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**The effect of disinfectant exposure on *Campylobacter* spp. adaptation;
A study on *Campylobacter* survival and on proteome and virulence
genes expression.**

A thesis presented to the National University of Ireland for the degree of
Doctor of Philosophy

by

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September 2013

Kochanym Rodzicom, Siostrze i Dziadziusiowi
w podziękę za wsparcie każdego dnia

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List of abbreviations

°C	Degrees Celsius
2D	Two Dimensional
A	Adenine
API	Analytical Profile Index
ASCh	Sodium chlorite acidified with 0.16% w/v citric acid
ATCC	American Typed Culture Collection
atm	Atmospheric pressure
C	Cytosine
CA	Citric acid
<i>cdtB</i>	Cytolethal distending toxin gene
CFU	Colony Forming Unit
CHEF	Contour-Clamped Homogeneous Electrophoretic Field
<i>ciaB</i>	<i>Campylobacter</i> invasion antigen
CLSI	Clinical Laboratory Standard Institute
cm	Centimetres
<i>cmeA</i>	Efflux pump gene
CO ₂	Carbon dioxide
D	Dilution rate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
<i>flaA</i>	Flagellin A gene
<i>flgS</i>	Flagellar sensory histidine kinase
G	Guanine
g	Gram(s)
<i>g</i>	Gravity
GHP	Good Hygienic Practice
GMP	Good Manufacturing Practice
h	Hour
HACCP	Hazard Analysis and Critical Control Points
HO·	Hydroxyl radical
H ₂ O ₂	Hydrogen peroxide
IEF	Isoelectric focusing
kDa	kilo-Daltons
kV	kilo-volts
LA	Lactic acid
µg	Micrograms
µg ml ⁻¹	Microgram per millilitre
µl	Microlitres
µm	Micrometre
µ _{max}	Maximum specific growth rate

M	Molar
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MHB	Mueller-Hinton broth
MHA	Mueller-Hinton agar
MIC	Minimum Inhibitory Concentration
min	Minutes
mg	Milligram
ml	Millilitre
mM	Millimolar
MQ	MilliQ water
ms	Milisecond
N ₂	Nitrogen
NaCl	Sodium chloride
NCCLS	National Committee for Clinical Laboratory Standards
NCTS	National Collection of Typed Cultures
nm	Nanometre
O ₂	Oxygen
O ₂ ⁻	Superoxide anion
OD	Optical Density
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
psi	Pounds per Square Inch
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RpoS	Global stationary phase stress response factor
rRNA	Ribosomal Ribonucleic acid
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SoxRS	Oxidative stress response factor
T	Thymine
Taq	DNA polymerase obtained from <i>Thermus aquaticus</i>
TBE	Tris/Boric acid/ EDTA
Tc	Tetracycline
TEMED	N,N, N', N' Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloric acid
V	Voltage
VBNC	Viable But Non-Culturable
TSPH	Trisodium phosphate
w/v	weight per volume
%	Percentage

Thesis structure and Declaration of contribution

Thesis structure

Each of the four chapters (Chapters II-V) have been written in the manuscripts format with a view to submitting them to international journals for publication. First drafts of two articles have been completed and it is expected that they will be submitted shortly. The first article will contain the results from the Chapter II and the Chapter III, while the second article will contain the results from the Chapter IV and Chapter V.

Declaration of contribution

I declare that I have not obtained a degree in this University, or elsewhere, on the basis of this work. All work presented in this thesis was performed by myself, with the following exceptions:

Chapter 3: the scanning electron microscopy was performed under supervision of Pierce Lalor, Centre for Microscopy and Imaging (CMI), who provided the necessary reagents. The CMI is under directorship of Professor Peter Dockery (Anatomy Department, NUI Galway).

Chapter 5: the mass spectrometry of proteins identification was carried out by Dr Catherine Botting and her Colleagues in BSRC Mass Spectrometry and Proteomics Facility, University of St Andrews.

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Abstract

Campylobacter spp. are a common source of food contamination, especially in poultry products. To reduce the level of campylobacteriosis occurrence, different intervention strategies at poultry farms and processing plants (among them the application of disinfectants) have been proposed. The use of disinfectants can only be officially approved following a risk assessment. This study set out to investigate a possible adaptation of campylobacters to disinfectants recognised as safe for poultry decontamination: lactic acid (LA), citric acid (CA), acidified sodium chlorite (ASCh) and trisodium phosphate (TSPH).

From an initial pool of seventeen campylobacters, six of them were chosen for adaptation studies to disinfectants, after genotypic and phenotypic screening. *C. jejuni*, following continuous culture in the presence of gradual incremental concentrations of disinfectant, showed an ability to grow up to 8-fold above the initial MIC value when ASCh was examined and up to 2-fold when LA and CA were examined. Chemostat derived strains adapted to CA and ASCh were less susceptible to supra-MIC concentrations upon repeated exposure to the disinfectants, while a chemostat derived strain adapted to LA showed increased susceptibility to LA at supra-MIC concentration. In the case of TSPH, although *Campylobacter* failed to grow at the MIC value in the chemostat culture, strains obtained were less susceptible to TSPH than their WT.

TSPH and CA challenged strains revealed overexpression of all virulence genes studied (*ciaB*, *cmeA*, *cdtB*, *flgS*) while ASCh challenged strains revealed overexpression of *ciaB* only. In the case of strains challenged with LA no significant changes in gene expression were observed. The study on proteome changes showed that a majority of the proteins were down-regulated with an increase in the disinfectant concentrations. The changes in protein expression indicated an oxidative stress defence response indicating an overlap between the acid and oxidative stress response mechanisms.

CHAPTER I

General introduction

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Key words: biosecurity; *Campylobacter*; genetic diversity; poultry decontamination;
pathogenicity; stress response

1.1. *CAMPYLOBACTER* SPP. OVERVIEW

The first report of *Campylobacter* detection dates from 1886, when Theodor Escherich observed organisms resembling *Campylobacter* in children's stool samples (Park, 2002). Originally, *Campylobacter* was assigned to the *Vibrio* genus due to its morphological similarity to *Vibrio* and was named as *Vibrio fetus*. Advanced studies on vibrios associated with enteric diseases allowed two different types of bacteria to be distinguished. One of them was *Vibrio fetus* and the second one was found to be thermophilic in nature. In addition, this thermophilic microorganism revealed different G+C content to that of *Vibrio* spp. and lack of ability to utilize sugars (Moore *et al.*, 2005). These findings led to the creation a new genus *Campylobacter* in 1963. Its name in Greek means "curved rod" ("campylo" means curved/twisted and "bacter" means rod) (Ketley, 1995). *Campylobacter* was isolated the first time in patients with diarrhoea in Belgium in 1972 (Altekruse *et al.*, 1999).

1.1.1. Physiology

Campylobacter spp. are Gram-negative, spiral, flagellated bacteria (Parkhill *et al.*, 2000) belonging to the class of epsilon-proteobacteria (Okoli *et al.*, 2007). Their average width is between 0.2–0.9 µm and length between 0.2–5.0 µm (Humphrey *et al.*, 2007). They are very fastidious bacteria, that require microaerophilic conditions (10% CO₂, 5% O₂ and 85% N₂) and temperature between 37 °C - 42 °C for their *in vitro* growth (Gaynor *et al.*, 2005). As a source of carbon and energy *Campylobacter* spp. use amino acids such as: serine, proline, glutamic acid and aspartic acid. These asaccharolytic bacteria can also use fermentation by-products from the gut and low molecular organic acids as a source of carbon and energy (Jackson *et al.*, 2009). Among *Campylobacter* species assigned to the genus *Campylobacteraceae* (<http://www.uniprot.org/taxonomy/194>), the most common include *C. jejuni* subsp. *jejuni*, *C. coli*, *C. fetus*, *C. upsaliensis*, *C. hyointestinalis*, *C. lari* and *C. jejuni* subsp. *doyley*.

1.1.2. Sources of *Campylobacter* infections and disease

At present, *Campylobacter* spp. are the most common bacterial cause of gastroenteritis worldwide. In Ireland, 1,808 cases of campylobacteriosis were reported to the Health Protection Surveillance Centre (HPSC) in 2009, while a total of 198,252 confirmed cases of

campylobacteriosis were reported in European Union (EU) that year (FSAI, 2011). In 2011, 2,440 campylobacteriosis notifications were reported to Irish HPSC corresponding to a crude incidence rate of 52.9/100,000 population. This represents an increase of 46.2% compared to 2010 (*Annual Report 2011. Health Protection Surveillance Centre, 2011*). In the EU, diseases caused by this pathogen remain the most reported zoonotic infection in humans since 2005 (EFSA, 2012).

Likewise, *Campylobacter* species are the third leading cause of domestically acquired bacterial food-borne illness in the United States, with an estimated 845,024 cases occurring annually (FDA, 2012).

The most common mode of *Campylobacter* transmission to humans is through the consumption of improperly prepared food, particularly undercooked poultry products or raw contaminated dairy products such as unpasteurised milk (MacKichan *et al.*, 2004). It has also been reported that consumption of contaminated beef, pork, lamb, water and seafood are sources of infection (Ge, 2002; Konkel *et al.*, 1998). The transmission of thermophilic *Campylobacter* to a host might also occur by direct contact with infected pets suffering from diarrhoea (Alter & Scherer, 2006). *C. jejuni* and *C. coli* are the main species infecting humans, but *C. lari* and *C. upsaliensis* also contribute to human infections to a minor degree (Alter & Scherer, 2006). *C. jejuni* has a low infection dose at 500-800 cells (Jackson *et al.*, 2009) and symptoms of disease caused by this bacterium vary from asymptomatic to severe enteritis: abdominal cramping, fever and bloody diarrhoea (Allos, 2001; Blaser *et al.*, 1982). The symptoms are usually self-limiting (Siringan *et al.*, 2011) but can occasionally lead to serious sequelae illnesses like reactive arthritis, Reiter's Syndrome (include the following triad of symptoms: an inflammatory arthritis of large joints, inflammation of the eyes in the form of conjunctivitis or uveitis), Hemolytic Uremic Syndrome (a disease characterized by haemolytic anaemia caused by destruction of red blood cells, acute kidney failure and a low platelet count) and Guillain-Barré Syndrome (a disease affecting the peripheral nervous system) (FDA, 2012; Nachamkin *et al.*, 2008). Meningitis and urinary tract infection may also occur as a result of *Campylobacter* infection (Nachamkin, 2002; Pickett, 1996).

1.1.3. Genome diversity between *Campylobacter jejuni* isolates

The first complete genome sequence for *Campylobacter jejuni* NCTC11168 (a clinical isolate) has been determined at 1,641,481 base pairs which was predicted to encode 1,654 proteins (Parkhill *et al.*, 2000). The genome of this bacterium is characterised by low G+C content (30.6%). The genome sequencing for another three isolates, gave a better understanding about genome diversity among *Campylobacter* strains. Two of them were isolated from humans and they are *C. jejuni* 81-176 and *C. jejuni* CG8486, while the third one is *C. jejuni* RM1221 and was isolated from a chicken carcass. Analysis and comparison of these genomes showed that *C. jejuni* NCTC11168 possesses 8 unique genes, strain *C. jejuni* 81-176 possesses 35 unique genes, while 38 have been determined in *C. jejuni* CG8486. In strain *C. jejuni* RM1221 four large elements called *Campylobacter jejuni*-integrated elements (CJIEs) have been isolated (Parker *et al.*, 2006). CJIE1 is characterised as a *Campylobacter* Mu-like phage, while CJIE2 and CJIE4 are probably responsible for encoding phage related endonucleases, methylases or repressors. CJIE3 probably is an integrated plasmid. These elements were not found in the *C. jejuni* NCTC11168 strain. Parker (2006) analysed 67 *C. jejuni* isolates in terms of the presence of CJIEs. Results showed that in 55% of strains tested at least one of the CJIEs elements as in *C. jejuni* RM1221 was observed.

In each of the *C. jejuni* strains, loci with highly diverse genetic materials were found. Between two conserved genes namely, *cj0564* (integral membrane protein) and *cj0570* (ATP/GTP binding protein) in *C. jejuni* 81-176, five open reading frames (ORFs) encoding a putative protein of unknown function were determined. In addition, strains *C. jejuni* NCTC11168 and *C. jejuni* RM1221 showed the presence of other ORFs, unique to each of the isolates (Hofreuter *et al.*, 2006). Two inserts were also found in strain *C. jejuni* 81-176 between *cj1687* and *cj1688* (gene encoding putative efflux protein and protein translocase subunit SecY in reference strain NCTC11168, respectively). The functions of these two ORFs are probably to encode a permease of the major facilitator superfamily, while the second one possibly encodes peptidase named *cju38*. Although, these kinds of insertions were not observed in *C. jejuni* NCTC11168 and *C. jejuni* RM1221 strains, they were present in some other strains tested by Hofreuter *et al.* (2006). Besides the presence of highly diverse genetic material between genes, evidence of hypervariable regions is also reported (Dorrell *et al.*, 2001). The plasticity regions in *C. jejuni* involve genes coding flagellin, capsule and lipooligosaccharide.

In the highly virulent *C. jejuni* 81-167 clinical isolates, two plasmids pVir (37.4 kb) and pTet (45.2 kb) were found, while these DNA molecules were absent in *C. jejuni* NCTC11168 and *C. jejuni* RM1221. The presence of pVir in *C. jejuni* 81-167 may indicate its role in *Campylobacter* pathogenicity (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006) as pVir encodes several genes of type IV secretion system (T4SS). This secretion system has been found to be involved in DNA export, bacterial conjugation and protein secretion (Batchelor *et al.* 2004). However, the exact role of pVir in *Campylobacter* pathogenicity has yet to be determined. The other plasmid that was found in the *C. jejuni* 81-167 strain was pTet, which confers tetracycline resistance. Sequencing of the pTet showed that majority of the genes present in this plasmid are involved in its replication and conjugative transfer. Some sequences, similar to the T4SS of *Brucella* species, a pathogen causing brucellosis and *Actinobacillus actinomycetemcomitans*, a periodontal pathogen, have also been reported (Batchelor *et al.*, 2004). However, the discovery of 30 ORFs of unknown function suggest the possible existence of more genes related to pathogenicity (Batchelor *et al.*, 2004). Genome heterogeneity and plasticity among *Campylobacter jejuni* isolates makes them interesting and at the same time challenging organisms to work with.

1.2. CAMPYLOBACTER PATHOGENICITY

Advanced research in understanding the molecular mechanisms of host invasion by *Campylobacter* spp. in order to combat the diseases caused by this pathogen have been carried out. The challenge to achieve this is increased due to genotypic diversity between *Campylobacter* spp. isolates (Hänninen *et al.*, 1999; Wassenaar *et al.*, 1998). Many studies have shown this diversity among different strains using Pulse-Field Gel Electrophoresis, PFGE, a commonly used typing method (Camarda *et al.*, 2000; Fitzgerald *et al.*, 2001; Han *et al.*, 2007; O'Leary *et al.*, 2011; Owen *et al.*, 1995). Although, the mechanism of *Campylobacter* spp. pathogenicity is not yet fully understood, the major virulence factors including the flagella expression, host cell adhesion and invasion, and toxin production have been implicated in influencing *Campylobacter* pathogenicity.

1.2.1. Flagellar and secretion system

Flagella is a major motility factor in bacteria. In *Campylobacter* spp. it also plays an important function in pathogenicity. It has been identified as being involved in the adhesion and invasion of the host epithelial cells (Carrillo *et al.*, 2004). A study by Konkel *et al.* (2004)

indicates also a possible importance of flagella in protein secretion called *Campylobacter* invasion antigen (CiaB). While the mechanism of *C. jejuni*-mediated enteritis is proposed to be multifunctional, flagella expression and function is believed to play an important role in the ability of *C. jejuni* to colonise the intestinal tract of animals.

The complex mechanism of flagella expression in *Campylobacter* spp. is well described. For flagella to be expressed, integration of two systems: the flagellar Type III Secretion System (T3SS), the FlgSR two-component system, and gene *flhF* are required (Gilbreath *et al.*, 2011). The genes *flhA*, *flhB*, *fliP* and *fliR* (flagellar biosynthetic proteins) are responsible for encoding the T3SS system, which activate a two-component signal transduction system FlgSR (Figure 1.1.). Phosphorylation of the FlgS (a flagellar sensory histidine kinase) to FlgR (a signal-transduction regulatory protein) with the presence of FlhF protein (flagella-associated GTP-binding protein) results in the activation of regulon σ^{54} also known as RpoN (Dasti *et al.*, 2010; Gilbreath *et al.*, 2011; Malik-Kale *et al.*, 2007). All these genes, including *rpoN*, belong to the class 1 genes. As a result of σ^{54} activation, expression of class 2 genes (flagellar rod, ring, and hook proteins) and σ^{28} occur. Following activation of the σ^{28} regulon (also known as *fliA*), the expression of class 3 genes which include flagellin protein, FlaA take place (Gilbreath *et al.*, 2011).

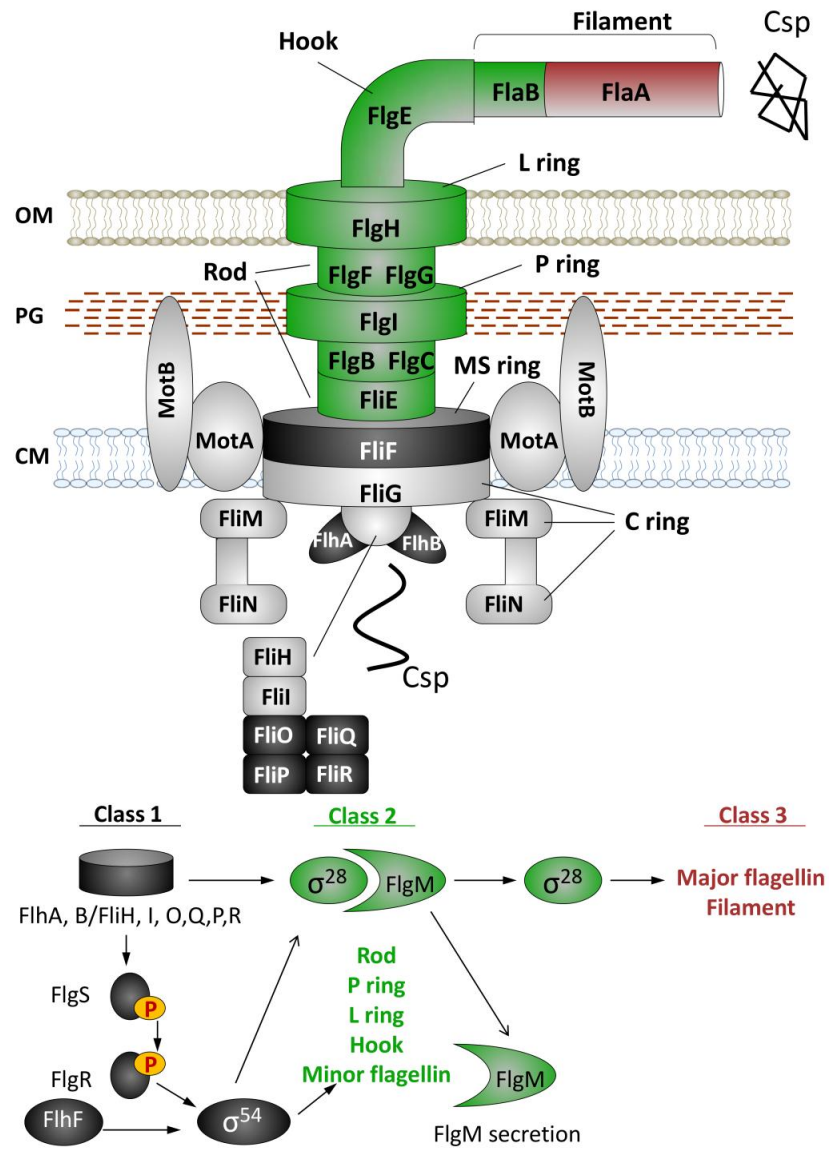


Figure 1.1. Flagella expression system and type III secretion system in *Campylobacter* spp. Three classes of genes were distinguished. **First class** – represented in **black**, **second class** - **green** and **third class** - **maroon**. **Silver** represents outside the flagellar transcriptional hierarchy. Secretion of Cia protein (*Campylobacter* invasion antigen), a subset of the *Campylobacter* secreted proteins (Csp) that required maximal invasion, occurs via flagellum. (Adapted from Gilbreath *et al.*, 2011; Rajagopala *et al.*, 2007, Larson *et al.*, 2008).

One of the protein secretion systems through the bacterial inner and outer membranes in *Campylobacter* spp. requires T3SS. This flagellar T3SS system is essential for the secretion of *Campylobacter* invasion antigen (CiaB) proteins, a subset of *Campylobacter* secreted proteins (Csp). Cia proteins have been found to play a significant role in invasion of the host intestine (Konkel *et al.*, 2004). It has been reported that in the *Campylobacter* spp. *ciaB* mutant strains, the invasion of epithelial cells by bacteria was significantly lower, when compared with *Campylobacter* strains where CiaB secretion was present (Konkel *et al.*,

2004). A significant reduction in virulence of *C. jejuni* *flaA* and *flaB* mutants was also observed (Yao *et al.*, 1994). This indicates that the secretion of CiaB protein is possible in either the presence of active *ciaB* gene and flagellar genes. However, there is still some controversy about the secretion function of flagella, as the study by Novik *et al.* (2010) reported that no measurable differences in host invasion between *C. jejuni* 81-176 wild type and its *ciaB* deficient mutant were detected. The authors suggest that these differences in results might be due to various strains or protocols used in the studies.

1.2.2. Adherence and invasion

Following adhesion to epithelial cells, *Campylobacter* spp. invade the host. The flagellar apparatus is involved for both these processes. Direct connection of flagellar apparatus and epithelial cell adherence was observed in a study by McSweegan and Walker in 1986. They found a significant reduction of *Campylobacter jejuni* aflagellated strains in their adhesion to human epithelial cells, INT407 (McSweegan & Walker, 1986). The use of human intestinal epithelial (INT407) and human colon (Caco-2) cell lines allows for the *in vitro* study on *Campylobacter* adherence. As a result of this, the activation of proteins such as CadF, fibronectin-binding protein (Konkel *et al.*, 1997); CapA, an autotransporter protein (Ashgar *et al.*, 2007); PEB1, a major cell-binding factor (Pei & Blaser, 1993) and JlpA, a surface-exposed lipoprotein (Jin *et al.*, 2001) during adhesion to the cells, were found. CadF is a 37 kDa outer membrane protein that binds fibronectin and is so far the best characterised adhesion protein. The binding process of bacteria to its host is via the last four amino acids of the CadF: phenylalanine, arginine, leucine and serine. CadF is an essential factor of both human and poultry adhesion. *Campylobacter cadF* mutants showed a reduction in INT407 cell invasion (Monteville, 2003). Besides this, a mutation in another *Campylobacter* gene involved in adhesion, *capA* an autotransporter protein, showed a reduced ability in the invasion of Caco-2 cells and lack of colonisation in chickens (Ashgar *et al.*, 2007). Another protein that has also been found to play an important role in epithelial adhesion and invasion is PEB1. PEB1 oscillates between 27-28 kDa protein. It has been reported that *Campylobacter jejuni* PEB1 mutants showed a 50 to 100-fold decrease of adherence and 15-fold decrease of invasion of HeLa cells (Pei *et al.*, 1998). A 43 kDa *Campylobacter jejuni* surface-exposed lipoprotein (JlpA) is also involved in adherence. Jin *et al.* (2001) showed that JlpA protein binds to Hep-2 (HeLa derivative) epithelial cells concluding that this protein might play a role in *Campylobacter* adherence.

Adherence of *Campylobacter* to the epithelial cells of the host is the first step in its invasion. However, among bacteria crossing the monolayers of the intestinal track, only a minority are able to invade the host (Dasti *et al.*, 2010). Following bacterial attachment to the cells, protein secretion occurs. These proteins are named CiaB and are described in Chapter I in Section 1.2.1. The secretion of CiaB is via the flagella and any mutation in this apparatus causes decreased invasion in the host (Konkel *et al.*, 2004).

Besides the proteins that have been described above, the 37 kb pVir plasmid has also been found to be significant for bacterial host invasion. Bacon *et al.* (2002) identified homologues of type IV secretion on *C. jejuni* 81-176 pVir plasmid. *C. jejuni* 81-176 in the presence of plasmid showed invasion of INT407 cells, while mutation resulted in a 2 to 11-fold reduction in INT407 cell invasion.

1.2.3. Toxin production

Campylobacter genome studies indicate that bacterium can produce a variety of toxins. These include: a 70-kDa cytotoxin, a shiga-like toxin, a haemolytic cytotoxins, a hepatotoxin and cytolethal distending toxin, but their role in pathogenesis is still contentious (Parkhill *et al.*, 2000; Wassenaar, 1997). It seems that only the CDT (cytolethal distending toxin) of *C. jejuni* is important in the affecting host cell cycle. This multi-subunit toxin requires the activity of three genes, *cdtA*, *cdtB*, *cdtC* (Whitehouse *et al.*, 1998). Each subunit (with molecular weights of about 30, 29 and 21 kDa, respectively) is required for cytotoxin activity but *cdtB* as the active subunit is thought to induce host cell apoptosis (Mortensen *et al.*, 2011; Young *et al.*, 2007). Its role is involved with blockage of the G1/S or G2/M transition phase of the cell cycle (Dasti *et al.*, 2010; Young *et al.*, 2007). In HeLa cells, Chinese hamster ovary (CHO) cells and Caco-2 cells, CdtB toxin causes cell deformation. These cells become lengthened or swollen. Contact of cells with the toxin may cause their death (Whitehouse *et al.*, 1998). When reaching the nucleus, CdtB works like the deoxyribonuclease (DNaseI) enzyme (Dasti *et al.*, 2010). Subunits CdtA and CdtC are responsible for delivery of CdtB subunit into the cell. They have been found to have homology to the B chain of the ricin toxin (Young *et al.*, 2007). This B chain of ricin has cytotoxic activity and is responsible for the transport of the ricin into the cell. Lee *et al.* (2003) observed that CdtA and CdtC bind to HeLa cells, while this effect was not observed in subunit CdtB. Unfortunately, clear functions of CdtA and CdtC still have to be determined. Eyior *et al.* (1999) showed that among 105 isolates from 91 fresh chicken carcasses, 94%

contained active CDT and highlighted the potential problem that may arise from cross-contamination or consumption of undercooked meat. However, the production of CDT toxins was found in invasive as well as in non-invasive strains (Müller *et al.* 2006). It has been suggested that the majority of *Campylobacter* strains produce CDT toxins, although its expression may be varied among different species (Wassenaar, 1997). This indicates that the production of CDT during colonization and invasion is not yet well known.

1.3. *CAMPYLOBACTER* GLOBAL RESPONSE MECHANISMS TO STRESS

Unlike other food-borne pathogens, *Campylobacter* spp. are apparently fragile organisms that are unable to multiply outside the animal host and are highly susceptible to a number of environmental conditions. This fastidious organism is also characterised by the lack of most common stress response factors such as RpoS – global stationary phase stress response factor or SoxRS – oxidative stress response factor (Park, 2002). In spite of this, these bacterium can survive many different environmental stress conditions such as those encountered during meat processing. This implies that *C. jejuni* have developed mechanisms for the survival in adverse conditions that are not commonly found in other enteric bacteria such as *Salmonella* and *Shigella* (Davis *et al.*, 1996). Many studies have shown genotypical diversity and genomic instability of *C. jejuni* (Dorrell *et al.*, 2001; Hänninen *et al.*, 1999; Hofreuter *et al.*, 2006; Poly *et al.*, 2007; Wassenaar *et al.*, 1998). Possibly genetic instability in *C. jejuni* makes them capable of genome modification and gives the bacteria the ability to survive in a wider range of environments (Manning *et al.*, 2001). Although, a specific response mechanism has not been described, a few stress survival mechanisms have been reported.

1.3.1. Viable but non-culturable state

Campylobacter has been found to possess two morphologies; culturable spiral forms and non-culturable coccoid forms. The viable but non-culturable state (VBNC) was first described by Rollins and Colwell in 1986. Bacteria can enter this VBNC state in response to exposure to different environmental stressors such as nutrient starvation, osmotic shock, temperature or pH changes (Jackson *et al.*, 2009). Entering a VBNC state causes a reduction in metabolic activity and gene expression, and a modification of cell wall structures (Nachamkin *et al.*, 2008). Bacteria may remain in this VBNC state and survive until more favourable environmental conditions have returned. The study by Rollins and Colwell

(1986) proposes that animal passage supports the transformation from the VBNC to the culturable state. Lázaro *et al.* (1999) observed the transformation to the VBNC state as a response to cold stress when incubated at 4 °C and at 20 °C in PBS. In addition, up- and down-regulation of proteins between culturable and non-culturable cells was investigated in their research but unfortunately, the authors did not identify the proteins involved, but only gave their molecular sizes. The conversion to the VBNC state was also observed in low osmolality nutrient media (Reezal *et al.*, 1998). A study by Moran and Upton in 1986, however, proposed that the coccoid form of *C. jejuni* was a degenerate cell form which is undergoing cellular degradation (Moran & Upton, 1986).

1.3.2. Stringent control

Although, *Campylobacter* is characterised by the lack of RpoS or SoxRS (Park, 2002), it has been found to possess other stress response factors. One of them is called stringent control and it been shown to be regulated by *spoT* (Gaynor *et al.*, 2005). This important stress response is required for several specific stressors: stationary phase survival, growth and survival under low CO₂/high O₂, antibiotic resistance (rifampicin), transmission (adherence and invasion epithelial cells) and was the first *Campylobacter* gene shown to be involved in longer-term survival in epithelial cells (Gaynor *et al.*, 2005). This global stress response mechanism, activated by environmental stressors, alters gene expression which results in an abundance of uncharged tRNA molecules at the ribosomal acceptor site. The lack of aminoacylated tRNAs during protein synthesis, results in catalysing the synthesis of alarmone guanosine 5'-triphosphate 3'-dihosphate (pppGpp). pppGpp is then hydrolysed to guanosine tetra-phosphate (ppGpp), which in turn is thought to bind RNA polymerase and alter gene expression by affecting promoter specificity, transcription initiation and elongation (Gaynor *et al.*, 2005; Poole, 2012).

1.3.3. Two-component signal transduction systems

The two-component signal transduction system (TCSTS) is a stimulus-response coupling system, allowing bacteria to adapt to different environmental conditions. TCSTS has been found in various bacterial species. It plays a role in regulating bacterial cell processes such as osmoregulation, chemotaxis, sporulation. In *Campylobacter* TCSTS has been found to be important to its pathogenicity (MacKichan *et al.*, 2004; Stock *et al.*, 2000). In *Campylobacter*, the TCSTS system is likely to contain genes encoding nine putative response

regulators and six sensor histidine protein kinases (Parkhill *et al.*, 2000). Sensory histidine kinases are located in the membrane and are responsible for monitoring the environmental signals and for autophosphorylation. Following autophosphorylation, the response regulator is activated by the transfer of the phosphate from the histidine kinase to a conserved aspartate residue, and in this state the activated response regulator is able to bind to the promoter region of its target genes. As a result of this, changes in gene expression may occur (MacKichan *et al.*, 2004). TCSTs are frequently involved in the co-ordinate expression of virulence-associated genes in pathogenic bacteria, ensuring that the genes required for colonization and virulence in a host, are expressed specifically *in vivo* (Barrett & Hoch, 1998).

Besides FlgSR, a two-component system that is required for flagella expression (see Section 1.2.1. of this chapter), two more two-component systems that have an impact on *Campylobacter* pathogenicity have been described. One is termed RacRS (reduced ability to colonise) and second one is Cj1223c-Cj1222c. RacRS is a temperature-dependent signalling pathway, required for optimal colonization of 1-day-old chicks (Brás *et al.*, 1999). The RacRS system can act both as an activator and repressor to regulate gene expression. RacRS mutants have been found to have a growth defect at 42°C and a reduced ability to colonise the host (Young *et al.*, 2007).

A second two-component system in *C. jejuni*, Cj1223c-Cj1222c is known as dccR-dccS (diminished capacity to colonise regulator and sensor). Mutants in the DccRS two-component system, for which an activating signal is unknown, are poor colonizers of chicks compared with the wild type. The DccRS-regulated genes that have been identified possessed no known or predicted functions but mutations in this system lead to chick colonization defects (Young *et al.*, 2007).

1.3.4. Biofilm formation

As mentioned previously, flagella play an important function in host cell adhesion and invasion. Flagella are also involved in biofilm formation (Kalmokoff *et al.*, 2006; Merino *et al.*, 2006). It has been reported that biofilm formation is a bacterial response to unfavourable conditions and one of the survival mechanisms within the gastrointestinal tract (Svensson *et al.*, 2009). Biofilms may form on a wide variety of surfaces from living tissues, medical devices, industrial or potable water system piping to natural aquatic

systems (Donlan, 2002). The four main phases of the biofilm formation: attachment, accumulation, maturation and detachment have been distinguished (Figure 1.2.). For biofilm formation to occur, planktonic cells have to adhere to the surface first of all. Attached cells accumulate to form multiple layers. At this stage bacteria start to coat them with an extracellular polymeric substance (EPS), consisting mainly of the exopolysaccharides. The composition of EPS also include nucleic acids, proteins, globular glycoproteins and lipids (Donlan, 2002; Gilbert *et al.*, 2002). The EPS cover a multicellular layer of bacteria attached to the surface. This layer of matrix protects bacteria from stressful environmental conditions allowing them to be still extant (Kalmokoff *et al.*, 2006). The next step of biofilm formation is called maturation. This phase is identified by the creation of a characteristic structure by adherent bacteria. The shape of the biofilm resembles statues or mushrooms (Pcae *et al.*, 2006) and the bacteria in mature biofilm are characterised by the lack of flagella. Detachment from the biofilm can occur leading to the release of planktonic cells in the environment which may then initiate another cycle of biofilm development.

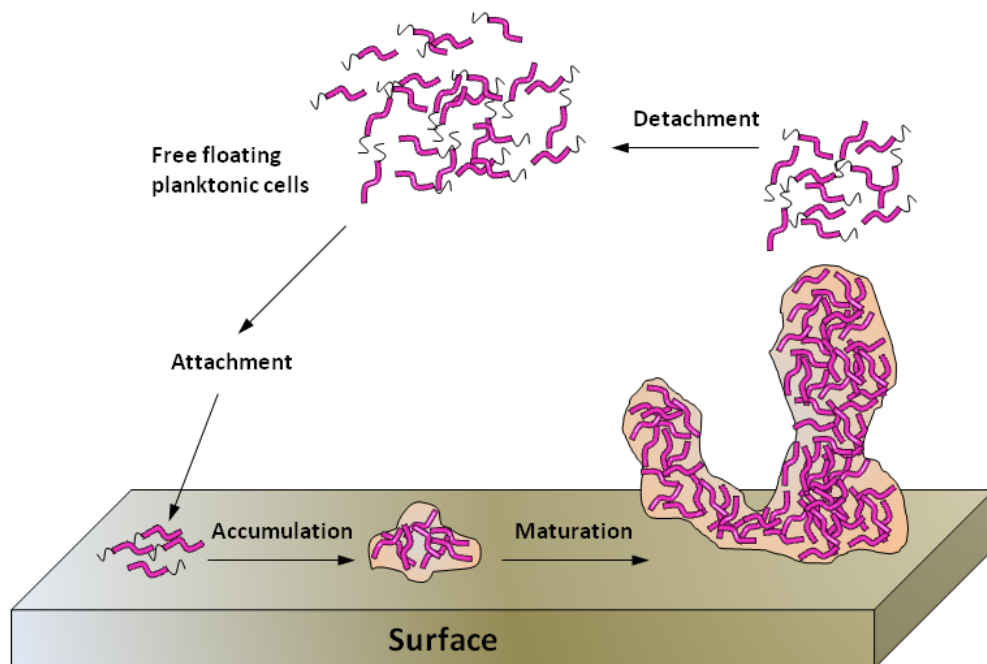


Figure 1.2. Model of biofilm formation. Adapted from Pace *et al.*, 2006.

It has been reported that biofilm formation also has an influence on expression of proteins. In the study carried out by Kalmakoff *et al.* (2006) a higher expression of general stress response proteins (GroEL, GroES), oxidative stress response proteins (Ahp, Txp), adhesion

proteins (FlaC, Peb1) and proteins involved in biosynthesis (nucleotide diphosphate kinase, the β chain of riboflavin synthase, and ribosome recycling factor) were obtained. Besides this, flagellins proteins (FlaA and FlaB), the filament-associated protein (FlaG), the putative hook-associated protein (FlgK), the filament cap (FlhD), two proteins probably associated with the basal body (FlgG2 and FlgG), and the chemotaxis protein CheA were found to be overexpressed in biofilm (Kalmokoff *et al.*, 2006).

The protective effect associated with biofilm formation on bacterial survival has been investigated. A study by Joshua and colleagues, using a monoculture of *C. jejuni*, they reported that cells can form three distinct biofilms: aggregates, pellicles at the liquid-gas interface and attached to the glass (Joshua *et al.*, 2006). The author compared viable counts of planktonic cells and aggregate cells during the long-term storage in ambient temperature (30 °C) and atmospheric conditions. Increased resistance to adverse environments was observed in the aggregate biofilms that were able to survive for up to 24 days while the planktonic cells survived only 12 days. Another study investigated quaternary ammonia compounds, peracetic acid, and a mixture of peracetic and peroctanoic acids in terms of effectiveness in biofilm inactivation (Trachoo *et al.*, 2002). The above compounds could not sufficiently kill *C. jejuni* in the biofilm at a concentration of 50 $\mu\text{g ml}^{-1}$ and 200 $\mu\text{g ml}^{-1}$ for 45 seconds. However, when chlorine was used at a concentration of 50 $\mu\text{g ml}^{-1}$ and applied for 45 seconds complete inactivation of the *C. jejuni* biofilm occurred (Trachoo *et al.*, 2002). Another study presented by the same author indicates that biofilm formation enables the longer survival of *C. jejuni* at 12 °C and 23 °C (Trachoo, 2003). These data support the hypothesis that biofilm can protect bacteria from unfavourable environmental conditions (Trachoo, 2003).

1.4. CAMPYLOBACTER SPECIFIC SURVIVAL MECHANISMS TO DIFFERENT STRESS CONDITIONS

Although, *Campylobacter* has been reported to be sensitive to different environmental conditions, it nevertheless has the ability to dynamically adapt and survive environmental stressors (McDougald *et al.*, 1998). To date, the different stress mechanisms studied on *Campylobacter* spp. include response to nutrient starvation (Gaynor *et al.*, 2005), osmotic shock (Doyle & Roman, 1982; Phongsisay *et al.*, 2007), oxidative stress (Atack *et al.*, 2008;

Hwang *et al.*, 2011), thermal stress (Brøndsted *et al.* 2005; Nachamkin *et al.*, 2008; Stintzi, 2003) and pH stress (Chaveerach *et al.*, 2003; Murphy *et al.*, 2003b; Ricke, 2003).

1.4.1. Response mechanisms to temperature stress

The optimal growth of thermophilic *C. jejuni* is between 37 °C and 42 °C, and replication occurs within a narrow temperature range of approximately 32 °C - 47 °C. A few degrees below or above that range causes a very significant decrease in bacterial growth rate to zero (Alter & Scherer, 2006). *Campylobacter* lack the cold shock response mechanism related with the expression of a cold shock proteins, Csp (Alter & Scherer, 2006), a common response mechanism in other pathogens such *Listeria*, *Escherichia* (Bayles *et al.*, 1996; Goldstein *et al.*, 1990; Hébraud & Guzzo, 2000). However, it can survive storage for long time periods at 4 °C (Chan *et al.*, 2001). In a study by Lázaro (1999), *Campylobacter jejuni*, following incubation, remained alive for up to 7 months at 4 °C by transforming to a cocoid form representing a transition to a viable but non-culturable stage.

Elevation of the temperature from 37 °C to 42 °C resulted in activation of the heat shock proteins (HSPs) such as ClpP, HrcA, HtrA/DegP and chaperone proteins: GroESL, DnaK, GrpE, DnaJ, ClpB (Nachamkin *et al.*, 2008; Parkhill *et al.*, 2000; Stintzi, 2003). Chaperones are responsible for correct folding of proteins and proteolysis of misfolded ones (Stintzi, 2003; Van Vliet & Ketley, 2001). Chaperone proteins have an important function not only during the response to temperature changes but also in the response to other stressors. According a study by Brøndsted *et. al* (2005) HtrA, a periplasmic protease, is required for growth above 44 °C. Work presented by Stintzi Alain (2003) illustrates approximately 20% of significant up or down-regulation of the *C. jejuni* genes after transferring the culture from 37 °C to 42 °C for 50 min. Genes encoding ribosomal proteins were in the majority of down-regulated genes, while genes encoding chaperones and heat shock proteins were up-regulated after the temperature change. It has been suggested by the author that a short growth arrest upon temperature stress, allows the bacteria to reshuffle their energy toward survival and adapt at the new growth temperature. Murphy *et al.* (2003a) investigating the survival of *Campylobacter* at 42 °C in fresh and spent mediums (a medium that previously contained cells, the medium was heat inactivated at 55 °C), found a 100-fold increase in *Campylobacter* survival in the spent medium. The authors suggest that this may be due to the presence of protective extracellular compounds in the used medium. This phenomenon was strain specific.

1.4.2. Response mechanisms to osmotic stress

Campylobacter is more sensitive to hyper-osmotic conditions in comparison to other food-borne pathogens (Alter & Scherer, 2006). Minor differences in cell turgor might result in inhibition of physiological processes which affect the cell transforming into a VBNC state (Jackson *et al.*, 2009). As a response to hyper-osmotic stress various bacteria may start to activate the transport system to uptake the compatible solutes such as proline, glycine, betaine, K^+ (Jackson *et al.*, 2009). Phongsisay and co-workers (2007) highlighted up-regulation of the putative lipid A biosynthesis of lauroyl acetyltransferase gene (*htrB*) under acid, heat, oxidative and osmotic stresses in *C. jejuni*. In addition, in *C. jejuni htrB* mutants, inhibition of growth under osmotic stress conditions was observed, indicating its role in *C. jejuni* survival. *C. jejuni* growth at 42 °C in the presence of $\geq 2.0\%$ w/v NaCl, decreases its culturability (Jackson *et al.*, 2009). Decreasing the incubation temperature from 25 °C to 4 °C was found to enhance *C. jejuni* survival for a longer time period (from 2 days to 2 weeks) in the presence of 4.5% w/v NaCl (Doyle & Roman, 1982). In contrast to hyper-osmotic stress, little is known about *C. jejuni* hypo-osmotic stress. It is known that generally Gram- negative bacteria produce oligosaccharides as a response to hypo-osmotic stress (Jackson *et al.*, 2009). However, *C. jejuni* can survive in this environment for a long period (Nachamkin *et al.*, 2008).

1.4.3. Response mechanisms to oxidative stress

C. jejuni as a microaerophilic organism, requires an environment of approximately 10% CO₂, 5% O₂ and 85% N₂ for optimal growth. During host invasion, campylobacters are most exposed to oxygen stress and damaging molecules caused by reactive oxygen: superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (HO·). However, *Campylobacter* possess a defence system that involves protein expression such as superoxide dismutase (SodB), catalase (KatA), alkyl hydroperoxide reductase (AhpC) and DNA-binding protein from starved cells (Dps) to neutralise reactive oxygen (Hwang *et al.*, 2011). The superoxide dismutase gene (*sodB*) breaks down the superoxide anions to H₂O₂ and dioxygen, the gene coding catalase (*katA*) converts H₂O₂ to H₂O and O₂, alkyl hydroperoxide (*ahpC*) reduces the alkyl hydroperoxides to alcohol (while being oxidised itself) and repairs damaged molecules that have been peroxidised (Murphy *et al.*, 2006; Van Vliet & Ketley, 2001).

Bacterioferritin comigratory protein (Bcp) and thiol peroxidase (Tpx), are other common peroxiredoxins in *C. jejuni* (Atack *et al.*, 2008). Bcp, has been suggested to be a general peroxide reductase, whilst Tpx is considered to be a specific hydrogen peroxide detoxification enzyme (Atack *et al.*, 2008). A study by Olczak (2003) showed that *tpx* and *ahpC* influence the pathogenicity of *Helicobacter pylori*. The *tpx* mutant had reduced ability to colonise, while the *ahpC* mutants failed to colonize the host mice. The activation of AhpC protein is regulated by iron (Park, 2002; Van Vliet & Ketley, 2001) but in *Campylobacter* spp., in contrast to other Gram-negative bacteria, oxidative stress and iron acquisition are regulated separately. The oxidative stress is regulated by the peroxide stress regulator (PerR), whilst iron accumulation is regulated by ferric uptake regulation protein, Fur (Van Vliet *et al.*, 2002). Another protein involved in oxidative stress protection is Cj0012c, a homolog of rubrerythrins, but its function and regulation has not been identified (Van Vliet *et al.*, 2002).

It has also been reported that *Campylobacter* oxidative stress regulator (CosR) is involved in negative regulation of SodB, Dps and positive regulation of AhpC. Probably CosR binds specifically to the promoter region of the oxidative stress genes and regulates their expression (Hwang *et al.*, 2011).

1.4.4. Response mechanisms to nutrient starvation

Campylobacter is a capnophile (requiring CO₂ for growth) and an asaccharolytic (unable to metabolise sugars for carbon due to the absence of the 6-phosphofructokinase) organism. *Campylobacter* spp. uses amino acid, as well as fermenting by-products from the gut such as: small organic acids and metabolic intermediates, as a carbon source (Jackson *et al.*, 2009). Amino acids such as L-serine and L-aspartate are the preferred carbon source (Leach *et al.*, 1997). The nutrient starvation might be a result of limited nutrient concentration in the environment or growth media and results in bacterial entry into the stationary phase (Lange & Hengge-Aronis, 1991). However, *Campylobacter* are characterised by the lack of the global stationary phase stress response factor RpoS, a common stress response factor in other bacteria. This suggests the existence of other response mechanisms to starvation stress in *Campylobacter*. At a carbon limiting nutrient stress *C. jejuni* uses a stringent response system that modulates gene expression and allocates resources from growth and division to amino acid synthesis (Gaynor *et al.*, 2005).

1.4.5. Response mechanisms to disinfectant stress: acid stress

Many studies have been carried out investigating the response of *Campylobacter* to acid shock in an attempt to understand their survival and colonisation in the host intestine (Chaveerach *et al.*, 2003; Murphy *et al.*, 2003a; Ricke, 2003). The antimicrobial mechanisms of disinfectant action are not fully understood due to their complex nature. A few possible mechanisms have been described by Ricke in his review (2003). One of the traditionally assumed modes of action indicates that organic acid in undissociated forms can easily penetrate the bacterial cell lipid membrane. However, in the neutral pH of the cytoplasm, acids dissociate into anions and protons. To maintain the optimum pH for *C. jejuni* growth between 6.5-7.5, the presence of higher level of protons are removed by the export of excess protons via the membrane (Chaveerach *et al.*, 2003). This mechanism requires adenosine triphosphate (ATP) that may cause a reduction of cellular energy. Another strategy indicates the existence of mechanisms that inhibit the influx of protons through the cell by inner membrane phospholipid modification (Reid, *et al.*, 2008b). As a result, the electron transport is impossible and ATP production is reduced. More recent studies indicate more specific mechanisms of pH stress response(s).

