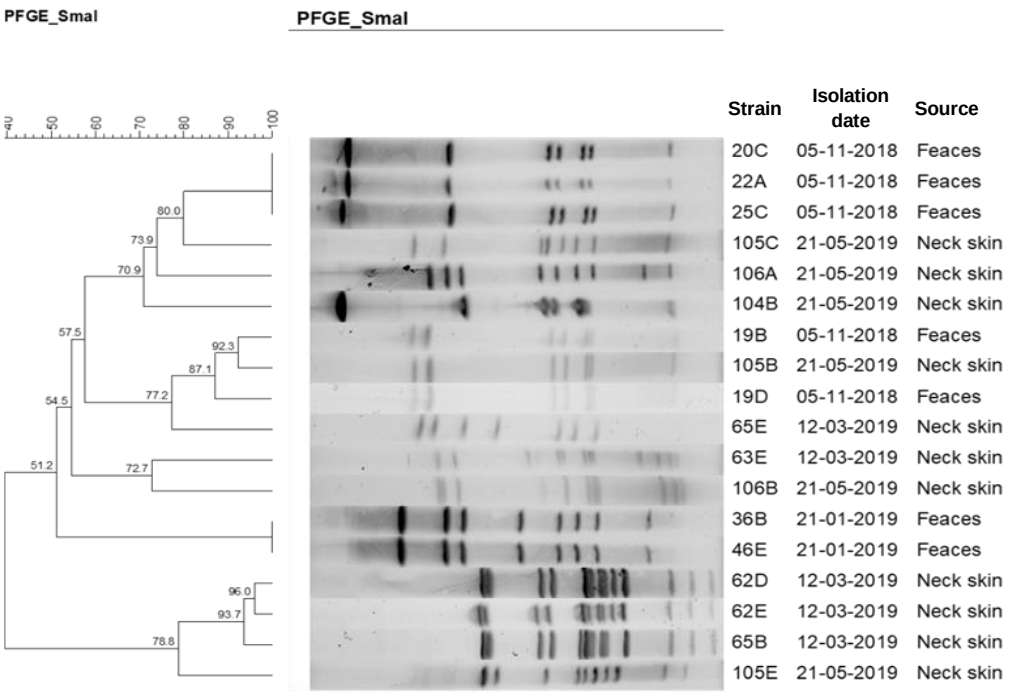


Figure 8. PFGE genotypes obtained for 18 isolates considered to have different flaA genotypes.

PFGE results clarified doubts and were useful to complement *flaA* typing results. Some of the profiles that were considered different in the dendrogram of *flaA* genotypes, were found to have identical PFGE genotypes. In this way, some *flaA* genotypes that seemed very similar by naked eye observation, were confirmed to have the same PFGE profile. This was the case for the following isolates:

- 25C: an isolate inserted in a small cluster in which the band pattern was very similar to the one found in cluster 5. Confirmation that this small cluster actually belonged to cluster 5 was shown by PFGE genotype. 25C had the same band pattern as 20C (an isolate from cluster 5) in PFGE results. This isolate was already suspected to have very similar *flaA* profile to 20C (inserted in cluster 5). This result led to the correction of cluster 8, which became part of cluster 5 (Figure 7).
- 36B: an isolate suspected to belong in cluster 3, also due to band pattern similarity. PFGE results confirmed its similarity to the band pattern found in cluster 3, since 36B had the same genotype as 46E (an isolate from cluster 3).
- 19D: an isolate suspected to belong to cluster 11. PFGE results demonstrated that 19D obtained the same genotype as 105B (an isolate from cluster 11), confirming again the misleading placement of this isolate in the dendrogram obtained for *flaA* genotypes. This way, cluster 11 became cluster 10 (Figure 7).

Even though PFGE genotypes allowed a better placement of some isolates that raised doubts as for their position in the *flaA*-typing dendrogram, results from this technique still had to be compared with *flaA* genotypes for the final selection of different profiles. The best approach for the selection of different strains was to consider results of both techniques. For example, 19D, 19B, and 105B obtained the same PFGE profile, but 19B had a very distinct *flaA* profile when compared to 19D and 105B. The

same situation occurred for 22A and 20C, which obtained the same PFGE profile but very different *flaA* genotypes. In these cases, *flaA* typing results determined that these isolates were still going to be considered as different from each other. The sensitivity of PFGE is dependent on the choice of the restriction enzyme, which could explain why some profiles that had the same PFGE *SmaI* profile had different *flaA* patterns (Ribot *et al.*, 2001). A better discrimination could be obtained with other restriction enzymes such as *KpnI* or *XbaI* (Neoh *et al.*, 2019), and a somatory analyses of clusters obtained with different restriction enzymes by Bionumerics.

After analysis of *C. jejuni* and *C. coli flaA* and PFGE genotypes, a total of 10 main clusters were considered (Figure7), and 5 unique profiles, in the *flaA* dendrogram (Figure 7). The different *flaA* genotypes considered are presented in Table 9. The most common profile (biggest cluster) was the number 3 (Table 9), followed by 8 and 5, comprised by 4 (cluster 3) and 5 bands respectively (cluster 8 and 5), with approx. molecular size ranging from 700-100bp. One strain from each cluster was selected, along with the 5 strains with individual profiles, for biofilm formation and antimicrobial susceptibility assays (n=15). To obtain more information regarding *C.coli* species, two more *C. coli* strains with *flaA* profile number 5 were added to the collection of 15 strains with different *flaA* patterns. Table 10 contains all the information related to the strains selected.

There are two main observations that can be made from results obtained in the *flaA* typing and PFGE dendrograms (Figure 7 and Figure 8): genetic variability, and persistence during the period studied. As for genetic variability, in *flaA* typing dendrogram, just for 21 March 2019 sample collection day (neck skin-March), 7 different *flaA* genotypes were obtained. Also, from neck skin samples taken on 5 of November 2018, 5 different genotypes were obtained. Similar results were already reported in *C. jejuni* and *C. coli* species isolated from poultry sources in several studies (El-Hamid *et al.*, 2019; Damjanova *et al.*, 2011; Wieczorek *et al.*, 2015; Zbrun *et al*., 2017; Zweifel *et al*., 2008), using different typing methods. This wide genetic diversity may be due to rapid adaptive changes along the processes of colonization and infection cycles. The underlying mechanisms are based on acquisition of foreign DNA by natural transformation (Gomes *et al.*, 2016), and random recombination of large DNA segments (Zbrun *et al.*, 2017). According to Vidal *et al.* (2016) commercial broiler farms provide “an ecological niche for a wide diversity of genotypes” causing impact on the structure of *Campylobacter* populations found in broiler production. In this context, it is safe to assume that bacterial populations of this pathogen, responsible for colonizing broiler flocks, are complex, containing multiple genotypes. Also, there is increasing evidence that some clonal complexes associated with human foodborne disease (campylobacteriosis) are widely distributed and dominant along poultry production food chain (Vidal *et al.*, 2016).

Besides the diversity observed, the hypothesis that genetically related isolates can persist throughout the period studied was assessed by analysing the sample type and date of collection. In cluster 3 we find the same *flaA*-RFLP profile in isolates obtained from faecal samples taken in January 2019, and in neck skin samples taken in June 2019. Also, in cluster 4, 5, 7, 8, and 10, we can find isolates obtained from faeces samples taken in November 2018, having the same band pattern as isolates obtained from neck skin samples collected in March 2019 (cluster 4, 5, 8), April (cluster 8), and

May (cluster 7, 10). These findings indicate the persistence of some *flaA* genotypes through time (November 2018- May 2019).

Table 9. Different band patterns of Campylobacter isolates (n=15).

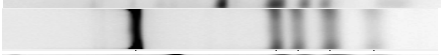
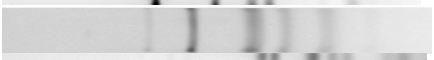
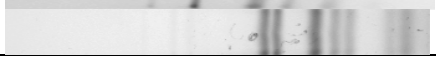
Profile	Bands *flaA* pattern	Cluster	Type of sample/date of collection
1		1	Neck skin-12 March 2019; Neck skin-21 May 2019
2		2	Neck skin-21 May 2019
3		3	Feaces-21 January 2019 ; Neck skin-6 June 2019
4		4	Faeces-5 November 2018; Neck skin-12 March 2019
5		5	Feaces-5 November 2018; Neck skin-12 March 2019
6		Single	Neck skin-21 May 2019
7		6	Neck skin-12 March 2019
8		7	Feaces-5 November 2018; Neck skin-21 May 2019
9		8	Feaces-5 November 2018; Neck skin-12 March 2019 and 16 April 2019
10		9	Neck skin-21 May 2019
11		Single	Neck skin-21 May 2019
12		10	Neck skin-21 May 2019
13		Single	Neck skin-21 May 2019
14		Single	Neck skin-12 March 2019
15		Single	Neck skin-12 March 2019

Table 10. Isolates (n=17) selected for antimicrobial resistance and biofilm assays.

Isolate	Species	Cluster/Band profile	Origin (sample type)	Slaugther date
1	*C. jejuni 64D*	Cluster 1	Neck skin	12/03/2019
2	*C. jejuni 105-1BR*	Cluster 2	Neck skin	21/05/2019
3	*C. jejuni* 46E	Cluster 3	Faeces	21/01/2019
4	*C. jejuni* 22A	Cluster 4	Faeces	05/11/2018
5	*C.jejuni* 20C	Cluster 5	Faeces	05/11/2018
6	*C. coli* 105E	Single band pattern	Neck skin	21/05/2019
7	*C. jejuni* 63E	Cluster 6	Neck skin	12/03/2019
8	*C. jejuni* 106E	Cluster 7	Neck skin	21/05/2019
9	*C. jejuni* 61C	Cluster 8	Neck skin	12/03/2019
10	*C. jejuni* 104B	Cluster 9	Neck skin	21/05/2019
11	*C. jejuni* 106A	Single band pattern	Neck skin	21/05/2019
12	*C. jejuni* 105B	Cluster 10	Neck skin	21/05/2019
13	*C. jejuni* 106B	Single band pattern	Neck skin	21/05/2019
14	*C. jejuni* 65E	Single band pattern	Neck skin	12/03/2019
15	*C. coli* 65B	Single band pattern	Neck skin	12/03/2019
16	*C. coli* 62E	Cluster 5	Neck skin	12/03/2019
17	*C. coli* 62D	Cluster 5	Neck skin	12/03/2019

Two *C. jejuni* strains with very similar PFGE genotypes (87.1% similarity) also highlighted this hypothesis: 19D, an isolate from faeces collected in November 2018, and 105B, an isolate from neck

skin collected in May 2019. The persistence ability of *Campylobacter jejuni* was also reported by Fraqueza *et al.* (2016) in a study conducted to evaluate *Campylobacter* frequency in quails at slaughterhouse level in two subsequent years (2009-2010). Genetic diversity was assessed by PFGE, in order to establish possible relationships among different flocks and producers. One pulsotype (pulsotype III) was obtained for isolates from neck skin and caecum samples taken on 2009, and for one isolate from a neck skin sample taken on 2010, indicating the possible persistence of pulsotype III through the period studied. Also, García-Sanchez *et al.* (2017) demonstrated with whole genome sequence typing, that highly clonal populations of *C. jejuni* were able to survive adverse conditions, even after cleaning and disinfection processes. Furthermore, *Campylobacter* was shown to persist for longer periods than expected, more specifically, at least 21 days, in the poultry plant environment (García-Sánchez *et al.*, 2017).

Additionally, *flaA* typing results also suggest that there is genetic similarity in the *flaA* gene found in strains obtained from neck skin and faeces samples, which in turn could indicate that cross contamination occurs at slaughterhouse, probably during evisceration step or even on other steps (such as plucking).

Overall, these genotyping techniques allowed the selection of different *Campylobacter* strains, considering the genetic variability existent at slaughterhouse level, for further assessment of biofilm formation ability and antimicrobial resistance.

6.2. Antibiotic resistance patterns of *Campylobacter* strains

Antibiotic resistance was tested for 10 antibiotics, each representing a different antimicrobial category: beta-lactam, namely the penicillins (ampicillin), macrolides (erythromycin), tetracyclines (tetracycline), amphenicols (chloramphenicol), aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin), naphthyridines (nalidixic acid), beta-lactamase inhibitors (amoxicillin+clavulanic acid), folate antagonists (trimethoprim-sulfamethoxazole) and carbapenems (ertapenem). Despite the low number of strains tested (n=17), results shown in Figure 9 were presented in percentage, because it was assumed that the sample, although small, is representative of the genetic variability found on the initial collection of isolates (n=145).