The first report of an adaptive tolerance response (ATR) in *C. jejuni* was described by Murphy *et al.* (2003b). Early stationary phase cells adapted at pH 5.5 showed a 100-fold increase in tolerance to pH 4.5 in comparison with survival of unadapted cells. Their study demonstrated that *C. jejuni* has the ability not only to induce an ATR to acid but also to a combination of aerobic and acidic conditions. The 100-1000-fold increase in survival as a result of the ATR was observed. Activation of ATR in response to acid stress was also observed in a study carried out by Ma *et al.* (2008). ATR allowed *C. jejuni* to survive pH 4.5 and increased its ability to withstand further acid challenge for over 200 min in comparison to non-stressed cells. It has been reported that ATR to acid stress induced up-regulation of general stress proteins: DnaK, GroEL (Murphy *et al.*, 2006). A study by Reid *et al.* (2008a) indicates further up-regulation of genes such as *clpB*, *grpE* (encoding heat shock proteins) and chaperones proteins: GroES as a response to acid stress.

Multidrug efflux pump, CmeABC, that belongs to the resistance-nodulation-cell division (RND) superfamily (Pumbwe & Piddock, 2002), has been suggested to have an impact in *Campylobacter* response to pH stress. Expression of *cmeABC* is related with the expression of three genes *cmeA*, *cmeB*, *cmeC*, where CmeA shows significant similarities to membrane

fusion proteins, CmeB shows significant sequence homology to inner membrane transporters and CmeC was found to be similar to the outer membrane proteins (Lin *et al.*, 2002). *cmeABC*, plays an important role not only in intrinsic resistance to antibiotics fluoroquinolones and macrolides (Cagliero *et al.*, 2006) but also confers resistance to bile salts or detergents (Martinez *et al.*, 2009). It is believed that overexpression of efflux pump could be a first step of bacteria becoming fully resistant (Pidcock, 2006). Investigating the *cmeABC* gene expression may give better understanding not only to pH stress response but also *Campylobacter* pathogenicity.

The studies on effects of different types of stressors on the *Campylobacter* response mechanism and acquired resistance play an important role especially when a new method of decontamination in the farms or processing plants would like to be used. Applied methods have to be safe for humans and the environment, and should not contribute to producing more virulent strains.

1.5. CAMPYLOBACTER CONTROL STRATEGIES ON FARMS AND IN PROCESSING PLANTS

Poultry and poultry products are considered to be the main source of *Campylobacter* food poisoning. Different intervention strategies have been applied both in the poultry farms and in the processing plants in order to reduce the contamination caused by this pathogen and thereby reduce the level of campylobacteriosis cases. The main purpose of applying the decontamination methods in the farm is to prevent bacterial spreading through the farm and between different farms, to reduce the level of bacterial contamination and the level of bacterial chick colonisation, while at the processing plants the purpose of applying various decontamination methods is to prevent bacterial cross-contamination.

1.5.1. Control strategies on farms

The main source of bacterial contamination on the farms is horizontal transmission. It is associated with environmental sources like contaminated water, wild birds, insects, litter, rodents, faecal contact, air or by farm personnel (Berndtson *et al.*, 1996; Lindblom *et al.*, 1986). It has been suggested that vertical transmission of *C. jejuni* from breeder flocks to their progeny could be another possible source of the farm contamination (Pearson *et al.*, 1996). However, there is no scientific evidence that this type of transmission exists. Besides

this, in newly hatched chicks and chicken up to 10 days old, the colonisation with *Campylobacter* was undetectable (Nachamkin & Blaster, 2000). Due to the fact that the bacteria was not isolated from chicks in the first 10 days, a vertical route of transmission from infected breeder flocks to their progeny is doubtful (Altekruse *et al.*, 1999; Van de Giessen *et al.*, 1992).

In order to control *Campylobacter* contamination at the farm level, different approaches have been investigated. At present, three strategies have been proposed (Lin, 2009). The first strategy focuses on the reduction of environmental exposure. These include biosecurity measures, that propose no other farm animals or pets should be present on the farm (Van Steenwinkel *et al.*, 2011). In addition, the farm should be free from wild birds and fly screens should be applied to prevent contamination and spread to adjacent broiler houses. Additionally, permanent rodent control should be carried out. Also, biosecurity control measures are focusing on a good hygiene of the farms' infrastructure, and of personnel and visitors, as well as hygiene precautions during transport to and from farms (EFSA, 2011a; Gibbens *et al.*, 2001; Van Steenwinkel *et al.*, 2011). The second intervention strategy to reduce *Campylobacter* contamination at the farm level focuses on increasing the poultry's host resistance to reduce campylobacters carriage in the gut. This involves the application of vaccinations. The third intervention strategy focuses on reducing the *Campylobacter* colonization level in chickens, and involves approaches including the use of antimicrobial alternatives such as bacteriophage therapy and bacteriocin treatment (Lin, 2009). The second and third strategies are described below in Section 1.5.1.1. of this chapter as part of the biological methods of poultry decontamination at the farm level.

1.5.1.1. Biological intervention strategies

Biological methods of intervention include the application of bacteriophages, bacteriocins or vaccinations. The use of bacteriophages and bacteriocins focuses on decreasing colonisation by *Campylobacter* on poultry already colonised, while vaccination focuses on increasing the resistance of broiler chickens to colonisation and preventing their colonisation. However, due to the lack of knowledge regarding the underlying mechanism of cecal poultry colonisation and the interaction between application of bacteriophages, bacteriocins or vaccinations for poultry, these methods have not been approved yet by the European Commission. In addition, the risk of consumption of the poultry treated with these methods needs also to be investigated.

1.5.1.1.1. Bacteriophages

Bacteriophages, naturally present in the environment, are bacterial viruses with the ability to infect and kill bacteria. The benefit of applying phage therapy as an intervention strategy against *Campylobacter* colonisation of broilers is their high strain specificity. Bacteriophages attack only their target bacteria and no interactions with the background microbial flora are observed. This advantage makes the phage therapy a great biocontrol measure and are considered a safe method (Hugas & Tsigarida, 2008). A study by Wagenaar *et al.* (2005) demonstrated an initial reduction of *C. jejuni* by 3 log in caecal content following application of the phage therapy while at the end of experiment (after 5 days) bacterial counts stabilised at a level of 1 log lower as compared to the control group. A reduction of *Campylobacter* by 2 log CFU g⁻¹ was observed after application of the phage cocktail by oral gavage and in poultry feed (Carvalho *et al.*, 2010). This suggested that administering phages to poultry via poultry feed could be successful on a commercial scale. However, due to the ability of bacterial levels to recover or increase again after phage treatment, this method can only successfully be applied in broilers before slaughter. An important issue relating to the application of phage therapy is the potential of bacteria to become resistance to the phage and research is ongoing in investigating the development or emergence phage-resistant bacterial mutants. Also, a possible influence of others factors such as feed pH and the bacterial physiological state on efficacy of the phage-therapy still have to be evaluated (Greer, 2005).

1.5.1.1.2. Bacteriocins

Bacteriocins are proteinaceous, antimicrobial compounds that are produced by bacteria and that usually inhibit closely related species (Carolissen-Mackay *et al.*, 1997). Svetoch and Stern (2010) studied various bacterial isolates, among them *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Escherichia* to determine the effects of the different bacteriocins against *Campylobacter jejuni*. A few bacteriocins were described and tested. It has been reported that *Paenibacillus polymyxa* produce NRRL B-30509 bacteriocin that effectively reduce *C. jejuni* colonisation. In 80 birds treated with the bacteriocin no colonisation was detected 3 days following application of the bacteriocin. This indicated that at least a six log reduction occurred. Similar results were observed when the bacteriocin NRRL B-30514 from *Lactobacillus salivarius* was used. While another bacteriocin, E 760 produced by *Enterococcus durans/faecium/hirae*, gave a reduction

greater than 8 log CFU g⁻¹. Bacteriocin E 760 supplemented in treatment water, resulted in the elimination of 90% of pathogens from 600 naturally infected birds (35-42 days old) after 3 days' application (Svetoch & Stern, 2010). *Lactobacillus salivarius* has been found to produce stable bacteriocin OR-7, effective against campylobacters colonisation (Stern *et al.*, 2006). In that study, day-old chicks were infected with *Campylobacter* by oral gavage. At day seven, chicks started to be fed with food supplemented with the OR-7 purified protein encapsulated in polyvinylpyrrolidone. At day 10, chicks were killed and enumeration of campylobacters from the cecal content was performed. A 6 log reduction in *Campylobacter* colonisation in comparison with the untreated control group was observed. Messaoudi *et al.* (2011) isolated lactic acid bacteria (LAB) from the 20 cecum of 6-week old Tunisian chickens. Among all LAB strains isolated, those with activity against *Campylobacter* were distinguished. Three strains of *Lactobacillus salivarius* have been found to produce bacteriocins that were still effective following either heat treatment (10 min at 80 °C) or catalase (5 mg ml⁻¹) treatment. This indicates that these proteinaceous antimicrobial agents are stable.

Although the application of bacteriocins seems to be a great method of reducing *Campylobacter* from the poultry at the farm level, further research investigating the long-term effect of its application has to be performed. It has been suggested that the application of bacteriocins might be affected by genomic instability in *Campylobacter*. In addition the effect of the poultry production environment on efficacy of bacteriocins application has to be also reviewed (Hermans *et al.*, 2011).

1.5.1.1.3. Vaccination

One of the proposed control measures for reducing *Campylobacter* colonisation at farm level is vaccination. The purpose of this method is to increase resistance of broiler chicken to *Campylobacter* colonisation (EFSA, 2011a). A number of studies were carried out to investigate the use of vaccines in reducing the amount of *C. jejuni* in broiler chickens. To date, various results were obtained depending on the vaccine used. Reduction of between 16 - 93% of *C. jejuni* was observed during oral vaccination with *C. jejuni* whole cells killed with formalin in combination with the *Escherichia coli* heat-labile toxin (Rice *et al.*, 1997). A 1.4 log CFU g⁻¹ reduction in one-day and in two-week old oral gavage chickens was observed after vaccination with a *Salmonella enterica* serovar Typhimurium Δ aroA mutant (Buckley *et al.*, 2010).

Although, some promising results have been obtained, a vaccine to prevent cecal colonisation in poultry by *Campylobacter* is not yet available. Furthermore, before applying them commercially, the mechanisms of cecal colonisation of each host (poultry and human) have to be understood because the vaccines created need to be immunogenic in chickens and not pathogenic in humans (Young *et al.*, 2007). It has been reported that as a result of the vaccination of chicks by injection of heat-killed *C. jejuni* closed in the amniotic fluid, an increase of the immunoglobulin A (IgA) antibodies occurred (Hermans *et al.*, 2011). Besides IgA, *Campylobacter* colonised chicks also produced increased levels of immunoglobulin IgG and IgM (Cawthraw *et.al.*, 1993). The effect on humans of poultry consumption that contains increased levels of immunoglobulin is unknown.

1.5.2. Control strategies at processing plants

The main concern with regards to processing poultry contaminated with *Campylobacter* at the processing plants is to prevent cross-contamination of the poultry meat. The risk factors include slaughter, dressing and processing. Both pre and post-slaughter intervention strategies play an important role. A feed withdrawal before poultry is transported to the slaughter house as well as cleaning of the poultry transportation crates seems to effectively reduce the risk of cross-contamination (EFSA, 2011a; Keener *et al.*, 2004). During the slaughter process, *C. jejuni* can easily be spread from the intestinal contents to the carcasses (Van de Giessen *et al.*, 1992). Besides the slaughter process, the evisceration process is of high risk in poultry contamination by *Campylobacter* spp. (EFSA, 2011a). Compliance with the rules of food safety management systems, based on the good hygienic process (GHP), good manufacturing process (GMP) and Hazard Analysis and Critical Control Points system (HACCP) play a crucial role in reducing contamination. In addition to these quality systems, interventions during the slaughter process at certain stages of the production are also highly significant in the control of *Campylobacter* contamination at the processing plant. This include: the prevention of leakage of intestinal contents, the detection of highly contaminated carcasses or the slaughter of the *Campylobacter*-negative batches before positive batches (EFSA, 2011a). Other sources of cross-contamination include the presence of contaminated equipment or work surfaces. Therefore, proper cleaning of the whole processing equipment is of high significance (EFSA, 2011a).

To reduce and prevent the contamination caused by *Campylobacter*, different methods of post-slaughter carcass decontamination have been investigated. These include both

chemical and physical methods. At the moment, no chemical treatments beside the use of lactic acid, are authorized in the European Union. However, some chemicals are permitted in other countries worldwide. Details about legislation are described in Section 1.6. of this chapter.

1.5.2.1. Physical methods of decontamination

The main physical methods that have been investigated in order to reduce the level of contamination at the processing plants and prevent bacterial cross-contamination include: water treatment, irradiation, ultrasound, freezing and cold storage and food packaging.

1.5.2.1.1. Water treatment

In the processing plant water treatment methods have been used to clean and reduce bacterial contamination from poultry carcasses. Li and colleagues (2002) investigated the used of different temperatures of tap water, using a birdwasher spray system (12 s at 80 psi, equivalent to 5.4 atm) to reduce *Campylobacter* levels. Increasing the water temperature from 20°C to 55°C and 60°C resulted in a decrease of *Campylobacter* by 0.78 log CFU per carcass and 0.93 log CFU per carcass, respectively. The authors also studied the difference between the uses of tap water only and tap water supplemented with 50 µg ml⁻¹ of chlorine. The conclusion was made that no significant difference between these two methods was observed. A significant bacterial reduction in 6 week old broiled after the evisceration process following treatment with distilled hot water was reported by Sinhamahapatra and colleagues (2004). The dressed carcasses were either sprayed or dipped in 70 °C distilled water for 1 min and then they were allowed to dry for 5 min. The authors observed a reduction of total plate counts by 1.28 log CFU cm⁻² and 1.2 log CFU cm⁻² following dipping and spraying the carcass in hot water, respectively. In addition, a reduction of coliform counts by 1.34 log CFU cm⁻² and 0.73 log CFU cm⁻² after dipping and spraying was detected, respectively.

1.5.2.1.2. Irradiation

Farkas (1998), in his literature review compared the effectiveness of irradiation on different suitable products. A dose at 3–5 kGy applied for frozen poultry, and 1.5–2.5 kGy for chilled poultry, reduced *Salmonella* and *Campylobacter* by 3 log-cycles. The application of 1 kGy on

steak tartare (meat dish made from raw beef) reduced the level of *Campylobacter* by more than 4 log-cycle.

Despite the fact that the irradiation method efficiently decreased *Campylobacter* levels in poultry meat, due to the complexity of the irradiation implementation, this method is considered difficult to apply. The target microorganism, the type of the food, the presence of water and oxygen in the food product, and the irradiation temperature all influence the effectiveness of the irradiation process (Hugas & Tsigarida, 2008). Due to the controversy about the safety of this method, it has not been widely used, however, different legislation in various countries has been applied. Section 1.6. in this chapter highlights the different legislation in various countries.

1.5.2.1.3. Ultrasound

The biological structure of bacteria can be destroyed when appropriate high doses of ultrasound are applied (Earnshaw *et al.*, 1995). A study by Haughton and co-workers published in 2012, indicates that high intensity sonication (HI) combined with thermal treatment reduced *Campylobacter* counts on broiler skin by 4.72 log CFU ml⁻¹ and was more efficient than thermal or sonication treatment alone (Haughton *et al.*, 2012). Better results were achieved while three treatments were combined: a 16 min thermal treatment at 53 °C, HI sonication and thermal sonication. These treatments resulted in a drop of *Campylobacter* and *Enterobacteriaceae* below detectable levels. A study by Boysen and Rosenquist (2009) reported a *Campylobacter* reduction by 2.51 log CFU per carcass, when hot-steam combined with ultrasound treatments were applied. This treatment was operated after evisceration but before the inside-outside bird washing procedure.

1.5.2.1.4. Freezing and cold storage

Poultry meat freezing and cold storage practices are used to extend the shelf-life of the product. In addition, these methods have also been found to reduce bacterial counts. Reduction of *C. jejuni* counts between 0.34-0.81 log CFU ml⁻¹ in ground chicken, and between 0.31-0.63 log CFU g⁻¹ in chicken skin was observed after storage at 4 °C for 3-7 days (Bhaduri & Cottrell, 2004). The viability of *Campylobacter* is decreased during storage at freezing temperatures, however they can still be isolated from frozen poultry (Malik-Kale *et al.*, 2007). According to a study carried out by Zhao and colleagues in 2003, a

reduction at 1.3 and 1.8 \log_{10} CFU g^{-1} of *C. jejuni* on chicken wings was achieved during storage at -20 and -30°C for 72 hours, respectively. Likewise, long-term freezing for 52 weeks at -20 °C, leads to further *C. jejuni* reductions of 4 \log_{10} CFU g^{-1} on chicken wings (Zhao *et al.*, 2003). In another study, a decrease of *C. jejuni* from 0.6 to 2.2 log within the first seven days of the long-term storage (112 days) at -18 °C was achieved (Moorhead & Dykes, 2002). This level of reduction remained the same for the whole incubation period. These results highlight that appropriate shelf-storage plays an important role in reducing *Campylobacter* level.

1.5.2.1.5. Packaging

Two European Union (EU) regulations exist pertaining to the types and properties of packaging materials in contact with foodstuff. The first one is 90/128/EEC “Plastic materials and articles intended to come into contact with food-stuff” and the second one is 94/62/EEC “Packaging and packaging waste directive” (Petersen *et al.*, 1999). These regulate the use of recycled plastic in materials in contact with food. There are however no regulations with regards to packaging of poultry meat products. The Food Safety Authority of Ireland called for an introduction of leak proof packaging of poultry-meat products after *Campylobacter* was found on the surface of surrounding packs (http://www.fsai.ie/news_centre/press_releases/17032010.html). Researchers worldwide are also looking at the use of different poultry-meat packaging regimes to prevent further bacterial spreading after poultry processing and to prolong shelf-life, different types of packaging and packaging atmosphere were examined. One of the proposed methods is through the use of modified atmosphere packaging (MAP) or vacuum packaging (VP). Typical gases applied in MAP, oxygen, nitrogen and carbon dioxide are used. However, it has been reported that preservative results of the product have only oxygen and carbon dioxide (Bakar, 2013). When modified atmosphere packaging or vacuum packaging were used, a minor reduction of bacteria in beef and chicken meat was observed (Alter & Scherer, 2006). Additionally, storage of naturally contaminated beef under either vacuum and 100% of carbon dioxide conditions for 41 days at -1.5 °C, resulted no significant changes of *Campylobacter* numbers (Dykes & Moorhead, 2001). Although, no relevant changes of *C. jejuni* counts were obtained, a MAP or VP combined with low temperature storage may extend the shelf-life of the products (Bakar, 2013).

1.5.2.2. Chemical methods of decontamination

At present, no chemical decontamination treatments, with the exception of lactic acid, are authorised in the EU. However, different legislation restrictions exist in different countries in the world in terms of the types of disinfectants that can be used and their applicable doses (see Section 1.6 in this chapter). Chemical methods of decontamination have been widely studied in order to prevent the spread of bacterial contamination at the poultry-meat processing plants. In particular, a lot of focus has been put on the use of chemical supplementation of water at pre and post-evisceration during poultry processing at the plant.

Weak organic acid, chlorine-based compounds, peroxyacid, trisodium phosphate, sodium hydroxide (NaOH) are the main compounds that have been studied in order to investigate their effectiveness in reducing microbial contaminations by applying them at different stages after the poultry slaughter process (Del Río *et al.* 2007; Jasass, 2008; Li *et al.*, 2002; Rajkovic *et al.*, 2010; Whyte *et al.* 2001). As a result of such disinfectant addition at different stages of production, the spread of bacteria at the processing plant can be prevented. The other advantage of the application of these compounds as food additives is the prevention of food deterioration and extension of the shelf-life of the final products (Hugas & Tsigarida, 2008; Ricke, 2003).

1.5.2.2.1. Weak organic acids: citric acid and lactic acid

The effectiveness of weak organic acids in the product decontamination, depends on the type of acid used, its concentration, the temperature of the acid solution, the type of food product, the type of decontaminated surface and initial microbial load of the food or surface. The application of weak organic acids (citric acid, $C_6H_8O_7$ and lactic acid $C_3H_6O_3$) for the decontamination of food products, were found to be most acceptable (Smigic *et al.*, 2010), as they can be found as natural compounds in many foods, in particular fruits and fermented milk products (EFSA, 2011b).

Several studies have reported on the effectiveness of weak organic acids in the reduction of different bacteria in meat products. In the case of lactic acid, a 2% concentration at 15 min exposure at 4 °C reduced *C. jejuni* cells by 1.5 log CFU g⁻¹ in chicken wings. The level of reduction increased to 3.87 log CFU ml⁻¹ when lactic acid treatment was followed by 24 h

storage at 5 °C (Rajkovic *et al.*, 2010). A study by Bosilevac *et al.* (2006) compared the antimicrobial effect of hot water treatment and the use of hot water supplemented with 2% lactic acid applied for beef carcasses. Using a hot water wash cabinet (74 °C) for 5.5 s resulted in the reduction of both aerobic plate counts and *Enterobacteriaceae* counts by 2.7 log CFU per 100 cm², while treatment of pre-eviscerated carcasses to a 2% lactic acid solution in a spray cabinet at 42 °C reduced aerobic plate counts by 1.6 log CFU per 100 cm² and *Enterobacteriaceae* counts by 1.0 log CFU per 100 cm². However, the combination of these two treatments resulted in a bacterial reduction to the same level as obtained by hot water treatment only. Results obtained by Del Río *et al.* (2007) showed aerobic bacteria, coliforms, and *Enterobacteriaceae* reduction by 1.2, 1.3, and 1.5 log CFU g⁻¹, respectively, following naturally contaminated chicken legs immersed in a 2% lactic acid solution for 15 min at 18 °C. A study by Bautista *et al.* in 1997, investigated the bacteriocidal effect of lactic acid on faecally contaminated turkey carcasses and found a 95% reduction of the total microbial load and coliforms achieved when a 4.25% w/w solution of lactic acid was used (Bautista *et al.*, 1997). The use of lactic acid on *Campylobacter jejuni* decontamination following incubation in various modified atmospheres has also been investigated (Smigic *et al.*, 2010). Bacterial cell suspension was treated with lactic acid at a concentration of 3% for 2 min. After treatment bacteria were centrifuged and resuspended in the broth. Then plates with *C. jejuni* were incubated at 4 °C for 7 days under modified atmospheres: 80% O₂, 20% N₂; 80% CO₂, 20% N₂; air and microaerobic atmosphere (10% CO₂/85% N₂/5% O₂). A reduction below detectable level following 5 days incubation under microaerobic atmosphere and air was observed, while incubation under 80% O₂, 20% N₂ resulted in a decrease below detectable level after 2 days of incubation.

Meredith (2013), besides trisodium phosphate investigated the effectiveness of lactic acid and citric acid on *Campylobacter* reduction in artificially contaminated fresh broiler cloacal contents. Application of 1%, 5% and 10% of either lactic acid or citric acid resulted in reduction of *Campylobacter* isolated from the cloacal contents of the chickens. A reduction by 1.08 log CFU g⁻¹, 2.17 log CFU g⁻¹ and 2.24 log CFU g⁻¹ was observed following treatment for 10 min with 1%; 5% and 10% of citric acid, respectively. Also a similar level reduction was observed when lactic acid was used at the same concentration. When this treatment was transferred to the processing plant, no statically significant differences between treated and untreated samples were observed. Another study, investigated the effectiveness of different antimicrobial agents on the reduction of different bacteria present in poultry meat, showed a significant reduction of bacterial counts after immersion

of naturally contaminated chicken legs in 2% citric acid for 15 min at 18 °C (Del Río *et al.*, 2007). Reductions of mesophilic aerobic bacteria, coliforms, and *Enterobacteriaceae* by 1.70, 1.98, and 2.15 log CFU g⁻¹ of skin were also reported, respectively.

1.5.2.2.2. Chlorine based compounds

Chlorine-based compounds such as chlorine dioxide and acidified sodium chlorite have also been studied as measures to reduce microbial levels and prevent cross-contamination in the fresh meat products. Chlorine dioxide (ClO₂) is very reactive and is rapidly transformed to chlorate ions (ClO₂⁻), while the main active ingredient of acidified sodium chlorite (ASCh) solution is chlorous acid (HClO₂) which is a very strong oxidizing agent, stronger than either chlorine dioxide or chlorine (EFSA, 2005a). These oxidizing disinfectants destroy microorganisms by direct action on the cellular membrane, disrupting the permeability of the outer membrane (Oyarzabal, 2005). Treatment of poultry neck skin samples with 25 µg ml⁻¹ of chlorinated water significantly reduced the total viable counts by log 4.98 CFU g⁻¹ and log 4.52 CFU ml⁻¹ for pre-evisceration and post-wash samples (Whyte *et al.*, 2001). A study by Yang *et al.* (2002) investigated the *Campylobacter jejuni* survival in pre-chill broiler carcasses taken from a commercial poultry processing plant at the end of an eviscerating line. Both tap water spray and a water spray containing 50 µg ml⁻¹ of chlorine on carcasses at different temperatures were researched. A 1.21 log most probable number (MPN) per carcass, 1.81 log MPN per carcass, 2.16 log MPN per carcass reduction was observed following spraying the carcass with chlorine at 20 °C, 55 °C, 60 °C, respectively. Further reduction of *Campylobacter* counts at 1.89, 2.05 and 2.53 log MPM per carcass was achieved on chicken carcasses sprayed with 50 µg ml⁻¹ chlorinated water at 20 °C, 55 °C and 60 °C and then chilled at 4 °C for 50 min. Research by Northcutt *et al.* (2004) investigated the use of water-only and water supplemented with 50 µg ml⁻¹ of chlorine at 21.1 °C, 43.3 °C and 54.4 °C on the reduction of bacterial counts. Following the carcasses washing in a bird washer at 5.4 atmospheric pressure for 5 s, with water-only and chlorinated water at different temperatures resulted in no significant reduction levels of total aerobic bacteria, *E. coli*, and *Campylobacter*.

Kemp *et al.* (2001) reported a 2.6 log CFU ml⁻¹ reduction of *Campylobacter* after spraying poultry carcasses for 18 seconds with 1100 µg ml⁻¹ of acidified sodium chlorite (ASCh). Furthermore, ASCh treatment was reported to be an effective disinfectant for significantly

reducing the naturally occurring microbial contamination of broiler carcasses. After immersion dipping at 500, 850, 1200 $\mu\text{g ml}^{-1}$ of ASCh a significant reduction at all concentrations was observed (Kemp *et al.*, 2000). Reduction of 1.5 log CFU g^{-1} of *Enterobacteriaceae* was reported when naturally contaminated chicken legs was immersed in 1200 $\mu\text{g ml}^{-1}$ of ASCh (Del Río *et al.*, 2007).

1.5.2.2.3. Trisodium phosphate

Trisodium phosphate (Na_3PO_4), a phosphate-based compound is a highly alkaline compound. Its mechanism of action is not yet fully understood. It probably interacts with fatty molecules in the cell membrane, causing bacterial cell leakage (Oyarzabal, 2005). The use of trisodium phosphate (TSPH) as a poultry decontamination treatment has been studied. A 1.7 log CFU g^{-1} reduction of *Campylobacter* was published by Whyte *et al.* (2001) when 10% of TSPH was applied for 18 min by immersion of the broiler carcass. Another study showed a reduction of *E. coli* on artificially contaminated chicken meat portion by 0.5, 1.2 and 1.6 log CFU cm^{-2} after dipping in 8, 10 and 12% of TSPH, respectively (Jasass, 2008). The effectiveness of 10% trisodium phosphate on *Campylobacter jejuni*, following a 10 min incubation of chicken breast skin was also investigated (Arritt *et al.*, 2002). The skin was inoculated with *C. jejuni* before the chemical treatment application. Following treatment a reduction by 1.63 log CFU per skin was observed. In addition, authors investigated the ability of trisodium phosphate to prevent the attachment of bacteria. The skin was inoculated with *C. jejuni* after chemical treatment. Reduction of *C. jejuni* at 1.28 log CFU per skin after chemical spraying was observed.

1.5.2.2.4. Peroxyacids

Peroxyacids are also used in poultry decontamination in some countries. Peroxyacetic acid, hydrogen peroxide, peroxyoctanoic acid, acetic acid, octanoic acid and HEDP (1-hydroxyethylidene-1,1-diphosphonic acid, used as stabiliser) are the main compounds in peroxyacids solution. The solution is used at a maximum concentration of total peroxyacid, expressed as peroxyacetic acid of 220 $\mu\text{g ml}^{-1}$ a maximum concentration of hydrogen peroxide of 110 $\mu\text{g ml}^{-1}$, and a maximum concentration of HEDP of 13 $\mu\text{g ml}^{-1}$ (EFSA, 2005a). All of them have a strong oxidizing function where disruption in the permeability of cell membranes and alteration of proteins and nucleotides synthesis occur (Oyarzabal, 2005).

Del Río *et al.* (2007) investigated the use of a peroxyacids solution after the poultry evisceration process. Samples were collected and stored at 3 °C for up to 5 days. Following the peroxyacids treatment by dipping the carcass, bacterial counts were investigated. Peroxyacids at a concentration of 220 µg ml⁻¹ reduced mesophilic aerobic counts by 0.53 CFU g⁻¹, psychrotrophs by 0.11 CFU g⁻¹, *Enterobacteriaceae* levels by 1.34 CFU ml⁻¹, coliforms level to 1.18 CFU ml⁻¹ and enterococci to 2.17 CFU ml⁻¹. A reduction by 0.6 log CFU ml⁻¹ and 1 log CFU ml⁻¹ in *Escherichia coli* O157:H7 and *Salmonella* serotype Typhimurium were obtained, respectively, while fresh trim beef pieces was dipped in a peroxyacetic acid at concentration of 200 µg ml⁻¹ at 43 °C for 15 s (Ellebracht *et al.*, 2005). Peroxyacids, following spraying for 15 s at a concentration of 200 µg ml⁻¹, resulted in a 2 log reduction of *Campylobacter jejuni* in an artificially contaminated poultry carcass (EFSA, 2005b).

1.5.2.2.5. Sodium hydroxide

Sodium hydroxide (NaOH) has also been investigated as a potential disinfectant to reduce different bacterial contamination in meat products. Reduction of *Listeria monocytogenes* using 0.175%, 0.200% and 0.220% (w/v) of NaOH in artificially inoculated chicken legs was observed. After inoculation and storage for up to 21 days at 21 °C, a reduction between 1.80 and 3.28 log for NaOH treated samples was achieved (Capita *et al.*, 2002). However, Zhao and Doyle in their study in 2006, showed more than 5 log CFU ml⁻¹ reduction of *Campylobacter jejuni* after treatment by 0.05 N (0.2%) and 0.1 N (0.4%) sodium hydroxide for 2 min. The authors suggest that this finding may be a useful treatment in chill water tanks for poultry processors to reduce *Campylobacter* on poultry skin after slaughter (Zhao & Doyle, 2006). Although, the authors obtained promising results, further studies were not carried out to investigate the different means of applying this disinfectant.

1.6. LEGISLATION

Enhanced problems related to the contamination of poultry products by bacteria during production, contributed to the creation of the first regulations on preventing their spread. These regulations were produced in 1980 by the Worldwide Health Organisation (WHO), and originally were concerned with controlling *Salmonella* contamination during poultry processing. Due to their timeless versatility, they could be easily extended to other

pathogens related to poultry contamination. The regulations consider three control strategies. The first strategy, concentrated on controlling the bacterial contamination in the flock and preventing the spread between flocks by implementation of biosecurity measures, maintaining good hygienic practices (GHP) and the separation of contaminated flocks from uncontaminated flocks. The second strategy focused on the prevention or reduction of contamination of the carcass at the processing plant, by the implementation of good manufacturing practices (GMP) and Hazard Analysis Critical Control Point (HACCP). The third strategy, focused on prevention of contamination during the final preparation of the food by the industry and consumers (Hugas & Tsigarida, 2008).

The European Food Safety Authority (EFSA) is the main organisation in the European Union, responsible for providing a risk assessment with regard food and safety. This includes risk assessment of using different methods for controlling pathogens in food and sanitation methods including the use of detergents, their concentrations, exposure times etc. Following chemical and microbiological risk assessment, the European Commission might approve the use of such substances or methods (Hugas & Tsigarida, 2008). The equivalent to the European Commission in the United States of America is United States Food and Drug Administration, FDA (Del Río *et al.*, 2007).

1.6.1. Legislation in the United States of America

With regard to the application of HACCP systems in the USA and Canada, intervention strategies in reducing bacterial carcasses contamination at slaughter are currently in use. The intervention approach includes the use of physical, chemical and biological methods (Loretz *et al.*, 2010).

Carcass decontamination at the processing plant is a regular occurrence, where a combination of different methods for microbial reduction is applied. Most of the methods are authorised by the FDA and generally recognised as safe (GRAS). Among the various substances recognised as safe and approved for use include: weak organic acid at a concentration of 1.5%-2%, trisodium phosphate at 8%-12%, acidified sodium chlorite at 500 $\mu\text{g ml}^{-1}$ -1200 $\mu\text{g ml}^{-1}$ and peroxyacid up to 220 $\mu\text{g ml}^{-1}$ (Del Río *et al.*, 2007).

Under some conditions, food irradiation as a control measure has been deemed safe (Lee, 1998). However, due to the high risk of chemical changes in the food and its packaging,

some strict regulation in terms of packaging materials and irradiation dose are applied. In addition, an appropriate packaging label has to be supplied by the manufacturer. The radura symbol and the phrase “treated with radiation” or “treated by irradiation” has to be clearly visible in the products. Fresh pork can be irradiated with dose up to 1 kGy, poultry with dose up to 3 kGy and frozen meat with dose up to 7 kGy (Lee, 1998).

1.6.2. Legislation in the European Union

Presently, there are two regulations in the European Union regarding the use of chemicals for food decontamination. According to Directive 71/118/EC, the use of substances other than portable water are not permitted (Del Río *et al.*, 2007; Loretz *et al.*, 2010). However, the European Union Regulation (EC) No. 853/2004, laying down specific hygiene rules for food of animal origin, highlights chemicals that might be used for bacterial decontamination of the meat product but recommended withholding their use until a risk assessment is provided by the EFSA (Loretz *et al.*, 2010). So far, the use of lactic acid to reduce microbiological surface contamination on bovine carcasses was adopted by the European Commission on the 4th of February 2013 and entered into force on the 25th February 2013 (<http://europa.eu/rapid/midday-express-04-02-2013.htm>) after a risk assessment by EFSA. The aim of this procedure is to maintain GHP and GMP practises rather than using antimicrobials to hide human error and the lack of proper training. The European Food Safety Authority (EFSA) with the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) reported that carcass decontamination by chlorine dioxide, acidified sodium chlorite, trisodium phosphate and peroxyacids has no toxicological risk to human health (EFSA, 2005a).

In case of using irradiation as a decontamination method, Directive 1999/2/EC regulates the type of food that can be irradiated and covers general and technical aspects for carrying out the irradiation processes, labelling of irradiated food and conditions for authorising food irradiation (Hugas & Tsigarida, 2008). Irradiation is only allowed for use on certain foods: dried aromatic herbs, spices and vegetable seasonings. Some countries have an authorisation for using irradiation on particular products. France may use up to 3.5 kGy on Camembert cheese and 4.0 kGy on egg white, while the Netherlands may use up to 5.0 kGy on frog legs and up to 1.0 kGy on dried fruit, and the UK may use up to 0.2 kGy on roots and tubers and 3.0 kGy on shellfish (Lee, 1998).

1.7. TECHNIQUES USED IN THIS STUDY

This study was set out to investigate the long-term effect of weak organic acids like citric acid and lactic acid, and phosphate based compounds such as trisodium phosphate, and chlorine based compounds such as acidified sodium chlorite on *Campylobacter* spp. survival and adaptation. The long-term effects of disinfectant challenge were studied in a bio-processor-controlled chemostat culture system which maintains defined growth rates. Long-term chemostats (>850h) were run with and without disinfectant selection pressures. During the course of exposure to the disinfectant representative samples from the chemostat cultures were also taken and stored for proteomic analysis and virulence gene expression.

However, before carrying out the long-term *Campylobacter* adaptation study, isolates were genotypically identified using molecular typing techniques and phenotypically characterised by antimicrobial susceptibility testing (MIC) and time-kill kinetics.

1.7.1. *Campylobacter* genotyping

Various methods of *Campylobacter* genotyping have been developed in the last 30 years. The modern methods of genotyping include: Pulse-Field Gel Electrophoresis (PFGE); Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Flagellin Genes (*fla* PCR-RFLP); Random Amplification of Polymorphic DNA Analysis (RAPD); Amplified Fragment Length Polymorphism Fingerprinting (AFLP) or Multilocus Sequence Technique (MLST). In this study two genotyping methods, PFGE and *fla* PCR-RFLP were used to genetically fingerprint *Campylobacter* spp. These two methods were chosen as they have been widely used in other studies for strain characterisation (Camarda *et al.*, 2000; Fitzgerald *et al.*, 2001; Han *et al.*, 2007; O'Leary *et al.*, 2011; Ono *et al.*, 2003) and they will be described in greater detail in comparison to the other modern methods of genotyping.

1.7.1.1. *Pulse-Field Gel Electrophoresis, PFGE*

The whole genomic DNA is used in the PFGE technique, where bacterial DNA is immobilised in the chromosomal grade agarose plugs. Cell lysis is carried out in the plugs in order to prevent DNA sharing. Following accurate plugs washing in order to remove compounds that may inhibit the restriction enzymes activity, a digestion by a rare-cutting enzyme is applied.

SmaI, *SaII*, *KpnI* are the most discriminatory enzymes used in the restriction of *Campylobacter*. Separation of digested DNA is performed by a specialised electrophoresis method which uses an alternating voltage gradient to improve the resolution of larger DNA molecules. The DNA cut fragments are separated by their molecular weight. *SmaI* restriction enzyme generates from 5 up to 10 bands from 40 to 480 kb, while *KpnI* easily produces up to 13 bands from 40 kb to 200 kb (Gibson *et al.*, 1997; Owen *et al.*, 1995). The smallest amount of DNA fragments, between 2 and 4, after digestion by *SaII* was reported (Hänninen *et al.*, 1999).

Digestion of genomic DNA by one of the above mentioned restriction enzymes, makes PFGE a great discriminatory method, that easily shows differences within *Campylobacter* spp. Han and co-workers (2007), genotyped 100 *C. jejuni* strains by PFGE and obtained 73 different PFGE types. Another study reported eight PFGE patterns from the 19 *C. jejuni* isolates tested (Camarda *et al.*, 2000). Fitzgerald and colleagues (2001) in their study, genotyped 22 *C. jejuni* isolates using the PFGE technique. Following digestion of the whole genomic DNA by a *SmaI* restriction enzyme, 11 different PFGE profiles were obtained. O'Leary and co-workers genotyped 600 *Campylobacter* spp. isolated from retail foodstuffs and patients with gastroenteritis as a part of epidemiological study (O'Leary *et al.*, 2011). Among 507 strains that were successfully analysed following *SmaI* digestion, 236 different patterns were obtained, of which 22 profiles were found to be common amongst these isolates.

PFGE is considered a gold standard method in epidemiological studies of pathogenic organisms. The PulseNet program was established in 1996 in the USA and since its foundation has developed into a network on an international scale (PulseNet International) for the molecular subtyping of food-borne disease surveillance. However, it is in the USA that PulseNet is most advanced by way of an integrated surveillance network. The PulseNet USA is a collaboration between various institutions such as the Association of Public Health Laboratories (APHL), the Centres for Disease Control and Prevention (CDC), the Council of State and Territorial Epidemiologists (CSTE) and the Food and Drug Administration's Center for Food Safety and Applied Nutrition (FDA-CFSAN). The advantage of this network of local and state health laboratories, which use PFGE as a genotyping tool, lies in its ability to detect, investigate and control outbreaks of food-borne infection. Subtyping allows scientists to see the differences between bacterial strains of the same species and monitor the trends of those bacteria (Gerner-Smidt *et al.*, 2006). PulseNet USA database contains

PFGE profiles for organisms such as *Salmonella*, *Listeria monocytogenes*, *Vibrio cholera* and *Campylobacter*. The *Campylobacter* database was established in 2004. However, due to high genetic diversity and plasticity of *Campylobacter* genome, it is difficult to detect the outbreaks caused by this pathogen (Gerner-Smidt *et al.*, 2006). Nevertheless, a national-scale *Campylobacter* spp. genotyping can be a useful tool in detection of different sources of human infection (Sheppard *et al.*, 2009).

1.7.1.2. Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Flagellin Genes, *fla* PCR-RFLP

RFLP is a technique that exploits variations in homologous DNA sequences. *Campylobacter* spp. is a flagellated organism that possesses a single polar flagellum at one or both ends of the cell (Alm *et al.*, 1993). There are two genes coding the flagellum: *flaA* and *flaB* at approximately 1.7 kb size, each. Between *fla* genes there is an approximately 170 bp intergenic spacer region. These genes are highly conserved, with 92% homology between the *flaA* and *flaB* genes in individual isolates. However, polymorphic regions in the flagellin genes exist, which provide the basis of a typing scheme (Nachamkin & Blaster, 2000; Nachamkin *et al.*, 2008). Comparison of the published sequences of the *flaA* genes of *C. jejuni* and *C. coli* revealed common regions at the N-terminal (170 amino acids) and C-terminal (100 amino acids) ends of the protein (Fischer & Nachamkin, 1991; Nuijten *et al.*, 1990).

In the Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Flagellin Genes (*fla* PCR-RFLP) technique, amplified *fla* region by PCR reaction is purified and cut by a restriction enzyme. The 1.7 bp amplicon is digested by a restriction enzyme or combination of enzymes. Common endonucleases, used to distinguish variability in *fla* sequences, include *AluI*, *DdeI*, *EcoRI*, *HinfI* and *PstI*. Then the fragments are separated by their molecular weight by electrophoresis.

The relatively low cost, easy accomplished and high discriminatory level between strains make the method an excellent tool in identifying various genotypes. Han and colleagues reported that following *DdeI* restriction enzyme digestion, 30 *flaA* types were identified among 100 *C. jejuni* strains that have been genotyped (Han *et al.*, 2007). Camarda and colleagues (2000) genotyped 22 *Campylobacter jejuni* strains and found 10 different *flaA* patterns from the isolates tested. Another study that used the *flaA*-typing technique,

following digestion by the *DdeI* restriction enzyme of 22 *C. jejuni* isolates, 7 different patterns were obtained (Fitzgerald *et al.*, 2001).

Despite the fact that *fla*-typing is a good discriminatory method for *Campylobacter* isolates, the major disadvantage of *fla* PCR-RFLP is the genetic instability of *fla* genes. It has been reported that *C. jejuni* is capable of intergenomic recombination between *flaA* genes of different strains as well as between *flaA* and *flaB* genes in the same strain (Harrington & Thomson-Carter, 1997). Due to this genetic instability these methods should not be used alone for the long-term study of *Campylobacter* pathogenesis. Other genotypic methods should be applied to achieve correct results. The combination of *fla*-typing and PFGE gives a good solution in order to achieve the optimal typing of *Campylobacter* (De Boer *et al.*, 2000).

1.7.1.3. Random Amplification of Polymorphic DNA, RAPD

The Random Amplification of Polymorphic DNA (RAPD) use randomly constructed primers to bind to genomic DNA and then the fragments are amplified by regular PCR. The size of amplicons and their number can be manipulated by reaction parameters. RAPD technique was found to have a similar discriminatory power in comparison with PFGE (Ono *et al.*, 2003).

1.7.1.4. Amplified Fragment Length Polymorphism, AFLP

Two restriction enzymes (*HindIII* and *HhaI* or *BglII* and *Csp6I*) are used to digest genomic DNA in the Amplified Fragment Length Polymorphism (AFLP) technique (Klena & Konkel, 2005). Following DNA cutting, specific oligonucleotide adapters are ligated to restriction sites. Finally, the PCR of adapter-fragment is carried out by using adapter-specific primers.

1.7.1.5. Multilocus Sequence Typing, MLST

Multilocus Sequence Typing (MLST) is a technique using sequencing housekeeping genes (typically seven) to distinguish differences between *C. jejuni* genotypes and the source. Each sequence variant will be differentiated between different allelic types. The MLST method can give better understanding of epidemiological investigation (Klena & Konkel, 2005). Sequence typing by MLST is internationally recognised as the new gold standard for

national and international epidemiological characterisation of major pathogenic microorganisms such as *C. jejuni* (Sullivan *et al.*, 2005; Urwin & Maiden, 2003).

1.7.2. Chemostat culture technique

In this study, the effect of several disinfectants on the survival, growth and death characteristics of *Campylobacter* strains was investigated. The ability of different *Campylobacter* strains to adapt to disinfectants was carried out under continuous culture conditions using a chemostat technique. This technique for the first time was described in 1950 by Monod (Hoskisson & Hobbs, 2005; Monod, 1950). An advantage of continuous culture is that the microbial population can be carried on in a constant environment and constant parameters as a growth rate. Another benefit of this culture is that the environmental factors such as pH, nutrient concentration and oxygen level can easily be controlled. In addition, the influx of sterile media and efflux of spent media create a balance between live and dead cells while a gentle mix allows the creation of homogenous steady state culture.

1.7.2.1. Practical application of the chemostat technique

The chemostat technique has been used in different research fields like ecology, microbiology or chemical engineering (Cheng-Che & Sze-Bi, 2005). Fleming *et al.* (1988) used chloramphenicol selective pressure in a chemostat in order to produce stable plasmid in *Bacillus subtilis*. pUB110, the plasmid that was used in the study, confers chloramphenicol and kanamycin resistance. In addition, it encodes α -amylase. Due to the high instability of the plasmid, bacteria can easily lose it. It has been reported that after 96 h growth in batch culture, the bacteria lost the plasmid. However, applying the selective pressure to the continuous culture by additional chloramphenicol, resulted in the maintenance of the plasmid after 160 h of culture. The chemostat culture technique was also used by Noack and co-workers (1984) to maintain the stable plasmids pBR322 and pBR325 in *Escherichia coli*. The chemostat cultures were run under glucose or ammonium chloride limitation at two different dilution rates. Results obtained showed that pBR322 was stable under all growth conditions tested. However, the plasmid pBR325 separated from the host under glucose restriction and a low dilution rate.

Continuous culture has also been used for investigating the effect of limited nutrient concentration on bacterial growth. The purpose of this type of study was to look at the physiological adaptation of the bacteria to nutrient concentration through the flux changes by looking at the molecular response mechanism for the stress conditions and associated gene expression. Shehata and Marr (1971) investigated the effect of a limited concentration of glucose, phosphate or tryptophan on *Escherichia coli*. The authors concluded that the growth rate is dependent on the concentration of the tested compounds when they are at the low level. A study by Hua and co-workers (2004) investigated the effect of limiting concentration of glucose and ammonia on *Escherichia coli* growth. When the bacteria were grown in ammonia limited concentration at growth rate of 0.1 h^{-1} , the DNA microarray analysis showed overexpression of 32 genes involved in amino acid metabolism. Higher expression of other genes involved in central intermediary metabolism, energy and nucleotide metabolism, and transport/binding protein were also found but at the lower level. In the case of glucose deficiency the higher percentage of gene expression was found to be associated with the central carbon and energy metabolisms, and transport systems (Hua *et al.*, 2004). These kind of studies on bacterial growth dynamics had a great impact on practical application of the chemostat techniques such as the creation of the appropriate selection media to control growth conditions which can lead to the overexpression of particular enzymes that then can be extracted as a product. The continuous bacterial culture has been used for example for α -amylase production. Some studies investigated different species of *Bacillus* and their growth conditions using a chemostat culture in order to construct a α -amylase producing strain. The results showed that *Bacillus* spp. under a carbon-limited environment and appropriate dilution rate produce the enzyme (Davis *et al.*, 1980; Emanuilova & Toda, 1984; Meers, 1972).

Chemostat technique has been also used to investigate the bacterial response to antimicrobial agents. Apart from its use in bacterial adaptation studies, it is a good technique to investigate the evolution of mutant cells with resistance and cross-resistance to compounds. Bacteria that grow continuously over long periods (multiple generation) under unfavourable conditions can alter (adapt) to these conditions and grow faster than the parent strain. In such instances, the bacterial population in the chemostat evolve from the wild type to the a new strain (Novic & Szilard, 1950). The long-term effect of antibiotic levofloxacin on *Escherichia coli* resistance was investigated by Fleming (2002). Incremental increases of antibiotic form $0.25 \mu\text{g ml}^{-1}$ to $40 \mu\text{g ml}^{-1}$ resulted in the emergence of

resistance strains. The MIC for the antibiotic increased 47-fold after 91 generations. These mutants remained stable following serial antibiotic-free batch and continuous cultures.

Chemostat studies can also be used to look at the molecular mechanism for resistance development in populations upon exposure to antimicrobials. In a study by McCay *et al.* in 2010 which looked at the effect of exposure of benzalkonium chloride (BKC) on *Pseudomonas aeruginosa* resistance, reported a 12-fold increase in resistance in comparison with the wild type strain (McCay *et al.*, 2010). Sequencing analysis of *gyrA* gene showed mutation in Thr-83→Ile. The authors also looked at the implication of cross-resistance to other antimicrobials. They observed a decrease in sensitivity to ciprofloxacin by 256-fold in the chemostat derived BKC resistant strain. Conversely, carry out the chemostat culture on ciprofloxacin selection pressure produced mutants that showed increased resistance to BKC. Adapted population increased their MIC to BKC at 4-fold.

1.7.2.2. *Mathematical explanation of the chemostat technique*

In the chemostat, a critical factor controlling the behaviour of bacteria is the dilution rate (D) described as the rate of medium flow into the vessel to its volume.

$$D = \frac{F}{V} \quad (1)$$

where,

D – dilution rate [h^{-1}]; **F** – flow rate [ml h^{-1}]; **V** – working volume of the fermenter [ml]

Each culture characterises the specific growth rate (μ) as well. This factor is defined as the increase in cell mass per unit time. The changes of the biomass (x) during time in the chemostat can be expressed as:

$$\frac{dx}{dt} = \mu x - Dx \quad (2)$$

During the steady state the changes in biomass are constant thus $\frac{dx}{dt}$ equals zero and the equation creates the form of:

$$\mu = D \quad (3)$$

Investigating the appropriate dilution rate of the culture is important. D as the parameter that can be controlled by adjusting the speed of the peristaltic pump and has to be maintained below the D_c (dilution rate critical). D_c , equivalent to μ_{max} (maximum specific growth rate), when it is higher than D ($D_c > D$), washing out of the culture from the fermenter occurs. When D_c is lower than D ($D_c < D$), the steady state is being achieved by bacteria.

When the culture is carried out at the steady state, where $D_c < D$, the limiting nutrient concentration has to be considered. The link between nutrient concentration and specific growth was presented by Monod (1942) with following equation:

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (4)$$

where,

μ - growth rate; S - nutrient concentration; K_s – the nutrient concentration at which the growth rate is half that achieved under nutrient-sufficient conditions (μ_{max}), i.e. equal to S when the growth rate is $0.5\mu_{max}$.

When the culture is performed in steady state, then μ can be replaced by D . The model presented by Monod is not ideal in terms of bacterial behaviour. The equation (4) was created by empirical observation between growth rate and nutrient concentration, where the limiting nutrient was a source of carbon (Dykhuizen & Hartl, 1983; Hoskisson & Hobbs, 2005). Other models were proposed to improve the existing one. Droop presented a model that seems to consider limiting the concentration of nitrogen, phosphorus or vitamins (Droop, 1973, 1975; Dykhuizen & Hartl, 1983). The advantage of the Droop model was measuring of the internal substrate concentration (substrate concentration in the cells), while in the Monod model the external substrate concentration (in the media) was calculated. Droop model for monoculture presents equation (5) below.

$$\mu = \mu_{max} \left[\frac{Q(t) - K}{Q(t)} \right] \quad (5)$$

where,

μ_{\max} - maximum specific growth rate; K - minimum concentration of limiting nutrient per cell required before growth can be proceed; $Q(t)$ - concentration of limiting nutrient per cell which equals the reciprocal of the yield when excretory losses can be neglected.

Presented models and equations are valid only when the chemostat is performed for the monoculture. While two or more strains are present not only limiting nutrient concentration has to be considered but as well as competition between strains. Long-term culture becomes more challenging also due to difficulties with regards to dilution rate set up. It is more likely that various tested strains will have different maximum specific growth rates and different lag phase.

Figure 1.3. illustrates the chemostat system used throughout this study. Before performing the chemostat cultures, the bacteria were grown in a batch culture until the logarithmic growth phase was reached.



Figure 1.3. The structure of the chemostat used for the long-term disinfectant challenge study. A 545 ml working volume chemostat was used in this study. The fermenter (A) was fed from a 10 litre glass reservoir (E) via a peristaltic pump (C) and feed tubing (H). The constant temperature was maintained by using an internal stainless steel heating coil fed with water from a bath controlled by a water heater (D) and was checked using the thermometer (I). The culture was mixed using magnetic follower on stirrer (B). To make sure that the culture will not be air blocked, the chemostat culture contained medium break (K) and filters (L). The samples for the analysis were collected via sampling port (F). The pH-meter (J) was not used in this study.

1.8. AIMS

At present, in the European Union, the use of substances other than potable water is not permitted for the decontamination of poultry meat products, at broiler farms or at poultry meat processing plants. However, EU Regulation (EC) No. 853/2004 highlights chemicals that might be used for bacterial contamination of meat products. Before these chemicals can be commercially approved by the European Commission, a risk assessment will need to be carried out relating to the response of bacteria to exposure to these antimicrobial agents and the risk of generating more virulent strains. This study set out to investigate *Campylobacter* response to exposure to disinfectant by way of its ability to survive and adapt to disinfectants and the consequences for the bacterial cell at genome and proteome level.

The specific aims were:

- 1)** to screen a pool of food and clinically derived *Campylobacter jejuni* and *Campylobacter coli* strains genotypically by PFGE and *flaA*-typing, and phenotypically by determining the MIC to a number of disinfectants (citric acid, lactic acid, acidified sodium chlorite, trisodium phosphate) and antibiotics (ciprofloxacin, tetracycline and nalidixic acid) as well as determining time-kill kinetics of the disinfectants for the test strains. From this initial characterisation, a shortlist of candidate strains was selected to carry out a study of the effect of disinfectant exposure on their adaptation;
- 2)** to conduct long-term chemostat culture studies on different *Campylobacter* strains in order to determine the effect of a gradual and continuous incremental exposure to disinfectants (citric acid, lactic acid, acidified sodium chlorite and trisodium phosphate) on the ability of the bacteria to survive and/or adapt to the disinfectants and develop cross-resistance to antibiotics (ciprofloxacin, tetracycline and nalidixic acid);
- 3)** to evaluate *Campylobacter* virulence genes expression in response to long-term exposure to disinfectants at sub-MIC, MIC and supra-MIC concentrations. The relative expression of four genes: *flgS* (flagellar sensory histidine kinase), *ciaB* (*Campylobacter* invasion antigens), *cmeA* (multidrug efflux pump) and *cdtB* (cytolethal distending toxin) were investigated;
- 4)** to investigate and determine *Campylobacter* proteome response to long-term disinfectant challenge using the two-dimensional acrylamide gel electrophoresis technique and MALDI-TOF mass spectrometry analysis.

1.9. REFERENCES

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CHAPTER II

Genotypic and phenotypic characterisation of *Campylobacter* spp. strains

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Key words: disinfectants; *Campylobacter*; PFGE, *flaA* typing; MIC

2.1. SUMMARY

Various methods of *Campylobacter* genotyping have been developed in the last 30 years. Modern methods of subtyping include Pulse-Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism of PCR-Amplified Flagellin Genes (*fla* PCR-RFLP), Random Amplification of Polymorphic DNA Analysis (RAPD) or Multilocus Sequence Technique (MLST). In this study, two genotyping methods: PFGE and *fla* PCR-RFLP were used to characterise *Campylobacter* isolates. In total, 17 *Campylobacter* spp. strains, 13 strains from food and 4 from clinical sources were genotypically fingerprinted. Strains were also characterised by way of susceptibility to antibiotics: tetracycline, nalidixic acid and ciprofloxacin and to the disinfectants citric acid, lactic acid, trisodium phosphate and acidified sodium chlorite.

In total, 11 different patterns, among 15 isolates where PFGE was successfully obtained, were determined. Two strains, *C. jejuni* 323BC and *C. jejuni*, 1495BF did not reveal any PFGE pattern. It is probable that these two isolates were DNase-positive, degrading their chromosomal DNA during standard preparation procedures. In addition, 14 distinct patterns were achieved following *DdeI* restriction enzyme digestion used for *flaA* PCR-RFLP analysis. However, following *HinfI* restriction enzyme digestion of the PCR-amplified *flaA* gene only 7 different patterns were detected. Digestion by *DdeI* restriction enzyme resulted in better differentiation between *Campylobacter* genotypes than digestion by *HinfI*.

Antimicrobial susceptibility testing (MIC) and time-kill kinetics analysis were used to phenotypically characterise the 17 *Campylobacter* spp. strains. The results obtained show that tetracycline agar MIC values for all isolates ranged from 0.125 to 128 $\mu\text{g ml}^{-1}$; nalidixic acid ranged from 0.5 to >128 $\mu\text{g ml}^{-1}$ and ciprofloxacin from <0.00025 to 128 $\mu\text{g ml}^{-1}$. The disinfectant agar MIC for citric acid and lactic acid ranged between 0.125-0.25%; for trisodium phosphate from 0.15% to 2.5% while for acidified sodium chlorite from 500 $\mu\text{g ml}^{-1}$ to 850 $\mu\text{g ml}^{-1}$. The time-kill kinetics reflecting the time required to reduce *Campylobacter* by 3 log (99.9%) were dependent on the strain, the type of disinfectant and its concentration. The time-kill varied from 5 min to >120 min.

2.2. INTRODUCTION

Campylobacter is the most common bacterial cause of gastroenteritis worldwide. The main source of transmission to humans is through the consumption of improperly prepared food, particularly undercooked poultry products (MacKichan *et al.*, 2004). Symptoms of disease caused by this food-borne pathogen are usually self-limited but occasionally lead to serious sequela illnesses like Guillain-Barré Syndrome, reactive arthritis or Reiter's Syndrome (FDA, 2012; Nachamkin *et al.*, 2008). In order to reduce the risk of campylobacteriosis, intervention strategies in both poultry farms and processing plants, such as the application of disinfectants, have been proposed. The European Union Regulation (EC) No. 853/2004 highlights substances as being suitable for bacterial decontamination of meat products but recommended withholding their use until a risk assessment is provided by the EFSA (Loretz *et al.*, 2010). The work presented in this thesis concentrates on the *Campylobacter* adaptation study of disinfectants that are highlighted by the EU and the response of its genome (in particular virulence genes) and proteome. However, before the study could commence, the preliminary screening of the initial *Campylobacter* spp. strains had to be performed. The research presented in this chapter concentrates on genotypic characterisation of 17 test *Campylobacter* strains by PFGE and *fla* PCR-RFLP and their phenotypic characterisation by the way of minimum inhibitory concentration (MIC) determined for different antibiotics and disinfectants. In addition, as a part of the phenotypic characterisation, the time-kill kinetic studies were carried out in order to determine effectiveness of antimicrobial ability of disinfectants.

All 17 *Campylobacter* spp. strains used in this study (13 food isolates and 4 clinical isolates) were genotypically fingerprinted using Pulse-Field Gel Electrophoresis (PFGE) and *fla*-typing in order to discriminate the various strains that had been isolated from different sources. These techniques were chosen as they have been reported to give good genetic diversity among *Campylobacter* strains isolated both from animals and humans. These techniques are also widely used in other studies for strain identification (Camarda *et al.*, 2000; Fitzgerald *et al.*, 2001; Han *et al.*, 2007; O'Leary *et al.*, 2011).

For *Campylobacter* phenotypic characterisation, the disinfectants that are highlighted in the European Union Regulation (EC) No. 853/2004, as being suitable for bacterial decontamination of the meat products, were examined. The disinfectants tested were: citric acid (CA), lactic acid (LA), acidified sodium chlorite (ASCh) and trisodium phosphate

(TSPH). In addition, the antimicrobial susceptibility testing was carried out on the following antibiotics: ciprofloxacin (Cipro), nalidixic acid (Nx) and tetracycline (Tc), that have been used for patient treatment with campylobacteriosis (Ge, 2002).

On the basis of results obtained from the genotypic and phenotypic characterisation of the initial pool of 17 *Campylobacter* strains, 6 strains were chosen for further study. Three food and three clinical isolates were selected based on a unique PFGE pattern, the MIC value and the time-kill kinetics to disinfectants. The further study was set up to investigate their ability to adapt to the four disinfectants. To achieve this, continuous cultures were performed, where gradually increasing concentrations of disinfectants were added.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial isolates

Initially, seventeen *Campylobacter* spp. isolates (sixteen *C. jejuni* and one *C. coli*) were selected for the study (Table 2.1.). Isolates were collected from food or clinical sources in Ireland (from Galway, Belfast and Dublin) as a part of a comprehensive *Campylobacter* spp. epidemiological study. Samples were collected monthly from March 2001 to October 2002. Full details of sample collection and isolation can be found in Dr Paul Whyte and colleagues' publication from 2004. Briefly, the majority of samples were collected from large retail outlets (pre-packed supermarket products) and consisted of: chicken, turkey, duck, pork, beef, lamb, shellfish (mussels and oysters) or raw milk. In total, 2,391 food samples were screened for the presence of *Campylobacter*. In addition, during that period samples were also collected from humans with gastroenteritis (O'Leary 2011). 600 of these isolates were taken for further epidemiological study carried out by Dr Aoife O'Leary (2007) and PFGE and *flaA* typing was performed in order to investigate the links between human and foodstuff genotypes.

In the naming of the strains, the first letter of the bacteria's name indicates where the bacteria was isolated: G from Galway; D from Dublin; B from Belfast, while the second letter indicates the source: F-food, C-clinical. Third letter refers to whether the isolates were selected on mCCD agar (modified charcoal cefoperazone deoxycholate agar) procured from Mast Diagnostics (M letter) or from Oxoid (O letter). The strain *C. jejuni* ATCC (American Typed Culture Collection) 33560 was used in this study as a control reference isolate for the antimicrobial susceptibility study.

Table 2.1. List of *Campylobacter* spp. isolates initially used in the study.

Strain	Source	Species
2124GF	Chicken	<i>C. jejuni</i>
1354DF	Chicken	<i>C. jejuni</i>
1369DF	Chicken	<i>C. jejuni</i>
1493DF	Pork	<i>C. jejuni</i>
1147DFM	Chicken	<i>C. jejuni</i>
1662DF	Chicken	<i>C. coli</i>
1495BF	Chicken	<i>C. jejuni</i>
2118GF	Beef	<i>C. jejuni</i>
1136DFM	Chicken	<i>C. jejuni</i>
1135DFO	Chicken	<i>C. jejuni</i>
1318DFM	Chicken	<i>C. jejuni</i>
1140DFO	Chicken	<i>C. jejuni</i>
1146DFM	Chicken	<i>C. jejuni</i>
23GC	Clinical	<i>C. jejuni</i>
354DC	Clinical	<i>C. jejuni</i>
301BC	Clinical	<i>C. jejuni</i>
323BC	Clinical	<i>C. jejuni</i>
ATCC 33560 *	Bovine feces	<i>C. jejuni</i>

* Strain *C. jejuni* ATCC 33560 was used as a control referee strains for the antimicrobial susceptibility study only.

2.3.2. Routine bacterial culture conditions and storage conditions

The *Campylobacter* strains were routinely cultured/subcultured on either *Campylobacter* blood free selective agar base (Oxoid) or blood agar, Mueller-Hinton Agar (LabM) supplemented with 5% v/v horse blood (Charles River Laboratories, Inc) and incubated microaerobically through the use of a gas generating sachet (BR038, Oxoid) in a gas jar at 42 °C for 48 h.

For long term storage, bacteria from *Campylobacter* blood free selective agar base were aseptically harvested in a 1 ml mixture of Tryptone Soya Broth (LabM) and 40% v/v glycerol, acting as a cryoprotectant, and transferred into cryotubes. Bacteria were stored at -80 °C.

2.3.3. Pulse-Field Gel Electrophoresis

Genomic DNA for PFGE was prepared from *Campylobacter* spp. isolates, according to the protocol described by Ribot *et al.* (2001). Following 48 h of incubation under microaerobic conditions on a *Campylobacter* blood free selective agar base plate, cells from the plates were harvested in 3 ml of ice cold PETH IV buffer (1 M NaCl, 10 mM Tris and 10 mM EDTA). The cell densities were adjusted to McFarland 6. A 1% SeaKem[®] Gold Agarose for Pulse-Field Gel Electrophoresis (Lonza) was prepared in Milli-Q water and cooled to 56 °C for 1 h before use. Cell suspensions of 300 µl were pipetted into eppendorfs containing 700 µl of molten SeaKem[®] Gold Agarose. The cells were mixed with the agarose and pipetted into 5 chambers of a plug mould (Bio-Rad) and allowed to set at 4 °C. Then the agar plugs were transferred into sterilin containing 3 ml of ESP buffer (0.5 M EDTA, 1% Sarkosyl Salt and 1 mg ml⁻¹ proteinase K). The EDTA and sarkosyl salt mixture was pre-heated to 56 °C prior to the addition of the 1 mg ml⁻¹ Proteinase K (Sigma-Aldrich). Plugs were incubated overnight at 56 °C.

Following overnight incubation, the ESP buffer was decanted from the samples and the plugs were washed for 20 min, 8 times, in 10 ml of TE buffer (10mM Tris and 1mM EDTA). The plugs were stored at 4 °C in 3 ml of fresh TE buffer. A 1 mm portion of a single agarose plug per isolate was carefully cut using a glass coverslip. The portion of plug was restricted by *Sma*I restriction enzyme (FastDigest, Fermentas) according to the manufacturer's protocol. Following restriction the plug slices were removed from the restriction cocktail and placed on the comb edge. As a molecular weight marker, ProMega Markers[®] Lambda Ladders (Promega) was used. The plugs and markers were sealed to the comb using 1% SeaKem[®] Gold Agarose. The 1% agarose was poured into the gel tray and allowed to solidify. 0.5X TBE running buffer was added to the running chamber and allowed to cool down to 14 °C. The gel was allowed to equilibrate for 20 min prior to running.

Restriction fragments produced by *Sma*I were separated by PFGE using a CHEF DRII apparatus (Bio-Rad) at 5.5 V/cm, ramped from 0.5 to 40 s over 22.5 hours at 14 °C in 0.5X TBE buffer (45 mM Tris HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0). Following electrophoresis, the gel was stained in ethidium bromide (Sigma-Aldrich) at a concentration of 0.5 µg ml⁻¹ for 25 min and washed in Milli-Q water for another 25 min. The image of the gel was captured and visualised under UV transillumination (G:Box, Syngene).

2.3.4. *flaA* PCR-RFLP analysis

The CAMPYNET protocol (<http://campynet.vetinst.dk/Fla.htm>) for *flaA* PCR-RFLP analysis, based on the Nachamkin *et al.* protocol (1993) with modifications presented in Nachamkin *et al.* (1996) was used for PCR amplification of the flagellin (*flaA*). A full loop of 48 h bacterial growth on *Campylobacter* blood free selective agar base plate (Oxoid) was taken and cells were washed into 1 ml SET buffer (150 mM NaCl, 15 mM EDTA, 10 mM Tris-HCl; pH 8.0) in a 1.5 ml eppendorf, followed by centrifugation at 3000 x *g* for 5 min. The pellet was resuspended in a 570 μ l SET buffer supplemented with SDS (VWR) and proteinase K (Sigma-Aldrich) at a final concentration of 0.5% SDS and 100 μ g ml⁻¹ proteinase K for the cell lysis. After overnight incubation at 50 °C, the protein removal step was applied using the phenol: chloroform: isoamylalcohol at of ratio 25:24:1 (Sigma-Aldrich) method. DNA was precipitated using 1/10 volume of 3M sodium acetate (pH 5.3, Sigma-Aldrich), and 2 volumes of absolute ethanol (Sigma-Aldrich). The tubes were placed at -20 °C overnight. Following washing the pellets in 70% ethanol, the RNA removal step was carried out using RNase, DNase-free (Roche). After incubation at 37 °C for 1 h the DNA was allowed to solvate overnight at 4 °C and was stored at -20 °C. The concentration of 20 ng μ l⁻¹ of DNA for *flaA* PCR reaction was used. The final volume of the reaction at 75 μ l, contained 200 μ M of each dNTP, a primer concentration of 0.5 μ M (*flaA* Forward: 5'-GGA TTT CGT ATT AAC ACA AAT GGT GC; *flaA* Reverse: 5'- CGT TAG TAA TCT TAA AAC ATT TTG), 1X reaction buffer, 2.5 mM MgCl₂ and 1.5 units of Taq DNA polymerase. PCR reaction was carried out at initial denaturation of 94 °C for 2 min and then cycled 34 times at 92 °C for 1 min, the primers annealed at 45 °C for 1 min and then the DNA extension stage at 72 °C for 2.5 min. A final extension step at 72 °C was carried out for 10 min. Following the PCR reaction, a purification step was carried out with overnight incubation at 2 volume of absolute ethanol and 0.1 volume of 3M sodium acetate pH 5.3 (Sigma-Aldrich), to prevent inhibition of the restriction enzyme by the PCR reaction mix. A 5 μ l of each purified PCR product were digested in separate eppendorfs with the restriction enzymes *Ddel* (FastDigest *Ddel*, Fermentas) and *Hinfl* (FastDigest *Hinfl*, Fermentas) following the manufacturer's protocol. Digested PCR product was separated on the 2% SeaKem[®] LE agarose gel (Lonza). Electrophoresis was carried out for 90 min at 60 V with the gel containing ethidium bromide at a concentration of 0.5 μ g ml⁻¹. Molecular Weight Marker XIV (Roche) was used as a marker. The image of the gel was captured and visualised under UV transillumination (G:Box, Syngene).

2.3.5. Disinfectants

Low-molecular weight organic acids, citric acid (CA; BDH) and lactic acid (LA; Sigma-Aldrich) were used to determine the minimum inhibitory concentration (MIC) for all tested strains. In addition, sodium chlorite - acidified with 0.16% w/v of citric acid (ASCh; Sigma-Aldrich), and trisodium phosphate (TSPH; Merck) were examined as a chlorine-based and as a phosphate-based compound respectively (Hugas & Tsigarida, 2008).

The disinfectants were tested at the following concentration range: from 0.015 to 2% in case of citric acid and lactic acid; from 75 to 1200 $\mu\text{g ml}^{-1}$ in case of acidified sodium chlorite; from 0.04 to 10% in case of trisodium phosphate.

2.3.6. Antibiotics

Ciprofloxacin (Bayer Schering Pharme), tetracycline (Sigma-Aldrich) and nalidixic acid (Sigma-Aldrich) were selected as representatives of the fluoroquinolone, tetracyclines and quinolone agents, respectively, in order to determine the MIC value for all tested strains.

The antibiotics were tested at a concentration range from 0.004 to 4 $\mu\text{g ml}^{-1}$ for ciprofloxacin, from 0.015 to 128 $\mu\text{g ml}^{-1}$ for tetracycline and from 0.06 to 128 $\mu\text{g ml}^{-1}$ for nalidixic acid.

2.3.7. Antimicrobial susceptibility testing, MIC

The agar dilution procedure was carried out as approved by the CLSI (Clinical and Laboratory Standard Institute, formerly NCCLS) in order to determine the antimicrobial susceptibility of *Campylobacter* spp. to disinfectants and antibiotics (CLSI, 2002). Pure 18 h blood agar culture was used to prepare culture for antimicrobial susceptibility testing. Phosphate buffer saline (PBS, Oxoid) was inoculated with this culture to give inoculums with an equivalent cell density to a 0.5 McFarland turbidity. Mueller–Hinton agar (MHA, LabM) plates supplemented with 5% horse blood (Charles River Laboratories, Inc.) and the appropriate concentrations of antimicrobial agent (disinfectant or antibiotic) were prepared and inoculated with 100 μl of the *Campylobacter* cell suspensions. The plates were incubated at 42°C under microaerobic conditions for 48 h. The antimicrobial plate on

which no growth was observed was recorded as the minimum inhibitory concentration (MIC) for that antimicrobial agent for that strain. *Campylobacter jejuni* ATCC 33560 was used as a reference control (CLSI, 2002). Each experiment was independently repeated twice, in duplicates, with two separate 48 h *Campylobacter* cultures.

2.3.8. Time-kill kinetics

In order to establish the percentage survival of bacteria after exposure to the different disinfectants at their MIC and above MIC value, viable counts were performed. Time-kill kinetics were performed for 11 selected *Campylobacter jejuni* strains (2124GF, 1354DF, 1369DF, 1147DFM, 1136DFM, 1135DFO, 1140DFO, 1146DFM, 23GC, 354DC, 301BC). An overnight culture was taken from a *Campylobacter* blood free selective agar base plate and a 0.5 McFarland bacterial cell suspension in MHB was prepared. An appropriate dose of disinfectant was added to the broth culture. At time zero, just before addition of the disinfectant, and then after 5, 10, 20 and 30 min and then after 1, 2, 3 and 24 h, samples were removed for colony counts. Collected samples were serially diluted 10-fold in phosphate-buffered saline (PBS) pH 7.3, and 100 µl of each dilution was plated onto *Campylobacter* blood free selective agar base. A disinfectant-free growth control was included for each strain. The bacteria were counted after 48 h incubation at 42°C under microaerobic conditions, and the time-kill kinetics were plotted as percentage survival over time. A bactericidal effect (99.9% kill) is defined as a 3 log decrease in CFU ml⁻¹ (equivalence to 1000-fold reduction) (CLSI, 1999). Each experiment was repeated twice in duplicates, (n=4).

2.4. RESULTS

2.4.1. Pulse-Field Gel Electrophoresis, PFGE

In this study, PFGE typing was carried out on 17 *Campylobacter* spp. strains (13 food isolates and 4 clinical isolates). PFGE typing demonstrated a high level of discrimination between strains. In total, 12 different patterns were identified among 15 strains where PFGE was successfully achieved. Figure 2.1. shows the results of fourteen *C. jejuni* and one *C. coli* pattern obtained as a part of the genotyping study. Two *C. jejuni* strains, 323BC and 1495BF did not reveal any PFGE pattern (results not shown). Most probably, these two *Campylobacter jejuni* strains were DNase-positive, thus degrading their chromosomal DNA during standard preparation procedures. The genotype pattern of strains *C. jejuni* 2124GF (lane 2) and *C. coli* 1662DF (lane 6) were found to be the same following *Sma*I digestion. As it turned out later *C. jejuni* 2124GF was a mix culture. *C. jejuni* strains 1493BF (lane 4), 1140DFO (lane 12) and 1146DFM (lane 13) shared the same pattern. A very similar pattern (similarity in 80%) was also observed for strains 1318DFM (lane 11) and 2118GF (lane 9). The PFGE patterns for the remaining strains were found to be unique for the particular isolate. The percentage of similarity between *Campylobacter* PFGE patterns is presented in the Section 2.6. Supplemental materials, Figure S2.1.

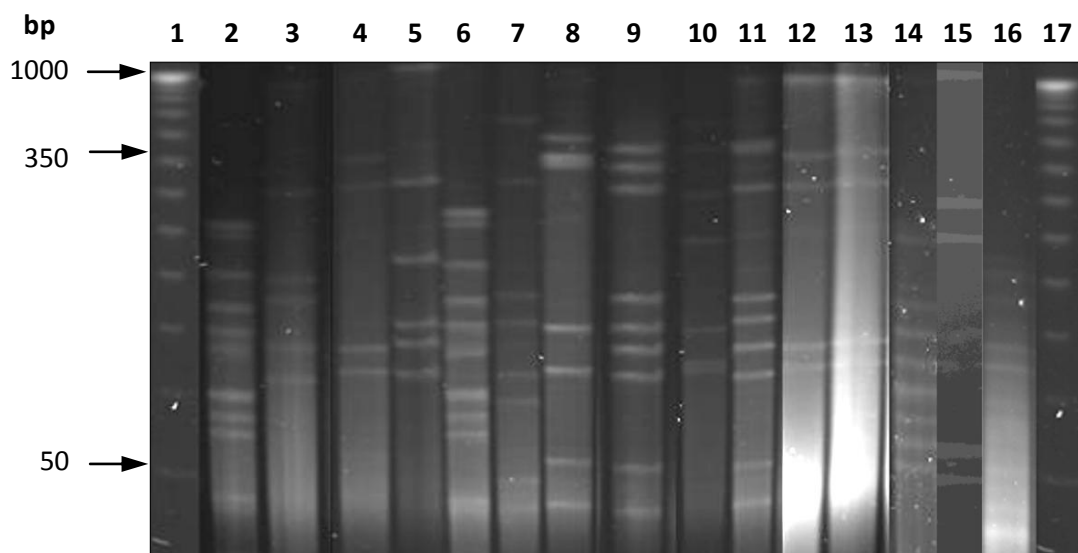


Figure 2.1. Pulse-Field Gel Electrophoresis results of DNA extracted from *C. jejuni/C. coli* and digested with *Sma*I. In lane 1-Molecular Marker-ProMega Markers Lambda Ladders, 2-2124GF, 3-1354DF, 4-1493BF, 5-1147DFM, 6-1662DF, 7-23GC, 8-354DC, 9-2118GF, 10-1135DFO, 11-1318DFM, 12-1140DFO, 13-1146DFM, 14-1369DF, 15-1136DFM, 16-301BC, 17-Molecular Marker-ProMega Markers Lambda.

2.4.2. Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Flagellin Genes (*fla* PCR-RFLP)

The subtyping technique, Restriction Fragment Length Polymorphism of PCR-Amplified Flagellin A gene locus (*flaA* PCR-RFLP), was also carried out on all test *Campylobacter* strains using *DdeI* and *HinfI* digestion. Figure 2.2. presents patterns following *DdeI* restriction enzyme digestion. *DdeI* digestion produced between 4 and 6 bands. In total 14 different patterns, from 15 strains were successfully analysed. Results obtained demonstrate wide phenotypic variety among *Campylobacter* strains. Only two strains, *C. jejuni* strains 1662DF (lane 12) and 1146DFM (lane 2), revealed the same *flaA* pattern. Very similar *flaA* patterns (similarity in 75%) following *DdeI* digestion were found in strains 1147DFM (lane 16) and 23GC (lane 4). The *flaA* pattern for the remaining strains, was found to be unique for the particular isolate. The percentage of patterns similarity between the *Campylobacter flA* gene digested by *DdeI* restriction enzyme is presented in the Section 2.6. Supplemental materials, Figure S2.2.

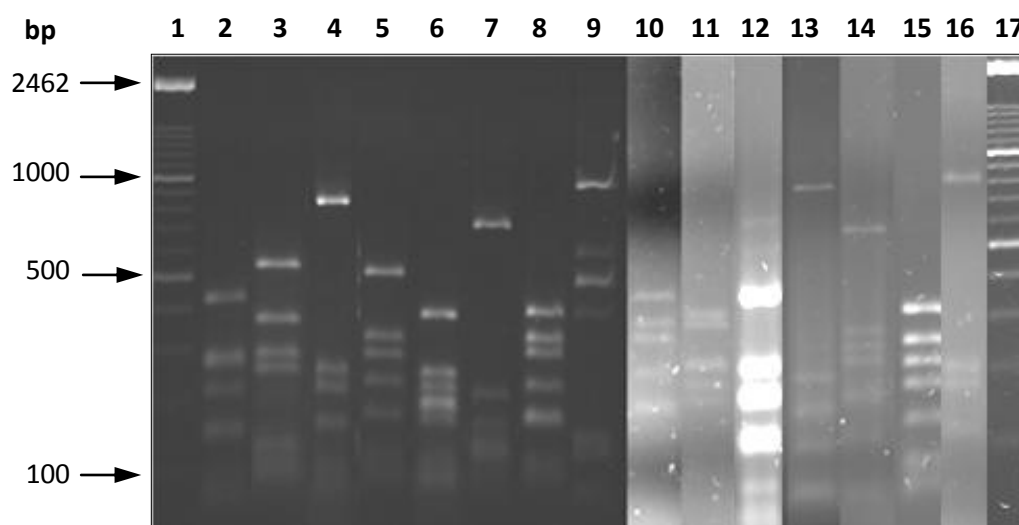


Figure 2.2. *FlaA* PCR-RFLP typing results after *DdeI* enzyme restriction digestion. In lane 1-Molecular Weight Marker XIV, 2-1146DFM, 3-1135DFO, 4-23GC, 5-1318DFM, 6-2118GF, 7-1354DF, 8-1136DFM, 9-1369DF, 10-1493BF, 11-354DC, 12-1662DF, 13-1140DFO, 14-301BC, 15-2124GF, 16-1147DFM, 17-Molecular Weight Marker XIV.

Figure 2.3. presents the *flaA* PCR-RFLP typing patterns achieved after *HinfI* digestion. Digestion by this restriction enzyme produced between 2 and 4 bands. In total, only 7 different patterns were observed. Strains 1146DFM (lane 2), 354DC (lane 11), 1662DF (lane 12), 1493BF (lane 10), 301BC (lane 14), 2124GF (lane 15) and 1147DFM (lane 16) all

revealed the same pattern. A similar pattern, was achieved for strains 1318DFM (lane 5), 2118GF (lane 6) and 1140DFO (lane 13), however, the molecular size of the obtained bands were found to be at different levels. Strain 1135DFO (lane 3), strain 1136DFM (lane 8) and strain 1354DF (lane 7) revealed a unique pattern, characteristic only to this particular strain. In the case of strains 23GC (lane 4) and 1369DF (lane 9), the patterns obtained were found to be very similar (similarity in 85%) and contained 3 and 4 bands respectively following *HinfI* restriction enzyme digestion. To conclude, PCR-Amplified Flagellin A gene locus digestion by *DdeI* restriction enzyme gave better differentiation between *Campylobacter* genotypes, than by *HinfI* digestion. The percentage of patterns similarity between *Campylobacter flaA* gene digestion by *HinfI* restriction enzyme is presented in the Section 2.6. Supplemental materials, Figure S2.3.

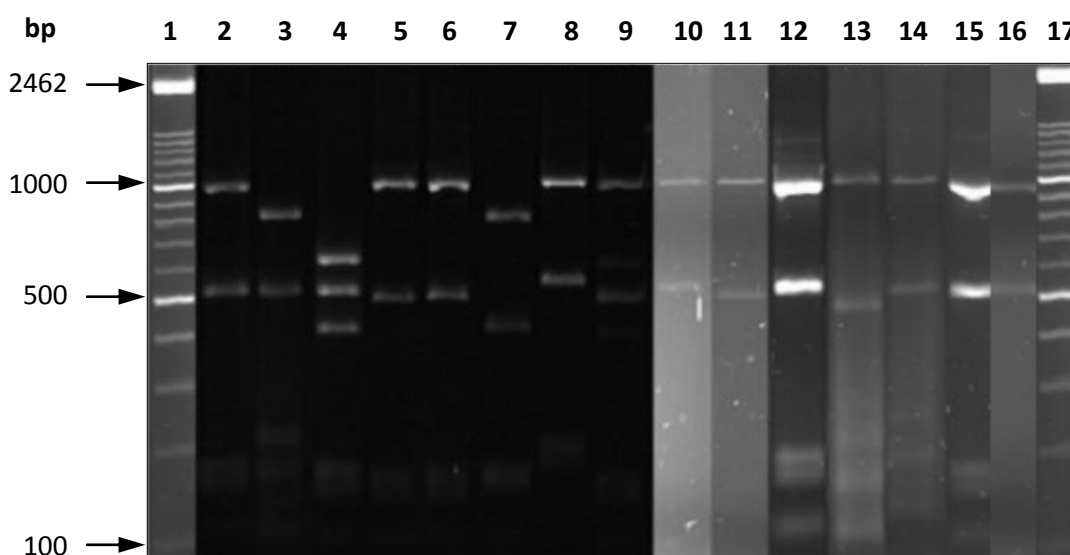


Figure 2.3. *FlaA* PCR-RFLP typing results after *HinfI* restriction enzyme digestion. In lane 1-Molecular Weight Marker XIV, 2-1146DFM, 3-1135DFO, 4-23GC, 5-1318DFM, 6-2118GF, 7-1354DF, 8-1136DFM, 9-1369DF, 10-1493BF, 11-354DC, 12-1662DF, 13-1140DFO, 14-301BC, 15-2124GF, 16-1147DFM, 17-Molecular Weight Marker XIV.

2.4.3. Antimicrobial susceptibility testing: agar MICs for disinfectants and antibiotics

In order to phenotypically characterise *Campylobacter* strains, agar MIC (minimum inhibitory concentration) for disinfectants and antibiotics were carried out. Agar MIC values tested for different disinfectants for all *Campylobacter* strains, were low with values between 0.125-0.25% recorded for both CA and LA; 500-850 $\mu\text{g ml}^{-1}$ for ASCh and 0.15-2.5% for TSPH (Table 2.2.). With regard to the antibiotics, the agar MIC values for the strains

were much more differentiated and varied. The MIC values for tetracycline were between 0.125-128 $\mu\text{g ml}^{-1}$, while the MIC values for nalidixic acid and ciprofloxacin respectively were obtained between 0.5->128 $\mu\text{g ml}^{-1}$ and between <0.00025-128 $\mu\text{g ml}^{-1}$. Three strains, *C. jejuni* 2124GF, *C. jejuni* 1369DF and *C. jejuni* 1135DFO, revealed low susceptibility to some antibiotics. With regards to *C. jejuni* 2124GF, the strain had an MIC at >128 $\mu\text{g ml}^{-1}$ for Tc. Strain *C. jejuni* 1369DF had an MIC value for Nx at 128 $\mu\text{g ml}^{-1}$, while *C. jejuni* 1135DFO had an MIC value at ≥ 128 for Nx and Cipro. Results obtained for strain *C. jejuni* 1354DF showed it to be highly susceptible to ciprofloxacin, with an MIC below 0.00025 $\mu\text{g ml}^{-1}$ (Table 2.2.).

Table 2.2. Minimum inhibitory concentrations (MICs) of the 17 test *Campylobacter* spp. isolates.

Strain	Agar MIC value for disinfectants				Agar MIC value for antibiotics		
	CA[%]	LA [%]	ASCh [$\mu\text{g ml}^{-1}$]	TSPH [%]	Tc [$\mu\text{g ml}^{-1}$]	Nx [$\mu\text{g ml}^{-1}$]	Cipro [$\mu\text{g ml}^{-1}$]
2124GF	0.125-0.25	0.25	500	0.6-1.25	>128	8	0.125
1354DF	0.25	0.25	850	0.3	0.25	0.5	<0.00025
1369DF	0.25	0.25	850	1.25	2	128	4
1493DF	0.25	0.25	500	0.6	0.25	8	0.06
1147DFM	0.25	0.25	850	1.25	0.125	4	0.06
1662DF §	0.25	0.125	850	2.5	1	8	0.125
23GC	0.25	0.125-0.25	500	0.3	0.5	4	0.125
354DC	0.25	0.125	850	0.3	0.25	4	0.25
301BC	0.25	0.125	500	0.6	0.25	8	0.125
2118GF	0.25	0.25	850	1.25	0.25	4	0.125
1136DFM	0.125-0.25	0.25	500	0.3	0.25	2	0.06
1135DFO	0.25	0.25	850	0.3	0.5	>128	128
1318DFM	0.125-0.25	0.25	850	0.15	0.25	1	0.03
1140DFO	0.25	0.125	850	0.3-0.6	0.5	4	1
1146DFM	0.25	0.125	500	0.3	0.25	4	0.125
323BC	0.25	0.25	850	0.3	0.5	8	0.125
1495BF	0.25	0.25	850	0.3	0.5	4	0.125
ATCC33560*	0.25	0.25	850	0.3	2	8	0.25

CA-citric acid; LA-lactic acid; ASCh-acidified sodium chlorite; TSPH-trisodium phosphate; Tc-tetracycline, Nx-nalidixic acid, Cipro-ciprofloxacin. Asterisk (*) indicates strain *C. jejuni* ATCC 33560 that was used as a control referee strains for antimicrobial susceptibility study only. § *Campylobacter coli* isolate, while the remaining strains are *Campylobacter jejuni*. A Blue colour indicates isolates selected for time-kill kinetics study, see Section 2.4.4.

Each experiment was independently repeated twice in duplicates (n=4). If the results obtained varied, the range of the MIC values are presented.

In the Table 2.2., strains highlighted in blue were chosen for further study on time-kill kinetics, investigating the effectiveness of disinfectants in *Campylobacter* reduction. The reasons for selecting these strains have been described below in Section 2.4.4.

C. jejuni ATCC 33560 was used as a quality control strain as approved by the CLSI for agar dilution methods (CLSI, 2002). According to CLSI, the MIC for this strain should be between 0.125–1 $\mu\text{g ml}^{-1}$ for Cipro; 8–32 $\mu\text{g ml}^{-1}$ for Nx and 1–4 $\mu\text{g ml}^{-1}$ for Tc. In this study, the MIC for *C. jejuni* ATCC 33560 was obtained at 0.25 $\mu\text{g ml}^{-1}$ for Cipro; 8 $\mu\text{g ml}^{-1}$ for Nx and 2 $\mu\text{g ml}^{-1}$ for Tc and were within the MIC tolerance range indicated by CLSI (CLSI, 2002).

To conclude, the range of MIC values of the 17 study *Campylobacter* strains for the disinfectants were not as varied in comparison to the MIC values for the antibiotics.

2.4.4. Selection of 11 *Campylobacter* spp. strains for the time-kill kinetics study

Genotypic and phenotypic screening of a number of *Campylobacter* isolates was used as an initial part of this study in order to identify differences between strains and to select strains with differences in genome, and MIC values to the test disinfectants and antibiotics. On the basis of the results, a selection of 11 strains were chosen for the next part of the study, which was to determine the killing effectiveness of disinfectants (time-kill kinetics). *Campylobacter* strains with a unique PFGE pattern was selected for further study. Where two or more strains possessed the same PFGE pattern, then the *fla* PCR-RFLP results and the MIC value were considered. The final call was made on the basis of the antibiotics MICs, as the MIC's values for disinfectants were determined at narrow range for all test strains. Strains with a much higher antibiotic MIC value, compared with the antibiotics' MIC for reference strain *C. jejuni* ATCC 33560, were preferred for further study.

Two strains, 323BC and 1495BF, were rejected due to difficulties in obtaining their genotype, possibly caused by DNase activity. *C. jejuni* strains 2124GF and 1662DF had the same PFGE and a different *DdeI* patterns. The tetracycline MIC for 2124GF and 1662DF at $>128 \mu\text{g ml}^{-1}$ and $1 \mu\text{g ml}^{-1}$ was obtained respectively. Due to a high MIC value for strain 2124GF, this strain was chosen for the disinfectant time-kill kinetics study. In another set of strains with the same PFGE pattern include 1493DF, 1146DFM and 1140DFO. Their *DdeI* patterns were found to be different, and their MIC was taken into consideration. The MIC values for ciprofloxacin were similar at 0.06; 1.0; 0.125 $\mu\text{g ml}^{-1}$ for 1493DF, 1140DFO and 1146DFM, respectively. For Tc the MIC values were also similar at 0.25 $\mu\text{g ml}^{-1}$; 0.5 $\mu\text{g ml}^{-1}$; 0.25 $\mu\text{g ml}^{-1}$, respectively, and for Nx 8 $\mu\text{g ml}^{-1}$; 4 $\mu\text{g ml}^{-1}$; 4 $\mu\text{g ml}^{-1}$, respectively. Due to lack of variation in MIC values for these strains, it was decided randomly that only *C. jejuni*

strains 1140DFO and 1146DFM would be selected for further study. Due to similarity in the genome and MIC values of strains 2118GF and 1318DFM, they were excluded from further study.

From an initial pool of 17 *Campylobacter* spp. strains, 11 isolates were selected for the time-kill kinetics study. These *C. jejuni* isolates were: 2124GF, 1354DF, 1369DF, 1147DFM, 23GC, 354DC, 301BC, 1136DFM, 1135DFO, 1140DFO, 1146DFM (Table 2.2., strains highlighted in blue). As only *C. jejuni* strains were chosen for further study, in order to avoid repetitions, only the number of the strain might be given.

2.4.5. Time-kill kinetics disinfectants against 11 *Campylobacter jejuni* strains

Eleven *Campylobacter jejuni* strains were used to study the bactericidal activity of citric acid, lactic acid, acidified sodium chlorite, and trisodium phosphate. Each *Campylobacter* strain was tested at the MIC concentration and supra-MIC value.