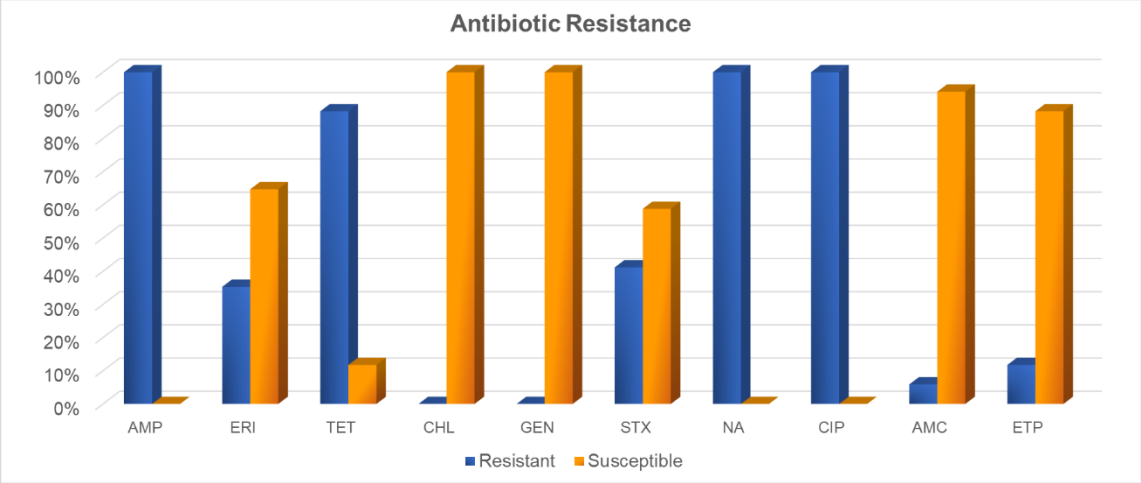


Figure 9. Antimicrobial resistance (%) in 13 C. jejuni and 4 C. coli strains.

All of the strains (n=17) were resistant to ampicillin, nalidixic acid and ciprofloxacin. On the contrary, all strains were susceptible to chloramphenicol and gentamicin. Ampicillin and nalidixic acid results were expected, due to the known widespread resistance to these antibiotics in Portugal (Duarte *et al.* 2013, Fraqueza *et al.*, 2016), as well as susceptibility to chloramphenicol and gentamicin, since resistance to these antibiotics is reported to be very rare (Whitehouse *et al.*, 2018, EFSA, 2020).

Results obtained for the 17 strains tested (*C. jejuni* =13; *C. coli*= 4) demonstrated that all the strains had at least 3 antibiotics to which they had acquired resistance.

EFSA (2020) has reported that the highest resistance rates on isolates recovered from poultry meat were found for ciprofloxacin, nalidixic acid and tetracycline (overall percentages: 54–83%). Most EU members states reported high to extremely high levels of resistance to these antibiotics (EFSA, 2020).

Ciprofloxacin resistance found in the strains tested was 100%. In Portugal, Duarte *et al.* (2013) also reported high frequency of resistance for ciprofloxacin (92.8%) in 125 clinical strains of *C. jejuni* and *C. coli* collected during the period of 2009-2012. Ciprofloxacin resistance trend is associated to fluoroquinolone use in poultry (Iovine & Blaser, 2004). In fact, in 2005, the Food and Drug Administration (FDA) in the United States banned the use of fluoroquinolones in poultry production, in order to reduce the prevalence levels of fluoroquinolone-resistant *Campylobacter* (Price *et al.*, 2007). Even so, the CDC National Antimicrobial Resistance Monitoring System (NARMS) reports that ciprofloxacin resistance keeps increasing until today. Preventive measures might have been taken too late, or they aren't being duly implemented. Fluoroquinolones use in poultry production is still permitted in most European countries (Roth *et al.*, 2019). The European Commission has not taken any actions to ban the veterinary use of fluoroquinolones at present. This could explain why ciprofloxacin resistance was so high in the strains tested in this work.

As for tetracycline resistance, it was the fourth highest acquired resistance rate observed (15 out of the 17 selected strains were resistant). Similar results were obtained by Elhadidy *et al.*, (2018) when testing antimicrobial susceptibility for 204 *C. jejuni* isolates obtained from broiler carcass samples. The highest frequency of resistance was also reported for ciprofloxacin (53.9%) and nalidixic acid (53.4%), followed by tetracycline (47%). EFSA's (2020) latest report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food, doesn't provide data about antibiotic resistance in *C. jejuni* isolates from broiler meat in Portugal. The only data reported by EFSA (2020) indicates that Portugal is the country with the highest occurrence of resistance reported for tetracycline (100%) in *C. jejuni* isolates obtained from fattening turkeys (EFSA, 2020). Tetracyclines were discovered in 1940s, and because of their heavy use in the past for both human and veterinary indications, currently, there is a widespread resistance that has been limiting their use (Iovine, 2013).

Following tetracyclines, the highest resistance levels were found for erythromycin and trimethoprim-sulfamethoxazole. Erythromycin resistance in the *Campylobacter* strains tested was found to be particularly concerning, since this antibiotic represents a treatment option for persistent *Campylobacter* infections (Aksomaitiene *et al.*, 2019). In this study, 6 out of 17 strains were resistant to erythromycin. EFSA reported that in human *C. jejuni* isolates, resistance to erythromycin was low in general (1.8%), however, it was remarkably higher in *C. coli* isolates (14.3%). In this study frequency of

resistance was approx. 35%, and of the four *C. coli* strains tested, only one was resistant. This result is more in agreement to the frequency of resistance reported by Fraqueza *et al.* (2014). In 82 *C. jejuni* isolates from poultry samples, the occurrence of resistance was 35% for the macrolide erythromycin, which was higher than the occurrence reported in *C. coli* isolates (n=85), correspondent to 13%.

As for trimethoprim-sulfamethoxazole (SXT) resistance was present in seven strains. A moderate resistance frequency to SXT in *Campylobacter* isolates from samples of quails was also found and reported by Fraqueza *et al.* (2016).

Only one strain was resistant to amoxicillin-clavulanic acid. In Europe, the resistance to this antibiotic is low in *C. jejuni* and *C. coli*. EFSA (2020) reported that low proportions of *Campylobacter* human isolates were resistant to amoxicillin-clavulanic acid, except in Luxembourg, Malta and Spain, where 20–27.3% of the *C. coli* isolates were resistant.

Only two *C. jejuni* strains had a potential resistance to ertapenem: *C. jejuni* 106E and *C. jejuni* 63E. To confirm this resistance, MIC should be accessed, for example with E-test gradient MIC strips. Resistance to carbapenems is very rare in *C. jejuni* and *C. coli*. This resistance isn't even well defined by the authorities. Furthermore, carbapenem antibiotics are not licensed for use in food-producing animals in the EU, north America, and Australia. In all circumstances, EFSA recommends that the use of such antibiotics in animal production should be actively discouraged globally (EFSA, 2013). Additionally, EFSA also suggested that use of compounds containing heavy metals such as zinc should also be minimised since genes encoding carbapenemase production are sometimes linked to genes encoding resistance to heavy metals.

The factors that support the emergence of carbapenem resistance include: a) increased consumption of carbapenems in humans, in part due to the worldwide spread of ESBLs in Enterobacteriacae; b) the location of carbapenem resistance genes on mobile genetic elements (genetic material, like plasmids or transposons, that is able to move within a genome and also, be shared with neighbouring bacteria); and c) positive selection caused by co-resistance with other frequently used antibiotics (EFSA, 2013). Indeed, rare cases of resistance occurring during treatment of persistent infections have been emerging. For example, a recent clinical case was reported in Japan subsequent to long-term oral antibiotic treatment with tebipenem and faropenem for a persistent infection caused by *C. coli* (Hagiya *et al.*, 2018).

After discussing resistance levels obtained for each antibiotic, another interesting analysis is to assess the different resistance profiles obtained. Results are shown in Table 11. The most frequent antibiotic resistance profile was AMP-TET-CIP-NA in 5 strains, followed by AMP-TET-CIP-NA-SXT (4 strains). This result emphasizes the high resistance levels for combined resistance to ciprofloxacin and tetracycline. However, the most concerning combined resistance was for both ciprofloxacin and erythromycin, found in six strains: *C. jejuni* 106E, *C. jejuni* 63E, *C. coli* 105E, *C. jejuni* 104B, *C. jejuni* 64D and *C. jejuni* 106B. Regarding the available data on this combined resistance, EFSA's report (2020) indicated that in *C. jejuni* isolates from broilers, combined resistance for ciprofloxacin and erythromycin was detected in 7 out of 29 countries of Europe in 2018. Among those was Portugal, registering a combined resistance of 16.4% (EFSA, 2020).

Table 11. Resistance profiles of 17 strains tested (C. jejuni=13; C. coli=4).

Strain	Specie	Resistance profiles
106E	*C. jejuni*	AMP-ERI-TET-CIP-NA-SXT-ETP
63E	*C. jejuni*	AMP-ERI-TET-CIP-NA-ETP
105E	*C. coli*	AMP-ERI-TET-CIP-NA-AMC
104B	*C. jejuni*	AMP-ERI-TET-CIP-NA-SXT
64D	*C. jejuni*	AMP-ERI-TET-CIP-NA
106B	*C. jejuni*	
22A	*C. jejuni*	AMP-TET-CIP-NA-SXT
20C	*C. jejuni*	
65B	*C. coli*	
62D	*C. coli*	
105-1BR	*C. jejuni*	AMP-TET-CIP-NA
61C	*C. jejuni*	
62E	*C. coli*	
106A	*C. jejuni*	
105B	*C. jejuni*	
46E	*C. jejuni*	AMP-CIP-NA-SXT
65E	*C. jejuni*	AMP-CIP-NA

AMP = Ampicillin (10 µg); ERI = Erythromicin (15 µg); TET = Tetracycline (30 µg); CHL = chloramphenicol (30 µg); GEN = Gentamicin (10 µg); CIP = Ciprofloxacin (5 µg); NA = Nalidixic acid (30 µg); AMC = Amoxicillin + Clavulanic acid (20 + 10 µg); STX = Trimethoprim + Sulfamethoxazole (25 µg); ETP = Ertapenem (10 µg).

In order to make a composite analysis combining antimicrobial resistance profiles with the different *flaA* profiles selected, a clustering analysis was performed in Bionumerics with all the resistance profiles linked to the 17 *flaA* genotypes. The obtained results are found in Figure 10. The dendrogram demonstrates that the same resistance profiles can be found for different *flaA* profiles. On the other

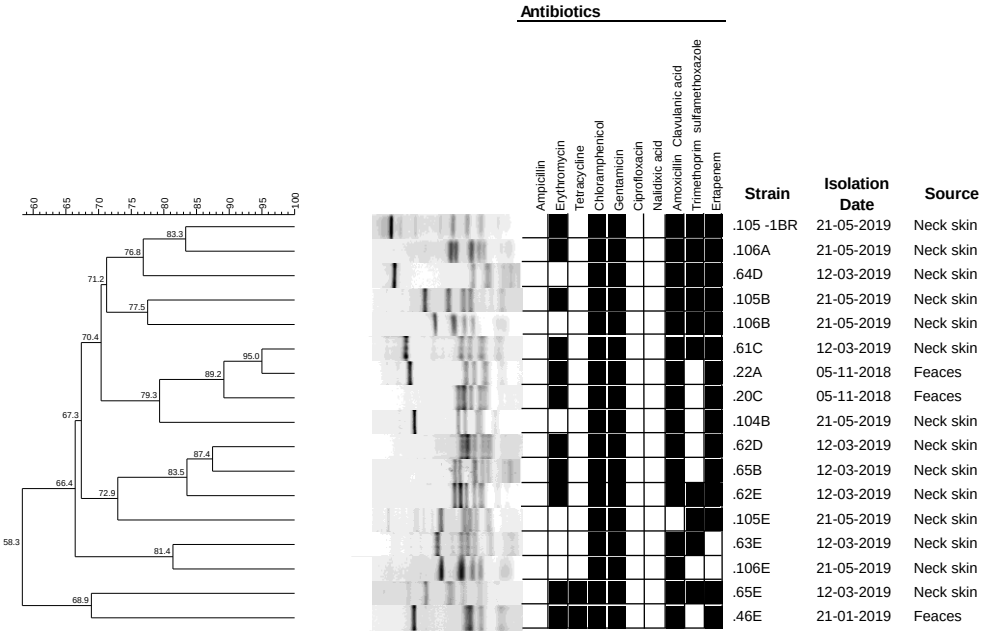


Figure 10. Campylobacter flaA-RFLP profiles of the 17 strains selected and their cluster association according to profile similarity and antibiotic resistance profile. Black squares represent sensitivity and white squares represent resistance.

hand, the strains *C. jejuni* 22A and *C. jejuni* 61C had very similar *flaA* profiles (95%), and they were confirmed as different strains once they obtained a different resistance profile.