2.4.5.1. Time-kill kinetics of citric acid against *C. jejuni*

The citric acid (CA) agar MIC values for the selected *Campylobacter jejuni* strains were obtained at 0.25%, with the exception of two isolates, 2124GF and 1136DFM, where the MIC values ranged between 0.125-0.25% (see Section 2.4.3.).

Figure 2.4. presents results of bacterial survival upon exposure to 0.25% and 0.5% CA as plotted as percentage survival over time. When time-kill kinetics experiments were performed for 0.125% CA, bacterial growth with counts above 10^7 CFU ml⁻¹ were still detected for all isolates after 24 h disinfectant exposure. A two-fold increase of the antimicrobial concentration to 0.25% CA, resulted in a significant decline in bacterial survival. Cell exposure to 0.25% of CA for 50 min resulted in a 50,000-fold reduction for strains 2124GF and 1369DF (Figure 2.4. A). A similar result was observed for strain 1354DF, where 60 min exposure to 0.25% CA resulted in a 10,000-fold reduction. Strains, 23GC, 1140DFO, 354DC and 1147DFM were less sensitive to 0.25% CA treatment, compared to the strains described above, and a 500,000-fold reduction was observed following 120 min of incubation. Only a 10-fold reduction for two strains, 1135DFO and 1136DFM, following 120 min incubation at 0.25% of CA was observed. However, a greater reduction at 100-fold and 1,000-fold respectively following 120 min for 1146DFM and 301BC were found.

An incremental increase of citric acid concentration up to 0.5% resulted in a decrease in bacterial survival (Figure 2.4. B). Within 15 min exposure to 0.5% CA an almost 100,000-fold reduction was achieved for strains 1147DFM and 1369DF, while for 1354DF strain a 50,000-fold reduction was observed after 20 min of 0.5% CA treatment. Strains, 2124GF, 1146DFM and 1135DFO showed between a 1,000-fold and 10,000-fold reduction following 40 min exposure to 0.5% CA. With regard to strain 23GC only a 100-fold reduction was obtained after 60 min of exposure to the disinfectant, while for the isolates: 1136DFM, 1140DFO and 354DF a 100,000-fold decrease was observed following 60 min of exposure.

Exposure to 1% CA, was more dramatic in its antimicrobial effect, with more than a 100,000-fold decrease in bacterial survivors observed after 5 min exposure.

Control tests were carried out in the absence of disinfectant for each of the tested strains. The percentage survival oscillated between 105% and 83%. In order to ensure better readability of the graphs, the control was shown only for the 2124GF strain, not for all 11 isolates.

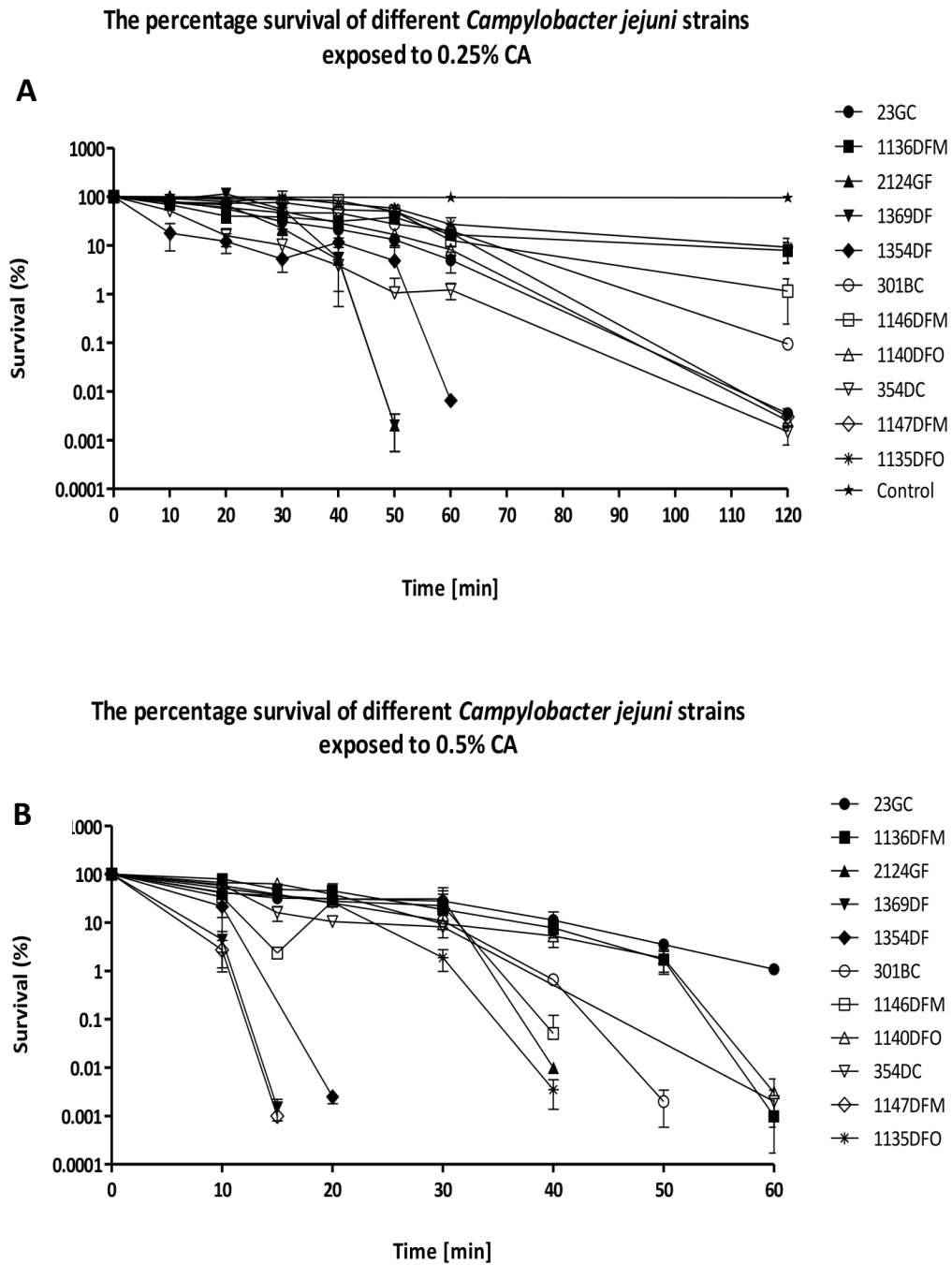


Figure 2.4. Time-kill kinetics for *Campylobacter* spp. strains exposed to 0.25% of CA (Graph A) and 0.5% of CA (Graph B). Error bars indicate standard deviation. In some cases they are present but cannot be seen.

2.4.5.2. Time-kill kinetics of lactic acid against *C. jejuni*

The agar MIC values for lactic acid (LA), for the selected 11 *Campylobacter jejuni* strains were obtained at 0.25%, with the exception of strain 23GC, where the MIC was detected between 0.125-0.25% (see Section 2.4.3.).

Figure 2.5. below, presents the time kill-kinetics for LA at MIC value of 0.25% and at 2-fold above. For strains 23CG and 1369DF, a 100,000-fold decrease of bacteria counts was observed following 20 min incubation in the presence of 0.25% LA, while for 2124GF a 50,000-fold reduction was obtained (Figure 2.5. A). Reduction at a level of 50,000-fold; 10,000-fold and 1,000-fold for 301BC, 1146DFM and 1354DF was achieved, respectively, after 40 min of exposure to the disinfectant. Strain 1140DFO was able to survive longer and a reduction at 50,000-fold was not observed until 120 min of the disinfectant exposure.

Doubling of the LA concentration resulted in a reduction of time-kill kinetics (Figure 2.5. B). Lactic acid at a concentration of 0.5%, showed a reduction by 100,000-fold for 23GC, 1147DFM and 354DF after 10 min incubation. The remaining strains demonstrated 10,000-fold or 100,000-fold reduction following 5 min of disinfectant exposure. These results when compared to CA results where a concentration of 0.5% was used, suggest that lactic acid could be a better disinfectant in terms of antimicrobial activity for *Campylobacter* spp. The range of time, where a 100,000-fold reduction for 0.5% CA was observed, was between 15-60 min, whereas for LA it was between 5-10 min.

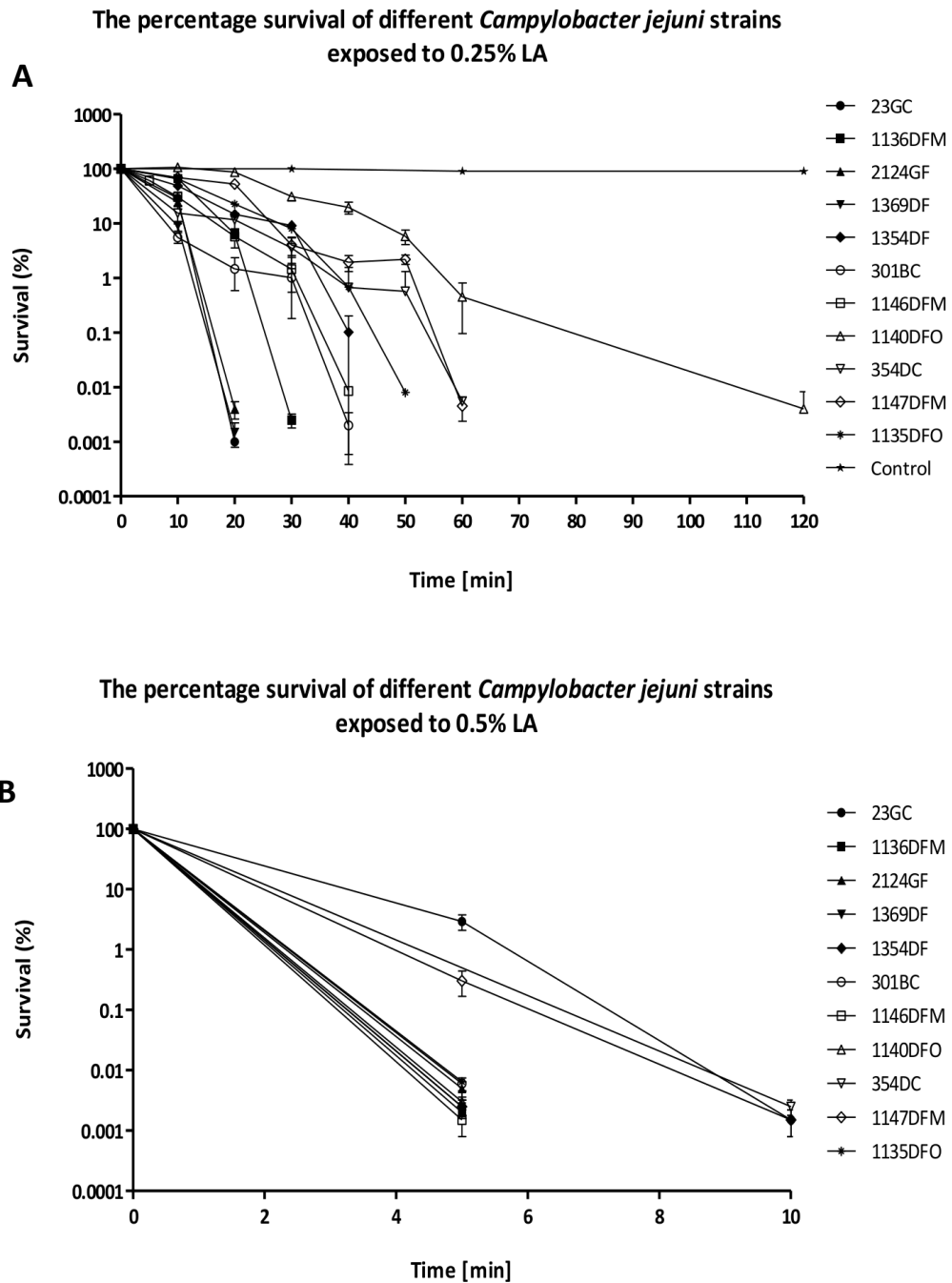


Figure 2.5. Time kill kinetics for *Campylobacter* spp. strains exposed to 0.25% of LA (Graph A) and 0.5% of LA (Graph B). Error bars indicate standard deviation. In some cases they are present but cannot be seen.

2.4.5.3. Time-kill kinetics of acidified sodium chlorite against *C. jejuni*

The agar MIC for acidified sodium chlorite (ASCh) for the 11 selected *C. jejuni* strains was between 500-850 $\mu\text{g ml}^{-1}$.

The effectiveness of ASCh in killing *Campylobacter* strains was studied at two concentrations: 850 $\mu\text{g ml}^{-1}$ and 1200 $\mu\text{g ml}^{-1}$ (Figure 2.6.). When exposed to the disinfectant at a concentration of 850 $\mu\text{g ml}^{-1}$, a 100,000-fold reduction in survivors was observed for the strains between 20 min to 60 min exposure.

During the investigation of the time-kill kinetics for 1200 $\mu\text{g ml}^{-1}$ of ASCh, 10 min exposure to disinfectant reduced bacteria counts by 50,000-fold for 1140DFO, while 20 min exposure resulted in a 10,000-fold decrease for 1146DFM and 1147DFM. The remaining strains showed a reduction of between 100- and 100,000-fold following 30 min incubation in the presence of disinfectant, with the lowest increase for 354DC and 1135DFO isolates.

2.4.5.4. Time-kill kinetics of trisodium phosphate against *C. jejuni*

The agar MIC for trisodium phosphate (TSPH) for the 11 selected *C. jejuni* isolates, varied from 0.3% to 1.25% depending on the strain tested (see Section 2.4.3.).

Figure 2.7. presents results for time-kill kinetics for 0.6% and 1.2% of TSPH. 100,000-fold reduction after 30 min exposure to 0.6% TSPH was observed for 1147DFM, while for 1140DFO the same fold reduction was observed following 40 min. Three strains: 1354DF, 1369DF and 1146DFM showed a 100,000-fold reduction following 60 min incubation, while remaining strains obtained a reduction of 10- and 100-fold following the same incubation time (Figure 2.7. A).

A 2-fold increase of TSPH concentration to 1.2% resulted in a significant decrease of time-kill kinetics. Seven of the tested *Campylobacter jejuni* strains: 301BC, 23GC, 2124GF, 1146DFM, 1140DFO, 354DC and 1147DFM, showed a 50,000-fold reduction following 10 min exposure (Figure 2.7. B). In the case of the 1369DF, 1136DFM and 1354DF isolates, this level of reduction was achieved after 15 min of incubation. Strain 1135DFO endured the longest with a 10,000-fold reduction obtained following 20 min of incubation.

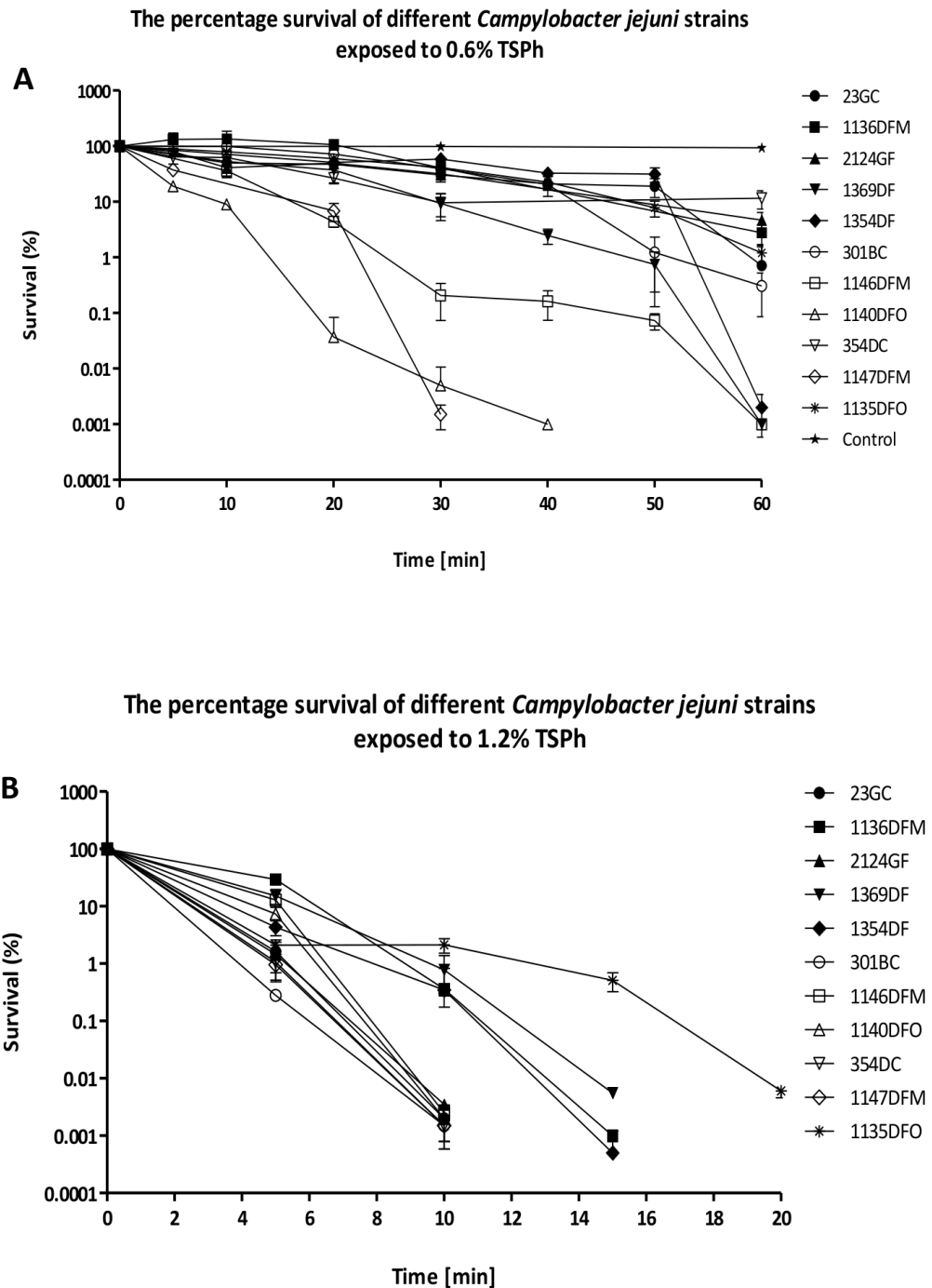


Figure 2.7. Time-kill kinetics for *Campylobacter* spp. strains exposed to 0.6% of TSPH (Graph B) and 1.2% of TSPH (Graph A). Error bars indicate standard deviation. In some cases they are present but cannot be seen.

To conclude, the time-kill kinetics results for the tested strains were dependent on the type of disinfectant used and its concentration. It was also strain specific as the tested disinfectants that belong to the same group of weak organic acids: CA and LA, gave different results for the survival ability of isolates. Strains that revealed a better ability to survive the presence of CA, did not necessarily show better survival to LA. The time-kill

kinetics for the four disinfectants that were performed at the bacterial MIC's values showed that these concentrations of disinfectants were not sufficient to kill bacteria or reduce them during short periods of time. The application of a higher concentration of disinfectants, however, resulted in a decrease of the time-kill of all strains tested.

2.4.6. Final selection of six *C. jejuni* strains for the long-term disinfectant adaptation study

From the pool of 11 *Campylobacter jejuni* strains selected for the time-kill kinetic study, six of them were chosen for long-term disinfectant adaptation studies. In this research, the *Campylobacter* strains were exposed to incremental concentrations of disinfectant over multiple generations using a chemostat technique, where their ability to adapt to and survive the disinfectants was determined. The results are presented in the next chapter (Chapter III).

The same amount of food and clinical isolates were selected for further study. All three clinical *C. jejuni* strains: 301BC, 23GC, 354DC were chosen. The choice of the remaining three strains was determined on the basis of their time-kill kinetics and the antibiotics MICs. Among the strains tested in the time-kill kinetics study, those that were either a sensitive or tolerant to the disinfectants were selected. In addition, the choice of the selected strains was supplemented with the information about the antibiotics MIC. It was decided that one strain with a high level of antibiotic resistance and two strains with a common MIC value would be selected. Thus, the following strains: 2124GF, 1136DFM, 1140DFO, 301BC, 23GC and 354DC were chosen for further study. These strains were used as a baseline reference point for the adaptation studies of disinfectants and for investigating possible antibiotic cross-resistance.

2.5. DISCUSSION

PFGE is considered to be a gold-standard method in epidemiology. This typing technique is commonly used to investigate *Campylobacter* outbreaks through identifying a source of infection (Clark *et al.*, 2003; Hanninen *et al.*, 2003; Olsen *et al.*, 2001). It has also been applied in monitoring critical points of cross contamination in farms and in processing plants (Hansson *et al.*, 2005; Potturi-Venkata *et al.*, 2007). *fla*-RFLP is also widely used as a typing method and discriminates well between isolates but due to intergenic rearrangements in the *fla* genes, this method could not have been used by itself (Petersen & Newell, 2001). Usually, *fla*-RFLP and PFGE are used together for *Campylobacter* spp. identification.

In this study, PFGE and *flaA* PCR-RFLP were used for genotypic characterisation of a set of 17 *Campylobacter* spp. strains, 13 poultry and 4 clinical isolates. These genotyping techniques allowed the genetic fingerprinting of the test strains and both of them give a good level of discrimination between isolates, although they differed in experimental approach. In PFGE, the whole genome was digested by the *Sma*I restriction enzyme, while in *flaA* PCR-RFLP typing only one locus, the *flaA* gene, was digested either by *Dde*I or *Hin*fl. These subtyping methods were used in this study as they have been found to be the most suitable methods for *Campylobacter* genotyping and have been widely used in other studies for strain characterisation (Camarda *et al.*, 2000; Fitzgerald *et al.*, 2001; Han *et al.*, 2007; O'Leary *et al.*, 2011; Ono *et al.*, 2003). From an initial pool of 17 strains, 15 of them were successfully identified. Two of the *C. jejuni* strains, 323BC and 1495BF, did not reveal any PFGE and *fla*-RFLP pattern during a standard preparation procedure. It is likely that these strains were DNase-positive.

Besides genotypic characterisation, phenotypic characterisation of *Campylobacter* spp. was also performed. In order to phenotypically characterise the initial pool of *Campylobacter* strains, the minimum inhibitory concentration (MIC) for four test disinfectants namely CA, LA, ASCh and TSPH was carried out. The disinfectants used in this study are highlighted in EU Regulation No. 853/2004 as suitable for poultry decontamination. These disinfectants were chosen, as the work presented here is mainly focused on investigating the risk assessment conducted with regards to their application as a decontaminant in farms and in processing plants. The results obtained for disinfectant susceptibility testing showed that

each of the tested disinfectants was able to inhibit *Campylobacter* growth almost at the same concentration. The narrow range of disinfectant concentration that inhibits the bacterial growth suggests that disinfectant application for decontamination purposes should effectively reduce the different strains of *Campylobacter*. This makes the disinfectants a potentially good antimicrobial agent. The MIC for antibiotics Tc (tetracycline group), Nx (quolone group) and Cipro (fluoroquolone group), that have been used for patient treatment with campylobacteriosis (Ge, 2002; Pichler *et al.*, 1986), were also investigated. The results obtained showed that the MICs for test antibiotics varied and were strain specific. Three *C. jejuni* strains, out of thirteen food isolates, were found to be highly resistant to one or two antibiotics tested, while all four clinical isolates revealed common MIC values that have been obtained for the majority of the test *Campylobacter* strains.

The time-kill kinetics, investigating the effectiveness of CA, LA and TSPH to reduce bacteria were performed at a concentration of MIC value and sub-MIC. Increasing the concentration of disinfectant resulted in a decrease in the time taken to reduce the level of *Campylobacter*. The concentrations of disinfectants analysed were lower in those recommended by EFSA for poultry farms and for processing plants decontamination. The CA and LA were tested at 0.25% and 0.5%, while the recommended dose for decontamination purposes is 2%. However, when the time-kill kinetics were performed for 1% CA, within 5 min a reduction by 5 log was observed for all strains. In the case of TSPH, tested doses were at 0.6% and 1.2%, while concentration typically used for poultry carcass decontamination is 8%-12% (EFSA, 2005).

The study of time-kill kinetics for ASCh was carried out for 850 $\mu\text{g ml}^{-1}$ and 1200 $\mu\text{g ml}^{-1}$ (the MIC was determined between 500-850 $\mu\text{g ml}^{-1}$). According to the results obtained, an application at a concentration of 1200 $\mu\text{g ml}^{-1}$ ASCh, still resulted in a good *Campylobacter* survival. A typical dose used for *Campylobacter* decontamination is between 500 $\mu\text{g ml}^{-1}$ and 1200 $\mu\text{g ml}^{-1}$ (EFSA, 2005). This suggests that the application of ASCh at 1200 $\mu\text{g ml}^{-1}$ might not sufficiently reduce the *Campylobacter* level. However, the effectiveness of acidified sodium chlorite is dependent on the concentration of citric acid, so that ASCh is activated. During the course of this study, the concentration of CA for ASCh activation was used at a lower concentration than its recommended for decontamination in farms and processing plants. The application of a lower concentration of CA resulted in decreasing

ASCh activity. As a result of it, this disinfectant (ASCh) possessed a reduced ability of bacterial decontamination, as its activity is a summary of decontamination properties associated with low pH (due to addition of CA) and with oxidative stress (due to release of HClO₂).

Analysis of the time-kill kinetics study for strains with high MIC value for antibiotics (2124GF 1354DF and 1135DFO) in a majority of cases did not show better survival compared to the remaining test isolates. Only *C. jejuni* 1135DFO which revealed an MIC above 128 µg ml⁻¹ for Nx and at 128 µg ml⁻¹ for Cipro showed slightly better survival at 850 µg ml⁻¹ and 1200 µg ml⁻¹ ASCh as well as to 1.2% TSPH. This suggests that the disinfectants used effectively inhibit *Campylobacter* growth independently to its antibiotics MICs.

This initial screening of *Campylobacter* spp. allowed familiarisation with them in their wild type state, before the study on *Campylobacter* adaptation to disinfectants was processed. Investigation, if disinfectants produce stronger and more pathogenic phenotypes, was carried out in a long-term chemostat culture challenged with disinfectant. The method and the results are described in the next chapter (Chapter III).

In addition, this initial screening allowed the selection of a set of 6 strains, among an initial pool of 17, for further study. This reduction in the number of test strains had to be carried out as studies on adaptation to four disinfectants were time-consuming. The long-term culture with disinfectant challenge was carried out between 845.8 h (35.5 days) and 1847.8 h (77 days). This long-term culture was required as *Campylobacter* is a slow-growing organism. The time that the bacteria needed for a quantity to double was 40 h. Each chemostat run took approximately 3 months to complete due to the complex nature of campylobacters.

To conclude, this initial study allowed for the genotypic fingerprinting and phenotypic characterising of *Campylobacter* spp. and for the chosen representative *Campylobacter* for the disinfectant adaptation study. Furthermore, the results obtained in this chapter have been used as a basic reference point regarding parent strains. Any changes in strains that have been obtained from the disinfectant adaptation study, presented in the following chapters, have been compared with these wild types. In addition, a cross-resistance study to antibiotics, between the wild types and the phenotype strains obtained from the adaptation study has been investigated.

Acknowledgments

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2.6. SUPPLEMENTAL MATERIALS

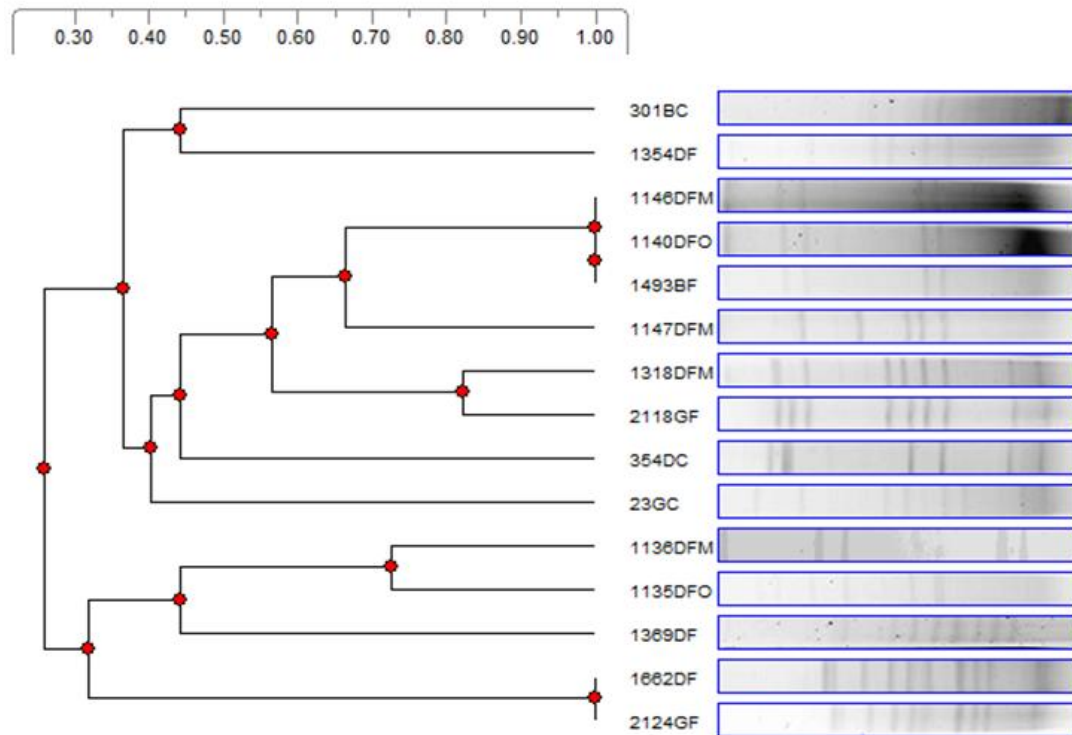


Figure S2.1. PFGE dendrogram of the *Campylobacter* spp. isolates.

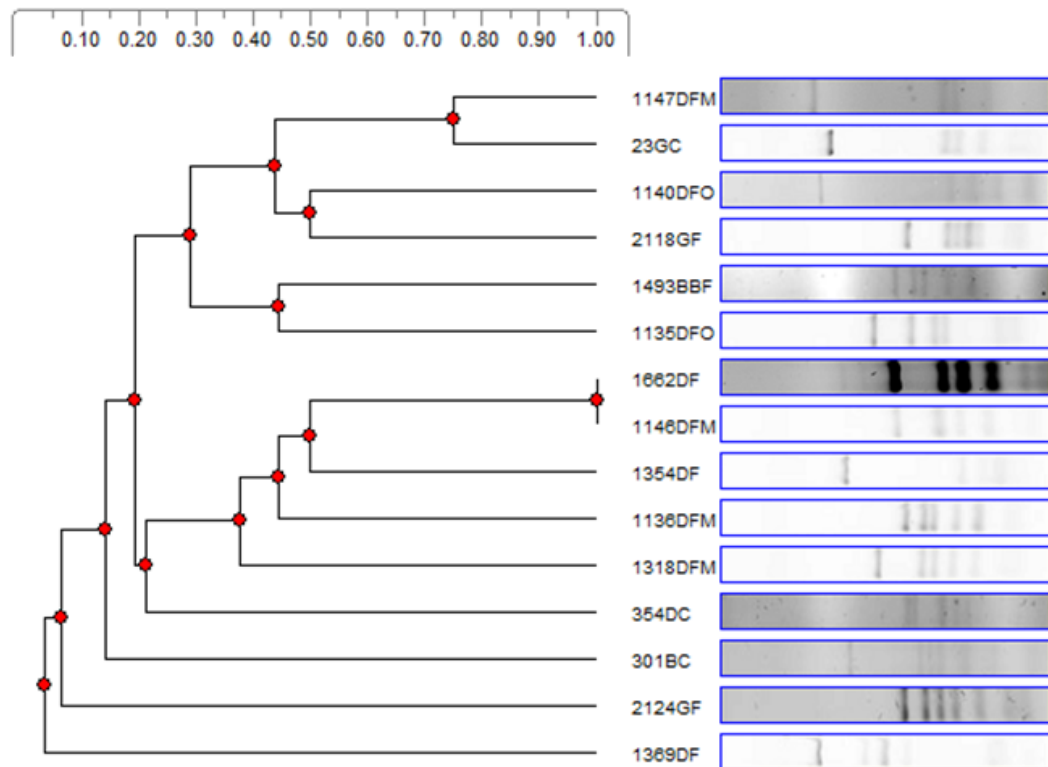


Figure S2.2. Dendrogram of *Campylobacter* spp. *flaA* gene digested by *DdeI* restriction enzyme.

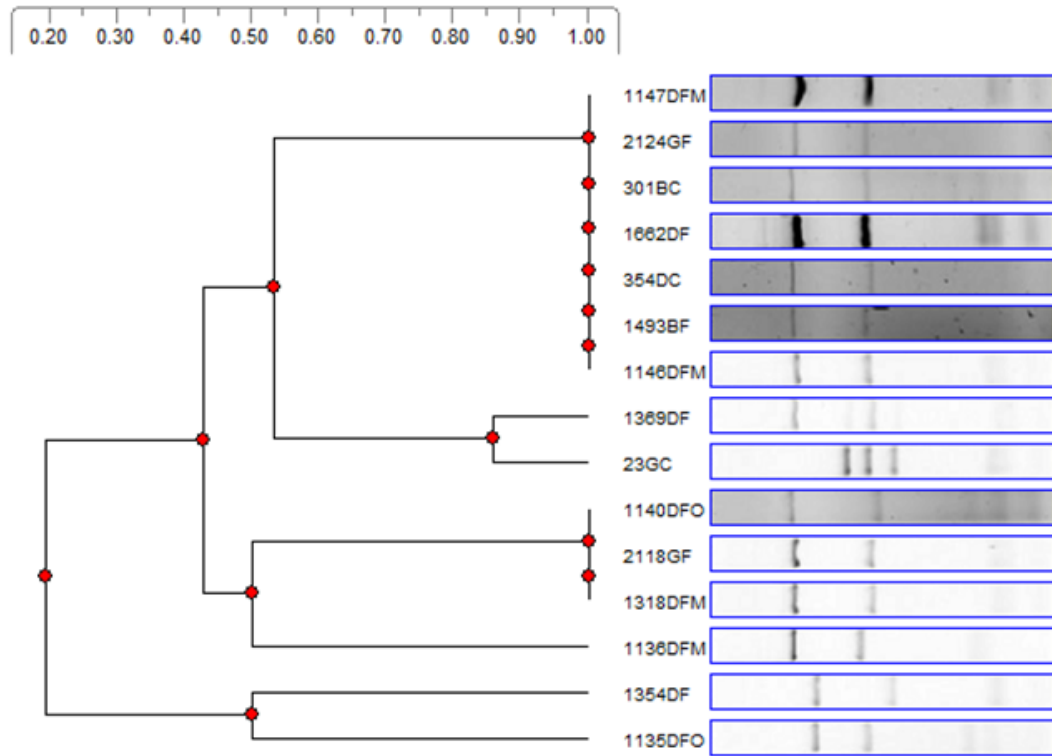


Figure S2.3. Dendrogram of *Campylobacter* spp. *flaA* gene digested by *HinfI* restriction enzyme.

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- <http://campynet.vetinst.dk/Fla.htm> (protocol for *flaA* PCR-RFLP analysis, viewed on the 31st of July 2013)

CHAPTER III

Study on *Campylobacter jejuni* adaptation to disinfectants using the chemostat technique

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Key words: chemostat technique; *Campylobacter*; disinfectants; ATR; cross-resistance.

3.1. SUMMARY

Campylobacteriosis is mainly associated with the consumption of contaminated, undercooked poultry products. To reduce the number of cases induced by campylobacters different strategies at the poultry farm and the processing plants have been generated. Besides fundamental methods of inhibiting and spreading the contamination (GHP and GMP), other solutions like the application of different disinfectants has been taken into consideration. However, at the moment in the EU the use of substances other than potable water, with the exception of lactic acid, are not commercially approved. Before approval of these substances, a risk assessment has to be carried out to determine if exposure of pathogenic bacteria, and among them *Campylobacter*, to sub-inhibitory concentrations of these disinfectants affects their virulence. During disinfection, the process may not always be performed adequately or organic debris may inactivate of the disinfectant. Bacteria then as a result of exposure to sub-lethal concentrations, may survive and adapt after repeated exposure. The use of sub-inhibitory concentration of disinfectants affect in decrease of their susceptibility, has been shown in other bacterial pathogens.

Work presented here, investigates the effect of long-term exposure to citric acid (CA), lactic acid (LA), acidified sodium chlorite (ASCh) and trisodium phosphate (TSPh) on *Campylobacter* survival and adaptation, and also to antibiotic cross-resistance. Long-term exposure to the disinfectants was performed using continuous culture conditions in a chemostat. Cultures were initially grown at sub-inhibitory levels of disinfectant and then incremental concentrations of antimicrobial agent were gradually added. Strains challenged with citric acid grew continuously at the MIC value and up to 1.4-fold and 2-fold above the MIC value depends on the test strain; a strain challenged with lactic acid grew at concentrations up to 2-fold above the MIC value; strains challenged with acidified sodium chlorite grew at concentrations up to 4-fold and 8-fold above the MIC value. In the case of trisodium phosphate, *Campylobacter* failed to grow at their MIC concentration. The strains obtained from the chemostat trials were also investigated to determine if acquired resistance to these disinfectants provided cross-protection against the activity of antibiotics tetracycline (Tc), ciprofloxacin (Cipro) and nalidixic acid (Nx). Depending on the strain tested and the disinfectant used, bacteria obtained from the chemostat culture increased or decreased its MIC values for the antibiotics. In addition, alterations in time-kill kinetics of *Campylobacter* strains challenged with the different disinfectants in the chemostat were performed.

3.2. INTRODUCTION

Poultry and poultry products are considered to be the main source of *Campylobacter* food poisoning (MacKichan *et al.*, 2004). In order to reduce the level of campylobacteriosis cases, different strategies to prevent the spread of contamination in poultry farms and processing plants have been recommended (Lin, 2009). The main strategies concentrate on maintenance of biosecurity, good hygienic practices (GHP) and good manufacturing practices (GMP). Extended interventions like the application of physical, chemical and biological treatments have also been considered (Hugas & Tsigarida, 2008; Loretz *et al.*, 2010). According to Directive 71/118/EC, in the European Union the use of substances other than potable water are not permitted (Del Río *et al.*, 2007; Loretz *et al.*, 2010). However EU Regulation (EC) No. 853/2004 laying down specific hygiene rules for food of animal origin, highlights chemicals that might be used for bacterial decontamination of the meat products but recommends withholding their use until a risk assessment is provided by the European Food Safety Authority and approved by the European Commission (Loretz *et al.*, 2010). The aim of this recommendation is to maintain the fundamental hygienic practises rather than opting for the use of antimicrobials that may hide human error or lead to a lack of proper training for personnel. The European Food Safety Authority (EFSA) with the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) reported that carcass decontamination by citric acid, chlorine dioxide, acidified sodium chlorite, trisodium phosphate and peroxyacids has no toxicological risk to human health (EFSA, 2005).

Before these disinfectants are approved by the European Commission, in addition to determining their decontamination effectiveness, a risk assessment on how the use of sub-inhibitory concentrations of these antimicrobial agents affect bacterial pathogen virulence also has to be investigated. The studies on bacterial response mechanisms to antimicrobial agents play a crucial role as there is a high risk, that application of inappropriate doses may produce more virulent strains. It has been shown in other food-borne pathogens (*Salmonella enterica*, *Escherichia coli*), that the use of sub-inhibitory concentrations of disinfectants leads to their adaptation and cross-resistance to other antimicrobials, (Braoudaki & Hilton, 2004; Braoudaki & Hilton, 2004; Karatzas *et al.*, 2007; Whitehead *et al.*, 2011). The studies on adaptation and cross-resistance to disinfectants are of great significance especially due to the fact that the antimicrobial mechanisms of disinfectant action, that alter the pH of the environment, are not fully understood due to their complex

nature. To date, the use of lactic acid to reduce microbiological surface contamination on bovine carcasses was adopted by the European Commission on the 4th of February 2013 and entered into force on the 25th February 2013 (<http://europa.eu/rapid/midday-express-04-02-2013.htm>).

The ability of bacteria to survive stress conditions is multifaceted and is dependent on both the nature of the stress and the bacterial species. In order to maintain the optimum cellular pH for growth between 6.5-7.5 under acid stress conditions campylobacters export a high level of protons from cytoplasm via the membrane (Chaveerach *et al.*, 2003). This mechanism requires adenosine triphosphate (ATP) and may cause a reduction of cellular energy. Another strategy indicates the existence of mechanisms that inhibit the influx of protons into the cell by inner membrane phospholipid modification (Reid, *et al.*, 2008b). As a result, electron transport is impossible and ATP production is reduced.

Other mechanisms indicate the presence of an adaptive tolerance response (ATR) in *C. jejuni* as a response to acidic stress (Murphy *et al.*, 2003). Early stationary phase cells adapted to pH 5.5 showed a 100-fold increase in tolerance to pH 4.5 in comparison with the survival of unadapted cells. Their study demonstrated that *C. jejuni* has the ability not only to induce an ATR to acid but also to a combination of aerobic and acid conditions. Between 100-fold and 1000-fold increase in survival as a result of the ATR was observed. Activation of ATR in response to acid stress, was also observed by Ma *et al.* (2008). ATR allowed *C. jejuni* to survive pH 4.5 and increased its ability to withstand further acid challenge for over 200 min in comparison to non-stressed cells. It has been reported that ATR to acid stress induces the up-regulation of general stress proteins DnaK (chaperone protein), GroEL (60 kDa chaperonin) (Murphy *et al.*, 2006). A study by Reid *et al.* (2008a) indicates further up-regulation of genes such as *clpB* (encodes ATP-dependent chaperone protein), *groES* (10 kDa chaperonin) and *grpE* (HSP-70 cofactor) as a response to acid stress.

The aim of the present study was to determine how long-term exposure of *Campylobacter* to disinfectants at sub-lethal concentrations affects their survival and adaptation. To achieve this, three food and three clinical *Campylobacter jejuni* isolates were exposed to increasing incremental concentrations of disinfectant using a chemostat technique. The effect of the exposure to the disinfectants on *C. jejuni* survival was determined by a time-kill kinetic analysis while the effect of disinfectant tolerance on cross-resistance to antibiotics was determined for tetracycline, nalidixic acid and ciprofloxacin.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial isolates

Six *Campylobacter jejuni* strains were chosen for this study. Isolates were collected either from food or from clinical sources. Strains 2124GF, 1136DFM and 1140DFO are food isolates (isolated from chicken), strains 354DC, 23GC and 301BC are clinical isolates, isolated from Dublin, Galway and Belfast respectively.

3.3.2. Disinfectants

Low-molecular weight organic acids, citric acid (CA, BDH) and lactic acid (LA, Sigma-Aldrich) were used to investigate possible changes in campylobacters' MICs as a result of long-term exposure and as well as to investigate their time-kill kinetics on *Campylobacter* chemostat derived strains challenged with these disinfectants. In addition, sodium chlorite - acidified with 0.16% w/v of citric acid (ASCh, Sigma-Aldrich), and trisodium phosphate (TSPH, Merck) were examined as a chlorine-based and phosphate-based compound, respectively (Hugas & Tsigarida, 2008).

3.3.3. Antibiotics

Ciprofloxacin (Bayer Schering Pharme), tetracycline (Sigma-Aldrich) and nalidixic acid (Sigma-Aldrich) were selected as representatives of the fluoroquinolone, tetracyclines and quinolone agents. They were used to determine a possible acquired cross-resistance in *Campylobacter* strains challenged with the different disinfectants under long-term continuous culture conditions. The antibiotics were tested at a concentration range of: 0.004 to 4 $\mu\text{g ml}^{-1}$ for ciprofloxacin, 0.015 to 128 $\mu\text{g ml}^{-1}$ for tetracycline and 0.06 to 128 $\mu\text{g ml}^{-1}$ for nalidixic acid.

3.3.4. Broth MIC for disinfectants

The microdilution broth method was carried out as approved by the CLSI (Clinical and Laboratory Standard Institute), formerly NCCLS (CLSI, 2002). The study was carried out in order to determine any differences in disinfectants' MICs, between agar and broth MICs

among wild type (WT) strains, before the long-term culture was established. 96 U-bottom well plates (Orange Scientific) containing 100 μl of increasing doubling concentration (in duplicate) of antimicrobial agent prepared in Mueller-Hinton Broth (MHB, LabM), were inoculated with 100 μl of overnight MHB culture adjusted to 0.5 McFarland. The plates were incubated at 40 °C in a Microtitre Plate Reader (TECAN Infinite™) while the optical density (OD) at 595 nm was measured every 2 hours for 48 hours. Each experiment was independently repeated twice using two separate cultures. When no results were obtained or different data was gained, the experiment was repeated more than twice. As a positive control, MHB without antimicrobial agents inoculated with *Campylobacter jejuni* was used, while MHB only, was used as a negative control.

3.3.5. Continuous culture

C. jejuni strains were grown under continuous culture conditions in MHB with the inclusion of disinfectant being gradually incremented. The chemostat vessels and apparatus were constructed based on the design described by Fleming *et al.* (1988). The chemostat culture was run in a 1 litre glass vessel (working volume 545 ml), stirred at 180 rpm and a constant temperature of 42°C was maintained by using an internal stainless steel heating coil, fed with water, controlled by a water heater (Figure 1.3. in Chapter I illustrates the chemostat system used throughout this study). The dilution rate was kept at 0.025 h⁻¹ the experiment. The first dose of disinfectant was added to the chemostat after culture stabilisation, initially at a sub-MIC concentration and thereafter incrementally at doubling concentrations or lower.

The concentrations of disinfectants used in the chemostat ranged from 0.008% to 0.18% or 0.5% for citric acid depending on the test strain; from 0.008% to 0.25% for lactic acid; from 0.75 $\mu\text{g ml}^{-1}$ to 200 $\mu\text{g ml}^{-1}$ for acidified sodium chlorite; and from 0.004% to 0.6 or 0.8% for trisodium phosphate depending on the test strain. Samples of the culture were routinely collected under aseptic conditions and stored at -80 °C in MHB and in the presence of 20% glycerol. The growth of the bacterial community was determined by measuring the optical density (OD) at 546 nm and 625 nm. In addition, the pH values were recorded throughout the duration of the chemostat run. As a control, a chemostat continuously run in the absence of disinfectant for strain 1136DFM was carried out.

Strains challenged with disinfectants under continuous culture conditions in a chemostat were termed as “Ch strains” i.e. Ch2124GF denotes *Campylobacter jejuni* strain 2124GF which was challenged with a disinfectant during the chemostat trial.

3.3.6. Maximum specific growth rate determination

The maximum specific growth rate (μ_{\max}) determination was carried out in a batch culture (in the chemostat vessel, with a peristaltic pump turned off) after the logarithmic growth phase had been obtained. The optical density (OD_{546}) was dropped to approximately 0.03 by inoculating the fermenter, via the sampling port, with sterile MHB heated to 42 °C. Samples were taken from the fermenter and OD was measured every 30 min for up to 8 h. From the plot, ($\mu_{\max} = \ln 2/t_d$, where μ_{\max} – maximum specific growth rate, t_d – doubling time) the μ_{\max} was calculated.

3.3.7. Pulse-Field Gel Electrophoresis of chemostat challenged strains

Pulse-Field Gel Electrophoresis (PFGE) was carried out on chemostat strains challenged with disinfectant, according to the Ribot's *et al.* (2001) protocol. The full protocol has been described previously in Chapter 2, Section 2.3.3. The only modification to the protocol was related to sample collection for the plug preparation. In this case, the direct culture taken from the fermenter, collected from the sampling port, at a volume of 1 ml was used. Following the electrophoresis, the patterns obtained were compared with these achieved for the wild types (non-challenged strains) to determine if changes in the PFGE patterns occur after the long-term disinfectant challenge. PFGE profile changes could indicate bacterial genome alteration or culture contamination.

3.3.8. Agar MIC determination for chemostat challenge stains

The agar dilution procedure was carried out as approved by the CLSI (Clinical and Laboratory Standard Institute, formerly NCCLS) to determine the antimicrobial susceptibility for the disinfectant challenged chemostat strains (CLSI, 2002). To prepare the inocula, the samples were collected from the fermenter via the sampling port, and the cell suspensions were adjusted to a 0.5 McFarland. Mueller–Hinton agar plates (MHA, LabM) supplemented with 5% horse blood (Charles River Laboratories, Inc.) and the appropriate concentrations of antimicrobial agent (disinfectant or antibiotic) were prepared, and

inoculated with 100 µl of the adjusted *Campylobacter* culture. The plates were incubated at 42°C under microaerobic conditions for 48 h. Results obtained were compared with those for the wild type strains in order to determine if possible acquired resistance provided cross-protection against the activity to antibiotics.

3.3.9. Time-kill kinetics study of chemostat challenged strains

After bacterial cells were challenged with disinfectant under long-term continuous culture conditions, the cells were examined in terms of their possible alterations in survival ability. Samples from the fermenter were directly collected aseptically via the sampling port and spread onto a *Campylobacter* blood-free selective agar base plates (CCDA, Oxoid). The plates were incubated for 48 h at 42 °C under microaerobic conditions. To recover bacteria from the disinfectant challenged chemostat culture, the bacteria were sub-cultured in a *Campylobacter* blood-free selective agar base at least twice more and incubated under microaerobic conditions for 48 h at 42 °C. To determine the time-kill kinetics on chemostat challenged strains, overnight culture incubated under microaerobic conditions at 42 °C, were taken. The full protocol has been described in Chapter II, Section 2.3.8.

3.3.10. Scanning electron microscopy

Scanning electron microscopy was carried out on *Campylobacter jejuni* Ch1136DFM “planktonic” and biofilm samples isolated from a chemostat run conducted without the use of disinfectant challenge (control chemostat). Samples of biofilm were collected from the sampling port of the fermenter vessel, from the heating coil immersed into the fermenter, the outlet and from the feeding tube at the end of the chemostat run (1750.8 h; 43.7 generations). Samples were resuspended in 1.5 ml of primary fixative solution (2% gluteraldehyde + 2% paraformaldefyde in 0.1M sodium cacodylate/HCl buffer pH 7.2) in 1.5 ml eppendorfs and were incubated overnight at 4 °C in order to fix the cells. The samples were centrifuged at 5,000 x g for 5 min and the supernatant was removed. Following this dehydration of sample using the gradient concentration of ethanol was carried out and solutions of 50%, 70%, 90% and 100% ethanol were used. The samples with different concentrations of ethanol were incubated for 5 min. Incubation for each ethanol concentration was repeated thrice. Then the pellets were allowed to dry in HMDS for 15 min. The drying step was repeated twice. The mechanical disruption of the sample was carried out before the cellular material was mounted onto 15 mm aluminium stubs (Hitachi

Ltd, Japan) and sputter coated with gold using an EMSCOPE SC500 coater under argon gas. Cells were then viewed using an HITACHI S-2600N Scanning Electron Microscope.

3.4. RESULTS

3.4.1. Broth MICs for the wild types *Campylobacter* strains

Before performing the long-term disinfectant challenge study, where the *Campylobacter jejuni* adaptation to disinfectant was investigated in a broth culture, the broth MICs were determined. Table 3.1. below presents results obtained for the broth MICs. In addition, the *Campylobacter* growth graphs (from Figure S3.1. to Figure S3.4.) generated by the Magellan Software from where the broth MIC was determined, are presented in the Supplemental Materials Section (Section 3.6.) in this chapter. Although the experiments were carried out at least twice, only an example graph for each strain exposed to each of the disinfectants are presented.

The broth MIC was carried out only for the 6 isolates that were chosen for the long-term disinfectant challenge study. The broth MICs data for CA ranged between 0.125-0.25%, while for LA it was between 0.06% and 0.125% (Table 3.1.). Results obtained for ASCh showed that the broth MICs oscillated between 25 $\mu\text{g ml}^{-1}$ and 100 $\mu\text{g ml}^{-1}$ depending on the *Campylobacter* isolate, while for TSPH was determined from 0.3% to 1.25%.

Table 3.1. Broth MIC results for *Campylobacter jejuni* wild type strains.

Strain	Broth MIC for disinfectants			
	CA [%]	LA [%]	ASCh [$\mu\text{g ml}^{-1}$]	TSPH [%]
2124GF	0.25	0.125	50	1.25
23GC	0.25	0.125	25	0.3
354DC	0.25	0.06	50	0.3
301BC	0.125	0.06	100	0.6
1136DFM	0.125	0.125	25	0.3
1140DFO	0.125	0.06	50	0.6

CA-citric acid; LA-lactic acid; ASCh- sodium chlorite acidified with 0.16% w/v of citric acid; TSPH- trisodium phosphate.

Comparison of the results obtained for broth MICs and those achieved for agar MICs (the agar MICs for all strains tested have been presented in the Chapter II, Section 2.4.3) indicate that the broth MICs for CA were the same as recorded for the agar MICs. A 2-fold decrease for all tested strains was observed when the broth MICs was carried out for LA. Also, a 2-fold reduction in broth MICs was obtained when compared with the agar MICs for strains *C. jejuni* 2124GF and *C. jejuni* 23GC tested to TSPH. In the case of broth MICs obtained for ASCh, a reduction by 5-fold for *C. jejuni* 301BC to 20-fold was obtained for *C. jejuni* 23GC and *C. jejuni* 1136DFM, when compared with the ASCh agar MICs.

3.4.2. Continuous culture study

The *Campylobacter jejuni* test strains were grown under continuous culture conditions (dilution rate at 0.025 h^{-1}) using a chemostat system. Culture conditions varied for each tested strain with regards to the type and concentration of disinfectant used, the duration of the culture run and the specific growth rate of the test strain (Table 3.2.). The maximum specific growth (μ_{\max}) for each strain was low, ranging from 0.25 h^{-1} to 0.4 h^{-1} . The duration of individual chemostat runs varied from 845.8 hours (21.1 generations) in the case of strain *C. jejuni* 2124GF exposed to CA to 1847.8 hours (46.2 generations) for *C. jejuni* 1140DFO exposed to TSPH.

In this study, the term “chemostat strain adapted to X dose of disinfectant” indicates, that the dose of disinfectant that the strain was grown in a chemostat culture was at the MIC value or above. Additionally, the term “chemostat strain challenged with X dose of disinfectant” indicates that the bacteria obtained from the chemostat culture, were grown below the MIC value.

Table 3.2. Summary of chemostat cultures' parameters for each *Campylobacter* strain.

Strain	μ_{\max} (h ⁻¹)	Total run time [h]	Total generations	Tested disinfectant	Wild type broth MIC value	Disinfectant concentration range in the chemostat	
						Initial	Final
2124GF	0.4	845.8	21.1	CA	0.25%	0.008%	0.5%
1136DFM	0.28	1051.6/1631.7*	26.9/40.7*	CA	0.125%	0.008%	0.18%
1136DFM	0.29	1316.2	32.9	ASCh	25 $\mu\text{g ml}^{-1}$	0.78 $\mu\text{g ml}^{-1}$	200 $\mu\text{g ml}^{-1}$
354DC	0.25	1213.8	30.3	ASCh	50 $\mu\text{g ml}^{-1}$	0.78 $\mu\text{g ml}^{-1}$	200 $\mu\text{g ml}^{-1}$
301BC	0.35	1313.7	32.8	TSPH	0.6%	0.004%	0.8%
1140DFO	0.4	1847.8	46.2	TSPH	0.6%	0.004%	0.6%
23GC	0.32	1456.6	36.4	LA	0.125%	0.008%	0.25%
1136DFM	0.29	1750.8	43.7	Control	-	-	-

μ_{\max} indicates the maximum specific growth rate before the long-term disinfectant challenge was examined. In the case of continuous culture performed with *C. jejuni* 1136DFM which was initially challenged with CA, values of total run time and total number of generations are presented (*). Two values are given. The first value represents strain following initial challenge with CA (for 1051.6 hours while 26.9 generations were obtained). Strain then was allowed to run for further 24.1 days (580 h) without CA selective pressure. The second value represents total run time (1631.7 h), while in total 40.7 generations were obtained.

***Campylobacter* adaptation to ASCh**

Figure 3.1. illustrates the optical density (OD) and pH alterations during the long-term chemostat culture runs of the test *Campylobacter* strains challenged with ASCh. An acidified sodium chlorite (ASCh) challenge was performed for two strains: *C. jejuni* 354DF as a representative of a clinical isolate (Figure 3.1. A) and *C. jejuni* 1136DFM as a representative of a food isolate (Figure 3.1. B). Following cultures stabilisation, the first sub-MIC dose at 0.78 $\mu\text{g ml}^{-1}$ ASCh was applied into the feed reservoir. As a result of ASCh application, the OD decreased as the bacteria were burdened but quickly adapted to the new conditions related to the presence of disinfectant and the OD increased again until the next incremental challenge. Arrows in the graphs indicate the time points in the continuous culture where the disinfectant was added. For both strains, 354DF and 1136DFM, challenged with ASCh the first dose was applied at 0.78 $\mu\text{g ml}^{-1}$ and gradually increased up to 200 $\mu\text{g ml}^{-1}$. The initial broth MIC for strain 354DF and 1136DFM for ASCh was obtained at 50 $\mu\text{g ml}^{-1}$ and 25 $\mu\text{g ml}^{-1}$. This indicates that *Campylobacter* strains adapted to the ASCh and were able to grow not only at MIC value but also up to 4-fold in the case of strain 354DF and up to 8-fold above the MIC in case of strain 1136DFM. In addition, the pH measurements during the chemostat run for the strain Ch1136DFM were recorded. The initial pH of the culture at 7.3 decreased marginally to 7.18, when 200 $\mu\text{g ml}^{-1}$ of the

disinfectant was applied. The pH measurements for the strain 354DF long-term ASCh challenged were not recorded.

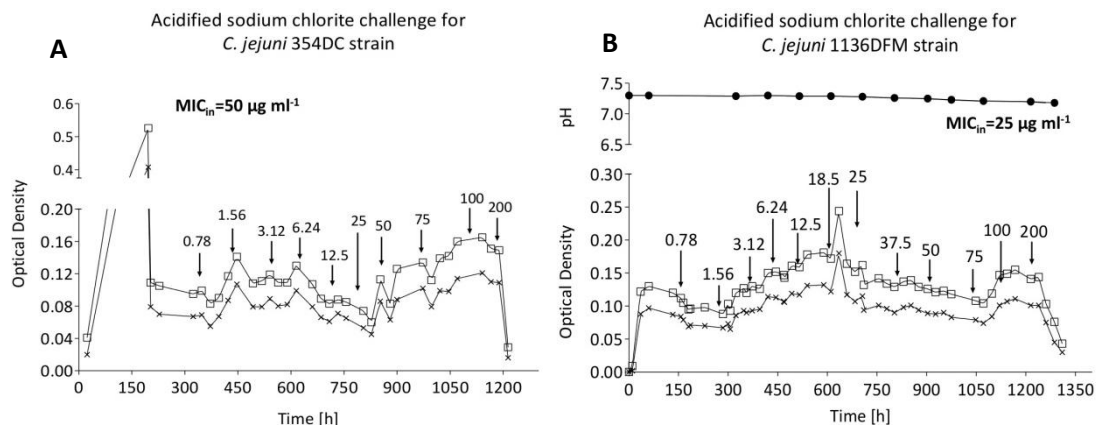


Figure 3.1. OD and pH changes during the long-term culture challenged with acidified sodium chlorite. Graph A presents changes for *C. jejuni* 354DC, while Graph B changes for *C. jejuni* 1136DFM. The optical density was measured at two different wavelengths: at λ 546nm (\square) and at λ 625nm (\times). Symbol (\bullet) indicates pH changes during the chemostat run. Also, the initial minimum inhibitory concentration for the broth (MIC_{in}) for the wild type counterparts are shown.

***Campylobacter* adaptation to TSPH**

Two strains, *C. jejuni* 301BC as a representative of a clinical isolate (Figure 3.2. A) and *C. jejuni* 1140DFO as a representative of a food isolate (Figure 3.2. B), were long-term challenged with trisodium phosphate (TSPH). Following the cultures stabilisation, the first sub-MIC dose at 0.004% TSPH was added. Upon each incremental challenge, the OD dropped as the bacteria were burdened but quickly adapted to the new concentration of disinfectant and the OD increased again until the next challenge. Arrows in the graphs indicate the time points in the continuous culture where the disinfectant was added. Dose at 0.6% TSPH added into the feed reservoir resulted in continuously decreased bacterial OD in the fermenter. The initial MIC for both strains challenged with TSPH was obtained at 0.6%. Results obtained from the long-term chemostat culture indicate that *Campylobacter* failed to grow at their MIC value. During the chemostat run for strain *C. jejuni* 301BC, the pH of the culture increased from the initial 7.2 in the absence of TSPH to 8.7 when TSPH was increased to concentration of 0.8%. Alterations for pH changes for 1140DFO while the TSPH challenge was investigated were expected to achieve the same range. The dilution rate during the long-term continuous culture for the *C. jejuni* 1140DFO strain was interrupted, in total for 143 hours (6 days). After 933 hours of the culture, bacteria started to wash out very quickly from the fermenter (OD₆₂₅ dropped from 0.024 to 0.013). Consequently, the pump was turned off (number A on the Figure 3.2. B) for 72 hours to prevent further

washing out. When *C. jejuni* started to grow afresh the pump was turned back on (number B on the Figure 3.2. B). The pump was turned off a second time (number C on the Figure 3.2. B) for 40 hours due to a technical problem.

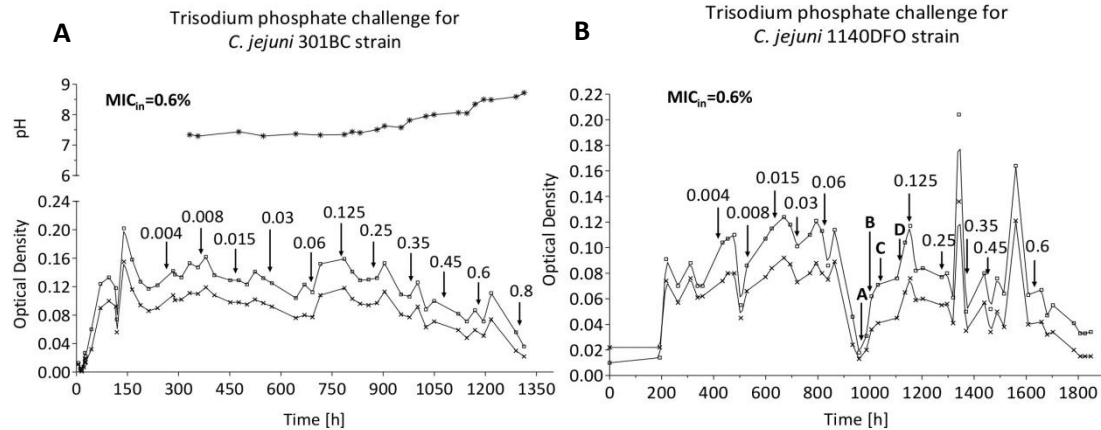


Figure 3.2. OD and pH changes during the long-term culture challenged with trisodium phosphate. Graph A presents changes for *C. jejuni* 301BC, while Graph B changes for *C. jejuni* 1140DFO. The optical density was measured at two different wavelength: at λ 546nm (\square) and at λ 625nm (\times). Symbol (\bullet) indicates pH changes during the chemostat run. Also, the initial minimum inhibitory concentration for the broth (MIC_{in}) for the wild type counterparts are shown. Letters A and C indicate on turning off the pump, while letters B and D indicate on turning on the pump.

***Campylobacter* adaptation to CA**

The adaptation study to citric acid (CA) was investigated for two food *Campylobacter* isolates, *C. jejuni* 2124GF (Figure 3.3. A) and *C. jejuni* 1136DFM (Figure 3.3. B). The first dose of citric acid added to the feed reservoir, after culture stabilisation, was at concentration of 0.008%. As a result of disinfectant addition, the OD of the culture initially decreased, but quickly increased again as bacteria adapted to the new conditions related to the presence of the disinfectant. Then a next dose of CA was added. The last dose of CA applied in the feed reservoir varied and was depended on the strain. While strain 2124GF was tested, the last dose added was at concentration of 0.5% CA but in the case of strain 1136DFM it was at 0.18% CA. Arrows in the graphs indicate the time when each of the CA doses were added. The broth MIC for CA obtained for strain 2124GF wild type, was at 0.25%. Gradually long-term exposure to incremental concentration of CA, resulted that strain 2124GF was able to growth at MIC value and up to 2-fold above. For strain 1136DFM, however, the broth MIC for CA was obtained at 0.125%. Following the long-term CA challenge in continuous culture, bacteria were able to sufficiently grow at the MIC value and up to 1.4-fold above it. During the chemostat runs, the pH of the culture for strains 2124GF and 1136DFM challenged with CA, decreased from the initial 7.2 to 4.5 and 5.0. When the

strains 2124GF and 1136DFM were challenged with CA, addition of 0.5% and 0.18% CA, respectively, resulted in a gradually increase of OD. In the case of the strain 1136DFM, the addition of 0.18% CA resulted in dropping the OD at 625 nm from 0.086 (907.1 h of the culture) to 0.020 (1051.4 h of the culture). Then the feed reservoir was replaced and a chemostat culture was carried out in the Mueller-Hinton broth in the absence of a disinfectant for a further 580 hours (24.1 days). Following replacement the media bacteria started to grow *de novo*. Within 170 hours (7 days) the OD₆₂₅ increased from 0.020 to 0.104.

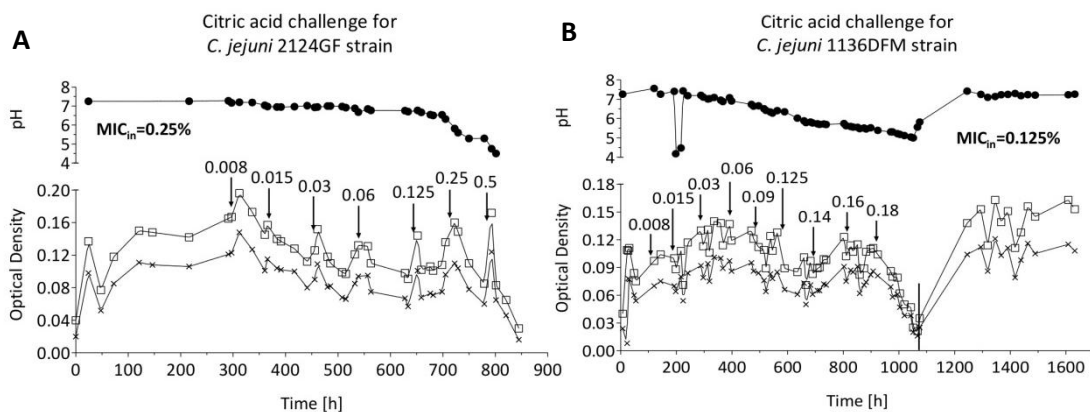


Figure 3.3. OD and pH changes during the long-term culture challenged with citric acid. Graph A presents changes for *C. jejuni* 2124GF, while Graph B changes for *C. jejuni* 1136DFM. The optical density was measured at two different wavelengths: at λ 546nm (□) and at λ 625nm (×). Symbol (●) indicates pH changes during the chemostat run. Also, the initial minimum inhibitory concentration for the broth (MIC_{in}) for the wild type counterparts are shown. The vertical line at 1051.4 hours of the graph B, indicates the time when the media reservoir was replaced and the culture was allowed to run for a further 24.1 days (580 hours) without CA selective pressure.

***Campylobacter* adaptation to LA**

The effect of the long-term lactic acid (LA) challenge was investigated on clinical isolates *C. jejuni* 23GC (Figure 3.4.). Following culture stabilisation, the first dose of LA was applied at 0.008%. The disinfectant addition resulted in the OD decreasing. When bacteria adapted and started to grow again, the next doses were applied. The final concentration added to the feed reservoir was at 0.25% LA. The initial broth MIC obtained for the wild type was at 0.125%. Results obtained from the long-term exposure to LA, indicate that the *Campylobacter* chemostat challenged strain were able to grow at MIC value and up to 2-fold above, at the same range as in the case of strain Ch2124GF exposed to CA. The initial pH of the culture decreased from 7.2 to 4.6 following the application of 0.25% lactic acid.

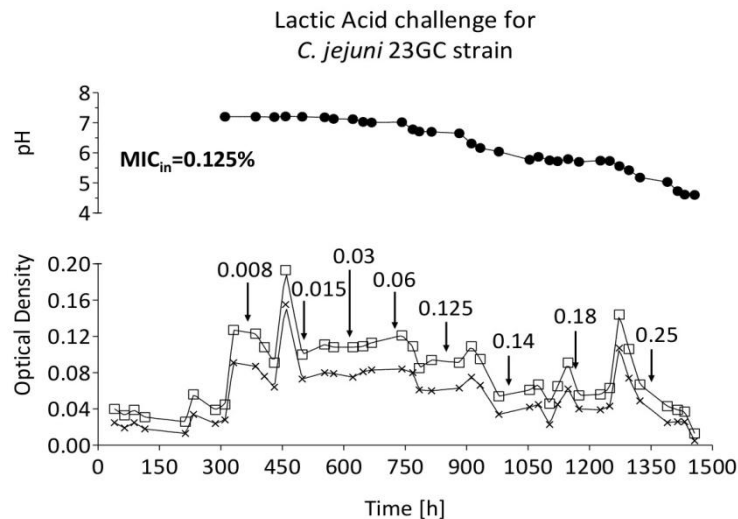


Figure 3.4. OD and pH changes during the long-term culture challenged with lactic acid. Graph presents changes for *C. jejuni* 23GC. The optical density was measured at two different wavelengths: at λ 546nm (\square) and at λ 625nm (\times). Symbol (\bullet) indicates pH changes during the chemostat run. Also, the initial minimum inhibitory concentration for the broth (MIC_{in}) for the wild type counterpart are shown.

Control chemostat

The control chemostat was performed for *Campylobacter jejuni* food isolate 1136DFM and was run without any disinfectant challenge. The culture was performed for total 1750.8 h, where 43.7 generations were obtained. The OD at 564 nm and 625 nm was measured and following the lag phase the OD_{546} was oscillating between 0.04 and 0.18 (Figure 3.5.). The pH was also measured during the chemostat trial and has been found to oscillate between 7.0-7.3 at the whole period of the chemostat culture. This control chemostat was used in order to investigate the effect of the long-term culture on MIC (this chapter), virulence gene expression (Chapter IV) and protein expression (Chapter V) alterations.

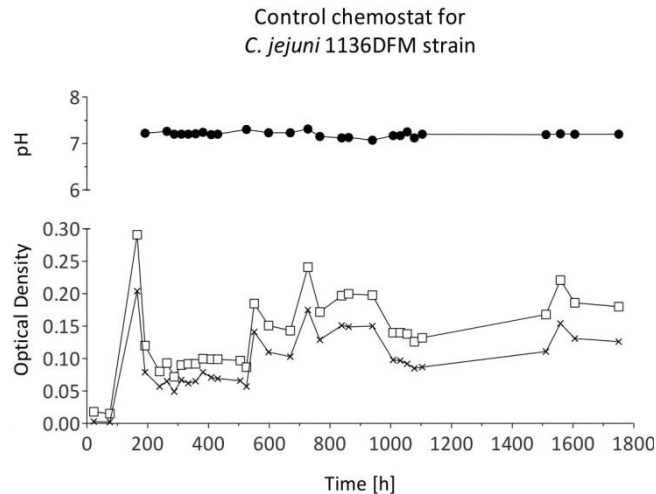


Figure 3.5. OD and pH changes during the long-term non-challenged culture. The optical density was measured at two different wavelengths: at λ 546nm (\square) and at λ 625nm (\times). Symbol (\bullet) indicates pH changes during the chemostat run.

3.4.3. Pulse-Field Gel Electrophoresis

The PFGE patterns obtained for the chemostat challenged strains to various disinfectants were compared with the PFGE patterns for their wild type (WT) counterparts. The PFGE patterns for the WT were described in Chapter 2, Section 2.4.1. The pattern similarity showed no changes for the strains with the exception of strain Ch2124GF exposed to CA. The reason for the PFGE pattern alteration in Ch2124GF was investigated. The PFGE results showed that all samples analysed at the different time points for the chemostat challenged strain Ch2124GF had the same pattern, a different pattern to that obtained for their WT. The patterns of Ch2124GF and the WT showed some missing bands in strains Ch2124GF exposed to different CA concentrations, possibly suggesting that the original WT could be a mixed culture. Differential sub-culture was performed in order to distinguish the morphological variety of bacteria. Two different types of colonies (big and small) were observed. Following isolation of these two types of colonies, the biochemical tests using the API Campy (BIOMÉRIEUX) was performed. The API test showed that the initial strain 2124GF was a mix of two cultures: *C. jejuni* and *C. coli*, while chemostat samples of the strain Ch2124GF were pure cultures of *C. jejuni*. In addition, the PFGE for *C. jejuni* 2124GF WT following differential sub-culture was carried out. No differences between *C. jejuni* 2124GF and chemostat strains were observed. Thus, the fermenter was inoculated with the mixed culture and two strains were initially present in the fermenter. In continuous culture competitive conditions, one strain was washed out (*C. coli*) and the experiment was carried out for *C. jejuni* only. No other changes of the pattern have been observed.

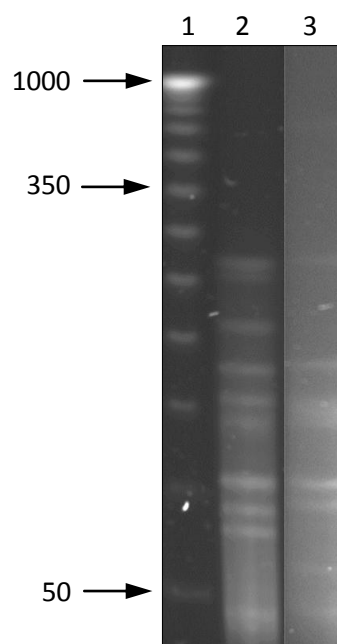


Figure 3.6. The PFGE results for *C. jejuni* 2124GF WT and Ch2124GF strains. Lane 1: Molecular Marker-ProMega Markers Lambda Ladders, Lane 2: *C. jejuni* 2124GF (WT), Lane 3: Ch2124GF challenge with 0.008% CA. The same pattern was obtained for all ranges of CA challenged Ch2124GF strains and only an example is presented above.

3.4.4. Time-kill kinetics study for disinfectant challenged chemostat strains

The long-term disinfectant challenge study showed that *Campylobacter* strains were able to efficiently grow at their MIC value and at values up to 1.4-fold to 8-fold above their MIC, with the exception of strains *C. jejuni* 301BC and *C. jejuni* 1140DFO exposed to TSPH. These results warranted an investigation into the effect of disinfectants on bacterial survival. Time-kill kinetics were determined for all the disinfectant challenged strains (collected at the end of the chemostat cultures) against the different disinfectants. These results were compared with those obtained for the wild types, in order to explore whether long-term sub-lethal exposure to disinfectants affected the bacteria's ability to survive disinfectant challenge *de novo*. The results obtained are presented below in Figure 3.7 to Figure 3.12.

3.4.4.1. The effect of citric acid on the survival of strains Ch2124GF, Ch1136DFM and their WT counterparts

Campylobacter strain Ch2124GF adapted to 0.25% citric acid (broken line in the Figure 3.7.) showed better survival in the presence of 0.25% and 0.5% citric acid than the original WT strain (solid line in the Figure 3.7.). A 3 log reduction (equivalent to 1000-fold), representing

99.9% killed bacteria, was obtained for strain 2124GF WT when the time-kill kinetics were performed at 0.25% CA after 45 min of incubation. The chemostat strain Ch2124GF adapted to 0.25% CA, however, required 95 min incubation with the presence of 0.25% CA to achieve the same 3 log reduction. This indicates that chemostat challenged strain were more resistant than its WT counterpart. In addition, following 50 min of the exposure to 0.25% CA the WT strain survival was reduced by 100,000-fold, while the Ch2124GF strain adapted to 0.25% CA showed almost 100% survival (Figure 3.7. B).

Increasing the concentration to 0.5% CA for the time-kill kinetics study showed reduction of the survival time for both the WT and chemostat challenged strain. A 3 log reduction was achieved after 35 min incubation for the WT, while for strain Ch2124GF adapted to 0.25% CA, the same level of reduction was observed after 55 min of incubation (Figure 3.7. B).

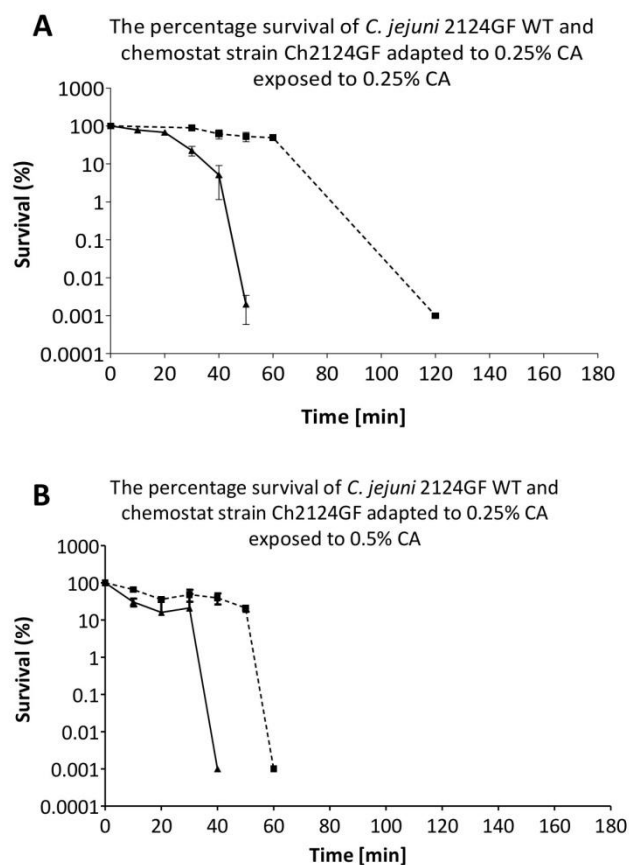


Figure 3.7. Time-kill curves of citric acid against *C. jejuni* 2124GF WT and Ch2124GF. The graphs demonstrate changes between strain *C. jejuni* 2124GF WT and strain Ch2124GF adapted to 0.25% CA, where 0.25% CA (graph A) and 0.5% CA (graph B) were examined. The solid line presents the results for the WT, while the broken line presents the results for the Ch2124GF CA adapted strains. Error bars are present but in some cases cannot be seen.

In the case of chemostat strain Ch1136DFM, the time-kill kinetics were carried out after removal of citric acid from the culture. In this instance the strain which was challenged with 0.18% citric acid, had the citric acid withdrawn and the strain was further cultured continuously for 24.1 days without citric acid challenge.

The time-kill kinetics study performed at 0.25% CA showed that the WT had a 3 log reduction achieved following 180 min exposure, while in the case of strain Ch1136DFM after the same exposure period only 1 log reduction was observed (Figure 3.8. A). This indicates that the chemostat challenged strain survived 100-fold better than its WT counterparts.

Increasing the concentration of CA to 0.5% for the time-kill kinetics study, resulted in a reduction of time to achieve a 3 log reduction. This level of reduction for the WT was achieved after 50 min of exposure, in comparison to 90 min exposure for the strain Ch1136DFM (Figure 3.8. B). This indicates that strain Ch1136DFM is able to survive 1.8-times longer than its WT counterpart. In addition, after 60 min of 0.5% CA exposure, a 100,000-fold of reduction for the WT was observed, while for the Ch1136DFM only a 100-fold reduction was observed.

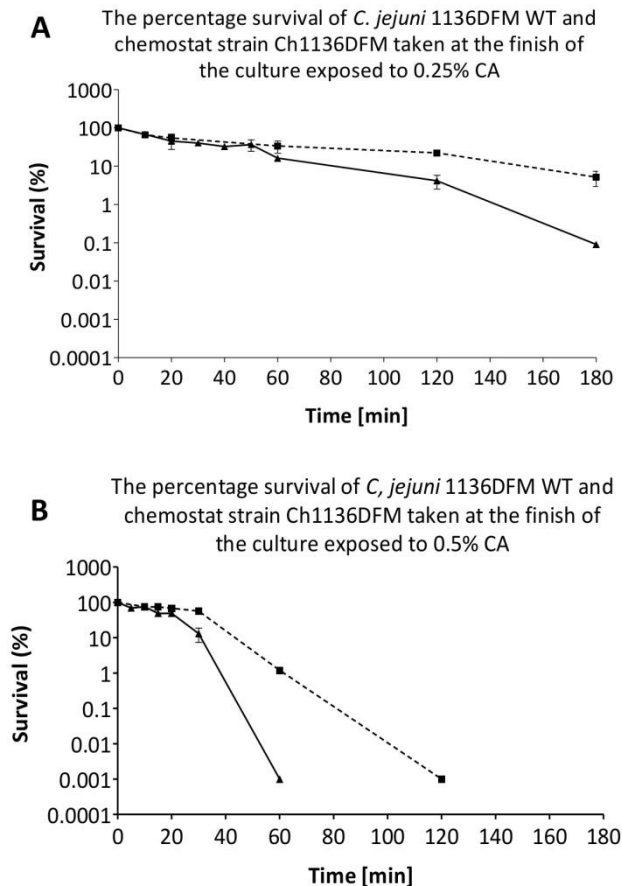


Figure 3.8. Time-kill curves of citric acid against *C. jejuni* 1136 WT and Ch1136DFM. The graphs demonstrate changes between strain *C. jejuni* 1136DFM WT and strain Ch1136DFM taken at the finish of the culture strains, where 0.25% CA (graph A) and 0.5% CA (graph B) were examined. The solid line presents the results for the WT, while the broken line presents the results for the Ch1136DFM CA adapted strains. Error bars are present but in some cases cannot be seen.

In summary, the long-term CA challenged at the MIC and at the sub-MIC concentrations for both strains, 2124GF and 1136DFM, resulted in generating bacteria that were able to better survive to a further CA challenge. The chemostat challenged strains were more resistant than their WT counterparts.

3.4.4.2. The effect of lactic acid on the survival of strain Ch23GC and its WT counterpart

Figure 3.9. presents the survival kinetics for strain Ch23GC adapted to 0.18% lactic acid and its unchallenged WT counterpart strain when exposed to 0.25% and 0.5% lactic acid. According to results obtained, the chemostat challenged bacteria were twice as susceptible to lactic acid exposure at these concentrations than their WT counterpart. When a concentration of 0.25% LA was used for the time-kill kinetic study, a 3 log reduction was

achieved following 6 min of incubation for strain Ch23GF adapted to 0.18% LA, while for its WT counterpart this level of reduction was not achieved until 15 min of incubation (Figure 3.9. A).

Strain Ch23GF adapted to 0.18% LA, achieved 3 log reduction in less than 5 min, when a concentration of 0.5% LA was examined, while the WT have the same level of kill after 8 min of exposure (Figure 3.9. B). Results obtained from the lactic acid time-kill kinetics analysis indicate that although strain Ch23GC could growth up to 2-fold above the MIC, the long-term culture did not produce more resistant bacteria able to survive unfavourable conditions longer.

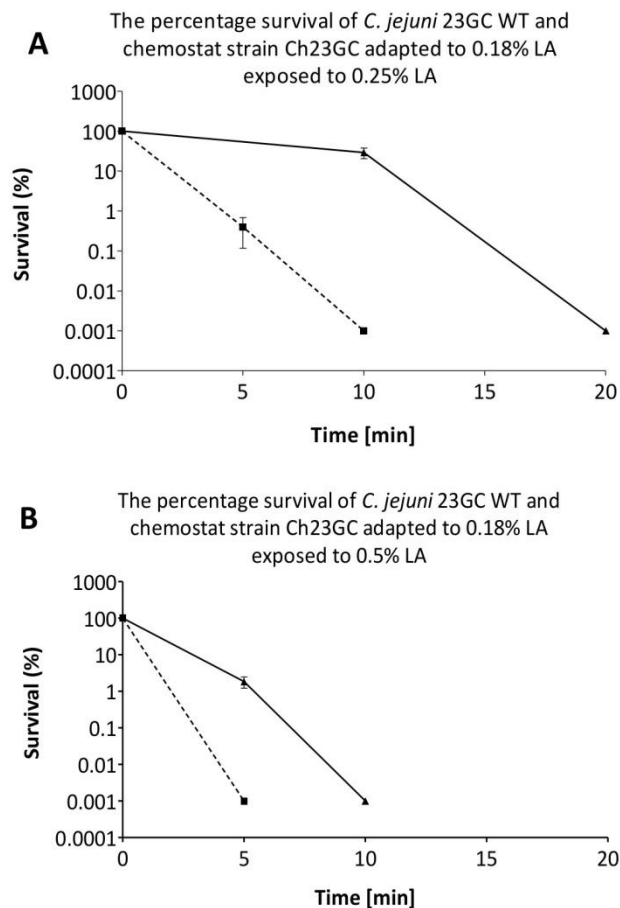


Figure 3.9. Time-kill curves of lactic acid against *C. jejuni* 23GC WT Ch23GC. The graphs demonstrate changes between strain *C. jejuni* 23GC WT and strain Ch23GC adapted to 0.18% LA, where 0.25% LA (graph A) and 0.5% LA (graph B) were examined. The solid line presents the results for the WT, while the broken line presents the results for the Ch23GC LA adapted strains. Error bars are present but in some cases cannot be seen.

3.4.4.3. The effect of acidified sodium chlorite on the survival of strains Ch354DC, Ch1136DFM and their WT counterparts

Figure 3.10. presents the survival kinetics for Ch354DC adapted to $100 \mu\text{g ml}^{-1}$ of ASCh and the unchallenged WT strain when exposed to $850 \mu\text{g ml}^{-1}$ (Figure 3.10. A) and $1200 \mu\text{g ml}^{-1}$ (Figure 3.10. B) of ASCh. According to the time-kill kinetics results, chemostat strain Ch354DC adapted to $100 \mu\text{g ml}^{-1}$ ASCh, showed a 3 log reduction (representing a 99.9% of bacterial reduction) after 55 min exposure to $850 \mu\text{g ml}^{-1}$ ASCh, while its WT counterpart showed the same log reduction after only 40 min of exposure (Figure 3.10. A).

Increasing the concentration of ASCh to $1200 \mu\text{g ml}^{-1}$, resulted in no major differences in survival in comparison with results obtained using $850 \mu\text{g ml}^{-1}$ ASCh. However, the strain Ch354DC adapted to $100 \mu\text{g ml}^{-1}$ ASCh showed a 10-fold better survival than its wild type counterpart (Figure 3.10. B).

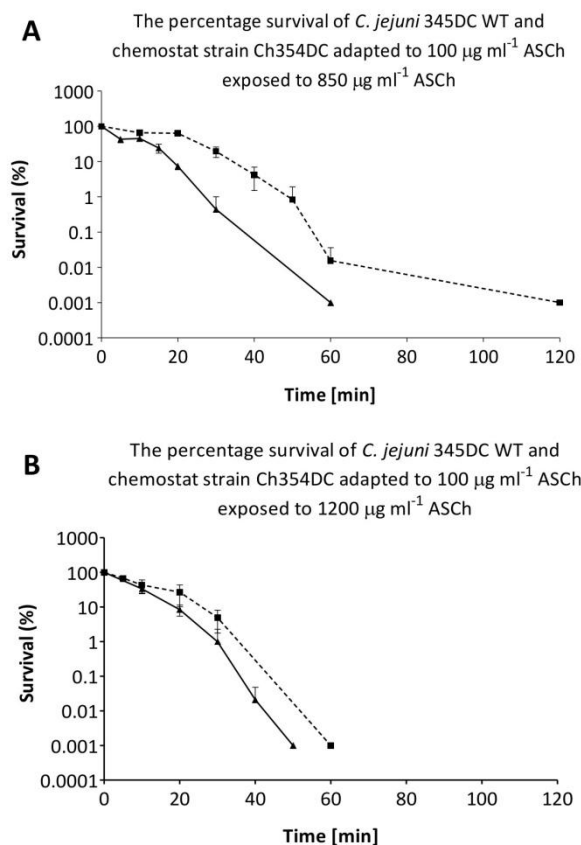


Figure 3.10. Time-kill curves of acidified sodium chlorite against *C. jejuni* 354DC WT and Ch354DC. The graphs demonstrate changes between strain *C. jejuni* 354DC WT and Ch354DC adapted to $100 \mu\text{g ml}^{-1}$ ASCh strains, where $850 \mu\text{g ml}^{-1}$ (graph A) and $1200 \mu\text{g ml}^{-1}$ (graph B) were examined. The solid line presents the results for the WT, while the broken line presents the results for the Ch354DC ASCh adapted strains. Error bars are present but in some cases cannot be seen.

In the case of strain Ch1136DFM adapted to $100 \mu\text{g ml}^{-1}$ of ASCh, initially a 100-fold better survival was observed when compared to its wild type variant (Figure 3.11. A). However, after 60 min of the exposure to $850 \mu\text{g ml}^{-1}$ ASCh, both strains (WT and Ch1136DFM adapted to $100 \mu\text{g ml}^{-1}$ ASCh) had the same level of survival. When a higher concentration of disinfectant ($1200 \mu\text{g ml}^{-1}$) was examined, the Ch1136DFM adapted strain showed 1000-fold better survival following 30 min of disinfectant exposure than its WT counterpart.

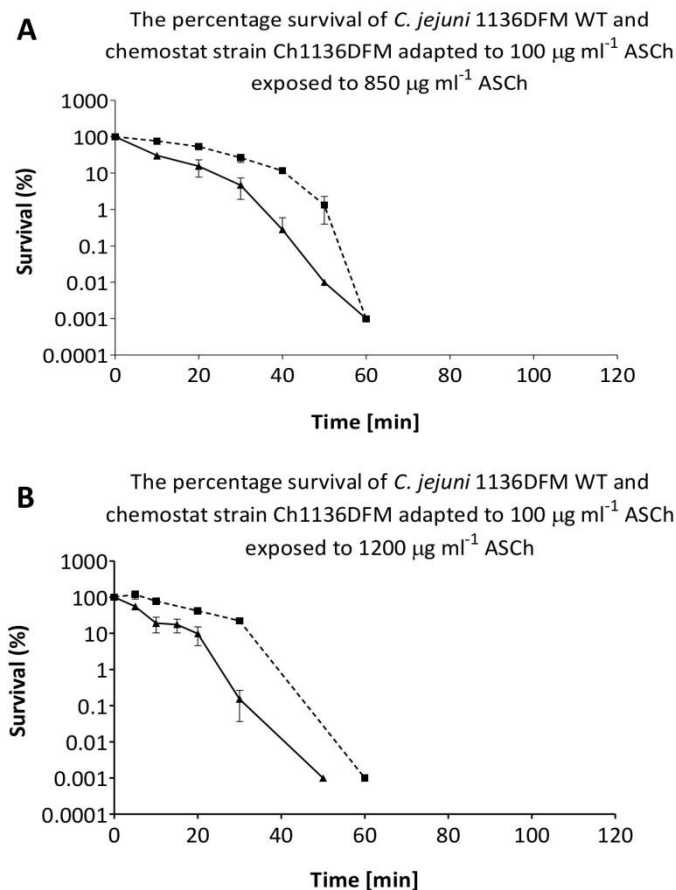


Figure 3.11. Time-kill curves of acidified sodium chlorite against *C. jejuni* 1136DFM WT and Ch1136DFM. The graphs demonstrate changes between strain *C. jejuni* 1136DFM WT and Ch1136DFM adapted to $100 \mu\text{g ml}^{-1}$ ASCh strains, where $850 \mu\text{g ml}^{-1}$ (graph A) and $1200 \mu\text{g ml}^{-1}$ (graph B) were examined. The solid line presents results for the WT, while the broken line presents the results for the Ch1136DFM ASCh adapted strains. Error bars are present but in some cases cannot be seen.

To conclude, the long-term culture with a gradual increase of *Campylobacter* exposure to ASCh, revealed strains with slightly better survival ability to the disinfectant, compared to the WT.

3.4.4.4. The effect of trisodium phosphate on the survival for strain Ch301BC, Ch1140DFO and their WT counterparts

Figure 3.12. below, demonstrates the time-kill kinetics results obtained for strain Ch301BC challenged with 0.6% of TSPH (Figure 3.12. A) and strain Ch1140DFO challenged with 0.6% of TSPH (Figure 3.12. B) when exposed to 0.6% TSPH (their initial MIC value).

A 3 log reduction for strain 301BC WT was observed following 70 min of exposure to 0.6% TSPH, while the strain Ch301BC challenged with 0.6% TSPH, the same level of reduction achieved following 150 min of the exposure to the disinfectant. This indicates that chemostat challenged bacteria became 2-times more resistant when compared with its WT. After incubation for 120 min, the WT strain showed a reduction of 100,000-fold (equivalent to 5 log reduction), while the chemostat TSPH challenged strain, showed only a 50-fold reduction after the same exposure interval.

In addition, the chemostat adapted strain Ch1140DFO challenged with 0.6% TSPH survived 3 times longer in the presence of 0.6% TSPH than its WT counterpart. Following 40 min the TSPH exposure the WT showed 100,000-fold reduction, while the chemostat challenged strain only showed a 100-fold reduction. These results are more interesting when the fact that both of these strains could not grow in a chemostat at their MIC value at 0.6% TSPH.