Strains with the most concerning resistance profiles were 106E (*C. jejuni*), 63E (*C. jejuni*) and 105E (*C. coli*), not only because they were multidrug resistant, but also because these strains possibly have a very rare acquired resistance to ertapenem (*C. jejuni* 106E and 63E) and to amoxicillin + clavulanic acid (*C. coli* 105E). 106E was identified as the strain with the most concerning resistance profile, since it acquired resistance to the highest number of antibiotics. This strain is inserted in cluster 7 (Figure 7), in which persistence throughout time is evidenced. In this cluster, most isolates were collected in May 2019, but there is one isolate from feaces collected in November 2018 with the same genotype. This result indicates that *C. jejuni* 106E *flaA* genotype persisted at slaughterhouse throughout the period of November 2018 to May 2019. As for *C. jejuni* 63E there is no evidence of persistence, since it belongs to a cluster only comprising isolates from March 2019. *C. jejuni* 105B in its turn has a unique profile, and therefore, it is also not possible to assess if there was persistence of its genotype throughout time.

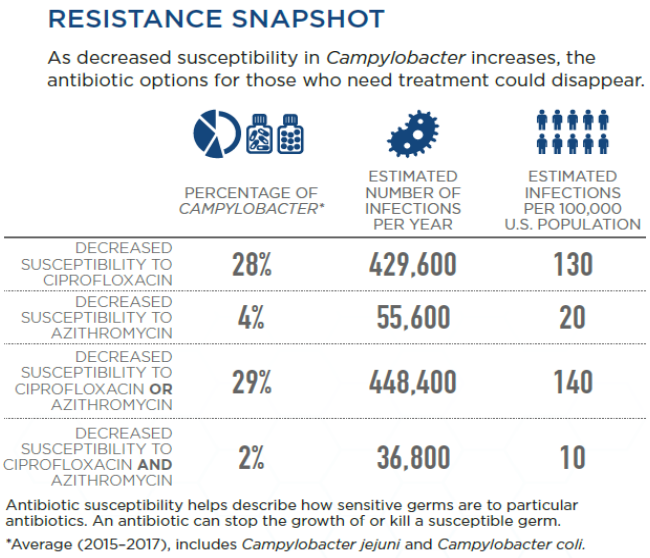
Furthermore, besides these strains, the other strains that have concerning profiles are the ones with combined resistance to erythromycin and ciprofloxacin. According to EFSA the occurrence of *Campylobacter* isolates with combined resistance to erythromycin and ciprofloxacin are of great importance to public health, since both these antibiotics are recognised as 'critically important antimicrobials' for the treatment of persistent *Campylobacter* infections in humans (EFSA, 2020). The *flaA* genotypes that these strains represent corresponded to small clusters and two single patterns (*C. coli* 105E and *C. jejuni* 106B). This result indicated that these genotypes weren't the most commonly found on the samples analysed, and even though they don't represent a major part of the initial collection (n=145), six strains (6 out of 17) is still a concerning number.

Concerning multidrug resistant strains, they were classified according to Magiorakos *et al.* (2012) definition: resistant to at least one agent in three or more antimicrobial categories. In this work, all strains were multidrug resistant, except *C. jejuni* 65E, because nalidixic acid and ciprofloxacin were considered to belong in the same class of antimicrobials, the quinolones. Duarte *et al.* (2013) also reported a high percentage of multidrug resistance (87,2%) in human clinical isolates (total of 125, *C. jejuni*=78 and *C. coli*=47) analysed between 2009 and 2012 for epidemiologic study. The percentage of multidrug resistance frequency obtained by Fraqueza *et al.* (2016) was also high (69%) in isolates obtained from quails.

The strains that were found resistant to the lowest number of antibiotics tested were *C. jejuni* 65E (AMP-CIP-NA) and *C. jejuni* 46E (AMP-CIP-NA-SXT). One of these strains, *C. jejuni* 46E, represented the biggest cluster formed (cluster 3) in the *flaA*-RFLP dendrogram. This result may indicate that isolates obtained at slaughterhouse, most frequently, are multidrug resistant. *C. jejuni* 65E in its turn had a unique *flaA*-RFLP profile.

Multidrug resistance should be considered a serious problem, especially when there is simultaneous resistance to fluoroquinolones and macrolides. CDC listed 18 pathogenic agents into one of three categories: urgent, serious, and concerning. Multidrug resistant *C. jejuni* and *C. coli* strains are considered by CDC (2019) as a serious threat alongside extended-spectrum beta-lactamase (ESBL)-

producing *Enterobacteriaceae*; vancomycin resistant Enterococci (VRE); methicillin-resistant *Staphylococcus aureus* (MRSA); among others. In total, 448,400 infections and 70 deaths were estimated during the year of 2019 in the united states, caused by drug resistant *Campylobacter*. Emphasize was given on the concerning decreasing levels of susceptibility in *C. jejuni* and *C. coli* to the current treatment options provided for persistent infections (ciprofloxacin and azithromycin) as shown in Figure 11 (CDC, 2019).



RESISTANCE SNAPSHOT

As decreased susceptibility in *Campylobacter* increases, the antibiotic options for those who need treatment could disappear.

	PERCENTAGE OF *CAMPYLOBACTER**	ESTIMATED NUMBER OF INFECTIONS PER YEAR	ESTIMATED INFECTIONS PER 100,000 U.S. POPULATION
DECREASED SUSCEPTIBILITY TO CIPROFLOXACIN	28%	429,600	130
DECREASED SUSCEPTIBILITY TO AZITHROMYCIN	4%	55,600	20
DECREASED SUSCEPTIBILITY TO CIPROFLOXACIN **OR** AZITHROMYCIN	29%	448,400	140
DECREASED SUSCEPTIBILITY TO CIPROFLOXACIN **AND** AZITHROMYCIN	2%	36,800	10

Antibiotic susceptibility helps describe how sensitive germs are to particular antibiotics. An antibiotic can stop the growth of or kill a susceptible germ.

*Average (2015–2017), includes *Campylobacter jejuni* and *Campylobacter coli*.

Figure 11. Decreased susceptibility to ciprofloxacin and azithromycin in C. jejuni and C. coli strains, in the period of 2015-2016 USA, reported by CDC in Antibiotic Resistance Threats in the United States, 2019 report (2019 AR Threats Report).

6.3. Assessment of *Campylobacter* biofilm ability under slaughterhouse condition

Currently, the most attractive hypothesis to explain *C. jejuni* and *C. coli* survival at the processing environment, is that strains possess biofilm formation ability. Most studies on *C. jejuni*/*C. coli* biofilm formation were performed using reference strains, instead of wild strains from the poultry food chain (Lamas *et al.* 2018). A small number of studies actually reported that strains from poultry sources can form biofilms (Balogu *et al.*, 2014; García-Sánchez *et al.*, 2019; Kim *et al.*, 2017; Melo *et al.*, 2017; Stetsenko *et al.*, 2019), however, conditions found on processing plant (aerobiosis, 10ºC) combined with medium mimicking nutrients available, weren't tested in assays for quantification of biofilm (e.g. crystal violet assay). In this way, the goal of this work was to assess the biofilm formation ability of wild strains from poultry origin, under conditions similar to those found in the industry, in order to explain how *Campylobacter* spp. strains persist during long periods of time at slaughterhouse.

6.3.1. Crystal Violet assay

Campylobacter strains ability to form biofilm was tested according to the conditions described in the section of material and methods (4.8.3.). Results obtained for each assay (low inoculum = assay 1 and high inoculum = assay 2) were presented in four separate Figures (Figure 12, 13, 14, and 15), representing both temperatures tested (10°C and 42°C). Measurements performed for the different

atmospheric conditions (aerobic and microaerophilic) were also displayed in the Figures in order to understand oxygen influence on the adherence levels of each strain.

The OD580nm means obtained for each strain at 10°C were presented in Figure 12 (assay 1), and Figure 13 (assay 2). In assay 1, where low inoculum concentration was tested (10^3 CFU/mL), the OD580nm mean values obtained for every strain were in general low (OD580nm <0.1).

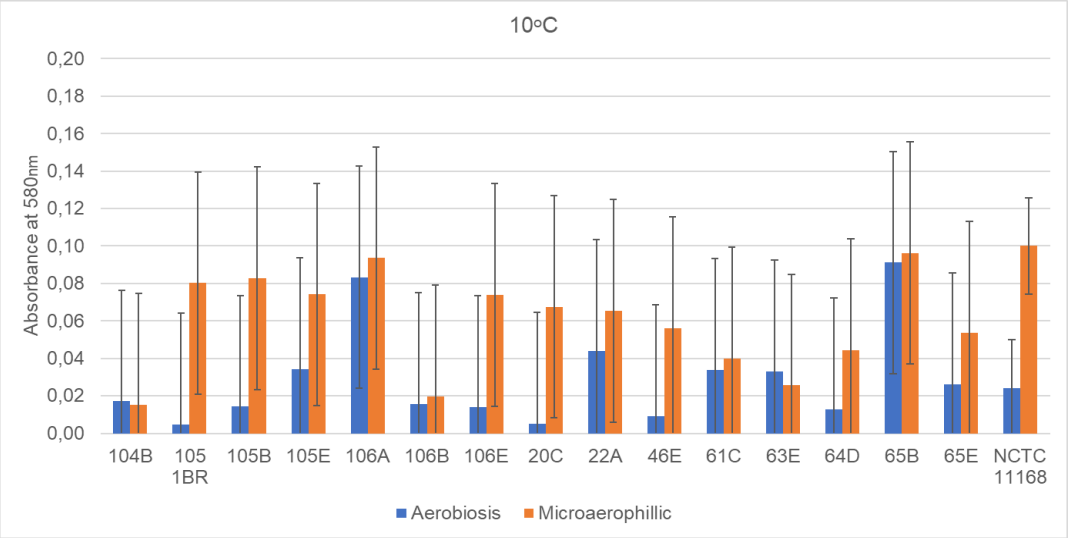


Figure 12. Assay 1 (inoculum= 10^3 CFU/mL): Biofilm formation at 10°C in either aerobic or microaerophilic conditions, in 15 Campylobacter strains along with positive control NCTC 11168.

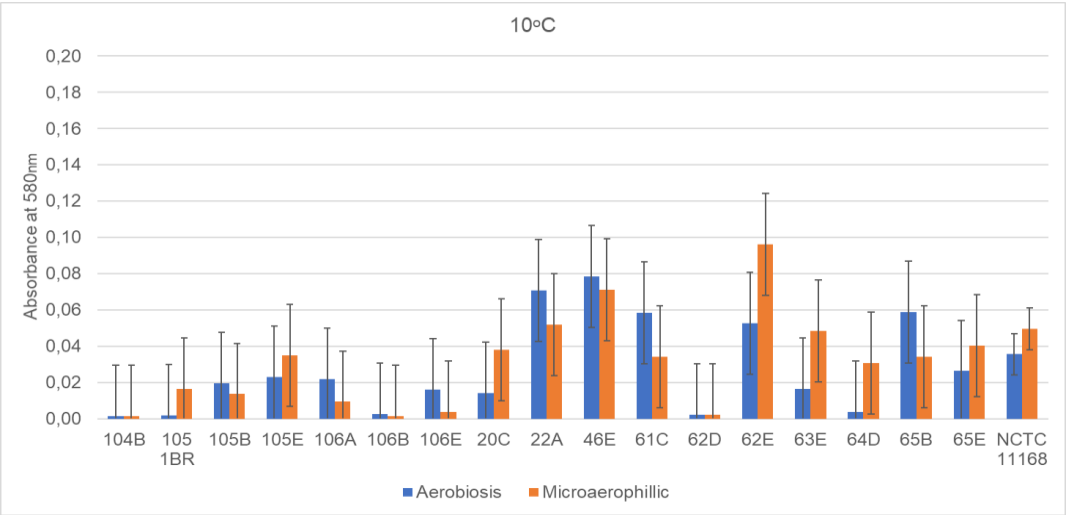


Figure 13. Assay 2 (inoculum= 10^6 CFU/mL): Biofilm formation at 10°C in either aerobic or microaerophilic conditions, in 17 Campylobacter strains along with positive control NCTC 11168.

Even though some authors report similar values for *C. jejuni* biofilms quantified via crystal violet staining method (Reeser *et al.*, 2007; Teh *et al.*, 2017), in general, OD cut-off values for biofilm classification range between 0.2-0.35 (Melo *et al.*, 2017; Zhang *et al.*, 2017). If OD580nm < 0.2 represents no biofilm formation, then it could be assumed that at 10°C no biofilm was produced in this work, either under aerobiosis or microaerophilic atmosphere. On the other hand, results shouldn't be overlooked, since the mean of negative controls was previously subtracted. In this way, even though it

cannot be assumed that biofilm formation occurred, some level of adherence should be considered as well as some potential to form biofilm.

Under aerobiosis, the strain with the highest adherence level was *C. coli* 65B (OD580nm≈0.09), followed by *C. jejuni* 106A (OD580nm≈0.08). Both these strains had single (individual) *flaA* profiles, and therefore they represent a minority of the isolates collected (n=145). These strains were also the strains with the highest adherence levels under microaerophilic atmosphere at 10°C.

Crystal violet ODs obtained in assay 1 (low inoculum), under aerobic and microaerophilic atmospheres, at 10°C, weren't significantly different in any strain, except for *C. jejuni* NCTC 11168 (p value =0.037). Adherence of *C. jejuni* NCTC 11168 was found to be significantly higher under microaerophilic atmosphere.

In assay 2 (high inoculum) (Figure 13), at 10°C, the OD580nm mean values obtained for every strain were low in general (OD580nm <0.1), as observed in the results obtained for assay 1 (low inoculum). The strains with the highest levels of adherence under aerobiosis were *C. jejuni* strain 46E (OD580nm≈0.08) and *C. jejuni* 22A (OD580nm≈0.07). *C. jejuni* 46E genotype represents the biggest cluster formed (cluster 3), while *C. jejuni* 22A was inserted in an average size cluster (cluster 4). Therefore, *C. jejuni* 46E genotype represents isolates that are commonly obtained from poultry samples (feaces and neck skin), whereas *C. jejuni* 22A represents a less common *flaA* profile. Additionally, the cluster where *C. jejuni* 46E was inserted (cluster 3), indicated persistence throughout the period of January-June 2019. *C. jejuni* 22A in its turn, was inserted in cluster 4, in which persistence throughout the period of November 2018 to March 2019 was found.

Under microaerophilic atmosphere, at 10°C, *C. coli* strain 62E obtained the highest (p value <0.05) adherence (OD580nm≈0.10), with a significant difference when compared to 7 other *C. jejuni* strains (104B, 106B, 105-1BR, 105B, 106A, 106B, 106E).

Under 10°C, differences between the ODs obtained under microaerophilic and aerobic atmospheres were not significant for any strain tested. In other words, oxygen concentration didn't cause significant differences in the adherence levels of any strain.

In sum, the adherence levels obtained at 10°C (Figure 12 and Figure 13) show that some *C. jejuni* and *C. coli* strains have ability to attach in surfaces present at slaughterhouse environment. For this reason, it is very important to maintain *Campylobacter* contamination levels safe with efficient hygiene programs in order to avoid biofilm formation, the next step after adhesion.

Regarding levels of adherence obtained at 42°C condition, results were shown in Figure 14 (low inoculum = assay 1) and Figure 15 (high inoculum = assay 2).

In assay 1 (Figure 14), for both aerobiosis and microaerophilic conditions, at 42°C, the strains with the significant (p <0.05) highest biofilm formation were *C. jejuni* 46E and 61C. The highest OD values were obtained under aerobiosis: 46E (OD580nm≈1.16) and 61C (OD580nm≈1.28). Interestingly, these isolates formed extensive floating aggregates in overnight cultures, as shown in Figure 16. To perform the biofilm assay, these aggregates were dissolved as much as possible, but nonvisible aggregates could have been present in the cultures used for biofilm formation assay. Biofilm formed in

polystyrene was visible by naked eye when performing crystal violet staining, as shown in Figure 17. It is pertinent to question whether the formation of these aggregates aided biofilm formation.

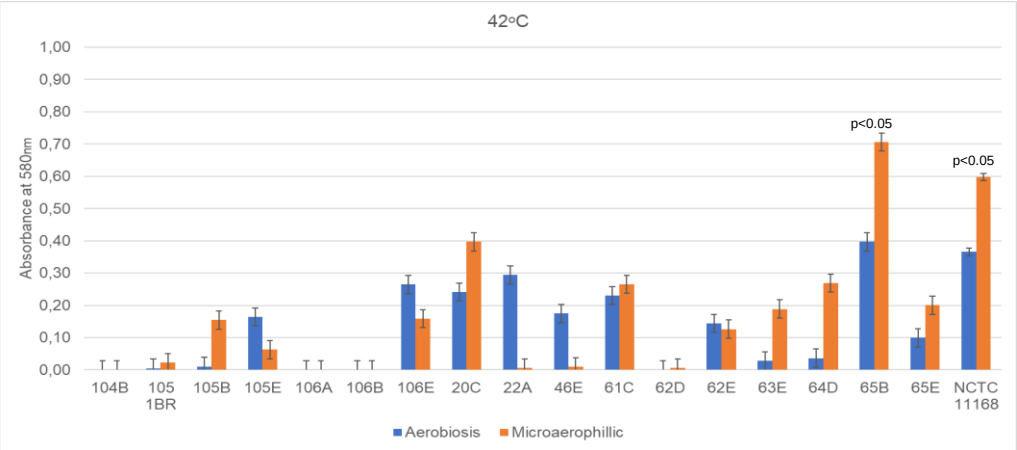


Figure 15. Assay 2 (inoculum= 10^6 CFU/mL): Biofilm formation at 42°C in either aerobic or microaerophilic conditions, in 17 Campylobacter strains along with positive control NCTC 11168. p value<0,05 is indicated for the strains that obtained significantly different OD values when compared to all other strains.

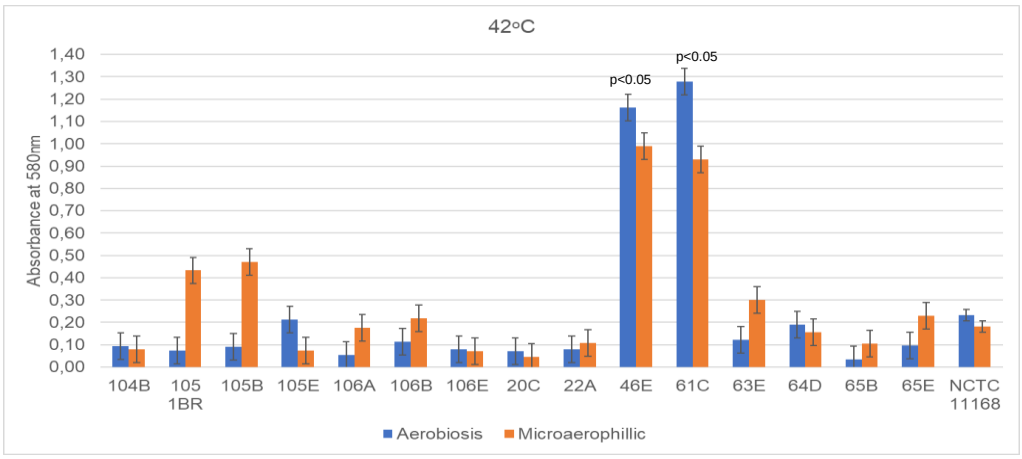


Figure 14. Assay 1 (inoculum= 10^3 CFU/mL): Biofilm formation at 42°C in either aerobic or microaerophilic conditions, in 15 Campylobacter strains along with positive control NCTC 11168. p value<0,05 is indicated for the strains that obtained significantly different OD values when compared to all other strains.

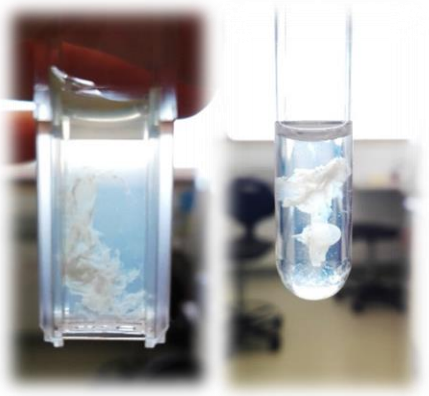


Figure 16. Aggregate formation in overnight culture by C. jejuni 46E strain.

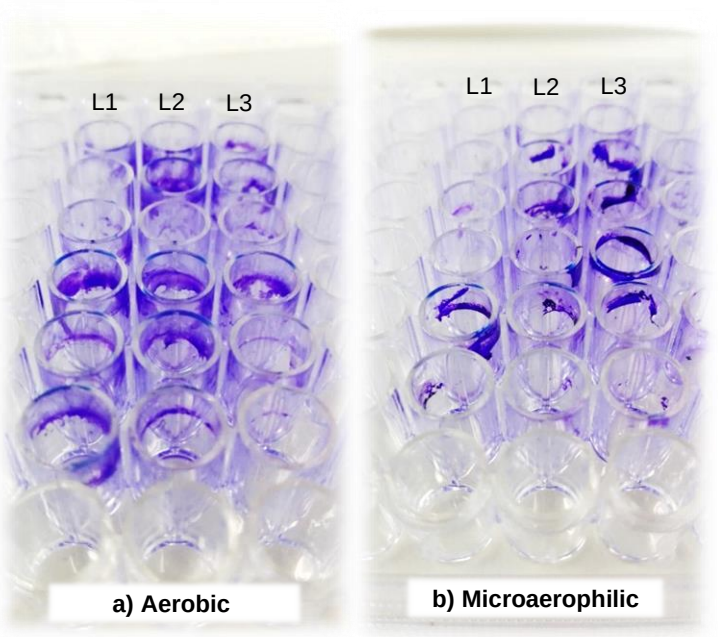


Figure 17. Biofilm formation by C. jejuni 61C strain at 42°C under a) aerobiosis, with three replicates (L1, L2, L3) and b) microaerophilic atmosphere, with three replicates (L1, L2, L3). 72h of incubation.

The biofilm formation ability presented by these strains was remarkable. Also, *C. jejuni* 46E and 61C genotypes represented the two biggest clusters (3 and 8) obtained in the *flaA*-RFLP dendrogram (Figure 7). Therefore, these genotypes represent the isolates that were most commonly present in the collection of n=145. Besides, inside these clusters there was evidence of these genotypes' persistence throughout time. Cluster 3 included samples from 21 January 2019 (feaces) and from 6 June 2019, indicating the persistence of this *flaA* profile from January to June 2019. In cluster 8, samples collected in 5 November 2018, 16 April 2019 and 12 March 2019 were found. Therefore, the *flaA* band pattern of *C. jejuni* strain 61C (inserted in cluster 8) suggests persistence at slaughterhouse during the period of November 2018 to April 2019. The biofilm formed by these strains, only corroborates their capacity to persist at slaughterhouse environment.

Additionally, the strains 105-1BR (*C. jejuni*), 105B (*C. jejuni*), and *C. jejuni* 63E at 42°C under microaerophilic conditions, presented OD580nm mean values significantly different when compared to most of the other strains (p value <0.05). These strains presented a significant higher adherence under microaerophilic atmosphere when compared with aerobic condition, which may indicate a stress to oxygen, inhibiting their ability to adhere.

Regarding assay 2 (high inoculum concentration) (Figure 15), at 42°C, the strain that significantly produced biofilm the most, under both aerobic and microaerophilic atmospheres, was 65B (*C. coli)*. The ODs obtained for *C. coli* 65B under both aerobic (OD580nm≈0.40) and microaerophilic (OD580nm≈0.71) conditions were significantly different (p <0.05) from the ones obtained for all other strains. This strain represents a single profile in the *flaA*-typing dendrogram, and therefore no assumptions can be made as for its persistence at slaughterhouse during the period studied.

As for oxygen influence, it appears to have inhibited biofilm formation in this strain (*C. coli* 65B), since adherence levels were significantly lower ($p < 0.05$) under aerobiosis when compared with microaerophilic condition.

When a higher inoculum concentration was tested at 42°C, *C. jejuni* strains 22A, 106E, 20C and 61C had the highest adherence levels after *C. coli* 65B. They all presented OD580nm means >0.2, either under aerobic atmosphere, microaerophilic, or both. All these strains represent clusters in the dendrogram (Figure 7) in which there is evidence of persistence throughout time (cluster 4, 7, 5, and 8 respectively). This result is very relevant because it suggests that adherence ability obtained in biofilm assay is "in agreement" with *flaA* typing results, since the strains that adhered the most, are also the ones with a *flaA* genotype that persisted throughout the period studied.

When comparing ODs obtained for each atmosphere condition in each strain, there were significant differences ($p < 0.05$) found, indicating stress oxygen influence on adherence levels. While oxygen seems to have stimulated adherence for the strains 105E (*C. coli*), 106E (*C. jejuni*), 22A (*C. jejuni*), and 46E (*C. jejuni*), ability was inhibited for the *C. jejuni* strains 105B, 20C, 63E, 64D, 65E, NCTC 11168 and *C. coli* strain 65B (p value < 0.05), in assay 2.