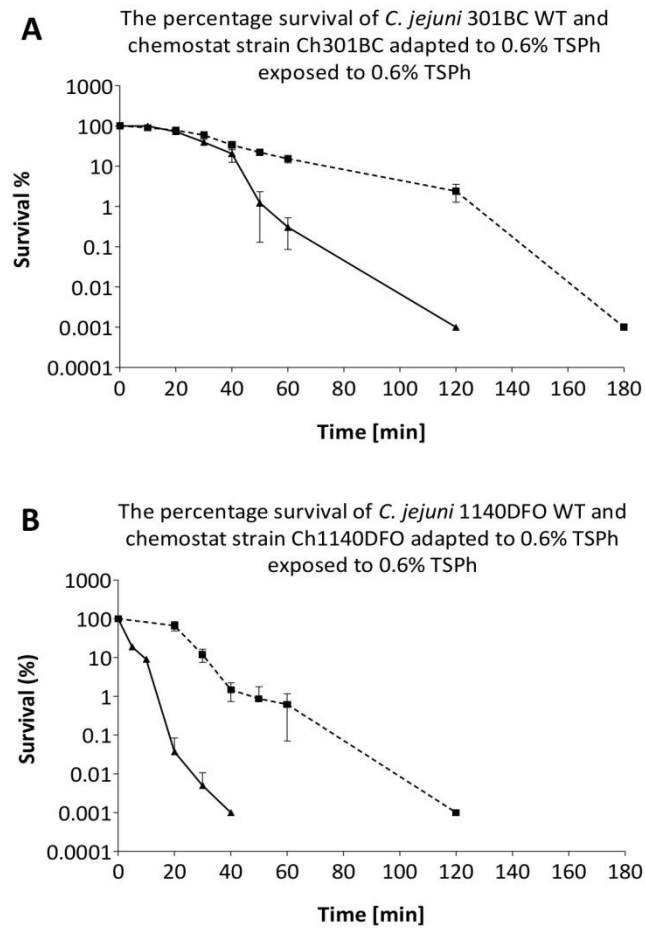


Figure 3.12. Time-kill curves of trisodium phosphate against *C. jejuni* 301BC and Ch1140DFO. The graphs demonstrate changes between strain Ch301BC challenged with 0.6% TSPH (graph A), strain Ch301BC challenged with 0.6% TSPH (graph B) and their WT counterparts where 0.6% of TSPH were examined. The solid line presents results for the WT, while the broken line presents the results for the chemostat TSPH challenged strains. Error bars are present but in some cases cannot be seen.

From the results obtained for all the time-kill kinetics experiments, it might be concluded, that the long-term exposure to CA, ASCh and TSPH enable these bacterium to survive longer to subsequent disinfectant exposure. In the case of the strain Ch23GC, that was growing in the chemostat at MIC value and up to 2-fold above, this strain did not show increased survival to lactic acid as a time-kill kinetics results demonstrate.

3.4.5. Alterations of antibiotics' MIC of *Campylobacter* strains exposed to disinfectant: a cross-resistance study

The MIC for *Campylobacter* chemostat strains that grew in the presence of disinfectant, were investigated with regard to their resistance to antibiotics/disinfectants as the long-term exposure of *C. jejuni* to sub-lethal doses of disinfectant might have had an influence on MIC alteration to antibiotics/disinfectants. Tables 3.3.–3.6. present the MIC values for disinfectants and antibiotics for WTs and chemostat challenged/adapted disinfectant strains.

Antimicrobial susceptibility for chemostat CA challenged strains

Campylobacter cells were re-examined for their MIC to the different disinfectants and to the antibiotics, tetracycline, nalidixic acid and ciprofloxacin after long-term exposure to increasing concentration of disinfectant, in order to investigate possible changes with their resistance/cross-resistance. Table 3.3. below shows the MIC values of the different disinfectants and antibiotics for two chemostat strains Ch2124GF and Ch1136DFM after being challenged with citric acid. MIC values for the wild type stains are also presented. The results show no MIC alteration for CA, LA and ASCh. In the case of TSPH, a 2-fold reduction in MIC value was observed between the WT and strain Ch2124GF adapted to 0.5% CA, indicating no significant changes. For strain Ch1136DFM challenged with 0.008% TSPH, an 8.3-fold reduced susceptibility was observed in comparison with the WT strain. In the case of antibiotics MICs, no cross-resistance for strains Ch2124GF and Ch1136DFM challenged with citric acid was obtained. However, a 4-fold increase in the Nx MIC was observed between 1136DFM WT and Ch1136 Finish (sample collected at the end of the chemostat, that had been carried out for 580 hours without presence of CA) was observed.

Table 3.3. Antimicrobial susceptibility for chemostat Ch2124GF and Ch1136DFM strains challenged with citric acid and their WT counterpart.

WT vs. chemostat challenged strains for CA	Agar MIC						
	Tc [$\mu\text{g ml}^{-1}$]	Nx [$\mu\text{g ml}^{-1}$]	Cipro [$\mu\text{g ml}^{-1}$]	ASCh [$\mu\text{g ml}^{-1}$]	CA [%]	LA [%]	TSPH [%]
2124GF WT	>128	8	0.125	500	0.125	0.25	0.6
Ch2124GF 0.008%	256	8	0.06	850	0.25	0.25	0.6
Ch2124GF 0.125%	256	8	0.06	850	0.25	0.25	1.25
Ch2124GF 0.25%	<16	8	0.06	850	0.25	0.25	0.6
Ch2124GF 0.5%	<16	4	0.03	850	0.25	0.25	0.3
1136DFM WT	0.25	2	0.06	500	0.125-0.25	0.25	0.3
Ch1136DFM 0.008%	0.25	8	0.008	500	0.25	0.25	2.5
Ch1136DFM 0.125%	0.25-0.5	4	0.06	500	0.25	0.25	2.5
Ch1136DFM 0.18%	0.5	8	0.06	500	0.25	0.25	1.25
Ch1136DFM Finish	0.25	8	0.06	850	0.25	0.25	0.6

Tc - tetracycline; Nx - nalidixic acid; Cipro - ciprofloxacin; CA - citric acid; LA - lactic acid; ASCh - acidified sodium chlorite; TSPH - trisodium phosphate. Each experiment was repeated twice (n=2). If results obtained varied, the range of the MIC values are presented.

Antimicrobial susceptibility for chemostat LA challenged strain

Table 3.4. below presents the MIC alterations for strain Ch23GC challenged with LA, collected at different tested LA conditions of the culture. No changes in MIC values for disinfectants were observed, with the exception of 8-fold reduction of the susceptibility for CA in all lactic acid chemostat challenge strains. In addition, an increase in susceptibility at 8-fold, 2.5-fold and 4-fold for Tc, Nx and Cipro was determined, respectively, indicating no cross-resistance had occurred.

Table 3.4. Antimicrobial susceptibility for chemostat Ch23GC challenged with lactic acid and its WT counterpart.

WT vs. chemostat challenged strains for LA	Agar MIC						
	Tc [$\mu\text{g ml}^{-1}$]	Nx [$\mu\text{g ml}^{-1}$]	Cipro [$\mu\text{g ml}^{-1}$]	ASCh [$\mu\text{g ml}^{-1}$]	CA [%]	LA [%]	TSPH [%]
23GC WT	0.5	4	0.125	500	0.25	0.25	0.3
Ch23GC 0.015%	0.25	4	0.06	500	0.06	0.125	0.3
Ch23GC 0.03%	0.06	1.56	0.03	500	0.06	0.125	0.6
Ch23GC 0.06%	0.06	1.56	<0.03	500	0.06	0.125	0.6
Ch 23GC 0.125%	0.06	3.06	0.03	500	0.06	0.125	0.3
Ch23GC 0.18%	0.06	1.56	0.03	500	0.06	0.125	0.3

Tc – tetracycline; Nx – nalidixic acid; Cipro – ciprofloxacin; CA - citric acid; LA – lactic acid; ASCh – acidified sodium chlorite; TSPH – trisodium phosphate. Each experiment was repeated twice (n=2)

Antimicrobial susceptibility for chemostat ASCh challenged strains

Two strains, 1136DFM (food isolate) and 354DC (clinical isolate) were used to investigate their adaptation to ASCh. As a result of carrying out the long-term disinfectant challenge culture, bacteria were able to grow above the broth MIC value, that was obtained at $25 \mu\text{g ml}^{-1}$ for 1136DFM WT and $50 \mu\text{g ml}^{-1}$ for 354DF WT. The cross-resistance study, however, was performed for the agar MIC (the agar MIC was achieved at $500 \mu\text{g ml}^{-1}$ for 1136DFM WT and $850 \mu\text{g ml}^{-1}$ for 354DF WT). Due to differences between agar and broth MIC for ASCh, no agar MIC changes for ASCh were observed (even though the chemostat challenged strains were able to grow above the MIC value in broth). In addition, no changes in MIC values for CA and LA for Ch1136DFM and Ch354DC strains challenged with ASCh were observed (Table 3.5.). An 8-fold increase to TSPH between 1136DFM WT and Ch1136DFM challenged with $6.24 \mu\text{g ml}^{-1}$ ASCh, Ch1136DFM adapted to $25 \mu\text{g ml}^{-1}$ ASCh and Ch1136DFM adapted to $50 \mu\text{g ml}^{-1}$ ASCh was observed, while a 4-fold increase to TSPH

for Ch1136DFM adapted to 100 $\mu\text{g ml}^{-1}$ ASCh was observed. Similar results were observed for Ch354DC challenged with TSPH. In strains, Ch1136DFM and Ch354DC, no cross-resistance to Cipro was observed. However, an initial 8-fold increase in resistance to Nx and Tc was detected, respectively.

Table 3.5. Antimicrobial susceptibility of chemostat strain Ch1136DFM and Ch354DF challenged with acidified sodium chlorite and their WT counterpart.

WT vs. chemostat challenged strains for ASCh	Agar MIC						
	Tc [$\mu\text{g ml}^{-1}$]	Nx [$\mu\text{g ml}^{-1}$]	Cipro [$\mu\text{g ml}^{-1}$]	ASCh [$\mu\text{g ml}^{-1}$]	CA [%]	LA [%]	TSPH [%]
1136DFM WT	0.25	2	0.06	500	0.125	0.25	0.3
Ch1136DFM 0.78 $\mu\text{g ml}^{-1}$	0.25	8	0.06	850	0.25	0.25	0.6
Ch1136DFM 6.24 $\mu\text{g ml}^{-1}$	0.5	16	0.125	850	0.25	0.25	2.5
Ch1136DFM 25 $\mu\text{g ml}^{-1}$	1	16	0.125	850	0.25	0.25	2.5
Ch1136DFM 50 $\mu\text{g ml}^{-1}$	0.5	16	0.06	850	0.25	0.25	1.25
Ch1136DFM 100 $\mu\text{g ml}^{-1}$	0.5	8	0.06	850	0.25	0.25	1.25
354DC WT	0.25	4	0.25	850	0.25	0.125	0.6
Ch354 DC 0.78 $\mu\text{g ml}^{-1}$	2	16	<0.125	1200	0.25	0.25	2.5
Ch354 DC 1.56 $\mu\text{g ml}^{-1}$	4	16	<0.125	850	0.25	0.25	2.5
Ch354DC 25 $\mu\text{g ml}^{-1}$	1	8	0.06	850	0.25	0.125	1.25
Ch354DC 50 $\mu\text{g ml}^{-1}$	1	8	0.06	850	0.25	0.25	2.5

Tc – tetracycline; Nx – nalidixic acid; Cipro – ciprofloxacin; CA - citric acid; LA – lactic acid; ASCh – acidified sodium chlorite; TSPH – trisodium phosphate. Each experiment was repeated twice (n=2).

Antimicrobial susceptibility for chemostat TSPH challenged strains

During investigating the effect of TSPH in the long-term culture for 301BC (clinical isolate) and 1140DFO (food isolate) on adaptation, no growth in the chemostat was observed at the MIC value of 0.6% TSPH. The concentration of 0.6% resulted in gradually washing out the bacteria from the fermenter. However, the cross-resistance study showed 4-fold increase in the TSPH MIC value for strain Ch301BC challenged with 0.6% TSPH and strain Ch1140DFO challenged with 0.4% TSPH (Table 3.6.). It is difficult to explain the reason why opposite results were achieved. Possibly, these results might be an effect of better survival under unfavourable condition in chemostat challenged strains as the time-kill kinetics study showed or it may be reflect differences in MIC values obtained using agar and broth determining methods.

A further study on cross-resistance indicates that clinical isolate 301BC became less susceptible to the tested antibiotics. Incremental exposure of *Campylobacter* 301BC to trisodium phosphate resulted in an 8-fold, 2-fold and 2-fold reduced susceptibility to tetracycline, nalidixic acid and ciprofloxacin, respectively. Food isolate 1140DFO, however, became more susceptible to tetracycline, nalidixic acid and ciprofloxacin with more than 2-fold, 2-fold and 66.6-fold MIC reduction observed, respectively. This opposite results might be depending on strain specificity. Clinical isolates seemed to adapt to unfavourable conditions easier (Table 3.6.).

Table 3.6. Antimicrobial susceptibility for chemostat strain Ch301BC and Ch1140DFO challenged with trisodium phosphate and their WT counterpart.

WT vs. chemostat challenged strains for TSPH	Agar MIC						
	Tc [$\mu\text{g ml}^{-1}$]	Nx [$\mu\text{g ml}^{-1}$]	Cipro [$\mu\text{g ml}^{-1}$]	ASCh [$\mu\text{g ml}^{-1}$]	CA [%]	LA [%]	TSPH [%]
301BC WT	0.25	8	0.125	850	0.25	0.125	0.6
Ch301BC 0.004%	2	16	0.125	850	0.25	0.25	2.5
Ch301BC 0.06%	2	16	>0.25	850	0.25	0.25	2.5
Ch301BC 0.125%	2	16	>0.25	850	0.25	0.25	2.5
Ch301BC 0.25%	2	16	1	850	0.25	0.25	1.25
Ch301BC 0.6%	2	16	0.25	850	0.25	0.25	2.5
1140DFO WT	0.5	4	1	850	0.25	0.125	0.3
Ch1140DFO 0.008%	2	>8	0.125	1200	0.25	0.25	2.5
Ch1140DFO 0.03%	2	32	0.125	850	0.25	0.25	2.5
Ch1140DFO 0.125%	1	<2	0.25	850	0.25	0.25	2.5
Ch1140DFO 0.25%	1	<2	0.25	850	0.25	0.25	1.25
Ch1140DFO 0.4%	<0.25	<2	<0.015	850	0.25	0.25	1.25

Tc – tetracycline; Nx – nalidixic acid; Cipro – ciprofloxacin; CA - citric acid; LA – lactic acid; ASCh – acidified sodium chlorite; TSPH – trisodium phosphate. Each experiment was repeated twice (n=2).

Antimicrobial susceptibility for non-disinfectant challenged *Campylobacter jejuni* 1136DFM

In order to investigate the effect of long-term culture of *Campylobacter* on possible changes to their antibiotics and disinfectants' MIC values, the MICs for both disinfectants (CA, LA, ASCh and TSPH) and antibiotics (Cipro, Nx and Tc) were performed on a non-disinfectant challenge long-term *Campylobacter* culture. This control chemostat was run continuously in the absence of disinfectant for strain *C. jejuni* 1136DFM for 1750.8 h (43.7 generations). Results obtained showed that no changes to the MICs for the

disinfectants or the test antibiotics occurred between the original WT strain and *C. jejuni* 1136DFM strains collected during continuous non-challenged culture after 525, 861 and 1510 h. This indicates that the performance of the long-term culture had no effect on *Campylobacter* MIC values and the MICs changes that occurred to strains that were challenged with disinfectants during the long-term cultures are effects of *Campylobacter* response to disinfectants only, not the long-term culture by itself.

In summary, the results indicate that although *Campylobacter* chemostat challenged strains were able to grow at MIC value and up to 1.4-fold and 8-fold above, depending on the strain and disinfectant used, there were also able to survive better disinfectant treatment as the time-kill kinetic results indicate, no major cross-resistance to antibiotics tested were detected. The cross resistance between 2-fold and 4-fold for Tc and Nx was obtained when ASCh was examined in the long-term challenge culture. In addition, between 2-fold and 4-fold higher MIC values for all antibiotics tested were found for strain 301BC long-term challenged with TSPH. The long-term challenge culture run in the absence of disinfectant for strain 1136DFM had no effect of antimicrobial agents' alterations.

3.4.6. Biofilm formation in chemostat cultures

During the course of this study, biofilm formation was observed both in the long-term disinfectant challenged chemostat cultures and in the control chemostat (non-disinfectant challenged culture). Following addition of 0.008% CA (the first sub-inhibitory dose) to the continuous culture of Ch2124GF (after 290 h) and Ch1136DFM (after 192 h), the first clearly visible pellets indicating biofilm development were observed. Biofilm formation was also detected when strains were challenged with LA, ASCh and TSPH. However, here the first visible signs of biofilm formation were observed at later stages of the continuous culture (even after 1155 h of the culture for strain 1140DFO challenged with 0.06% TSPH). These results suggest, that bacterium under presence of the disinfectant may form biofilm, as a response to stress. In the case of the non-disinfectant challenged chemostat culture, the first visible signs of biofilm formation were detected after 850 h of the continuous culture, with a well established biofilm present after 1000 h culture. The biofilm formation in non-disinfectant challenged culture can be caused by the presence of limited nutrient concentration. In addition, this observation may have an influence on the reason of biofilm formation in disinfectant challenged cultures. In the case of CA long-term exposure, the biofilm formation can result from stress caused by the disinfectant itself. However, in the

case of LA, ASCh and TSPH long-term challenged cultures, the biofilm formation can result from stress caused by the disinfectant and by limited nutrient concentration.

Figure 3.13. illustrates biofilm formation in chemostat fermenter vessels in a non-disinfectant challenged (A) and in a CA challenged (B). Differences in the amount of biofilm formed were clearly seen. In the disinfectant challenged culture the amount of biofilm was much lower, even after CA selective pressure removal and where the culture was allowed to run for further 24.1 days without the disinfectant challenge (total running hours of the culture 1631.7, where 40.7 generation were achieved).

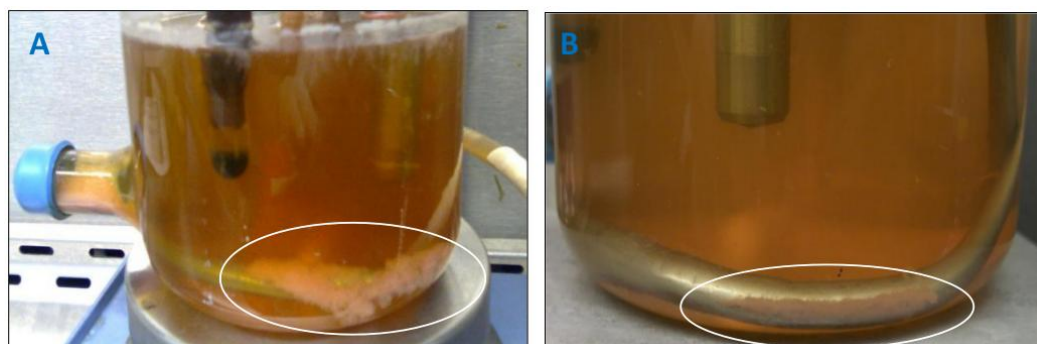


Figure 3.13. Biofilm presence in the non-challenged and CA challenged long-term *Campylobacter* cultures. Photo A, shows the biofilm created in the non-disinfectant challenge culture (Ch1136DFM after 1510 hours), while photo B for the CA challenged culture (Ch1136DFM adapted to 0.125% CA, after 685 hours).

The biofilm structure of a non-challenged culture, run for *Campylobacter* strain 1136DFM, was observed using electron microscopy. The samples were taken from different parts of the fermenter (port, heating coil, outlet tubing, feeding tubing and “planktonic” culture) at the end of the Ch1136DFM culture (after 1750.8 h). During samples preparation for electron microscopy, the first differences in the cell structure were observed. After centrifugation, the biofilm collected from the biofilm taken from the fermenter located on the heating coil, was characterised by its loose structure, while the biofilm collected from the port characterised very dense structure (Figure 3.14.).

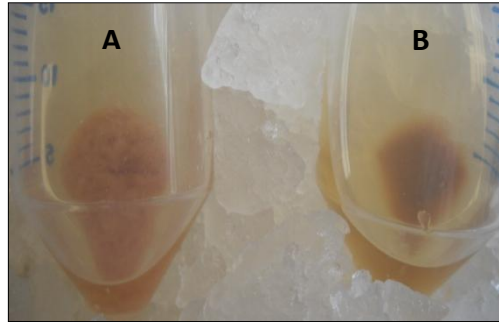


Figure 3.14. The differences of the biofilm structure after spun down of the culture. The A tube presents the biofilm taken from the fermenter located on the heating coil, while the B tube presents biofilm taken from the sampling port.

This variation may be due to biofilm localisation. In the fermenter where the culture is constantly stirred, the nutrients are better delivered into deeper layers of the biofilm, while the biofilm collected from the sampling port, nutrient may be more limited due to lack of media flow.

3.4.7. Scanning Electron Microscopy

The Scanning Electron Microscope (SEM) was used to distinguish dissimilarity in the structure of the biofilms collected from various parts of the fermenter. Figures 3.15 to 3.19 below present the electron microscopy results.

Figure 3.15. presents cells collected from the free floating culture from the fermenter. The cells are tightly packed and no thick EPS layer was detected following mechanical description of the sample. Although, the broth culture was taken from free spent media from the fermenter, no planktonic cells were observed under the microscope. The bacteria appeared to grow as a biofilm, even though no visible clumps were present in the collected samples. The spiral shape of the cells can be easily distinguished with an average length of 2-3 μm .

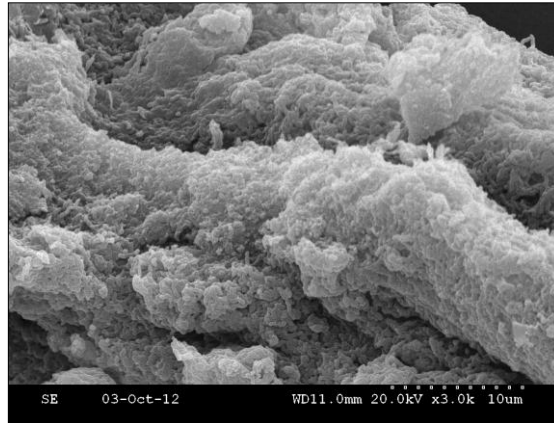


Figure 3.15. Electron microscopy of the “planktonic” culture taken from the fermenter under continuous culture conditions in Mueller-Hinton broth.

Both photos in the Figure 3.16. show biofilm samples collected from the fermenter, localised at the heating coil. Bacteria are densely packed and the structure of broken EPS is evident. Moreover, the presence of flagella (black arrows on the Figure 3.16. B) suggest that biofilm formation was still ongoing.

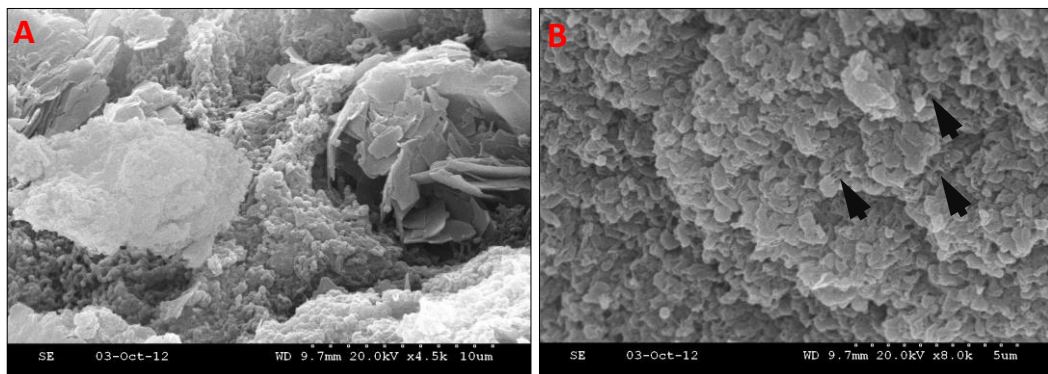


Figure 3.16. Electron microscopy of the biofilm taken from the fermenter. Black arrows in the Figure A indicate presence of flagella.

Both photos in the Figure 3.17. illustrate biofilm collected from the sampling port. The structure is more solid and EPS layers are thicker in comparison to the free floating cells and biofilm collected from the heating coil from the fermenter. Due to the thick EPS layers, the bacterial shape cannot be clearly distinguished. In the port, where no media flow was present, biofilm tended to accumulate and settle as opposed to actively form. In addition, due to lack of media flow at this site, limited nutrients were available, causing bacterial starvation. Possibly as a result of this, bacteria produced thicker EPS.

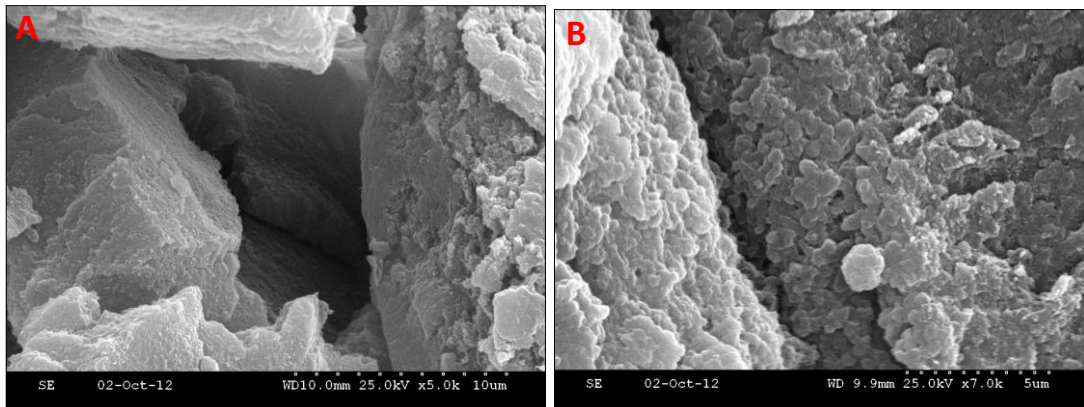


Figure 3.17. Electron microscopy of the biofilm taken from the chemostat sampling port.

The clear spiral shape of the bacteria can be distinguished in the Figure 3.18. presenting the biofilm collecting from the medium feed tubing. Flagella can be identified among the densely packed bacteria, (black arrows in the Figure 3.18 B). In addition, EPS layer was not as thick as found in the biofilm collected from the port. This biofilm and the biofilm taken from the fermenter had a similar structure.

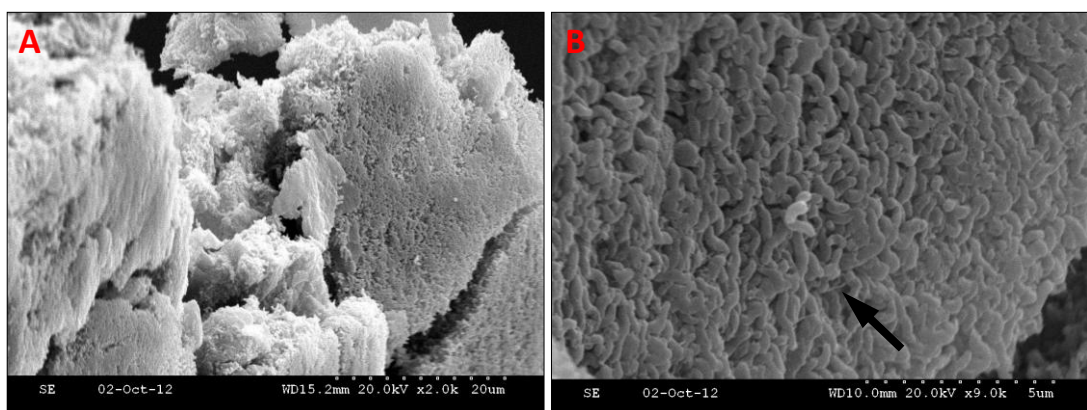


Figure 3.18. Electron microscopy of the biofilm collected from the chemostat medium feed tubing, immersed in the culture. Black arrows indicate presence of flagella.

Electron microscopy of biofilm that accumulated in the fermenter outlet tubing is presented in Figure 3.19. *Campylobacter* cells in this biofilm have a characteristic elongated shape, in comparison with the biofilm cells taken from the other parts of the fermenter. The length of bacteria is approximately double the size observed in bacterial biofilm collected from the fermenter. The estimating length is calculated at 5 μm . The tubing which was situated at the outlet of the fermenter, drained off spent media and cells, resulting in reduced biofilm formation.

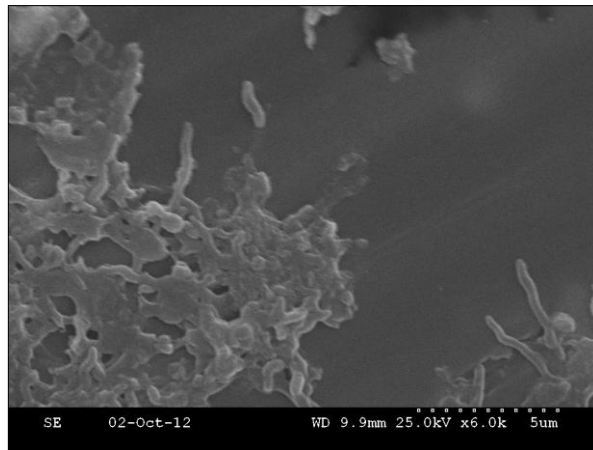


Figure 3.19. Electron microscopy of the biofilm collected from the chemostat outlet.

To conclude, stirring that maintained homogenous culture conditions inside the fermenter, resulted in no major differences in the structure between samples collected from the inside of the fermenter (“planktonic cells”), medium feed tubing and biofilm located at the heating coil. However, in some regions of the fermenter (sampling port and outlet tubing), the effect of limiting nutrient concentration was associated with increased cell stress as observed with an associated alteration on bacterial cell shape.

3.5. DISCUSSION

Different types of antimicrobials have been widely used in ordinary life. From triclosan, that is widely used in toothpaste, soaps, dishwashing liquids, deodorants, mouth rinses or toothbrush handles (Yazdankhah et al., 2006) to benzalkonium chloride - a quaternary ammonium cationic surfactant widely used in cosmetics, skin disinfectants and ophthalmic preparations (Basketter et al., 2004) and to chlorine that is used for water decontamination in a swimming pools. It is not surprising that a lot of studies on bacterial adaptation to different antimicrobials and their role in acquired cross-resistance have been widely carried out. Although it is believed that due to a broad spectrum of biocides activity acquired resistance to them are rare (Braoudaki & Hilton, 2005), there are some reports that the application of biocide can lead to creating highly resistance bacteria with acquired cross-resistance (Chuanchuen et al., 2001; Langsrud et al., 2004; Loughlin et al., 2002). Before application of disinfectant for decontamination purposes, especially disinfectants for food processing plants it is very important to investigate the development of resistance, as some bacteria might mutate very easily to a particular disinfectant and become highly resistant. In addition, it can acquire cross-resistance to other disinfectants and/or antibiotics. When this mutant is a source of food-borne infection it may cause serious sequela illness.

During this course of the study, *Campylobacter* adaptation to four disinfectants such as citric acid (CA), lactic acid (LA), acidified sodium chlorite (ASCh) and trisodium phosphate (TSPH) was investigated. The mode of action for CA and LA is decreasing the pH level in the cell's environment and in the cell, while ASCh decreases the pH and releases chlorous acid (HClO₂) causing additional oxidation of the cellular constituents (EFSA, 2005). It is worth noting here, that *Campylobacter* as a microaerophilic food-borne pathogens, have to survive a high level of oxygen in the air when outside the host, and after host invasion, exposure to a low pH of gastric juice. The bacterial response mechanism(s) when exposure to different disinfectants used for food decontamination, and mechanism(s) of disease caused by *Campylobacter* might be common. However, so far neither the response mechanism of pH/oxidative stress to disinfectant nor its survival mechanism in host organism is fully understood.

Work presented here concentrated on investigating if bacteria can adapt to disinfectants. *Campylobacter jejuni* strains were long-term challenged with CA, LA, ASCh and TSPH –

disinfectants highlighted in EU Regulation No. 853/2004. Isolates of *C. jejuni* grew sufficiently not only at MIC value, but in some instances up to 1.4-fold and up to 8-fold above, depending on the strain and disinfectant used (with the exception of TSPH, where bacteria were not able to grow at the MIC value). This indicates a possible risk related to acquired bacterial adaptation to unfavourable conditions. In addition, obtained *Campylobacter* phenotypes show better survival to stressful environments. This suggests that improper cleaning, related to inactivation of disinfectants by organic debris might result in the application of sub-inhibitory concentrations and may lead to the production of more resistant bacteria.

Among disinfectants tested in this study, to date, only lactic acid has been officially approved by the European Commission on the 4th of February 2013 and entered into force on the 25th February 2013 (http://europa.eu/rapid/press-release_MEX-13-0204_en.htm). Results from the work presented here showed that despite the ability of chemostat phenotypes to grow at MIC value and up to 2-fold above it, increased susceptibility to LA were achieved as the time-kill kinetics study showed.

Long-term citric acid challenge studies showed that campylobacters were able to grow between 1.4-fold and 2-fold above the MIC that have been obtained for the WT counterpart. This obtained adaptation results were similar to those for the LA. However, chemostat strains challenged with CA, in contrast to chemostat strains challenged with LA, could survive CA treatment better. As a time-kill kinetic results showed, CA adapted strains were able to survive between 1.5-2.4 times longer than their WT counterpart. The application of CA as a disinfectant might have its controversy. Improper preparation of the CA for decontamination purposes or its inactivation by organic debris might result in CA being used as a food ingredient by bacteria and thus supporting bacterial growth. Different bacteria can use this compound as a carbon source, as it has been reported for the example in the case of *Salmonella* (Gutnick, 1969). *Campylobacter* as a saccharolytic bacteria can also use low molecular organic acids as a source of carbon and energy (Jackson *et al.*, 2009). The conjugate base of citric acid, namely citrate, might be used in the Krebs Cycle, also known as tricarboxylic acid (TCA) and citric acid cycle. The TCA plays an important role in the cell metabolism. It generates biosynthetic starting compounds that are used in amino acid metabolism and it provides a metabolic energy source by creating intermediates that feed into the electron transport chain (Gaynor *et al.*, 2004). The studies on analysis of sequenced genome indicate that *C. jejuni* possessed a complete TCA cycle. However, little is

known about the TCA cycle pathway and enzymes involved in the process in *C. jejuni* (Gaynor *et al.*, 2004). On the other hand, citric acid is approved as an additive to food, to prevent its deterioration (European Union Register of Feed Additives. Pursuant to Regulation (EC) No 1831/2003, 2013) and it has been widely used.

The efficacy of sodium chlorite acidified with citric acid depends on the concentration of citric acid. The antimicrobial activation of sodium chlorite is derived from chlorous acid (HClO_2) that is determined by the pH of the solution. Gill and Badoni (2004) investigated the use of different disinfectants in reducing aerobes, coliforms and *Escherichia coli* on chilled beef carcass quarters delivered from a slaughtering to processing plant. They used 0.16% w/v ($1600 \mu\text{g ml}^{-1}$) sodium chlorite supplemented with 2% w/v citric acid to obtain a solution with a $\text{pH} \leq 3$. In this present study, the acidified sodium chlorite was supplemented with only 0.16% w/v of citric acid that resulted in a minor reduction in pH (from 7.3 to 7.18). This weaker solution of citric acid was incorrectly chosen, as a misinterpretation of data in an article published by Hugas and Tsigarida (2008). However, this misinterpretation led to an interesting observation. Even with decreased activity of ASCh, HClO_2 led to a sudden bacterial OD decrease following application of $200 \mu\text{g ml}^{-1}$ ASCh in both test strain cases. This might suggest that although the lower activity of the disinfectant was used, its oxidising ability still had a sufficient effect on bacterial growth. However, strains that were subjected to long-term challenge with acidified sodium chlorite were able to grow up to 4-fold and up to 8-fold above the MIC value. This finding indicates a better survival of chemostat phenotypes during ASCh re-treatment. Nevertheless, the time-kill kinetic results show minor changes in survival. The obtained chemostat strains were able to survive only 10-fold better than their WT counterpart at some stage of the experiment, however the time where bacteria achieved a 5 log reduction remained the same for chemostat strains challenged with ASCh and their WT strains. During chemostat runs, biofilm formation was observed at the later stages of the culture (after 900 h, following the application of MIC dose). The biofilm formation might have had a protective role on bacterial survival and allowed bacteria to withstand exposure to the disinfectant.

Trisodium phosphate action is based on its high alkalinity causing cell membrane disruption and also, it acts as a surfactant in eliminating bacteria that are not strongly adhered to the surface of poultry skin (EFSA, 2005). The pH of the culture medium, during the long-term challenge increased from 7.2 to 8.7 when a concentration of 0.8% TSPH was present. The typical dose of TSPH for poultry carcass decontamination in aqueous solution is ten times

higher, 8%-12% (EFSA, 2005), as was used in our study as the maximum test dose in the continuous culture. According to the results obtained, the long-term challenge study showed that bacterium was not able to survive at MIC value. However, the time-kill kinetic results indicate that chemostat adapted phenotypes were able to survive up to 3-times longer than their WT counterparts. This might be as a result of recovering bacteria following disinfectant selective pressure removal. It is worth mentioning that for the time-kill kinetics study, bacteria were subjected to least three sub-cultures on the agar without disinfectant before the time-kill kinetic experiments were carried out. This would suggest, that the application sub-inhibitory concentrations of disinfectant at different stages of poultry processing such as at farms and the processing plants, that its withdrawal might produce more resistant strains with the ability of survival longer under re-application of TSPH.

Chemostat strains, during the long-term challenge with the disinfectants, were also tested for acquired antibiotic cross-resistance. Chemostat adapted strains to disinfectants could become more resistant to antibiotics as there is some evidence based on studies carried out on different bacteria that the application of disinfectant may result in acquired cross-resistance to antibiotics. A study by McCay *et al.* (2010) showed that adapted *Pseudomonas aeruginosa* to benzalkonium chloride (12-fold increased to compared with the WT) had decreased sensitivity to ciprofloxacin by 265-fold (McCay *et al.*, 2010). In addition, *Salmonella enterica* serovar Typhimurium, following exposure to gradually increasing concentrations of triclosan, showed acquired cross-resistance to antibiotics such as chloramphenicol, tetracycline, ampicillin (Karatzas *et al.*, 2007).

In the work presented in this chapter, *Campylobacter* chemostat strains reveal a decreased susceptibility to Nx and Tc while ASCh and TSPH were examined. For the remaining cases, *Campylobacter* challenged strains showed a slightly decrease in their MIC values or the MIC remained the same level. For cross resistance to occur, either the disinfectant and the antibiotic have to possess the same mechanism of resistance (Joynson *et al.*, 2002). In this study only minor changes in the antibiotic cross-resistance were observed and disinfectant application should not have had a significant impact on producing *Campylobacter* that are highly resistant to antibiotics.

The biofilm formation that occurred during the chemostat runs might have had an effect on the results obtained. For example, from the time that the first dose of CA was added to the

chemostat vessel containing Mueller-Hinton broth, the bacteria started to form clumps, developing to a clearly visible biofilm. The biofilm formation was also observed when LA, ASCh and TSPH were examined but in later stages of the culture and at much smaller quantities. It has been reported, that *Campylobacter* under stress conditions are able to form a biofilm as a response mechanism to ambient environmental stressors (Guerry, 2007; Reuter *et al.*, 2010). In our study, the stress was possibly related initially to the addition of disinfectant and at the later stage of the continuous cultures (after 850 h) to limited nutrient concentration. This might suggest that the biofilm formation was a result of all these factors. Although biofilm offer a protective role from stress conditions, they might have an effect on the bacterial growth rate. It has been reported that the slower growth rate in biofilm is not associated with nutrient limitation by itself but with a general stress response initiated by biofilm formation (Trachoo *et al.*, 2002).

Although, biofilm could have a protective role under unfavourable conditions and have an influence on the decreasing bacterial growth rate, the bacteria that were long-term challenged with disinfectants, could not survive above a certain level of the disinfectants. In the case of TSPH, both strains tested were not able to growth at 0.6%. In the case of ASCh, both strains were not able to grow at 200 $\mu\text{g ml}^{-1}$. Similar results were also obtained when CA was tested. This suggest that the disinfectant addition could sufficiently inhibit *Campylobacter* growth when an appropriate dose of disinfectant was reached.

The scanning electron microscopy analysis of biofilm collected from the control chemostat 1136DFM, run in the absence of disinfectant, showed a diversity of biofilm structure between different samples collected from different locations. In the samples collected from the port sampling, the bacterial cell shapes could not be clearly distinguished in comparison with the samples collected from the fermenter vessel or from the medium feed tubing. The sample of the free floating cell collected from the fermenter, showed a lack of presence of single or isolated cells. All bacteria were grown as a biofilm and no planktonic cells were observed. This indicates that the biofilm took over and *Campylobacter* grew in a biofilm.

To conclude, *Campylobacter* might adapt to disinfectants and survive better repetitive applications of the sub-inhibitory concentration of antimicrobial agents, however it is likely that no major cross-resistance to antibiotics would occur.

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3.6. SUPPLEMENTAL MATERIALS

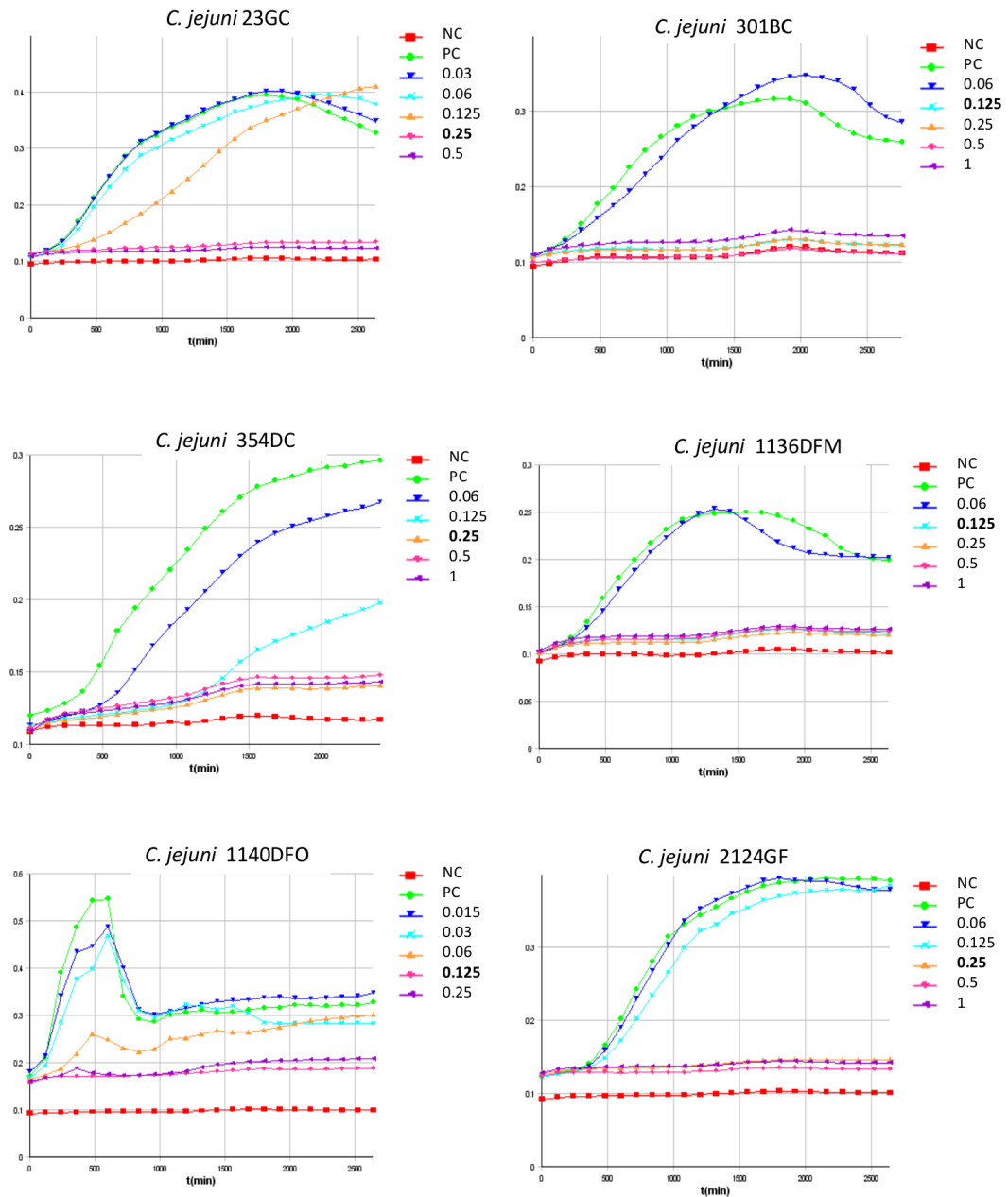


Figure S3.1. Antimicrobial susceptibility testing for citric acid carried out in a MHB. The first concentration of CA that inhibited *Campylobacter* growth was determined as broth MIC. The CA concentrations are given in [%]. For example from the graph, the MIC for *Campylobacter jejuni* 23GC to citric acid was 0.25%, highlighted in **bold**.