When comparing both assays performed (low and high inoculum), in general, there was great variability in adherence levels among the different strains tested. In other words, the formation of biofilm was strain dependent. Similar results were found by Kim *et al.* (2017) when testing 78 *Campylobacter* isolates obtained from raw chicken. Most isolates (64 isolates, 82%) did not have ability to form biofilm (OD590nm<0.05) as assessed by crystal violet staining method. Only 14 isolates presented some level of biofilm formation: 2 formed high level formation, 3 formed medium level, and 9 showed low levels. The study demonstrated that wild strains from chicken vary a lot in their biofilm formation ability on polystyrene (Kim *et al.*, 2017).

Regarding the impact caused by the different temperatures tested (10°C and 42°C), for both low and high inoculum assays, adherence levels were lower at 10°C. Oxygen on the other hand, had a different impact depending on the strains. Furthermore, statistical analysis was also used to assess the impact of low and high inoculum concentrations used, independently of temperature and atmospheric conditions. Results are found in Figure 18.

Results from SAS statistical analysis indicated that regardless of temperature and atmospheric conditions tested, high inoculum concentration led to significantly decreased adherence levels ($p < 0.05$) for the *C. jejuni* strains 105-1BR, 105B, 106A, 106B, 46E, and 61C. On the other hand, it had the reverse effect for the *C. jejuni* strains 20C and NCTC 11168, and *C. coli* strain 65B.

A possible explanation for the detrimental effect of high inoculum concentration in some strains could be the rapid nutrient scarcity. Culture media wasn't renewed; therefore, a higher inoculum concentration likely results in a more aggressive competition for nutrients. This could have led the bacterial population to enter a starvation state faster, not leaving enough time for the population to make a transition to the biofilm state. In other words, maybe a nutrient deficient medium inhibited adherence ability in these strains.

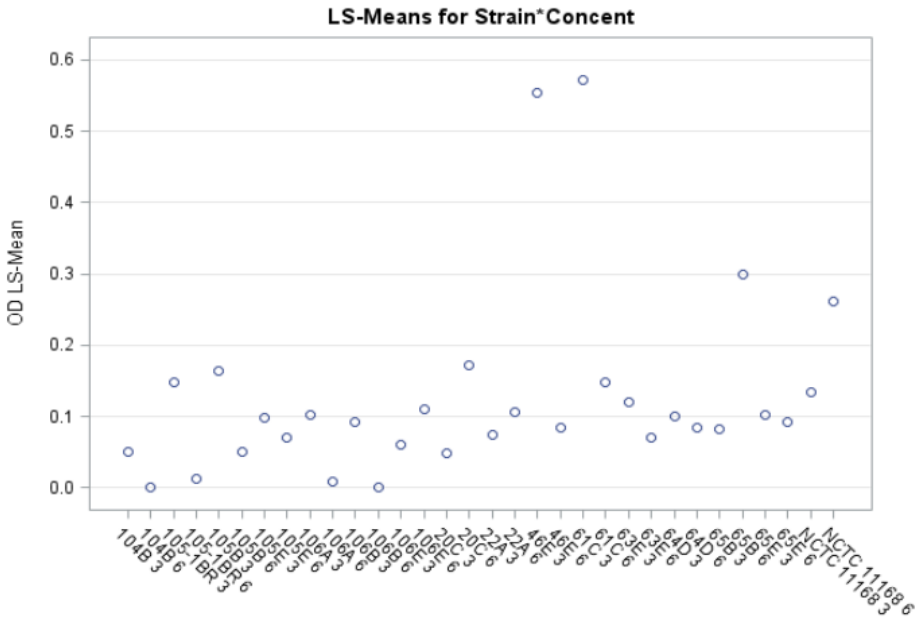


Figure 18. Campylobacter strains dispersion, displaying interactions between strains and inoculum concentration (10^6 CFU/mL), independently of temperature and atmosphere.

On the contrary, in the strains that obtained higher adherence levels when incubated with higher inoculum concentration, it could be speculated that bacterial population entered death phase very fast, leading to the accumulation of biomass composed of dead cells. This deposit of dead cells could have facilitated the adherence of surviving cells, leading to higher ODs obtained in crystal violet assay.

Overall, inoculum concentration was shown to be an important factor, since it appeared to highly influence adherence for some strains.

As for the association between biofilm assay results and antibiotic resistance rates presented on 5.2., it is important to discuss the adherence levels of the isolates that had the highest resistance levels. *C. jejuni* 106E, the strain with highest number of acquired resistances, including a possible rare resistance to ertapenem, even though it wasn't among the strains that presented the highest adherence level, it did reach OD580nm higher than 0.2, in assay 2 (high concentration inoculum), at 42°C, under aerobiosis. Also, its *flaA* profile belonged to a cluster demonstrating persistence throughout the period of November/2018 to May/2019. Results obtained suggested that this highly resistant strain not only had a genotype that persisted in time, but also had potential for biofilm formation capacity under aerobic atmosphere. The other two strains that were the most resistant after the strain *C. jejuni* 106E were 63E (*C. jejuni*) and 105E (*C. coli*). *C. coli* 105E had higher adherence levels in assay 1 (low inoculum), at 42°C under aerobiosis. On the contrary, *C. jejuni* 63E obtained higher adherence at 42°C under microaerophilic atmosphere, also in low inoculum assay (assay 1). The difference between levels obtained in each atmosphere was significant for *C. jejuni* 63E (p value <0.05) in assay 1, and for *C. coli* 105E in high inoculum assay (assay 2), at 42°C. For *C. jejuni* 63E strain, oxygen appears to have inhibited adherence. On the contrary, for *C. coli* 105E, oxygen seems to have stimulated adherence. *C. coli* strains were previously demonstrated to be are more aerotolerant than *C. jejuni* strains as reported

by Karki *et al.*, (2018), which could explain the more resilient ability of *C. coli* 105E to adhere under aerobiosis when compared to *C. jejuni* 63E.

On the other hand, the strains that stood out in biofilm assays, *C. jejuni* 46E, 61C and *C. coli* 65B, had the following resistance profiles:

61C: AMP-TET-CIP-NA; 46E: AMP-CIP-NA-SXT; 65B: AMP-TET-CIP-NA-SXT

These results indicated that these strains that demonstrated the highest adhesion levels, are among the ones with the least acquired resistances. Nevertheless, they were all multidrug resistant.

After analysing results obtained for the tested strains, it is important to discuss the results obtained for the strain used as a positive control: *C. jejuni* NCTC 11168. This strain was chosen because it is frequently used in crystal violet biofilm assays (García-Sánchez *et al.*, 2019; Jaakkonen *et al.*, 2020; Lynch *et al.*, 2019; Mahdavi *et al.*, 2014). *C. jejuni* NCTC 11168 strain had statistically significant differences from all the other strains in assay 2 (high inoculum) at 42°C under microaerophilic atmosphere. ODs obtained for the control strain were in accordance to Jaakkonen *et al.* (2020) study in which NCTC11168 was also used as a control strain in biofilm formation assay, at a concentration of 10^6 CFU/mL, under microaerophilic conditions, at 41.5°C for 48h. *C. jejuni* NCTC 11168 was reported to reach ODs that ranged between 0.4-0.6, obtained by crystal violet staining method (Jaakkonen *et al.*, 2020). Similar results were obtained in assay 2, where 10^6 CFU/mL inoculum concentration was tested, under the similar conditions. Other authors support this result, testing the same condition (42°C or 37°C under microaerophilic atmosphere) when performing similar biofilm assays with crystal violet staining method (Kim *et al.,* 2015; Mahdavi *et al.,* 2014; Shabbir *et al.*, 2018).

As for the influence of oxygen on biofilm formation, statistical analysis demonstrated that there were significant differences between both atmospheres in assay 2 (p value <0.05). This result led to the conclusion that the control strain formed more biofilm under microaerophilic atmosphere (p value <0.05). In contrast oxygen appears to have inhibited biofilm formation. This outcome was in disagreement to results obtained by Reuter *et al.* (2010) and Feng *et al.* (2018) studies, in which *C. jejuni* NCTC 11168 was reported to have increased biofilm formation under aerobic atmosphere as assessed by crystal violet staining method. Nevertheless, considering the similar conditions performed by Feng *et al.* (2018): inoculum concentration 10^7 CFU/mL, 200 µl added to each well in 96 well polystyrene plate, incubation under aerobic atmosphere at 37°C for up to 72h; the obtained OD from crystal violet staining method: OD595 nm ≈ 0.4, was very close to the one obtained in the present work (OD≈0.37) for *C. jejuni* NCTC 11168 under aerobic conditions in assay 2. On the other hand, Reeser *et al.* (2007) reported that aerobic conditions inhibited biofilm formation, whereas microaerophilic and thermophilic conditions were reported to increase biofilm formation (Reeser *et al.*, 2007).

Overall, *C. jejuni* NCTC 11168 was a good control strain when incubated at 42°C, with an inoculum concentration of 10^6 CFU/mL, under microaerophilic atmosphere. Only under these conditions biofilm formation could be visualized by naked eye on the bottom of the wells. Figure 19 shows the biofilm formed by *C. jejuni* NCTC 11168.

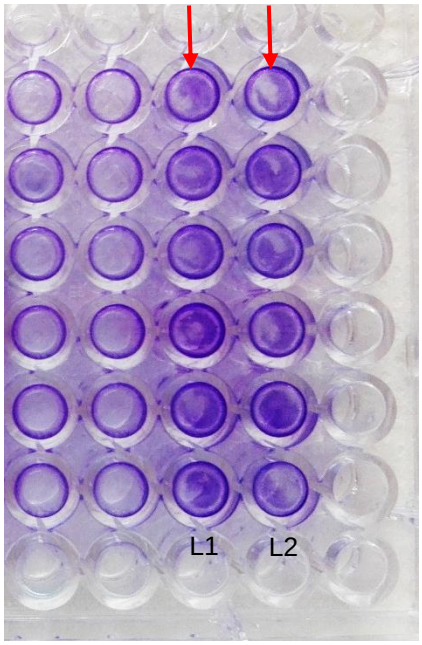


Figure 19. Biofilm formation by NCTC 11168, at 42°C under microaerophilic conditions after 72h of incubation, in 96-well polystyrene plate. Two replicates (L1, L2) were performed for every plate.

6.3.2. Strain classification for biofilm formation ability

In order to differentiate the adherence strength of the strains tested, they were classified as for their ability to adhere according to Stepanović *et al.* (2004). In Table 12 and Table 13, final classification for each strain in every condition is shown for both assays performed.

The cut-off value for low inoculum concentration (assay 1) was O.D.c. ≈ 0.178 and for for high inoculum concentration (assay 2), O.D.c. ≈ 0.350. The exact reason why the cut-off value for assay 2 was higher is unknown. Likely it is due to the inevitable variance caused by the washing steps. Crystal violet biofilm assay is well known for its great variation from experiment to experiment, even from well to well (Kragh *et al.*, 2019).

Table 12. Strains classified as for their ability to adhere in assay 1, according to Stepanović et al. (2004) criteria.

Assay 1								
Strain	10ºC Aerobiosis	Classification for biofilm formation	42ºC Aerobiosis	Classification for biofilm formation	10ºC Microaerophilic	Classification for biofilm formation	42ºC Microaerophilic	Classification for biofilm formation
64D	0,01	non-adherent	0,19	weakly adherent	0,04	non-adherent	0,15	non-adherent
105-1BR	0,00	non-adherent	0,07	non-adherent	0,08	non-adherent	0,43	moderatly adherent
46E	0,01	non-adherent	1,16	strongly adherent	0,06	non-adherent	0,99	strongly adherent
22A	0,04	non-adherent	0,08	non-adherent	0,07	non-adherent	0,11	non-adherent
20C	0,01	non-adherent	0,07	non-adherent	0,07	non-adherent	0,05	non-adherent
105E	0,03	non-adherent	0,21	weakly adherent	0,07	non-adherent	0,07	non-adherent
63E	0,03	non-adherent	0,12	non-adherent	0,03	non-adherent	0,30	weakly adherent
106E	0,01	non-adherent	0,08	non-adherent	0,07	non-adherent	0,07	non-adherent
61C	0,03	non-adherent	1,28	strongly adherent	0,04	non-adherent	0,93	strongly adherent
104B	0,02	non-adherent	0,09	non-adherent	0,02	non-adherent	0,08	non-adherent
106A	0,08	non-adherent	0,05	non-adherent	0,09	non-adherent	0,18	non-adherent
105B	0,01	non-adherent	0,09	non-adherent	0,08	non-adherent	0,47	moderatly adherent
106B	0,02	non-adherent	0,11	non-adherent	0,02	non-adherent	0,22	weakly adherent
65E	0,03	non-adherent	0,10	non-adherent	0,05	non-adherent	0,23	weakly adherent
65B	0,09	non-adherent	0,03	non-adherent	0,10	non-adherent	0,11	non-adherent
NCTC 11168	0,02	non-adherent	0,23	weakly adherent	0,10	non-adherent	0,18	weakly adherent

Table 13. Strains classified as for their ability to adhere in assay 2, according to Stepanović et al. (2004) criteria.