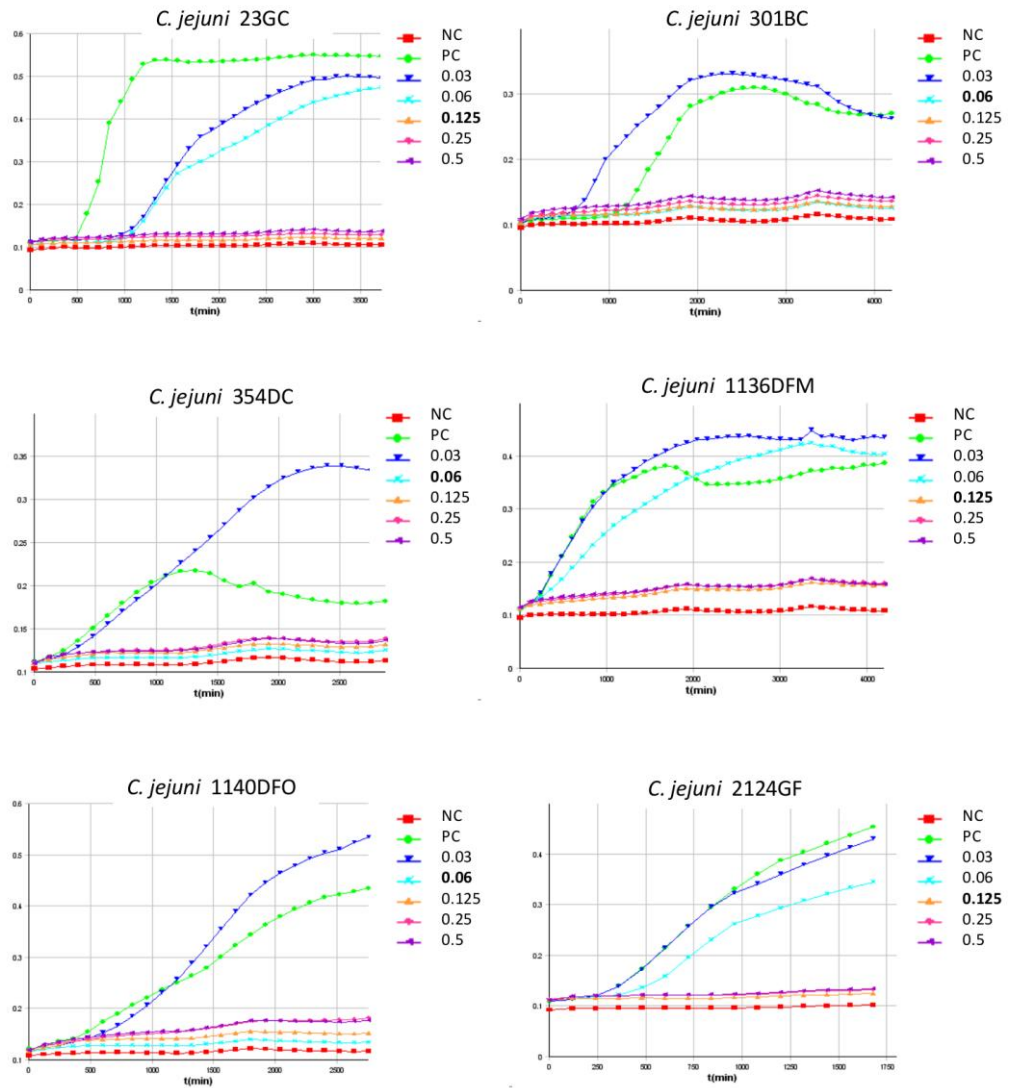


Figure S3.2. Antimicrobial susceptibility testing for lactic acid carried out in a MHB. The first concentration of LA that inhibited *Campylobacter* growth was determined as broth MIC. The LA concentrations are given in [%]. The MIC value highlighted in **bold**.

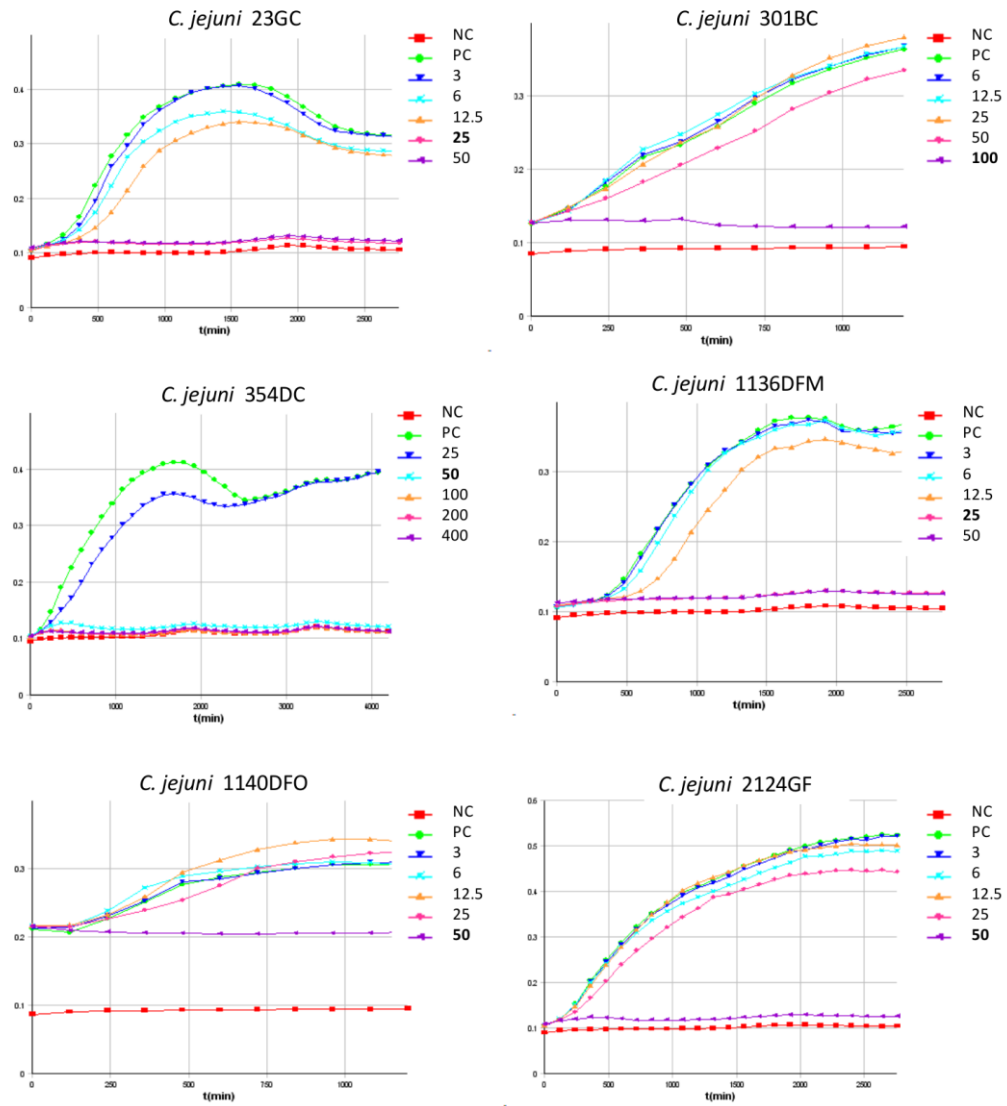


Figure S3.3. Antimicrobial susceptibility testing for acidified sodium chlorite carried out in a MHB. The first concentration of ASCh that inhibited *Campylobacter* growth was determined as broth MIC. The ASCh concentrations are given in $\mu\text{g ml}^{-1}$. The MIC value highlighted in **bold**.

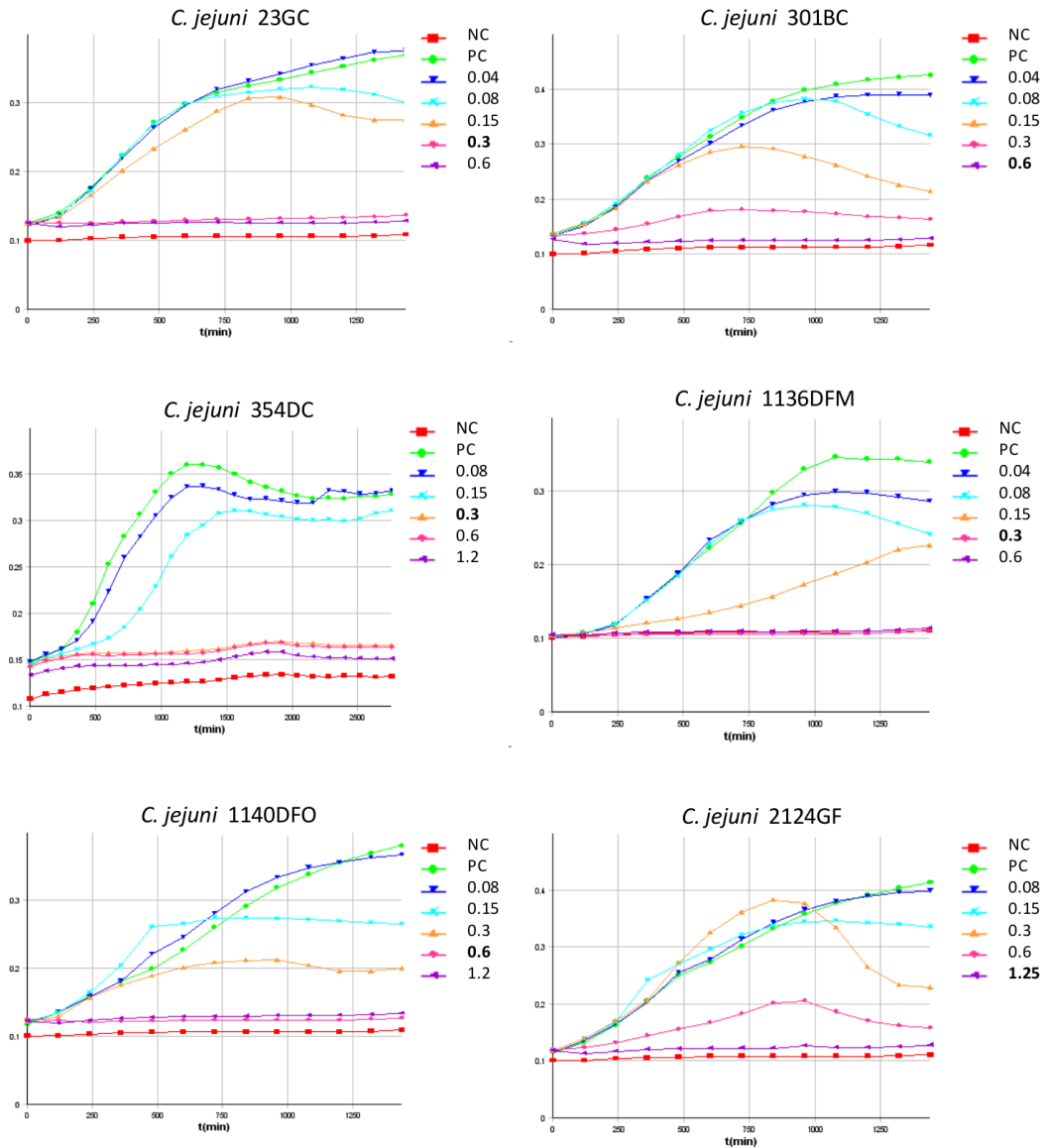


Figure S3.4. Antimicrobial susceptibility testing for trisodium phosphate carried out in a MHB. The first concentration of TSPH that inhibited *Campylobacter* growth was determined as broth MIC. The TSPH concentrations are given in [%]. The MIC value highlighted in **bold**.

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CHAPTER IV

The effect of the long-term disinfectant exposure on *Campylobacter* virulence gene expression

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Key words: *Campylobacter*; disinfectant exposure; virulence genes expression;
Campylobacter invasion antigen; cytolethal distending toxin; efflux
pump; flagella; pathogenicity

4.1. SUMMARY

The use of some types of disinfectants on bacteria has been reported to induce expression of virulence genes in pathogens such as *Salmonella* and *Escherichia* as a part of their general stress response. The work presented here, concentrates on changes in virulence gene expression in *Campylobacter* strains as a response to the long-term exposure to citric acid (CA), lactic acid (LA), acidified sodium chlorite (ASCh) and trisodium phosphate (TSPH), chemicals that have been found to be suitable for bacterial decontamination of meat products. These genes were, *Campylobacter* invasion antigen (*ciaB*), coding efflux pump (*cmeA*), cytolethal distending toxin (*cdtB*) and flagellar sensory histidine kinase (*flgS*). In this study, 8 chemostat runs in total were performed for four different disinfectants using 6 different *Campylobacter jejuni* strains. Strain *C. jejuni* 2124GF was challenged to CA, strain *C. jejuni* 354DC to ASCh, *C. jejuni* 301BC and *C. jejuni* 1140DFO to TSPH, *C. jejuni* 23GC to LA and strain *C. jejuni* 1136DFM to both CA and ASCh. It also included the control chemostat that was carried out in the absence of a disinfectant, in order to investigate the effect of the long-term culture on gene expression. The control chemostat was performed for strain 1136DFM. Results obtained for the control chemostat (running continuously for 1751 h in the absence of disinfectant) showed that the long-term culture does not have an effect on virulence gene expression until 861 h. After 861 h, overexpression at 4.8-, 2.9-, 2.0- and 4.7-fold for *flgS*, *ciaB*, *cmeA* and *cdtB*, respectively was observed, however these changes were not statistically significant. After 1054 h, continuous culture did lead to a significant increase in gene expression, ($p > 0.0001$), where overexpression at 29.4-, 17.0-, 10.4- and 17.5-fold for *flgS*, *ciaB*, *cmeA* and *cdtB*, respectively was detected. The virulence gene expression in the disinfectant challenged chemostat cultures varied and were dependent on the strain tested and disinfectant used. In the case of strain *C. jejuni* 23GC challenged with LA no significant increase in expression of the tested virulence genes was detected (with the exception of *ciaB* gene for strain Ch23GC adapted to 0.125% LA, where the overexpression of the gene was found at 2.5-fold, compared to the WT) while a significant overexpression (by 180-fold) of *cmeA* gene for Ch2124GF challenged with 0.125% CA was observed.

4.2. INTRODUCTION

In order to efficiently combat *Campylobacter* contamination in both poultry farms and processing plants, interventions strategies such as for example the application of disinfectants, have been proposed. Through the elimination of the risk related to consumption of contaminated poultry products by humans, the number of campylobacteriosis cases might be reduced. However, before approval of any antimicrobial agent, the possible effect of its use on bacterial adaptation and on the bacterial response mechanism to stress and also its effect on creating more virulent strains have to be at first investigated.

It has been reported that acid stress may produce more virulent strains. In a study by O'Driscoll and co-workers, who investigated the adaptive tolerance response (ATR) in *Listeria monocytogenes*, they found that acid adapted strains are more virulent to mice (O'Driscoll *et al.*, 1996). Mice injected with acid tolerant mutant strains of *Listeria monocytogenes* did not survive longer than 3 days, while a control group that was inoculated with the wild type strains did not show symptoms of disease. In addition, Humphrey and co-workers reported that acid-tolerant *Salmonella enterica* are more virulent in mice and more invasive in laying hens than an unadapted strain (Humphrey *et al.*, 1996).

The virulence genes that have been implicated in influencing *Campylobacter* pathogenicity, include the expression of genes such as *flgS* (flagellar sensory histidine kinase), *ciaB* (*Campylobacter* invasion antigen), *cdtB* (cytolethal distending toxin), *cmeA* (efflux pump) and *cadF* (fibronectin-binding protein). The flagellar apparatus in *Campylobacter* spp. has been found to possess a multifunctional role. Besides its motility function, it has been identified as being involved in the adhesion and invasion of host epithelial cells (Carrillo *et al.*, 2004). In addition, a study by Konkel *et al.* (2004) also indicates the importance of flagella in the secretion of protein such as *Campylobacter* invasion antigen (CiaB). To date, the best characterised adhesion protein is a 37 kDa outer membrane protein, namely CadF that binds fibronectin (Konkel *et al.*, 1997). *Campylobacter cadF* mutants showed reduced ability in INT407 cells invasion (Monteville, 2003). Following adhesion and invasion of the epithelial cell, *Campylobacter* produce cytolethal distending toxins (CDT). This multi-subunit toxin requires the activity of three genes, *cdtA*, *cdtB*, *cdtC* (Whitehouse *et al.*, 1998). Each subunit is required for cytotoxin activity but *cdtB* as the active subunit is thought to induce

host cell apoptosis (Mortensen *et al.*, 2011; Young *et al.*, 2007). Its role is involved in the blockage of the G1/S or G2/M transition phase of the cell cycle (Dasti *et al.*, 2010; Young *et al.*, 2007) by acting as deoxyribonuclease (DNaseI) enzyme and fragmenting the chromatin when it reaches the nucleus (Dasti *et al.*, 2010).

Multidrug efflux pump, CmeABC, belongs to the resistance-nodulation-cell division (RND) superfamily (Pumbwe & Piddock, 2002), has been suggested to have an impact in *Campylobacter* response to pH stress. *cmeABC*, plays an important role not only in intrinsic resistance to the antibiotics fluoroquinolones and macrolides (Cagliero *et al.*, 2006) but also confers resistance to bile salts and detergents (Martinez *et al.*, 2009). It is believed that overexpression of efflux pump could be a first step in bacteria becoming fully resistant (Piddock, 2006).

In this study *Campylobacter* strains were grown in a chemostat under continuous culture conditions, and were subjected to long-term disinfectant challenge. Four antimicrobial agents, highlighted in the European Union Regulation No. 853/2004 as chemicals that might be used for bacterial decontamination of the meat product were tested. These included, citric acid (CA), lactic acid (LA), trisodium phosphate (TSPH) and acidified sodium chlorite (ASCh). The results obtained from the previous chapter, showed that disinfectant challenged bacteria were able to grow at MIC concentrations of these disinfectants and above with the exception of TSPH challenged strains. In addition, chemostat challenged strains were able to survive better upon re-exposure to the disinfectants as time-kill kinetics results showed. These results suggest that the chemostat challenged strains might become more virulent. Investigating the sub-lethal treatments on virulence genes is necessary in the evaluation of food decontamination methods used in farms and processing plants in terms of safety.

The aim of this study was to determine any changes in *C. jejuni* virulence gene expression under disinfectant stress. The alterations in the expression of *ciaB*, *cmeA*, *cdtB* and *flgS* genes were studied.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial isolates

Campylobacter jejuni phenotypes strains collected during the long-term disinfectant challenge cultures in the chemostat were used. Six *C. jejuni* isolates, 23GC, 354DC, 301BC, 1136DFM 2124GF and 1140DFO were independently and continuously challenged in the chemostat cultures. Strains 2124GF and 1136DFM were challenged with citric acid (CA), strain 23GC was challenged with lactic acid (LA), strains 1136DFM and 354DF were challenged with acidified sodium chlorite (ASCh), while strains 301BC and 1140DFO were challenged with trisodium phosphate (TSPH). *Campylobacter jejuni* 1136DFM was also used to perform a control chemostat (run in the absence of disinfectant) to investigate the effect of the long-term culture on gene expression.

4.3.2. Sample collection for RNA extraction

Strains challenged with the different disinfectant under continuous culture, were collected at different time intervals for RNA extraction. A 150 ml of the culture was taken and centrifuged at $21,000 \times g$ (Beckman Coulter, Avanti™ J-25 Centrifuge) at 4°C for 10 min. The supernatant was removed and pellets were transferred into three eppendorfs and resuspended with 2-fold, ice-cold 5% phenol solution in ethanol to stop RNA synthesis and the degradation of the RNA already synthesised. The sample was incubated in ice for 15 min, following this, the sample was centrifuged at $12,000 \times g$ for 15 min. The supernatant was discarded and harvested cells were resuspended in $500 \mu\text{l}$ of 1 mg ml^{-1} of lysozyme (Pierce, Perbio) in TE buffer at pH 7.0 (Ambion). The sample was incubated for 3 min at room temperature and RNA isolation was carried out using an RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and purity of RNA was checked using a NanoDrop 2000c (Thermo Scientific). Values of $A_{260}/A_{280} \geq 2.0$ and $A_{260}/A_{230} > 2.0$ indicate of no protein and solvent contamination respectively. In addition, the RNA samples were run in 1% agarose gel (SeaKem® LE agarose gel, Lonza) contained ethidium bromide at $0.5 \mu\text{g ml}^{-1}$ to examine the quality of the extracted RNA and to check if the RNA samples were DNA-free. Any remaining DNA was digested with DNase (Turbo DNA-free™ Kit, Ambion) by adding $2 \mu\text{l}$ of 10X TURBO DNase Buffer and $0.3 \mu\text{l}$ TURBO DNase to $20 \mu\text{l}$ of the RNA sample at a concentration of $20 \text{ ng } \mu\text{l}^{-1}$. The sample was incubated at 37°C for 20 min. Following this, $2 \mu\text{l}$ of DNase Inactivation Reagent was added

and the sample was incubated at room temperature for 3 min with gentle agitation every 1 min. Following incubation, the sample was centrifuged at $10,000 \times g$ for 2 min and the supernatant was gently transferred into a fresh tube.

4.3.3. Reverse-transcription PCR

To obtain cDNA, the QuantiTect[®] Reverse Transcription Kit (Qiagen) was used. Following the manufacture's protocol, 14 μl of template RNA was added into the reverse-transcription master mix. The PCR was carried out at 42 °C for 30 min and this was then subjected to 95 °C for 3 min in order to inactivate Quantiscript Reverse Transcriptase. The concentration and purity of cDNA was checked using the NanoDrop. Values of $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} > 2.0$ indicate of no protein and solvent contamination, respectively. The cDNA was stored at -20 °C.

4.3.4. Real-time PCR

The LightCycler[®] 480 SYBR Green I Master (Roche) was used for the relative quantification of the expression of the genes. The real-time PCR was performed with 5 μl of cDNA at a concentration of 10 ng μl^{-1} ; 10 μl of 2x SYBR green PCR master mix and 0.5 μM of each primer (Table 4.1.) in a final volume of 20 μl . PCR grade water (Roche) was used to adjust the primers concentration (Eurofins MWG Operon). The experiment was carried out in a 96-well microtitre plate (Roche). The inoculated plate was placed in the LightCycler[®] 480 (Roche) and fluorescence was measured. The real-time PCR parameters for all genes detection reaction were carried out with an initial denaturation at 95°C for 10 min, following the amplification for 40 cycles for 15 s at 94°C, 30 s at annealing temperature of 64 °C and for 30 s at the extension temperature of 72°C. The specificity of amplification for each product was determined by a melting curve analysis at 95°C for 5 s and 65°C for 1 min, followed by a progressive increase of the temperature to 97°C with a ramp rate of 0.11°C/s, with continued measurement of fluorescence, and finally cooling of the plate to 40°C for 10 s. Each real-time PCR assay was conducted with a negative control using molecular-biology grade water instead of cDNA. In addition, the remaining RNA after TURBO DNase treatment was taken as a positive control for the first real-time PCR assay.

Each set of primers (Table 4.1.) was designed with specificity toward the genome of *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 strain (ATCC 700819); the accession no. NC_002163.

Table 4.1. List of primers and size of amplicons used to quantities *Campylobacter jejuni* expression of virulence genes in the long-term challenged and non-challenged strains.

Gene	Primers	Sequence (5'→3')	Amplicon length (bp)
<i>cjr01</i>	Forward	GTA CGG TCG CAA GAT TAA AAC TC	237
	Reverse	GTT AGC AAC TAA ATA CGT GGG TTG	
<i>flgS</i>	Forward	GGA GCG TAT CGT AAA TTC AAC TCT AC	317
	Reverse	CAA CCA TTA TCA TAC ACT CTG ATG C	
<i>cmeA</i>	Forward	CAT TTG CAA GCT GTG CTC TAG C	186
	Reverse	GTT GAT TCG GCT TAC GGA CAA G	
<i>ciaB</i>	Forward	CTA TAT TAT GCA CTC CTT GTA TTA AGG AC	230
	Reverse	CTA TGC TAG CCA TAC TTA GGC GTT TG	
<i>cdtB</i>	Forward	GCT CCT ACA TCT GTT CCT CCA TTA G	263
	Reverse	GCA ACA AGG TGG AAC ACC TAT TG	

cjr01-16S rRNA gene (housekeeping gene); *flgS* - flagellar sensory histidine kinase; *cmeA* - efflux pump; *ciaB* - *Campylobacter* invasion antigens; *cdtB* - cytolethal distending toxin.

The efficiencies of the real-time PCR primers were achieved using the LightCycler 480 software program. To obtain this, four decimal dilutions of cDNA were prepared and real-time PCR was run at the same parameters as above. The PCR primers efficiencies were established at 1.99 for the *cjr01* gene (encoding 16S rRNA gene; housekeeping gene), 1.96 for the *flgS* gene, 1.98 for the *cdtB* gene, 1.99 for the *ciaB* gene and 2.05 for the *cmeA* gene.

Relative expression was calculated as a ratio between expression of target genes and the expression of the housekeeping genes using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001). The relative expression ratio for control samples in all experiments were calculated as a expression of genes of *Campylobacter* strains for the parent strains and for strains grown under continuous culture conditions prior to disinfectant exposure.

4.3.5. Statistical analysis

Real-time experiments were independently repeated three times with three separate RNA extractions (n=9), performed on samples taken during the experiments at sub-MIC, at MIC and if possible at supra-MIC value. One-way ANOVA with Bonferroni's multiple comparison test was used to study the association of the level of genes expression between the control strains (chemostat strains prior to disinfectant exposure) and strains challenged with increasing incremental concentrations of disinfectant. Differences with P values of <0.05 were considered statistically significant.

4.4. RESULTS

In the previous study presented in Chapter III, 8 chemostat runs were carried out for four different disinfectants using 6 different *Campylobacter jejuni* strains, including the control chemostat culture run in the absence of disinfectant. Strain *C. jejuni* 2124GF was challenged with CA, strain *C. jejuni* 354DC to ASCh, *C. jejuni* 301BC and *C. jejuni* 1140DFO to TSPH, *C. jejuni* 23GC to LA and strain *C. jejuni* 1136DFM to both CA and ASCh. Strain *C. jejuni* 1136DFM was also run in a control chemostat where no disinfectant was applied. Strains were continuously cultured in the presence of increasing incremental concentrations of the different disinfectants for periods between 845.8 and 1847.8 hours depending on the strain, where between 21.1 and 46.2 cell generations were obtained. Results obtained, indicate that the long-term exposure of *Campylobacter* to disinfectants might have an effect on its virulence gene expression and on its pathogenicity. To prove or reject the hypothesis, quantitative real-time PCR (qRT-PCT) was performed for four virulence gene, *flgS* – flagellar sensory histidine kinase; *cmeA* – efflux pump; *ciaB* – *Campylobacter* invasion antigens; *cdtB* – cytolethal distending toxin, for long-term disinfectant challenge strains and for control non-challenge strains. Gene encoding 16S rRNA (*cjr01*) was chosen as a housekeeping gene.

However, before performing the qRT-PCR a number of preparation steps involving RNA extraction, reverse transcription reaction and optimisation of PCR primers had to be carried out.

4.4.1. RNA extraction and reverse transcription

RNA extraction was carried out using an RNeasy[®] Mini Kit, Qiagen, according to the manufacturer's instruction. Besides using the NanoDrop 2000c Software for calculating the concentration and purity of extracted RNA, the samples were also run in 1% of agarose gel contained ethidium bromide to check the quality of the extracted RNA and to examine if the RNA was DNA-free. The results obtained from the agarose gels, showed the presence of remaining DNA in the RNA samples. The DNA was removed by its digestion with DNase. Following DNA digestion, the agarose gel was run again in order to check if the DNA was fully removed. The pure RNA obtained was taken for reverse transcription. Figure 4.1. presents the example of the RNA conditions of *C. jejuni* chemostat disinfectant challenge strains before and after DNase treatment.

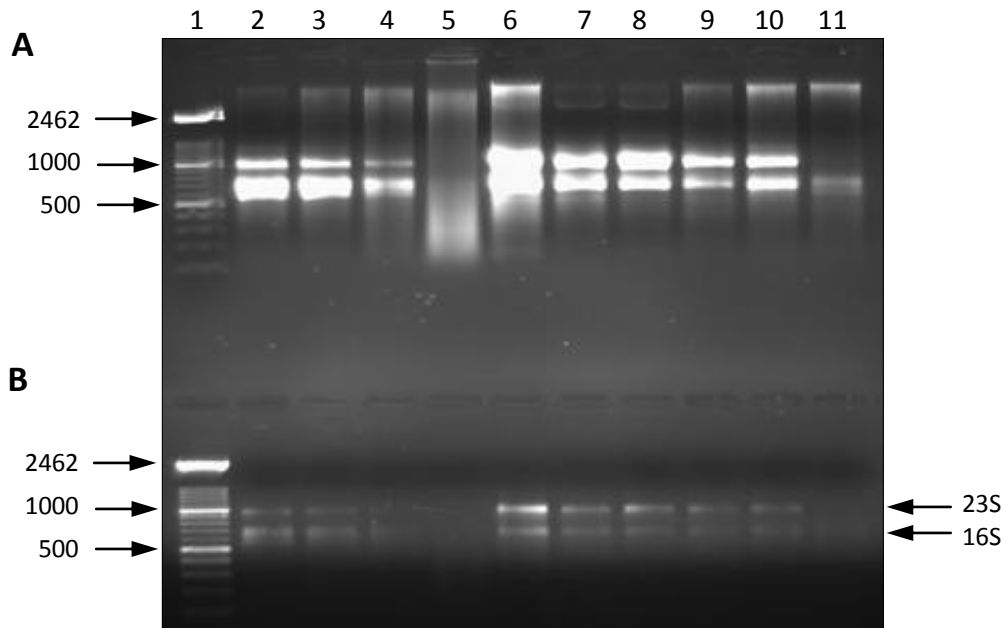


Figure 4.1. Electrophoresis of RNA extracted from *C. jejuni* disinfectant challenge strains on a 1% of agarose gel contained ethidium bromide. The top Figure A, presents bands of the RNA prior removal of genomic DNA traces, while Figure B presents the RNA samples following genomic DNA digestion. Lane 1: Molecular Weight Marker XIV, Lane 2: Ch23GC 0.008% LA, Lane 3: Ch23GC 0.125% LA, Lane 4: Ch23 GC 0.18% LA, Lane 5: Ch23GC Biofilm, Lane 6: Ch2124GF WT, Lane 7: Ch2124GF 0.008% CA, Lane 8: Ch2124GF 0.125% CA, Lane 9: Ch1136DFM 0.008% CA, Lane 10: Ch1136DFM 0.06% CA, Lane 11: Ch1140DFO 0.35% TSPH.

In Figure 4.1. the samples in lane 5 (representing Ch23GC Biofilm) and lane 11 (representing Ch1140DFO 0.35% TSPH) were not chosen for further analysis. The smear on the gel indicates that the RNA was digested during the extraction procedure or RNA was already digested. Taking into consideration that these samples for the RNA were obtained from the chemostat where the strains were challenged with a high concentration of disinfectant, the smear on the gel may suggest that the RNA of the sample was already digested. Even after repeating the extraction procedure with a new sample, no RNA was isolated. Similar results, where the smear was present at the gel, were challenged in other chemostat cultures with high doses of disinfectant (close or above the MIC values). In some instances, due to difficulties in obtaining RNA where strains were challenged with higher concentrations of disinfectant, for the qRT-PCR reaction, strains challenged with lower concentration than those were selected.

The next step of qRT-PCR optimisation after RNA extraction and reverse transcription, was to demonstrate that the selected parameters of qRT-PCR reaction were appropriate and the cDNA was to be given only one product of the reaction. To achieve this, the

conventional PCR reactions were carried out using thermocycler (Eppendorf Mastercycler, Labmerchant). The cDNA obtained from the reverse transcription reaction were used at a concentration of $10 \text{ ng } \mu\text{l}^{-1}$ and was added into the master mix containing $200 \mu\text{M}$ of each dNTPs, 10x Reaction Buffer, 1.5 units of Taq Polimerase, 2.5 mM of MgCl_2 and 0.5 pM of primers. Water instead of cDNA was used for negative control, while as a positive control $1 \mu\text{l}$ of *Campylobacter* cell suspension in PBS was applied. The PCR was carried out at the same conditions described in Material and Methods, Section 4.3.4. Figure 4.2. below presents examples of the result obtained.

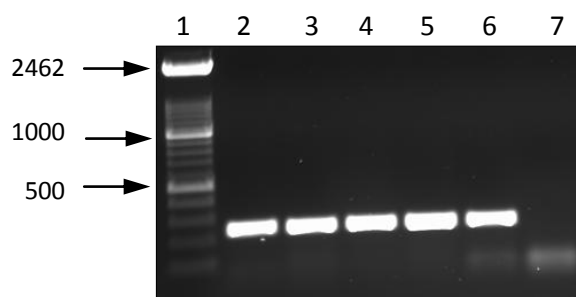


Figure 4.2. PCR amplification of cDNA with set of primers coding 16S rRNA (*cjr01*). Lane 1: Molecular Weight Marker XIV, Lane 2: Ch2124GF 0.25% CA, Lane 3: Ch354DC $75 \mu\text{g ml}^{-1}$ ASCh, Lane 4: Ch1140DFO 0.125% TSPH, Lane 5: Ch1140DFO% TSPH, Lane 6: Positive control, Lane 7: Negative control.

Figure 4.2. showed that selected PCR parameters were appropriate and gave a product. However, besides this extra PCR reactions with gradient annealing temperatures using the gradient thermocycler programme were performed in order to investigate the appropriate temperature for primers annealing. The PCR reactions were carried out from $52 \text{ }^\circ\text{C}$ to $68 \text{ }^\circ\text{C}$. Results obtained showed a good amplification product at the tested temperature range.

4.4.2. Calculation of the primers efficacy

In order to determine the virulence gene expression for the chemostat strains challenged with disinfectants, the qRT-PCRs were carried out. However, before performing the actual reactions, the efficacy of the designed primers was established. To obtain this, four decimal dilutions of cDNA ($100 \text{ ng } \mu\text{l}^{-1}$, $10 \text{ ng } \mu\text{l}^{-1}$, $1 \text{ ng } \mu\text{l}^{-1}$, $0.1 \text{ ng } \mu\text{l}^{-1}$) were prepared and qRT-PCR was run. Figure 4.3. to Figure 4.7. present examples of calculated data of primer efficacy, obtained from the LighCycler Software, for each set of primers that have been used for the study. The experiments were independently repeated trice, if necessary more than this. The

average value of primers efficacies were established at 1.99 for *cjr01*, 1.96 for *flgS*, 1.98 for *cdtB*, 1.98 for *ciaB* and 2.05 for *cmeA*.

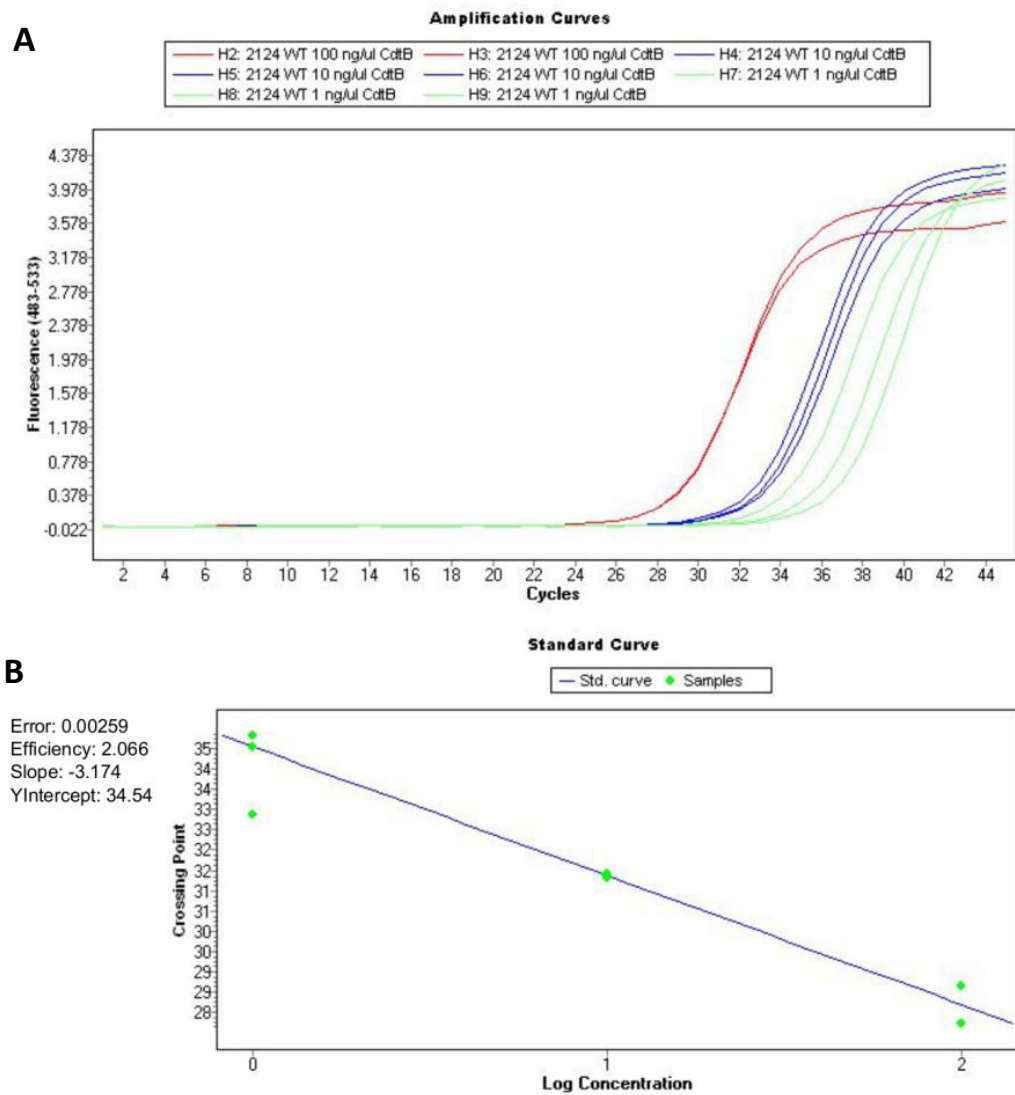


Figure 4.3. Real-time amplification of *cdtB* gene. Figure A presents the LightCycler quantification curves of cDNA standards, while Figure B the standard curve from where the primer efficiency was calculated. The primer's efficiency was calculated as $10^{-1/\text{slop}}$.

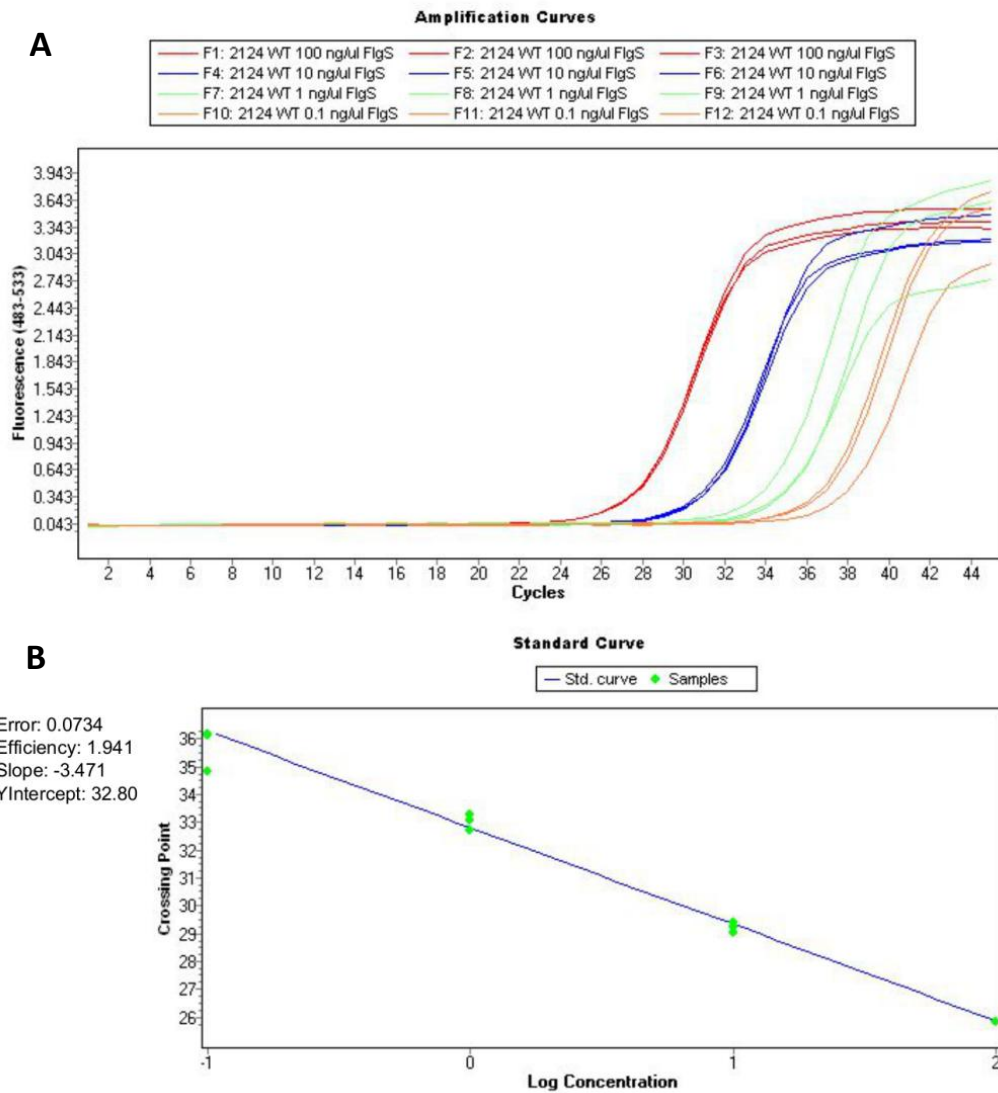


Figure 4.4. Real-time amplification of *flgS* gene. Figure A presents the LightCycler quantification curves of cDNA standards, while Figure B the standard curve from where the primer efficiency was calculated. The primer's efficiency was calculated as $10^{-1/\text{slope}}$.

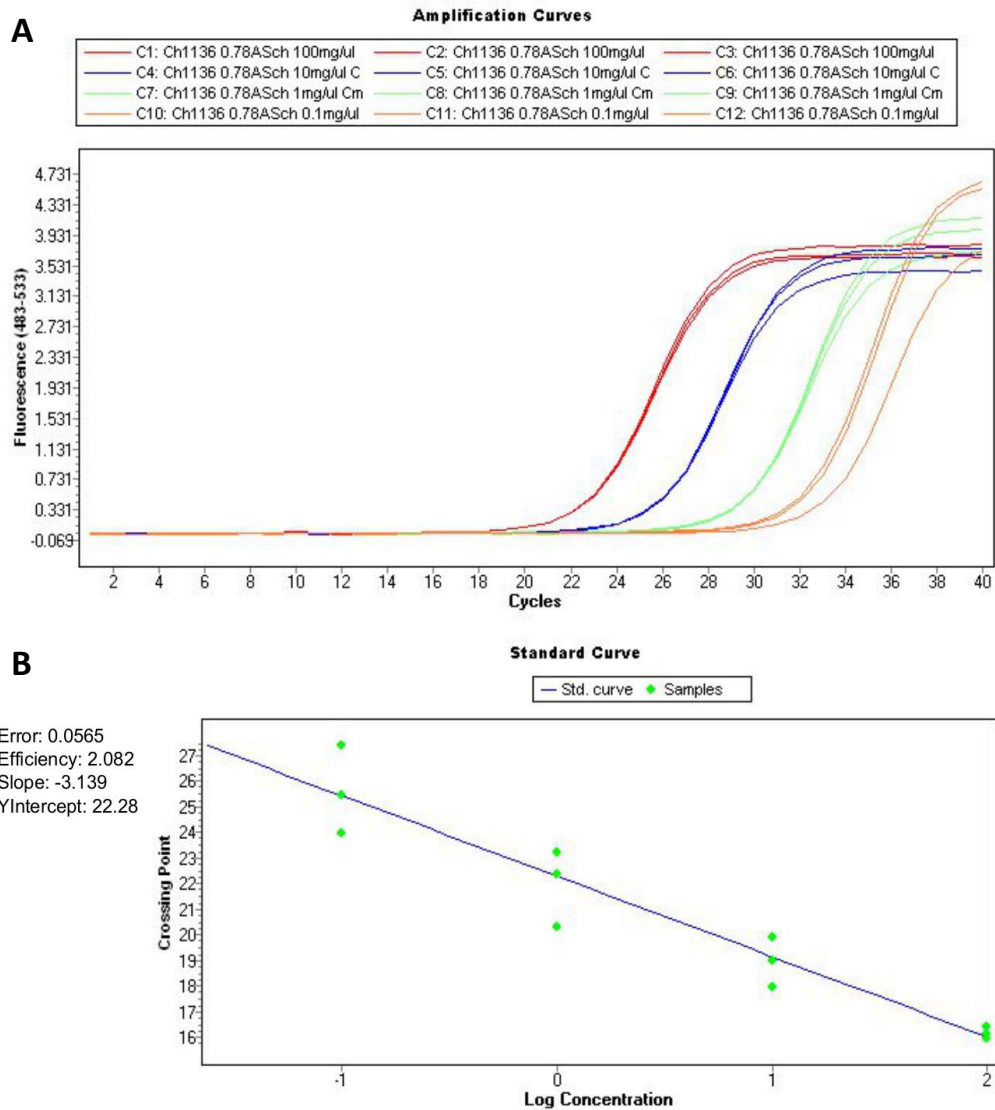


Figure 4.5. Real-time amplification of *cmeA* gene. Figure A presents the LightCycler quantification curves of cDNA standards, while Figure B the standard curve from where the primer efficiency was calculated. The primer's efficiency was calculated as $10^{-1/\text{slope}}$.

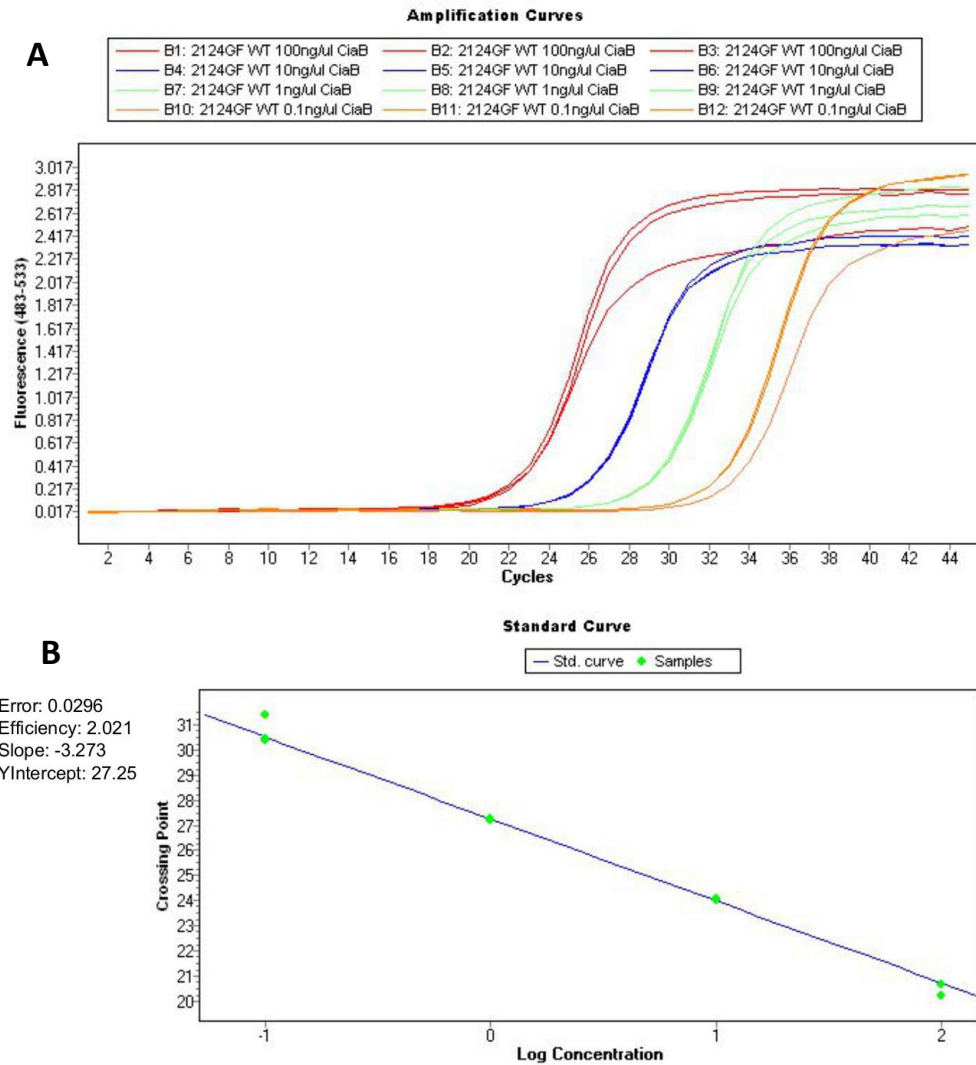


Figure 4.6. Real-time amplification of *ciaB* gene. Figure A presents the LightCycler quantification curves of cDNA standards, while Figure B the standard curve from where the primer efficiency was calculated. The primer's efficiency was calculated as $10^{-1/\text{slope}}$.