Assay 2								
Strain	10ºC Aerobiosis	Classification for biofilm formation	42ºC Aerobiosis	Classification for biofilm formation	10ºC Microaerophilic	Classification for biofilm formation	42ºC Microaerophilic	Classification for biofilm formation
64D	0,00	non-adherent	0,04	non-adherent	0,03	non-adherent	0,27	non-adherent
105-1BR	0,00	non-adherent	0,01	non-adherent	0,02	non-adherent	0,02	non-adherent
46E	0,08	non-adherent	0,17	non-adherent	0,07	non-adherent	0,01	non-adherent
22A	0,07	non-adherent	0,29	non-adherent	0,05	non-adherent	0,01	non-adherent
20C	0,01	non-adherent	0,24	non-adherent	0,04	non-adherent	0,40	weakly adherent
105E	0,02	non-adherent	0,16	non-adherent	0,04	non-adherent	0,06	non-adherent
63E	0,02	non-adherent	0,03	non-adherent	0,05	non-adherent	0,19	non-adherent
106E	0,02	non-adherent	0,26	non-adherent	0,00	non-adherent	0,16	non-adherent
61C	0,06	non-adherent	0,23	non-adherent	0,03	non-adherent	0,27	non-adherent
104B	0,00	non-adherent	0,00	non-adherent	0,00	non-adherent	0,00	non-adherent
106A	0,02	non-adherent	0,00	non-adherent	0,01	non-adherent	0,00	non-adherent
105B	0,02	non-adherent	0,01	non-adherent	0,01	non-adherent	0,15	non-adherent
106B	0,00	non-adherent	0,00	non-adherent	0,00	non-adherent	0,00	non-adherent
65E	0,03	non-adherent	0,10	non-adherent	0,04	non-adherent	0,20	non-adherent
65B	0,06	non-adherent	0,40	weakly adherent	0,03	non-adherent	0,71	moderatly adherent
62E	0,05	non-adherent	0,14	non-adherent	0,10	non-adherent	0,13	non-adherent
62D	0,00	non-adherent	0,00	non-adherent	0,00	non-adherent	0,01	non-adherent
NCTC 11168	0,04	non-adherent	0,37	weakly adherent	0,05	non-adherent	0,60	weakly adherent

According to this classification, *C. jejuni* NCTC 11168 control strain was considered in both assays (1 and 2), weakly adherent. Most strains were considered non-adherent and only two strains were considered strongly adherent in low inoculum assay: *C. jejuni* 61C and 46E. The number of occurrences representing any level of adherence (weak, moderate, or strong) is presented in Table 14. The number of occurrences was in a general way, low. Even though this classification did highlight the strains that perhaps deserve more attention, it is questionable if this criteria is adequate for *C. jejuni* and *C. coli* biofilms at all situations, or if it should be adjusted.

Table 14. Number of occurrences representing any level of adherence. Strains are classified as for their level of adherence according to Stepanović et al. (2004).

Strain	Species	Inoculum CFU/mL	Adherence	OD_{580nm}	Condition
64D	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.191	42°C Aerobiosis
105-1BR	*C. jejuni*	10^3 cfu/mL	moderatly adherent	0.432	42°C Microaerophilic
46E	*C. jejuni*	10^3 cfu/mL	strongly adherent	1.163	42°C Aerobiosis
46E	*C. jejuni*	10^3 cfu/mL	strongly adherent	0.989	42ºC Microaerophilic
105E	*C. coli*	10^3 cfu/mL	weakly adherent	0.214	42°C Aerobiosis
63E	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.299	42°C Microaerophilic
61C	*C. jejuni*	10^3 cfu/mL	strongly adherent	1.280	42°C Aerobiosis
61C	*C. jejuni*	10^3 cfu/mL	strongly adherent	0.931	42°C Microaerophilic
105B	*C. jejuni*	10^3 cfu/mL	moderately adherent	0.470	42°C Microaerophilic
106B	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.218	42°C Microaerophilic
65E	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.230	42°C Microaerophilic
NCTC 11168	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.232	42°C Aerobiosis
NCTC 11168	*C. jejuni*	10^3 cfu/mL	weakly adherent	0.182	42°C Microaerophilic
20C	*C. jejuni*	10^6 cfu/mL	weakly adherent	0.397	42°C Microaerophilic
65B	*C. coli*	10^6 cfu/mL	weakly adherent	0.397	42°C Aerobiosis
65B	*C. coli*	10^6 cfu/mL	moderately adherent	0.706	42°C Microaerophilic
NCTC 11168	*C. jejuni*	10^6 cfu/mL	weakly adherent	0.366	42°C Aerobiosis
NCTC 11168	*C. jejuni*	10^6 cfu/mL	weakly adherent	0.598	42°C Microaerophilic

For instance, by analysing ODs in Table 14 (assay 2), in which higher inoculum concentration was tested (10^6 CFU/mL), given that the mean of negative control was already subtracted, strains that had an OD higher than ≈0.2 should be considered adherent. Figure 20 was obtained from the statistical analysis made with SAS, and it reports the interactions between the OD580nm means obtained for each strain, in both atmospheres (aerobic or microaerophilic), and temperatures (10°C or 42°C)**.** Above 0.2, there is a clear difference between strains and their adherence levels. If the cut-off value established was 0.2 instead of 0.35, the strains *C. jejuni* 64D, 20C, 61C, and 65E would be considered adherent (instead of non-adherent) under microaerophilic atmosphere, and the strains *C. jejuni* 22A, 20C, 106E, 61C would also be considered adherent under aerobiosis, in assay 2. In fact, several authors report in their biofilm formation assays absorbance values as low as 0.2 (or lower) in *C. jejuni* strains when performing crystal violet staining method in 96-well microtiter plates (Oh *et al.*, 2018; Reeser *et al.*, 2007; Teh *et al.*, 2016; Zhong *et al.*, 2020).

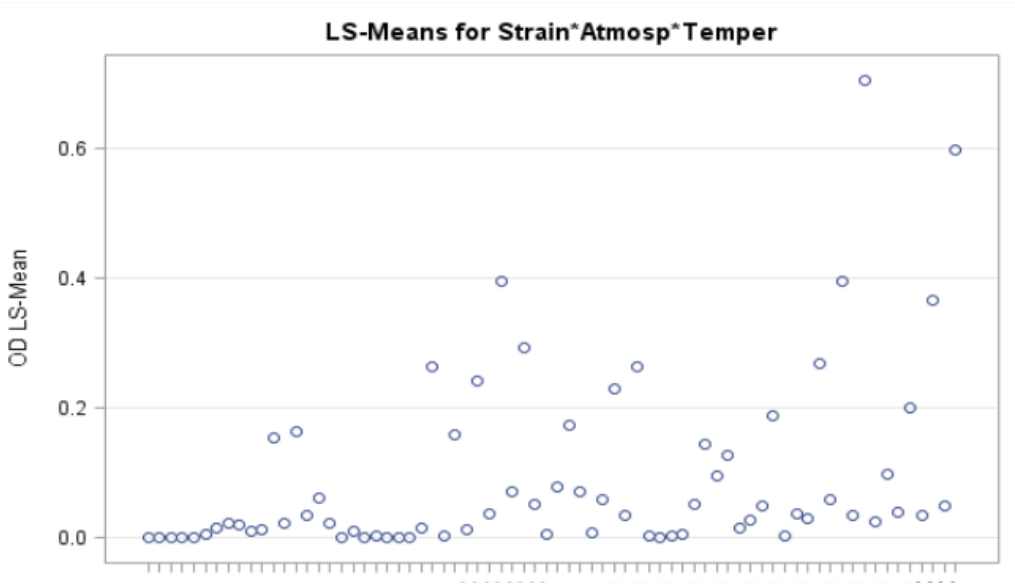


Figure 20. Campylobacter strains (n=18) dispersion, inoculated in high concentration (10^6 CFU/mL), and subjected to different temperatures (10ºC and 42ºC) and atmospheres (aerobic and microaerophilic).

Throughout the assays performed it was observed that *Campylobacter* biofilms are most likely, fragile, when compared to biofilms formed by other bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It has been mentioned that *C. jejuni* is in general a poor biofilm initiator on its own, forming biofilms only under specific favourable growth conditions (Teh *et al.*, 2014). Another hypothesis to explain *C. jejuni* persistence, is the association that it may have with pre-existing biofilms formed by other bacteria (Teh *et al.*, 2010), or even thriving in biofilms comprising aerobic species of bacteria that may confer an advantageous environment for *C. jejuni* by oxygen consumption such as *Pseudomonas aeruginosa* (Ica *et al.*, 2012).

As for the technique used for biofilm quantification, the crystal violet staining method, it was originally designed to study coagulase-Negative Staphylococci (Christensen *et al.*, 1985), and it is known to be precise for the quantification of high amounts of biofilm, providing an easy optical control

as well (Stiefel *et al.*, 2016). If the biofilm produced by *C. jejuni* and *C. coli* is in fact fragile under laboratory conditions, perhaps other methods should be used to assess *Campylobacter* biofilm formation, such as the one suggested by Brown *et al.* (2013), using 2,3,5 triphenyltetrazolium chloride (TTC) metabolic stain instead of crystal violet. It is based on the reduction of TTC to insoluble red crystals of 1,3,5-triphenylformazan (TFP). This method quantifies biofilm by staining specifically metabolically active *C. jejuni* cells that adhered to the abiotic surface. This is particularly important when chicken juice or other food-based models are used as a medium, since it has been demonstrated that crystal violet has high levels of unspecific binding in food matrices. Nevertheless, because TTC is a metabolic dye, growth conditions are usually required to be optimal. Suboptimal growth, or stressful conditions (such as aerobic atmosphere for a microaerophilic bacteria, or nutrient limitation) have been reported to result in inefficient reduction of TTC during staining (Brown *et al.*, 2013). Therefore, with this method, biofilm formation hardly could be tested in conditions other than optimal.

6.3.3. Aggregate formation

Aggregate formation was first noticed in *C. jejuni* NCTC11168 cultures, and so it was decided that aggregate formation ability would be assessed for all strains. Results for every strain were shown in Table 15. Commonly termed as flocs, aggregate formation of bacteria is described as a free floating and unattached form. Aggregates were reported to resemble attached biofilms, with a flattened and extensive extracellular polymeric matrix, when observed by scanning electron microscopy in *C. jejuni* 11168H strain (stable motile derivative of *C. jejuni* NCTC 11168) (Joshua *et al.*, 2006).

Table 15. Strains of C. jejuni and C.coli tested and their ability to form aggregates.

Strain	Species	Aggregate formation*
64D	*C. jejuni*	-
105-1BR	*C. jejuni*	+
46E	*C. jejuni*	+++
22A	*C. jejuni*	-
20C	*C.jejuni*	-
105E	*C. coli*	+
63E	*C. jejuni*	+
106E	*C. jejuni*	-
61C	*C. jejuni*	+++
104B	*C. jejuni*	+++
106A	*C. jejuni*	++
105B	*C. jejuni*	-
106B	*C. jejuni*	-
65E	*C. jejuni*	-
65B	*C. coli*	+++
62E	*C. coli*	+
62D	*C. coli*	+
NCTC 11168	*C. jejuni*	++

*-, absent; +, small (just visible); ++, intermediate; +++, extensive.

It is uncertain if there is a linear relationship between the formation of aggregates and the formation of biofilm. Nevertheless, the three strains that did stood out the most in 96-well plate biofilm assays: *C. jejuni* 46E, 61C and *C. coli* 65B, did formed extensive aggregates. Figure 21 present aggregate formation by a) *C. coli* 65B and b) *C. jejuni* 61C.

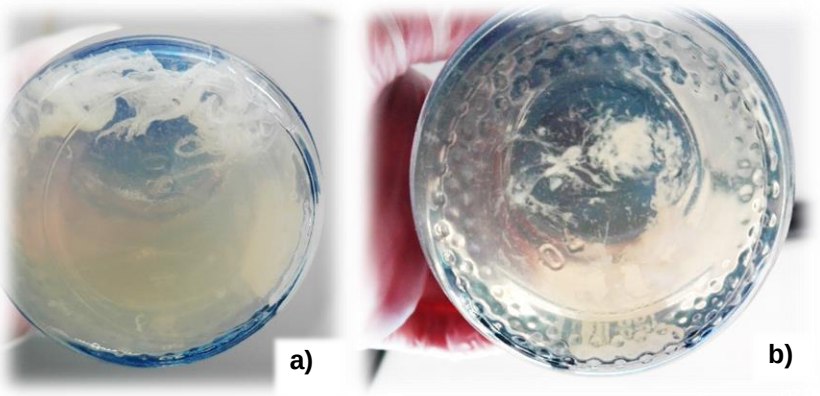


Figure 21. Aggregate formation in overnight culture by a) C. coli 65B and b) C. jejuni 61C.

Joshua *et al.* reported that aggregates of *C. jejuni* clinical strains had increased resistance to environmental stress. Bacteria in aggregates survived for up to 24 days in aerobic atmosphere at ambient temperature in comparison to planktonic bacteria that only survived up to 12 days as determined by viable bacterial counts (Joshua *et al.*, 2006). In the laboratory it was also verified that two strain that formed aggregates survived after 7 days, under aerobic atmosphere at 10ºC as determined by plate counts presented in Figure 22. This result was unexpected since *C. jejuni* is greatly affected by oxygen and low temperature (Chan *et al.*, 2001). In this work, these aggregate forms are proposed as a form of biofilm, in the same way that it was proposed by Joshua *et al.* (2006).

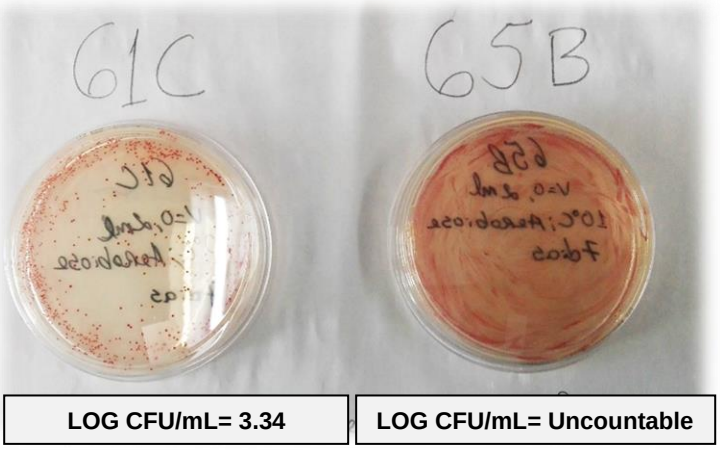


Figure 22. Plate counts of C. jejuni 61C and C. coli 65B (strains that form aggregates) after 7 days under aerobic conditions at 10°C. Volume inoculated=0.2mL on Campyfood agar plates.

6.3.4. Qualitative assessment of *Campylobacter* viability

This assessment was only performed from 96-well plates inoculated with higher inoculum concentration (10^6 CFU/mL) in order to understand if planktonic forms of *Campylobacter* strains survived at 10°C under aerobic conditions. Results are shown in Table 16.

Table 16. Campylobacter counts of planktonic cells after being submitted to aerobic atmosphere, at 10ºC during 72h.

Strain	Species	*Campylobacter* counts (LOG CFU/mL)
64D	*C. jejuni*	2.51
105-1BR	*C. jejuni*	3.29
46E	*C. jejuni*	uncountable
22A	*C. jejuni*	4.13
20C	*C.jejuni*	3.13
105E	*C. coli*	uncountable
63E	*C. jejuni*	uncountable
106E	*C. jejuni*	uncountable
61C	*C. jejuni*	3.34
104B	*C. jejuni*	uncountable
106A	*C. jejuni*	2.54
105B	*C. jejuni*	uncountable
106B	*C. jejuni*	3.77
65E	*C. jejuni*	uncountable
65B	*C. coli*	uncountable
62E	*C. coli*	uncountable
62D	*C. coli*	uncountable
NCTC 11168	*C. jejuni*	uncountable

Interestingly, the enumeration of CFUs varied from strain to strain. Giving that cells were exposed to aerobic atmosphere and low temperature (10°C), it was surprising that most of the strains barely suffered a reduction in viable cells after 72h of incubation, as demonstrated by the results of

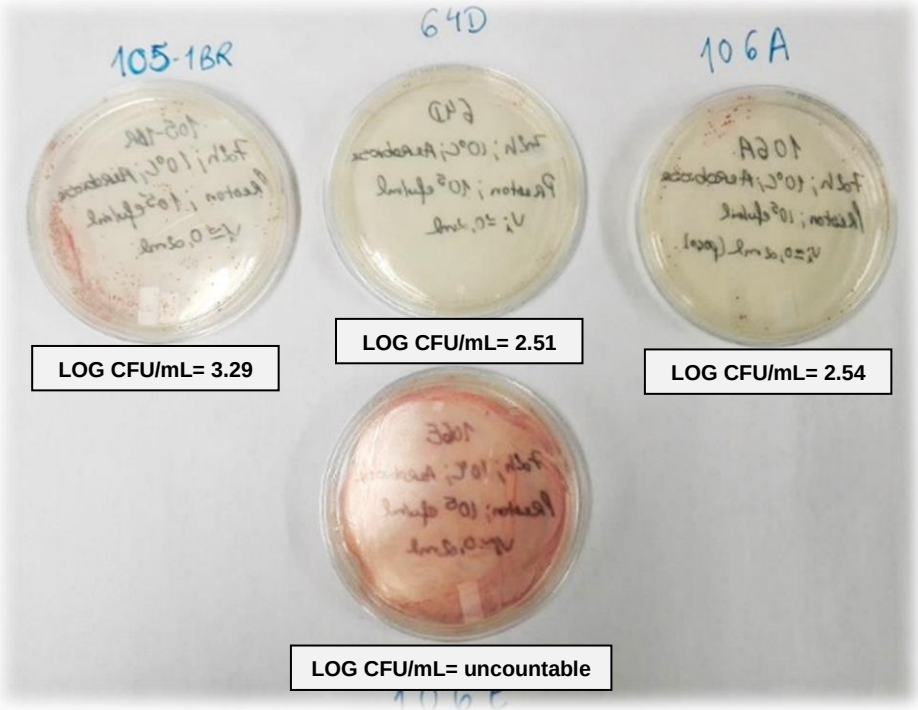


Figure 23. C. jejuni strains with different plate counts after 72h incubation, at 10°C under aerobiosis.

uncountable plate counts. Some of the strains however, clearly suffered an accentuated decrease in viable counts as demonstrated in Figure 23.

Overall, 10 out of the 17 (≈59%) strains tested showed high aerotolerance and tolerance to low temperatures (10°C). *C. jejuni* strains 64D and 106A were shown the be the most affected by oxygen and refrigeration temperature (10°C), having approximately a 4 log reduction. These *C. jejuni* strains (64D and 106A) however, do not represent a big part of the initial collection (n=145), because 64D genotype belonged to a very small cluster comprised only by 5 isolates, and 106A represented a unique profile. This result indicates that, most strains obtained at slaughterhouse, likely have tolerance to cold and aerobic atmosphere.

Regarding the strains that had highest biofilm formation, *C. jejuni* 46E, 61C, and *C. coli* 65B, two of them (*C. jejuni* 46E and *C. coli* 65B) had uncountable plate counts, and one (*C. jejuni* 61C) suffered approximately a 3 log reduction. The loss of viable cells found for *C. jejuni* 61C strain may have been associated to oxidative stress, however the strain overcame that by shifting to a biofilm form.

As for the strain with the highest number of acquired resistances (including a possible rare resistance to ertapenem), *C. jejuni* 106E strain, had uncountable plate counts. In the biofilm formation assay, 106E did showed a significant adherence capacity. It was the fourth strain with highest adhesion level when inoculated at 10^6 CFU/mL (assay 2), at 42°C under aerobiosis, after strains *C. coli* 65B, *C. jejuni* NCTC 11168 (control strain), and 22A (Figure 15). In this way, it is very concerning that this strain not only demonstrated ability to adhere, but also, had tolerance to refrigeration temperatures and aerobic atmosphere (conditions found at slaughterhouse environment) up to 72h.

Concerning the variability found for cold tolerance, similar results were obtained by Chan *et al.* (2001) when testing the ability of 19 *C. jejuni* isolates to tolerate prolonged exposure to low temperature over 14 days at 4°C. Of the 19 isolates tested, 10 isolates were of clinical origin and 9 isolates were of poultry origin. For some isolates there was no decrease in viable counts during the 14 days, while for others there was a decline by a factor of 10 to 100 on day 10. Clinical isolates had a tendency to be significantly more likely to remain viable following cold exposure. Giving that poultry at slaughterhouse may be contaminated with strains possessing different levels of tolerance to cold, perhaps refrigeration at slaughterhouse environment and storage conditions comprise a strong factor on strains selection, particularly those more tolerant, which ultimately will be the ones reaching the consumer (Chan *et al.*, 2001). This explanation, implies that strains demonstrating more tolerance to refrigeration temperatures, deserve a special attention. In this work most strains studied were unaffected by cold after 3 days, as demonstrated by viable cell plate counts. To further differentiate their tolerance to low temperature in the future, more plate counts should be performed, at two- or three-day intervals, until at least reaching 14 days.

Another factor to have in consideration is the influence of atmosphere. The plate counts shown on Table 16 were from the strains incubated at 10°C under aerobic atmosphere. The strains that didn't suffer a decrease in viable cell counts, not only demonstrated to be more tolerant to cold, but also, tolerant to oxygen. In this matter Oh *et al.* (2019) study is very relevant, because it tested refrigeration and freeze-thaw stresses in 70 *C. jejuni* strains isolated from retail chicken. Strains were previously grouped in three categories: oxygen sensitive, aerotolerant, and hyper-aerotolerant. Results indicated

that strains tolerant and hyper aerotolerant were also more tolerant to refrigeration at 4°C, and freeze thaw stress at −20°C after seven days (Oh *et al.*, 2019). A relation between atmosphere and temperature was clearly demonstrated.

Another question can be formulated on how temperature affected viable cell counts: Was cell viability the same under both temperatures tested (42°C aerobiosis and 10°C aerobiosis)?

In this matter, an interesting finding occurred in the laboratory. Even though it wasn't possible to carry out plate counts for every strain in every condition tested, sometimes they were performed randomly throughout the biofilm assays. For example, for *C. jejuni* NCTC 11168, 22A, and 46E, plate counts were assessed for every condition tested. Figure 24 shows CampyFood Agar plates inoculated with 200µl taken from one well of the 96 well plates. Interestingly, *C. jejuni* strains NCTC 11168, 46E and 22A, demonstrated a severe decline in viable cell counts after 3 days, at 42°C under aerobiosis. Also, refrigeration temperature (10°C) demonstrated to have a protective effect.

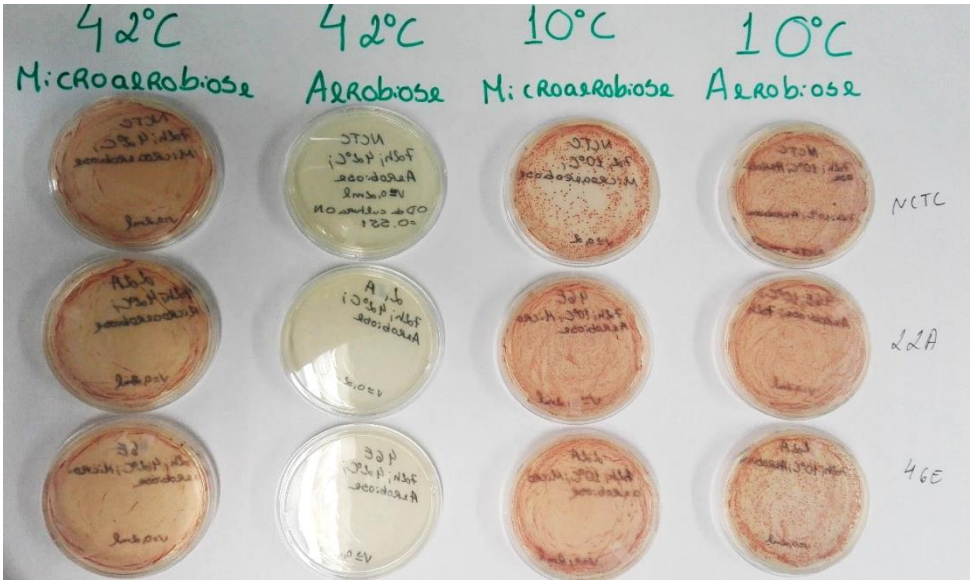


Figure 24. Plate counts for NCTC11168 (control strain), 46E and 22A. All conditions tested are presented: 42°C microaerophilic; 42°C aerobiosis, 10°C Microaerophilic; 10°C Aerobiosis.

This was not the case for every strain in which plate counts were performed. *C. coli* strains for instance, had uncountable plates at 42°C under aerobiosis, after 72h (results not shown). All *C. jejuni* strains however, did have the behavior previously described: 64D, 105-1BR, 104B, 106B (results not shown). Similar results were obtained by Garénaux *et al.* (2008) study, in which 13 *C. jejuni* strains, including NCTC 11168, were very sensible to oxidative stress at 42°C, but more resistant to it at 4°C. The optimal growth temperature (42°C) combined with aerobiosis lead to a decrease in viable cell counts within 3 days. At 4°C under aerobiosis, most strains had a low decrease in viable cell counts after 7 days. Results indicated that refrigeration temperature somehow, was linked to oxidative stress tolerance. Indeed, it has been demonstrated that *C. jejuni* response to cold shock includes the overexpression of several oxidative stress proteins, such as superoxide dismutase (Garénaux *et al.*, 2008). For this reason, the authors suggested that there may be a cross-protection between the cold-shock response and the oxidative-stress response. In other words, a global response could be induced

under refrigeration temperature combined with aerobiosis. That response could overlap the oxidative stress response, conferring cross-protection (Garénaux *et al.*, 2008). Furthermore, another interesting study on this matter was conducted for the identification of genes that altered their expression after *C. jejuni* exposure to low temperature (5°C) in chicken juice media. In this case, the analysis was focused on the protective effect provided by chicken Juice, since it was previously demonstrated that it prolonged the survival of *C. jejuni* at 5°C when compared to Brain Heart Infusion broth. Results demonstrated that there was increased expression of: *luxS* involved in quorum sensing; the activated methyl cycle; a gene involved in O-linked flagellin glycosylation; and an uptake system that may contribute to an increase in the uptake of cryoprotectants from the chicken juice, that may contribute to the survival of *C. jejuni* in low temperatures. In contrast there was reduced expression of haemin uptake system, and *ahpC, a* gene related to peroxide stress response (Ligowska *et al.*, 2011).

To summarize, in this work all strains survived after 72h at 10°C, under aerobiosis. Even though viable cell counts were strain dependent, this result indicated that most strains probably survive at slaughterhouse environment for an extended period of time. In fact, Several studies have showed that *C. jejuni* is able to survive on raw chicken meat and skin at 4°C and -20°C for at least 10 days (Proietti *et al.*, 2018; Davis & Conner, 2007; El-Shibiny *et al.*, 2009). If we consider that the ingestion of 500 to 800 *C. jejuni* cells might be enough to cause disease (Robinson, 1981; Black *et al.*, 1988), then it is clear that the survival of this pathogen for a prolonged period of time under refrigeration and aerobic atmosphere represents a risk to the consumer.

7. Conclusion and future perspectives

In this work, *flaA*-RFLP typing was effective for a preliminary characterization of the strains under study (*Campylobacter* spp.). It was possible to distinguish different *flaA*-RFLP profiles, demonstrating the genetic heterogeneity within isolates, and allowing the grouping of genetic types in clusters. Bionumerics was an essential tool for clustering analysis, even though it was noted that is still important to analyse results after obtaining a final clustering dendrogram. The analysis of clusters assigned, suggested that similar isolates (with identical *flaA*-RLFP type pattern) were present during a long period of time at slaughterhouse level. Moreover, *flaA*-typing allowed the selection of isolates with different band patterns for antibiotic susceptibility tests and biofilm assays, ensuring genetic variability.

Regarding resistance to antibiotics, all of the strains, except one, were multidrug resistant. This result is very concerning because multidrug resistance limits treatment options for persistent *Campylobacter* infections. Additionally, two *C. jejuni* strains were possibly resistant to ertapenem: *C. jejuni* 106E and 63E. Resistance to carbapenems is very rare in *C. jejuni* and *C. coli*, moreover, carbapenems are not allowed for usage in food producing animals.

As for biofilm formation assays, the strains that exhibited the most significant adherence capacity were *C. jejuni* 46E and *C. jejuni* 61C in assay 1 (low inoculum) and *C. coli* 65B in assay 2 (high inoculum). The *flaA* genotype of *C. jejuni* 61C, coincidently, belongs to a cluster that indicated persistence throughout time, by comprising samples collected in November 2018, and one sample from March 2019, from which *C. jejuni* 61C was isolated. 46E in its turn, belongs to a cluster exhibiting persistence throughout the period of January to June 2019. This result corroborates that the genotypes

of these isolates, possessing biofilm formation capacity, are shown in the *flaA*-RFLP dendrogram to have persisted during a long period of time. *C. coli* 65B had a unique *flaA* profile, so it wasn't possible to assess its persistence.

In general, in both assays (low and high inoculum concentration), there was great variability in adherence levels among the different strains tested. Oxygen demonstrated to have a different impact on adherence levels depending on the strain. In some cases, oxygen seems to have been a stimulus for biofilm formation, but in other cases it appears to have inhibited it. Low temperature (10°C) was clearly a detrimental factor for biofilm formation since adherence levels in both assays were always lower than 0.1 (OD580nm <0.1). Inoculum concentration, surprisingly, was also demonstrated to have an impact on biofilm formation in some strains. For six *C. jejuni* strains (105-1BR, 105B, 106A, 106B, 46E, 61C) a higher inoculum led to significantly lower adherence levels independently of the temperature and atmosphere tested. When considering all factors, it can be concluded that the studied conditions mimicking slaughterhouse environment did not favour biofilm formation, giving the adherence levels obtained at 10°C. Nevertheless, some strains demonstrated to have biofilm formation ability at 42°C. If a cut off value of 0.2 was considered for biofilm formation, then in low inoculum assay, 8 strains would have been considered to have biofilm formation ability, and in high inoculum assay, 7 strains would as well. Besides biofilm formation, many strains demonstrated high tolerance to aerobic atmosphere as assessed by viable cell plate counts, leading to the conclusion that levels of contamination continue to be a very important factor. If some strains have biofilm formation ability, and have tolerance to an aerobic environment, it remains very important to keep *Campylobacter* contamination levels low, and respect Reg. (EU) 2017/1495, in which the 10^3 CFU/g limit is imposed for carcasses of broilers.

It was possible to conclude that resistance levels weren't directly linked with biofilm formation capacity, since the strains that formed biofilm the most (*C. jejuni* 46E, 61C and *C. coli* 65B), were not the ones that had the highest number of acquired resistances (*C. jejuni* 106E, 63E and *C. coli* 105E). Even so, 106E (the strain with the most concerning resistance profile), did showed a significative adherence capacity at 42°C in assay 2. It was the fourth strain with highest potential ability to adhere, at 42°C under aerobiosis, after *C. coli* 65B, NCTC 11168, and *C. jejuni* 22A strains.

As for the goal of mimicking slaughterhouse environment, more studies using chicken juice as food base model should be conducted in order to understand its influence on *C. jejuni* survival. In this work, only 10% chicken juice was used in the media as a supplement, but it would be interesting to conduct biofilm assay testing 100% chicken juice to assess differences in biofilm formation.

Lastly, to understand the underlaying mechanisms for the protective effect on cell viability that low temperature (10°C) seemed to provide when compared to the optimal growth temperature (42°C), a transcriptome study would be interesting. Examples of techniques that could be performed are DNA microarray, a hybridization-based technique, RNA-seq, a sequence-based approach, and serial Analysis of Gene Expression (SAGE).

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Appendix I

Table 5. Latest articles (since 2014), testing biofilm formation by Campylobacter. Only articles relevant for food industry were included. Retrieved from PubMed database.

Number of strains and species	Temperature and Atmosphere for biofilm formation assay	Growth medium	Surface(s)	Method(s)	Conclusions	References
C. jejuni reference strains: NCTC 11168, 81116, 81-176, RM1221, NCTC 11168 aflagellate mutant (NCTC 11168 ΔflaAB); and *C. coli* clinical isolate 15-537360	37°C, under either microaerobic or atmospheric air conditions for 48h	Brucella Broth; Brucella broth supplemented with 5% (vol/vol) chicken juice or 100% chicken juice	Sterile borosilicate glass test tube or a 24-well polystyrene tissue culture plate, or six-well polystyrene tissue culture plate containing a sterile stainless steel coupon.	Crystal violet staining; Congo red staining; 2,3,5-Triphenyltetrazo-lium chloride (TTC) staining; scanning electron microscopy (SEM)	100% chicken juice as culture medium gave the highest level of biofilm formation; *C. jejuni* cells preferentially bind to chicken juice particulates rather than directly to the abiotic surface (as analysed by SEM); Chicken juice is a suitable laboratory model for the study of *C. jejuni* biofilm formation in the food chain, allowing investigators to more closely mimic the food chain conditions.	Brown *et al.* (2014)
Reference strain *C. jejuni* ATCC 33291 and seven *C. jejuni* strains isolated from poultry obtained from retail outlets, in Malaysia.	Microaerobic conditions without shaking for 6 days at 37 °C and at 42°C.	Nutrient broth no. 2 and Mueller-Hinton broth	96-well polystyrene microtiter plates;	Crystal violet staining	Biofilm formation by *C. jejuni* was affected by prior modes of growth (sessile or planktonic), nutrient conditions (Nutrient broth no. 2 or MHB), and initial growth temperature (37°C or 42°C). Results showed that *C. jejuni* strains were able to attach and form biofilm, but the quantity of the biofilm formed was low.	Teh *et al.* (2016)

Reference strain *C. jejuni* ATCC 33291 and seven *C. jejuni* strains isolated from poultry obtained from retail outlets, in Malaysia.	Microaerobic conditions or aerobic conditions at 37° C for 6 days.	Mueller-Hinton broth (MHB); Bolton broth; and Brucella broth	96-well polystyrene microtiter plates	Crystal violet staining	Ability of the *C. jejuni* strains to form biofilm varied depending on the growth medium used; Biofilm formation was enhanced under microaerobic conditions; different broths used for biofilm assay had different dissolved oxygen contents under different incubation conditions, which is likely to affect the biofilm forming ability of *C. jejuni*; Broth such as MHB, without reducing agents in their formulation, are a better choice to investigate the biofilm forming ability under aerobic conditions.	Teh *et al.* (2016)
Seven *C. jejuni* strains were isolated from cold and frozen poultry products	37°C and 25°C, Microaerobic conditions (10% CO2, 5% O2, 85% N2), for 24-72 h.	Mueller-Hinton broth (MHB)	Glass plates, slides, and coverslips, polymeric microtubes, Petri dishes, and polystyrene plates with different bottom profiles.	Crystal violet staining	Four of seven strains studied formed biofilms on the surfaces of polystyrene plates and Eppendorf tubes.	Efimochkina *et al.* (2017)
Thirty *C. jejuni* strains from the analysis of 280 cooled chicken carcasses from a Brazilian poultry exporting industry	37°C in microaerophilic conditions for 48 h.	Mueller Hinton broth (MHB) or MHB supplemented with 5% of chicken juice	96-well plates	Crystal violet staining	All of *C. jejuni* strains were capable of forming strong biofilms when supplemented with chicken juice by the crystal violet test.	Melo *et al.* (2017)
206 Campylobacter isolates, including 166 *C. jejuni* and *40 C. coli*, isolated from chicken farms and live poultry markets in central China.	37 °C under microaerobic condition for 48h	Brucella medium supplemented with 5% (v/v) chicken juice.	24-well polystyrene tissue culture plate	Crystal violet staining	Seventy-three isolates were non-biofilm producers and 133 isolates were biofilm producers. Among biofilm producers, 113 isolates were weak biofilm producers and 20 isolates were strong biofilm producers.	Zhang T. *et al.* (2017)

Seventy eight Campylobacter Isolates from chicken	37 °C under microaerobic conditions, 72 h	Mueller-Hinton Broth (MHB)	96-well polystyrene microtiter plates	Crystal violet staining	Most of the *Campylobacter* isolates tested (64 isolates, 82%) do not have the ability to form biofilm. 14 *Campylobacter* isolates (18%), (7 *C. jejuni* isolates and 7 C. coli isolates) were able to form biofilms on polystyrene surfaces.	Kim *et al.* (2017)
Four *C. jejuni* and six *C. coli* strains isolated from retail meat and liver products	37 °C in microaerobic conditions, 72h.	MHB and meat and liver juice	Borosilicate glass tubes and polystyrene 96-well microtiter plates	TTC dye staining	Chicken juice induced high levels of biofilm formation only for some *C. jejuni* and *C. coli* strains.	Karki A. & Fakhr (2019)
Forty *C. jejuni* strains isolated from broiler chickens (n = 26) and dairy cattle (n = 14).	37 °C for 72 h under microaerobic conditions	Mueller–Hinton (MH)	96-well tissue culture plates	Crystal violet staining	Only four strains formed biofilm.	Farfán M. *et al.* (2019)
Forty five *Campylobacter jejuni* strains isolated from slaughterhouse environment samples.	37 °C, 30 °C and 25 °C, under aerobic and microaerobic conditions.	Nutrient broth	stainless steel and 96-well polystyrene microtiter plates	Crystal violet staining	ST 904 and ST 607 CC isolated during 21 days in a previous work has showed the highest biofilm production and antimicrobial resistance.	García-Sánchez L., *et al.* (2019)