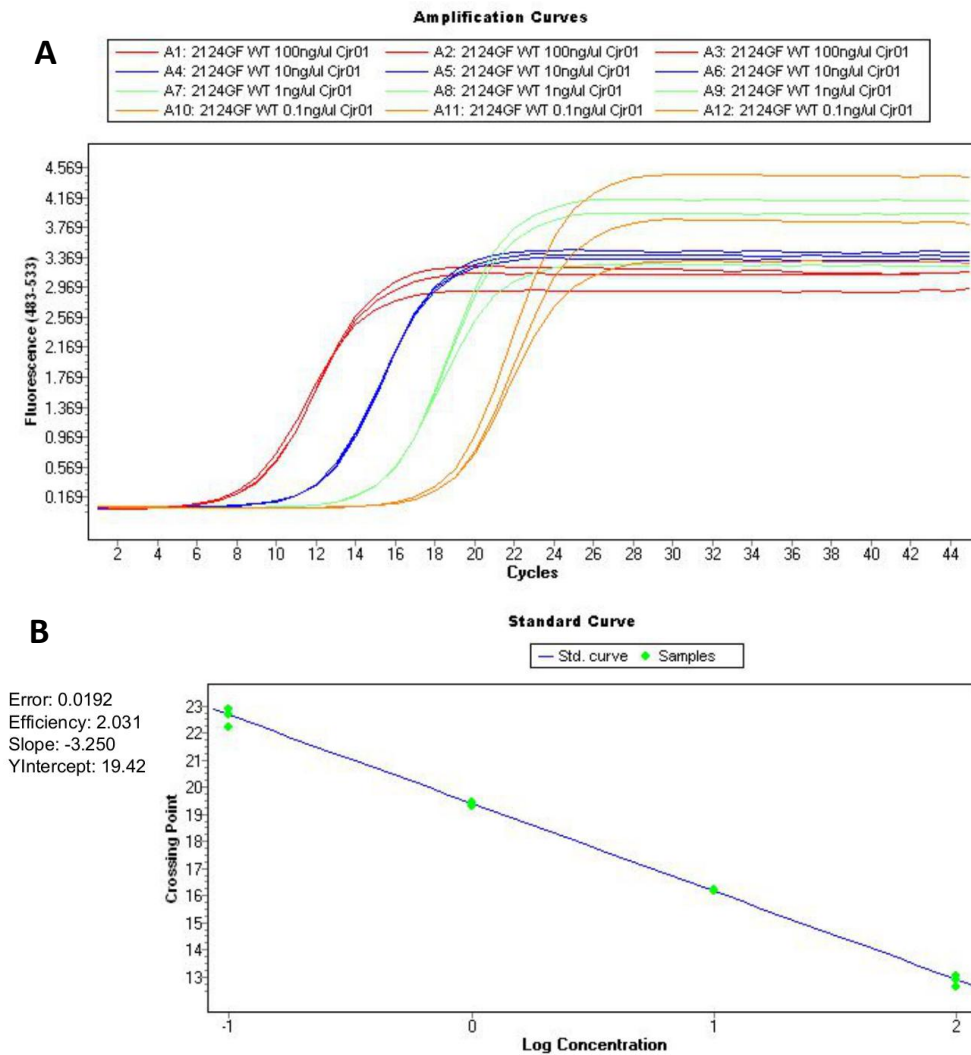


Figure 4.7. Real-time amplification of *cjr01* gene. Figure A presents the LightCycler quantification curves of cDNA standards, while Figure B the standard curve from where the primer efficiency was calculated. The primer's efficiency was calculated as $10^{-1/\text{slope}}$.

4.4.3. Quantitative analysis of the virulence genes expression in the chemostat challenged strains, qRT-PCR

Following the initial set up the PCR parameters and preparation of necessary PCR substrates, the quantitative Real-Time PCR could be performed. Virulence gene expression (*flgS*, *ciaB*, *cmeA*, *cdtB*) was determined for *Campylobacter* strains that grew at sub-MIC, MIC and if possible supra-MIC concentrations of the different disinfectants (CA, LA, TSPH, ASCh) under continuous culture conditions in a chemostat. In order to determine if disinfectant stresses had an effect on increased levels of virulence in *Campylobacter* disinfectant challenged strains, the expression level of four virulence genes were compared with the control sample collected before the disinfectant challenge studies began.

Furthermore, the expression of these genes were also investigated for a control chemostat, run in the absence of disinfectant, in order to determine the effect of the long-term culture on gene expression. The figures from 4.8 to 4.12 present the results of *ciaB*, *cmeA*, *cdtB* and *flgS* expression in *Campylobacter* strains challenged with the test disinfectants and also for control non-disinfectant challenged culture. As mentioned previously, in some instances the RNA extraction was not successful, probably due to degradation of the RNA from cultures exposed to concentration of disinfectants above their MIC value. In these cases, for the qRT-PCR strains challenged with lower concentration of disinfectant were taken.

4.4.4. Alterations in virulence gene expression in *Campylobacter* control chemostat carried out in the absence of the disinfectant

The control chemostat, run in the absence of disinfectant for strain 1136DFM for 1750.8 h (43.7 generations), was carried out to investigate the effect of the long-term culture on the genes expression alterations. Samples were taken at five different time points during continuous culture, collected at the start of the chemostat run, after 525 h, 861 h, 1054 h and 1510 h. At the end of the chemostat run biofilm material was also taken for analysis. Biofilm material was taken from two sites of the chemostat, from the sampling port and from the heating coil immersed in the fermenter vessel.

After 861 h of the continuous culture, 4.8-fold; 2.9-fold; 2.0-fold and 4.7-fold increases on gene expression were observed for *flgS*, *ciaB*, *cmeA*, *cdtB*, respectively in comparison to wild type controls (sample taken from the chemostat culture before the disinfectant challenge was commenced). The changes of the gene expression at the chemostat culture after 861 h over the wild type control were not statistically significant (Figure 4.8.). After 1054 h, however, continuous culture did lead to a significant increase in gene expression ($p > 0.0001$), where overexpression by 29.4-fold, 17.0-fold, 10.4-fold and 17.5-fold for *flgS*, *ciaB*, *cmeA* and *cdtB*, respectively was detected.

When gene expression for the sample collected after 1510 h, were compared with the genes expression of the sample of biofilm collected from the fermenter, a 9.5-fold, 10.4-fold, 10.2-fold decrease in expression for *flgS*, *cmeA*, *cdtB* in the biofilm was observed, respectively (Figure 4.8.). In addition, the *ciaB* gene in the biofilm sample taken from the fermenter was found to be down-regulated by 98.3-fold. In general, results obtained for the biofilm samples showed down-regulation of all virulence genes.

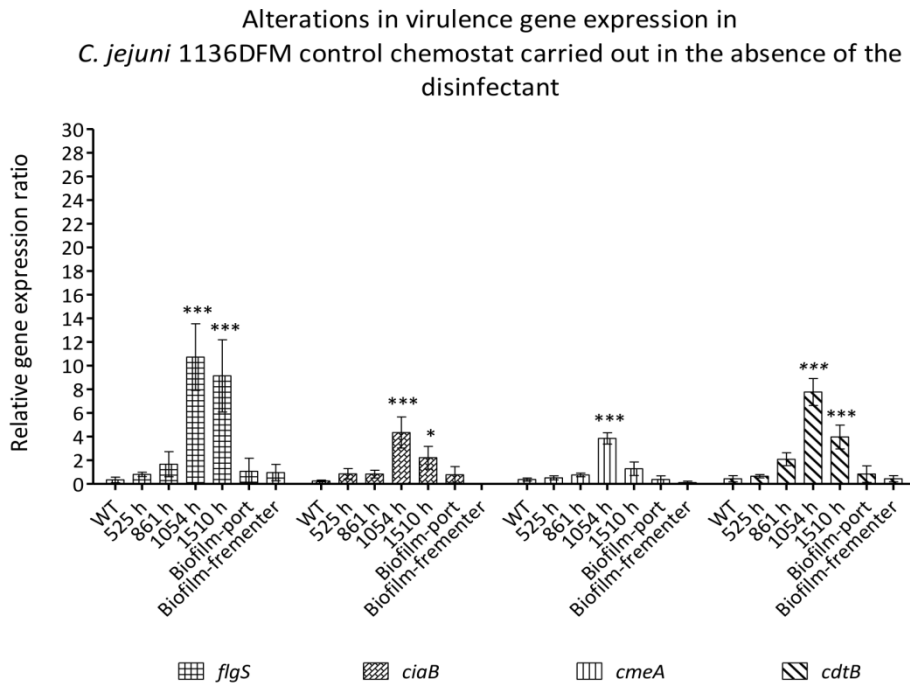


Figure 4.8. Alterations in virulence gene expression in *Campylobacter* control chemostat carried out in the absence of the disinfectant. The graph presents results for strains Ch1136DFM collected at different time intervals. (*) indicates $P < 0.05$, (***) indicates $P < 0.0001$.

During the course of the continuous culture of *Campylobacter jejuni* 1136DFM, biofilm formation was observed to occur. The first visible signs of clumping appeared after 850 h culture, while after 1000 hours, the biofilm was clearly formed and visible in the fermenter. Scanning electron microscopy results (see Chapter III, Section 3.4.7., Figure 3.11.) showed that at the end of chemostat run (1750.8 hours), no single bacterial cells were visible in “planktonic” culture. *Campylobacter* was grown as a biofilm. Analysis of the gene expression in “planktonic” culture taken after 1510 h showed their overexpression. It is probable in this sample collected for the qRT-PCR bacteria actively formed biofilm. However, when the clump of biofilm was analysed, no significant changes in gene expression were observed. It can be concluded, that the long-term challenge culture has an effect on the virulence gene expression after 861 h in the planktonic samples. However, in the fully formed biofilm no significant changes in the virulence gene expression were observed. Some studies indicate, that during the long-term culture at some stage the concentration of limited nutrient has to be considered (Dykhuizen & Hartl, 1983; Ellermeyer *et al.*, 2003). Probably, as a consequence of this, biofilm formation occurred and had an effect on the qRT-PCR results in the samples collected after 1054 h and 1510 h.

4.4.5. Virulence gene expression in *Campylobacter* strains challenged with CA

Two food isolates, *C. jejuni* 2124GF and *C. jejuni* 1136DFM were chosen for long-term exposure to citric acid. Strain 2124GF was run in the chemostat culture for 845.8 h where 21.1 generations were obtained, while strain 1136DFM was run for total 1631.7 h where 40.7 generations were achieved. Strains Ch2124GF and Ch1136DFM were grown up to 2-fold and up to 1.4-fold above the MIC value which was determined at 0.25% and 0.125% for the wild types. Figure 4.9. presents the level of gene expression in *C. jejuni* Ch2124GF and strain Ch1136DFM upon citric acid challenge. The overexpression of the gene coding the efflux pump (*cmeA*) were found to be up-regulated by 78.4-fold, 64.4-fold and 180.6-fold (to compare with the control sample collected before the disinfectant challenge was commenced) for Ch2124 challenged with 0.008%, Ch2124GF challenged with 0.125% and Ch2124GF adapted to 0.25% of CA respectively (Figure 4.9. A). These data were found to be highly statistically significant ($P < 0.0001$). In addition, up-regulation by 12.4-fold was also found for the *flgS* gene for Ch2124GF challenged with 0.008% of CA ($P < 0.0001$). The expression for remaining genes was not determined to be statistically significant from the control untreated *C. jejuni* 2124GF strain.

In the case of strain 1136DFM, a gradual increase of the expression for all virulence genes was achieved upon incremental increase of citric acid concentrations. During the course of this chemostat run, the presence of 0.18% CA in the chemostat, resulted in OD_{625} dropping from 0.086 to 0.020. It was decided at this stage to change the broth and run the chemostat without disinfectant. Continuous culture was carried out for a further 580 h (24.1 days). This disinfectant selective pressure removal allowed bacteria to recover and start to grow *de novo*. The expression of *flgS*, *ciaB*, *cdtB* genes after that period stayed at a high level. A 15.6-fold, 48.3-fold and 12.3-fold overexpression was detected for *flgS*, *ciaB* and *cdtB* genes respectively, for strain Ch1136DFM at the end of the experiment (1631.7 h), while in the biofilm, 32-fold, 64-fold and 39-fold up-regulation for *flgS*, *ciaB* and *cdtB* gene to compared with the control was found (Figure 4.9. B).

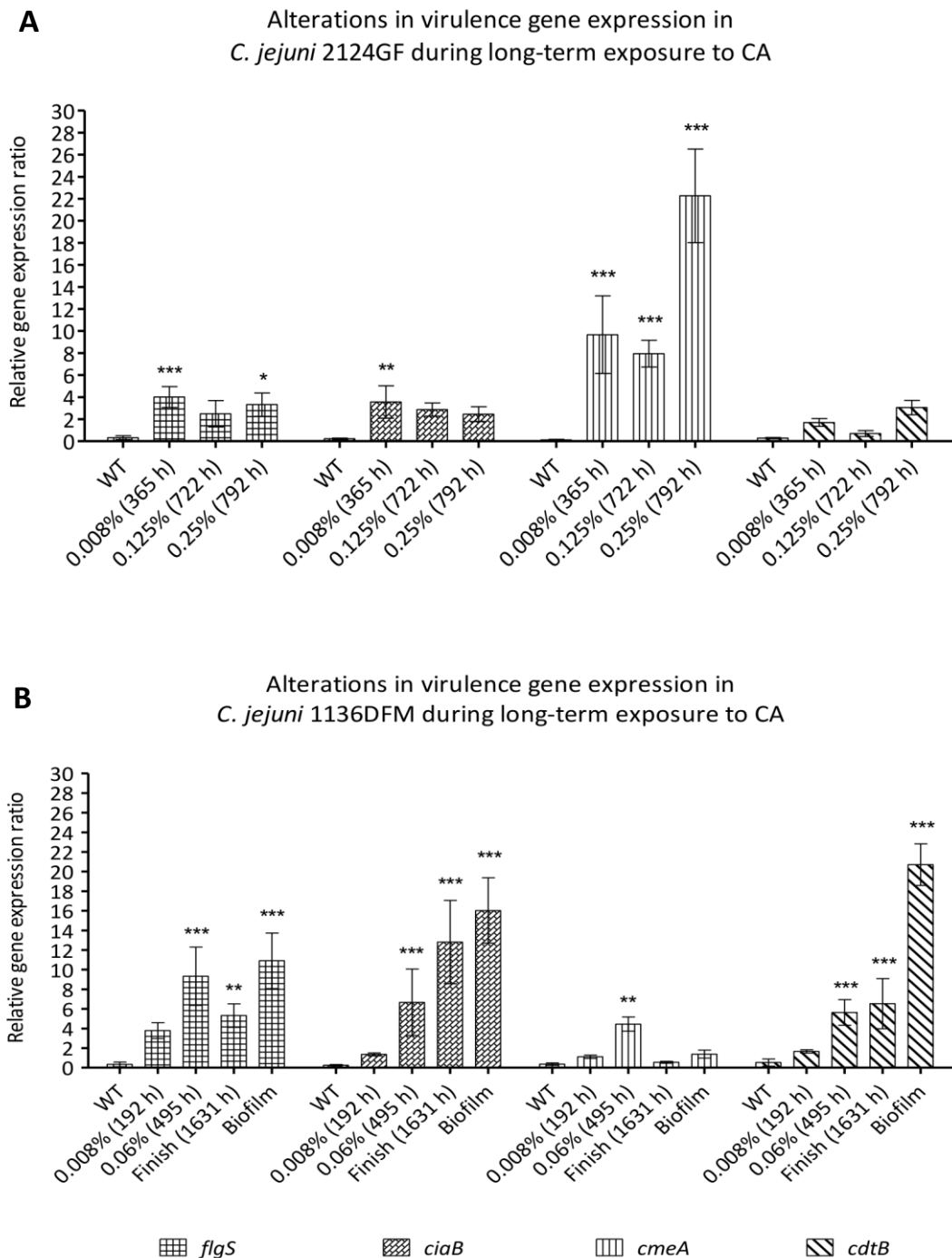


Figure 4.9. Alterations in virulence gene expression in *Campylobacter* strains during long-term exposure to CA. The Graph A presents results for strains Ch2124GF, while the Graph B for strains Ch1136DFM. (*) indicates $P < 0.05$, (**) indicates $P < 0.001$, (***) indicates $P < 0.0001$.

The results of the virulence gene expression in the long-term non-challenged with disinfectant culture, carried out for strain 1136DFM (see Section 4.4.4.), showed that up to 861 h the long-term culture did not have an effect on the overexpression of these genes. In the long-term exposure to CA cultures, the majority of the samples for qRT-PCR were collected before that time. This indicates that results obtained were the effect of

Campylobacter adaptation to CA, not the long-term culture by itself. Results obtained are the first signs that challenged strains might be more pathogenic.

Interesting results were observed when the two chemostat experiments were compared. Both chemostats were carried out for the same strain *C. jejuni* 1136DFM. The first chemostat run was carried out without any citric acid challenge while the second chemostat run was carried out with CA disinfectant challenge. Opposite results of virulence gene expression for biofilm was achieved. As mentioned previously, following initial challenge of Ch1136DFM strains to CA, while the OD at 625 nm dropped to 0.025 (after increasing citric acid concentration to 0.18%) the chemostat culture were allowed to run without citric acid selective pressure for 580 hours (24.1 days; 13.8 generations). In the control chemostat (continuously run without any disinfectant challenge), the virulence genes for biofilm were down-regulated and no statistically significant changes were observed (see Section 4.4.4 of this chapter), while for Ch1136DFM strain initially tested in the presence of CA, overexpression of *flgS*, *ciaB*, *cdtB* genes in biofilm was observed to be up-regulated by 32.1-fold, 62.7-fold, 38.9-fold, compared with the wild type. This demonstrates that the *Campylobacter* present in the formed biofilm that its formation took place during the long-term citric acid challenged culture, maintained high levels of the virulence gene expression even after removal the disinfectant. Results obtained demonstrate that the use of sub-inhibitory concentrations of CA might contribute to produce a more virulent phenotype in comparison with non-treated strains.

In addition, to prove that the expression of the virulence genes obtained during this study were the effect of bacterial adaptation to the disinfectant, two additional experiments were performed. Strain *C. jejuni* 1136DFM was run in a series of small volume batch cultures in the presence of different pH values adjusted with 0.5 M HCl (experiment 1) or with the presence of different CA concentrations (experiment 2). Figure 4.10. presents the experimental design of these experiments.

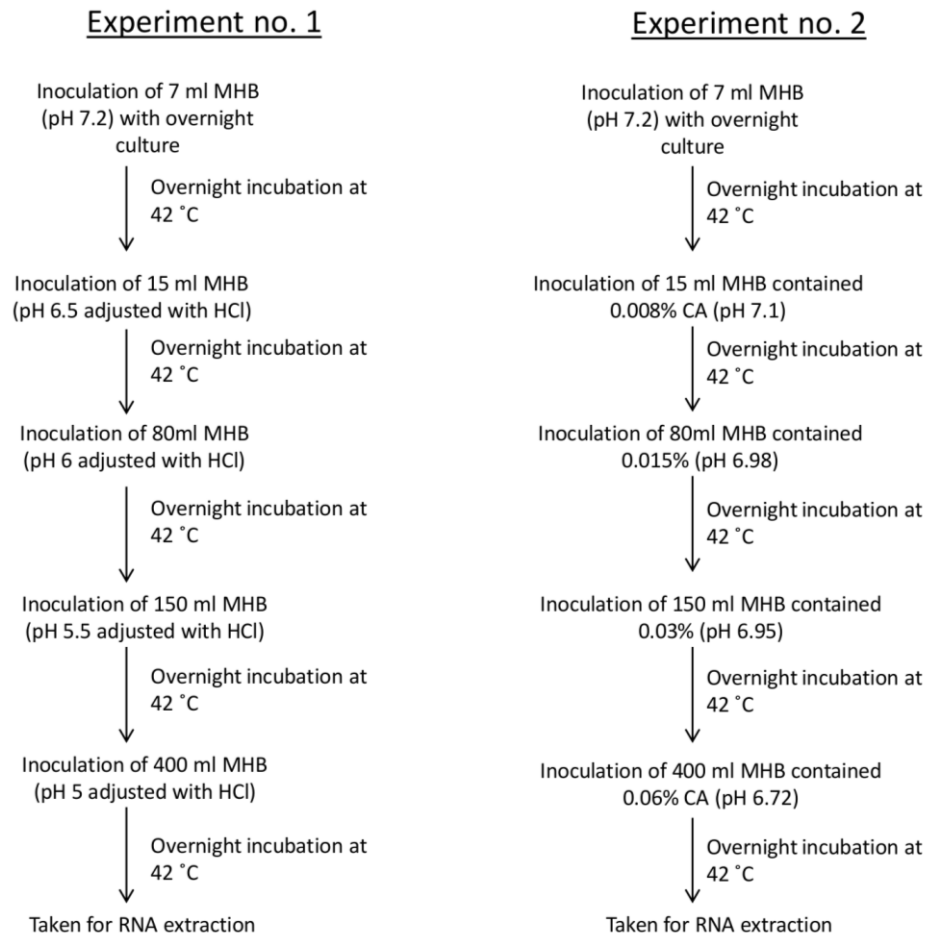


Figure 4.10. Experimental design of two supportive experiments investigating the effect of the short-term incubation in gradual decrease of pH on the virulence gene expression. In the Experiment 1 the pH of the cultures carried out in the Mueller-Hinton broth were adjusted with 0.5 M HCl, while in Experiment 2 the cultures were incubated with gradually decreased pH level adjusted with CA.

Following the performance of the culture, the bacteria were spun down and taken for RNA extraction according to procedure described previously in this chapter. qRT-PCR results showed no significant gene alteration in both experiments to compare to the WT. Results obtained from these two experiments, prove that the data of the virulence gene expression achieved from the long-term challenge with CA culture were the effect of *Campylobacter* adaptation to the disinfectant (Experiment 2). In addition, Experiment 1 showed that this adaptation was to CA, not to pH by itself.

4.4.6. Virulence gene expression in *Campylobacter* strains challenged with TSPH

The effect of the long-term TSPH exposure on virulence gene expression was investigated on a food and a clinical isolate, namely *C. jejuni* Ch1140DFO and *C. jejuni* Ch301BC. Strain 1140DFO was run in the chemostat for 1847.8 h where 46.2 generations were obtained, while strain 301BC was run for 1313.7 h where 32.8 generations were achieved. These strains were not able to grow in a chemostat above the broth MIC value that was calculated at 0.6% of TSPH for both strains. In the case of samples collected at concentrations greater than 0.125% TSPH, no RNA was successfully isolated. Probably the RNA was degraded by disinfectant.

Samples for qRT-PCT analysis for challenged strains Ch301BC were taken at the start of the chemostat run in the absence of a disinfectant challenge (control), at 0.008% TSPH, at 0.03% TSPH and at 0.125% TSPH. An initial gradual increase in *flgS* and *ciaB* genes expression was observed by 11-fold and 54-fold, respectively, when challenged with 0.03% of TSPH (Figure 4.11. A). This data indicates a highly statistically significant difference at $P < 0.0001$. Subsequently, the expression of these genes dropped for the Ch301BC challenged with 0.125% TSPH, indicating no statistically significant changes. In case of the *cmeA* and *cdtB* genes expression for strains Ch301BC, the overexpression was retrieved at a statically significant difference for all tested strains with the exception of the *cdtB* expression for Ch301BC challenged with 0.03% TSPH.

In the case of strains Ch1140DFO, samples for quantitative RT-PCR (qRT-PCR) analysis were taken at the start of the chemostat (before the long-term challenge culture was commenced) and was used as a control. Samples were also taken at 0.004% TSPH, at 0.06% TSPH and at 0.125% TSPH. qRT-PCR data showed, that gradually increased concentrations of TSPH resulted in a gradual increase of the expression of all four tested genes (Figure 4.11. B). However, a highly significant difference ($P < 0.0001$) for all genes was found only for strain Ch1140DFO challenged with 0.125% TSPH. A 4.7-fold; 4.1-fold; 8.9-fold and 4.5-fold overexpression for *flgS*, *ciaB*, *cmeA* and *cdtB*, respectively was observed.

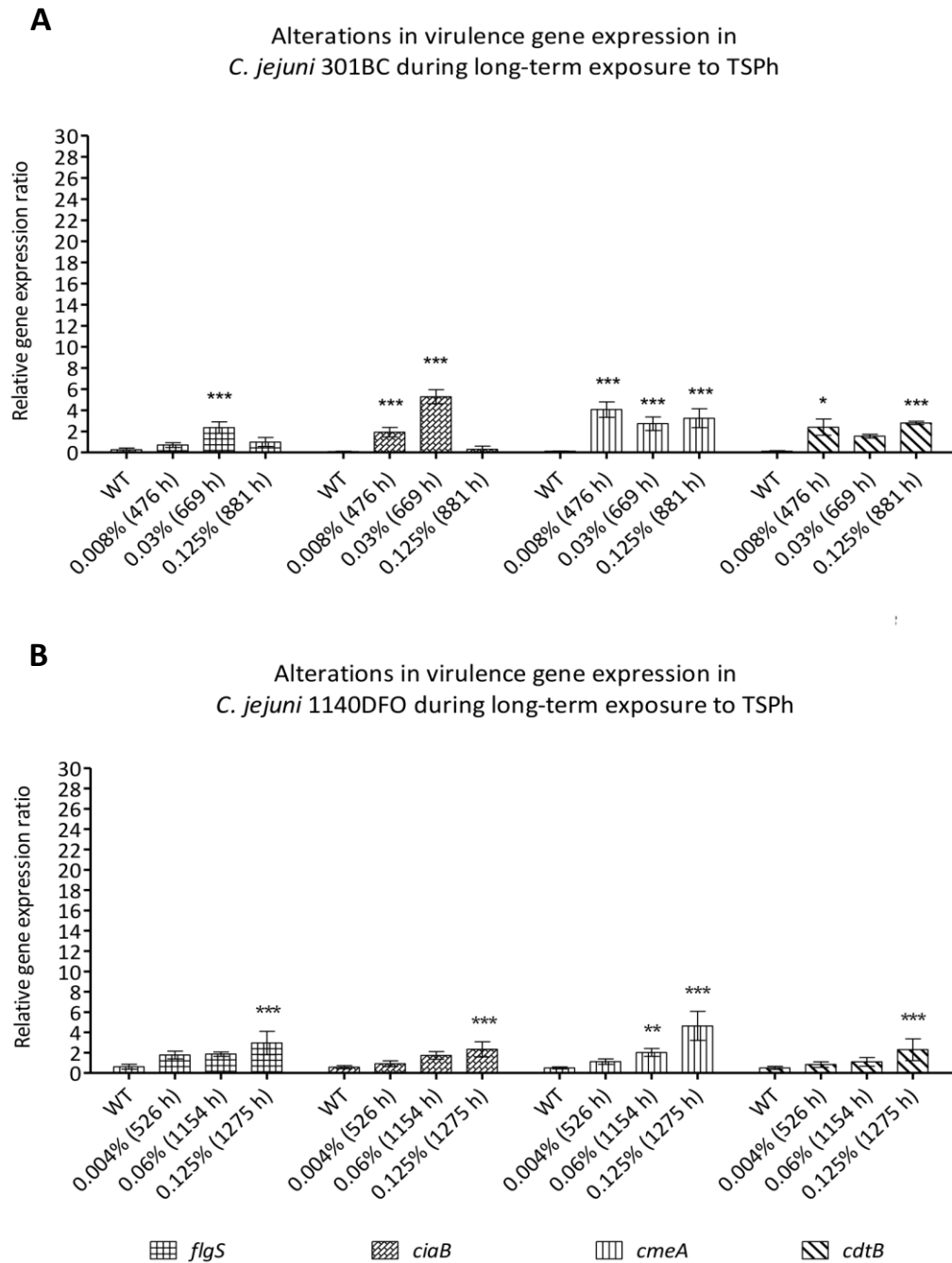


Figure 4.11. Alterations in virulence gene expression in *Campylobacter* strains during long-term exposure to TSPH. The Graph A presents results for strains Ch301BC, while the Graph B for strains Ch1140DFO. (*) indicates $P < 0.05$, (**) indicates $P < 0.001$, (***) $P < 0.0001$.

The up-regulation of the virulence genes in strain Ch301BC possibly are the bacterial response to TSPH stress as the sample for qRT-PCR were collected before or around 861 h, where no effect of the long-term culture on the gene was observed. However, the overexpression of the genes in strain Ch1140DFO (collected after 1154 h and 1265 h) might be a result of the expression related to the bacterial response to TSPH stress and as well the stress related to performance of the long-term culture.

4.4.7. Virulence gene expression in *Campylobacter* strains challenged with ASCh

Long-term ASCh challenged were carried out on two *C. jejuni* strains, 1136DFM (food isolates) and 354DC (clinical isolates). Strain 1136DFM was run in a chemostat culture for 1316.2 h where 32.9 generations were obtained, while strain 354DC was run for 1213.8 h where 30.3 generations were achieved. The ASCh MIC value for *C. jejuni* 1136DFM and *C. jejuni* 354DC strains was obtained at 25 $\mu\text{g ml}^{-1}$ and at 50 $\mu\text{g ml}^{-1}$. As a result of continuous cultures with incremental increase of ASCh concentrations, strain Ch354DC and strain Ch1136DFM were able to grow up to 4-fold and up to 8-fold above the MIC value.

Figure 4.12 presents the level of gene expression in *Campylobacter jejuni* Ch354DC and strain Ch1136DFM upon ASCh challenge. The qRT-PCR results showed that the transcription level of *ciaB* gene was gradually increased for both strains tested. In the case of strain Ch354DC adapted to 100 $\mu\text{g ml}^{-1}$ ASCh, an 11.8-fold increase in *ciaB* gene expression was observed, while an 41.8-fold expression increase of this gene was observed for strain Ch1136DFM adapted to 50 $\mu\text{g ml}^{-1}$ ASCh. These increased gene expression levels were found to be highly statistically significant ($P < 0.0001$). Changes of the remaining genes were found in the majority not to have a statistically significant expression. The exception constituted the expression of *flgS* and *cdtB* genes for strain Ch1136DFM adapted to 25 $\mu\text{g ml}^{-1}$ of ASCh, when 9.7-fold and 9-fold of overexpression of these genes at $P < 0.001$ and $P < 0.0001$ were observed.

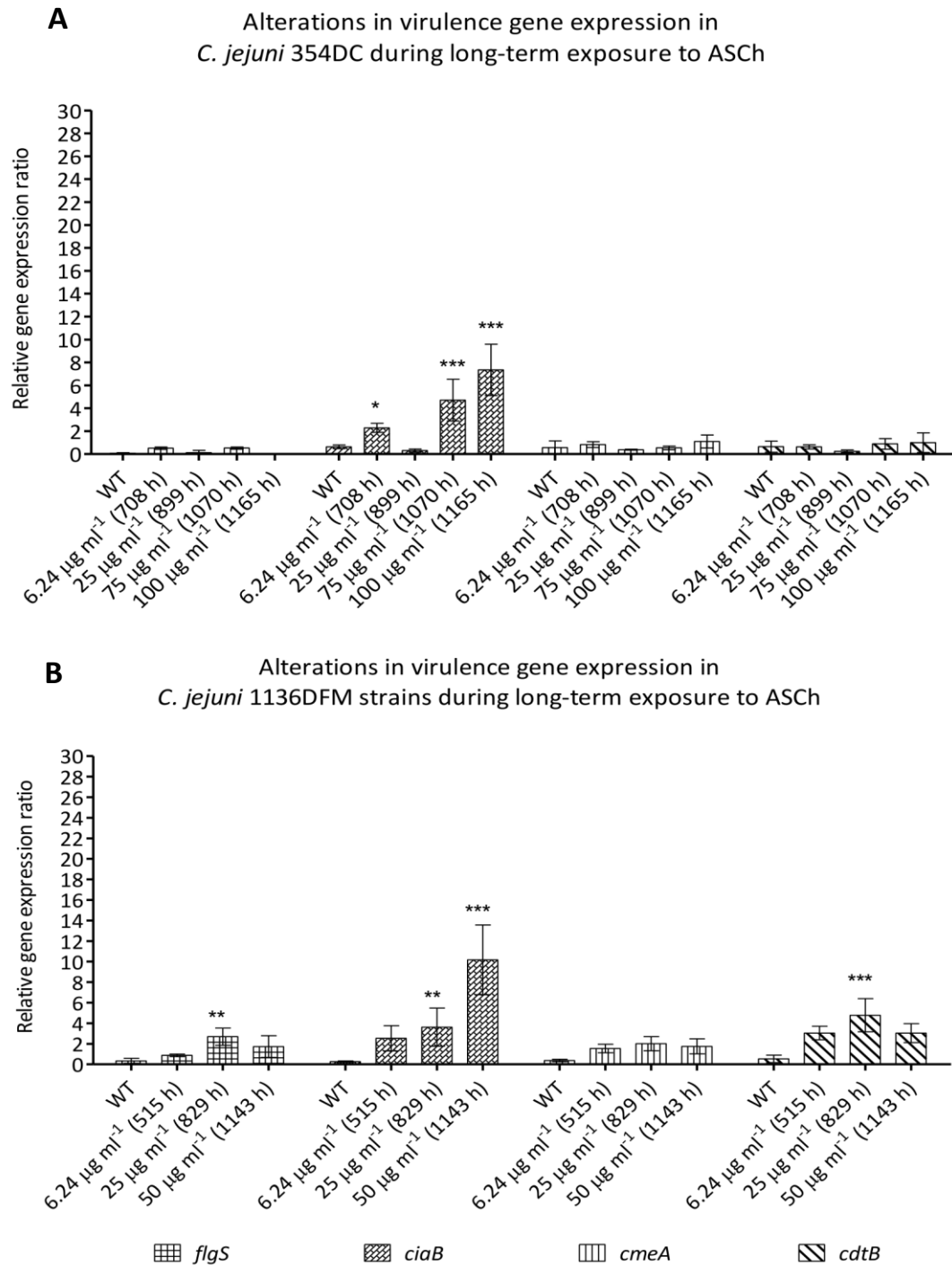


Figure 4.12. Alterations in virulence gene expression in *Campylobacter* strains during the long-term exposure to ASCh. The Graph A presents results for strains Ch354DC, while the Graph B for strains Ch1136DFM. (*) indicates $P < 0.05$, (**) indicates $P < 0.001$, (***) $P < 0.0001$.

Considering the effect of the long-term culture on virulence gene expression, the overexpression of *ciaB* gene in strains Ch354DC adapted to $75 \mu\text{g ml}^{-1}$, Ch354DC adapted to $100 \mu\text{g ml}^{-1}$ and Ch1136DFM adapted to $50 \mu\text{g ml}^{-1}$ might be the resultant of the stress related to presence of ASCh and the long-term culture by itself.

4.4.8. Virulence gene expression in *Campylobacter* strains challenged with LA

Long-term lactic acid challenged was carried out on *C. jejuni* 23GC (a clinical isolate) over 1456.6 h, where 36.4 generations were obtained. Chemostat challenged bacteria were able to grow at the MIC value (0.125%) and up to 2-fold above the MIC value. However, the qRT-PCT results showed no statistically significant overexpression for any of the virulent genes under any of the test lactic acid concentrations for all tested strains Ch23GC occurred. The exception constitutes statistically significant up-regulation for *ciaB* gene (2.5-fold increase) for strain Ch23GC adapted to 0.125% LA (Figure 4.13.).

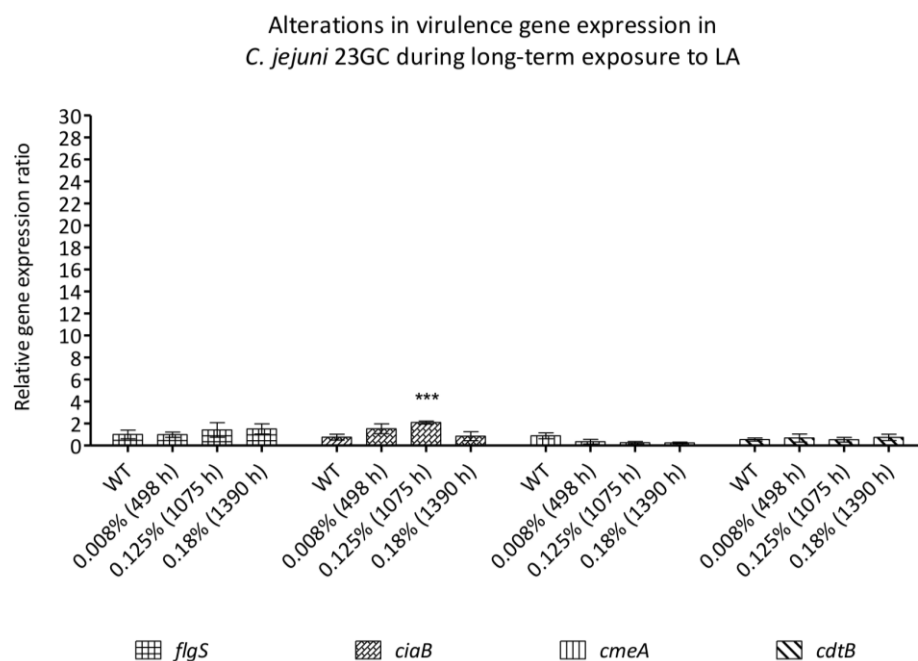


Figure 4.13. Alterations in virulence gene expression in *Campylobacter* strain during long-term exposure to LA. The graph presents results for strains Ch23GC. (***) indicates $P < 0.0001$.

To conclude, the expression of virulence genes in tested strains was dependent on the type of disinfectant used and the strain specificity. The use of sub-inhibitory concentrations of disinfectants may produce stable more virulent bacteria. However, during analysis of the gene expression alterations, the effect of the long-term culture had to be considered. In the case of strain Ch1140DFO challenged with TSPH and Ch354DC challenged with ASCh the overexpression could be attributed to the negative effect of the long-term culture. Furthermore, the process of biofilm formation that was observed in the control chemostat (run in the absence of disinfectant) and as well in the chemostat challenged strains, might have had an effect on the gene expression alterations. The biofilm formation while carrying out the challenged cultures possibly functioned as a *Campylobacter* protectant against pH

stress deriving from LA, CA and TSPH or oxidative stress deriving from ASCh. In addition, biofilm might create suitable environments for *Campylobacter* to become more pathogenic.

4.5. DISCUSSION

Performance of the long-term challenged cultures in the presence of different disinfectant resulted in bacterial growth not only at the MIC value but also up to 1.4-fold and up to 8-fold above it, depending on the strain tested and antimicrobial used. These results suggest that the expression of the virulence genes in these bacteria might be altered and *Campylobacter* disinfectant challenged strains might become more pathogenic. The study of the expression of the virulence genes, might not only allow evaluation of the risk connected with the application of disinfectants for decontamination purposes, but might also contribute to better understanding of the *Campylobacter* stress response mechanism.

The disinfectants used for bacterial decontamination should effectively eliminate them and not produce more virulence phenotypes. A study by Rodrigues *et al.* investigated the effectiveness of hypochlorite, benzalkonium chloride, hydrogen peroxide and triclosan on *Listeria monocytogenes* and *Salmonella enterica* biofilm removal (Rodrigues *et al.*, 2011). Their results showed that although the disinfectants used eliminated the cells that grew in biofilm, the stress-response genes in *L. monocytogenes* and virulence genes in *S. enterica* were found to be up-regulated in surviving cells. This indicates that the cells that survive the stressful conditions are more pathogenic. Kastbjerg *et al.* investigated the virulence gene expression in *L. monocytogenes* treated with sub-lethal concentrations of disinfectants from different groups: peroxy, chlorine and quaternary ammonium compounds (Kastbjerg *et al.*, 2010). They found that the virulence genes were down-regulated while peroxy and chlorine compounds were used. In the case of quaternary ammonium compounds the expression was found to be increased. In addition, they observed that disinfectants with the same active ingredients had a similar effect on gene expression.

In the work presented in this chapter, the expression of *Campylobacter* virulence genes was dependent on the disinfectant used for the long-term challenge culture and the tested strain. However, similarities of gene expression between two strains tested with the same disinfectants can be observed. As the disinfectants challenge studies were performed continuously between 845.8 h and 1847.8 h on the chemostat system, the effect of the long-term culture on the expression of the virulence genes had to also be investigated. According to the results obtained from the non-challenge chemostat trial carried out for strain 1136DFM for 1750.8 h, no significant changes in the gene expression up to 861 h

were obtained. When the next sample was collected after 1054 h highly statistically significant differences in genes expression were found. This indicates that the long-term culture does influence the expression of virulence genes after 861 h of the culture and this finding should have been taken into consideration while the changes in gene expression of disinfectant challenged cultures were being analysed. The differences in the expression in the control chemostat could possibly be an effect of biofilm formation during the chemostat run. The first visible signs of biofilm formation were observed after 850 h, while the sample collection after 1054 h, the biofilm was clearly visible. As a biofilm formation is recognised with a bacterial response mechanism to stress (Joshua *et al.*, 2006; Kalmokoff *et al.*, 2006; Merino *et al.*, 2006), the biofilm formation in a non-challenged chemostat may indicate the presence of other stress or stressors. Possibly, the stress was related to limited nutrient concentration. It has been reported that during performance of long-term culture, the limited nutrient concentration at some stage has to be considered (Dykhuizen & Hartl, 1983).

Analysis of the overexpression of all virulence genes when the TSPH challenge was carried out for *C. jejuni* 301BC, showed that the up-regulation is the effect of TSPH not other stress, as the samples for qRT-PCR were collected before 861 h. However in the case of the strain 1140DFO it is not clear if the gene up-regulation in strain Ch1140DFO challenged with 0.125% TSPH (collected after 1275 h) was related to TSPH stress or limited nutrient concentration or both of these stressors. A similar issue was observed in some case of the samples collected from ASCh long-term challenged cultures. In the case of CA challenged culture, the majority samples for qRT-PCR were collected before 861 h. The highly significant changes in the expression of virulence genes were possibly caused by CA only. However, in the case of CA the biofilm formation was observed following the addition of the first dose of disinfectant (after 290 h for strain Ch2124GF and after 192 h for strain Ch1136DFM). Considering the biofilm formation at an early stage of the culture, it might be concluded that the expression results obtained during the CA challenge might be not only caused by CA stress but also stress related to biofilm formation. In the control chemostat the biofilm formation was probably related to the occurrence of limited nutrient concentration, while in CA challenged culture the biofilm formation was observed at a very early stage. Probably, the biofilm formation was a result of bacterial response to stress. The key question is how much influence the biofilm expression had on the virulence gene expression and how much the expression of the genes were dependent on the disinfectant adaptation. Unfortunately, for the time being the question remains unanswered.

In addition, a comparison between the results obtained for the biofilm from the control chemostat carried out for 1136DFM and the biofilm from the chemostat challenged with the CA that was carried out for the same strain, 1136DFM, led to an interesting finding. The long-term non-disinfectant challenged culture for 1136DFM did not cause statistically significant overexpression in the biofilm for any of the virulence genes. However, the chemostat CA challenge culture for that strain showed a highly statistically significant difference in virulence gene expression in biofilm. This can be regarded as a worrying signal that the sub-lethal CA application may affect virulence gene expression in the human pathogen *Campylobacter jejuni*.

When the continuous culture was performed for strain 23GC challenged with LA, no significant differences in the gene expression was observed (with the exception of 2.5-fold overexpression of *ciaB* for strain Ch23GC adapted to 0.125% LA). Although the samples for qRT-PCR were collected after 861 h and later biofilm formation was observed, no significant changes in the expression were found. All these results and observations lead to a multifaceted problem of stress related to disinfectant application, biofilm formation and possibly limited nutrient concentration. Probably, each of the disinfectants has to be considered individually and no universal answers about the effect of these factors can be given.

Data from the gene expression study is presented as an average value of the gene expression within the entire population of planktonic bacteria or growing as a biofilm. The study on biofilm showed that its structure is heterogeneous. The level of gene expression varied within different zones of biofilm (Lenz *et al.*, 2008; Stewart & Franklin, 2008). In general, the bacterial activity in lower layers might be decreased to zero, while in the upper layers of biofilm the bacteria could still show a high activity. Decreased activity of bacteria in the lower layers results in a lower level of mRNA. According to Folsom *et al.* (2010), the number of RNA copies of the *Pseudomonas aeruginosa* housekeeping gene was approximately 60 times greater at the top of the biofilm compared to the bottom, while in another tested gene the difference between the top and bottom was approximately 30-fold. In order to identify the expression activity among different layers of the biofilm various techniques such as laser capture microscopy supplemented with qRT-PCR or reporter genes method can be used.

The heterogeneous structure of the biofilm and findings about the various levels of the gene expression within biofilm might indicate that results obtained in this study for the *Campylobacter* virulence gene expression might be even greater.

To conclude, tested disinfectants in this study, with the exception of LA, had an effect on virulence gene expression. Challenged bacteria were not only able to adapt and survive longer in the disinfectant's presence, as the results from the Chapter III illustrate, but probably also became more pathogenic.

Acknowledgments

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CHAPTER V

***Campylobacter* proteome response to long-term disinfectant challenged**

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Key words: 2-D gel electrophoresis; *Campylobacter*; chaperone and chaperonin proteins; mass spectrometry; protein expression

5.1. SUMMARY

The results obtained and presented in the previous chapters indicate that depending on the *Campylobacter* strain and disinfectant used, a sub-inhibitory level of disinfectant might contribute not only to better bacterial survival in the presence of antimicrobial agents but also might possibly produce more virulent strains. These results suggest that protein expression is also altered. Examination of these changes may allow a better understanding of *Campylobacter* pH/oxidation stress response mechanism. This knowledge might also contribute to combating severe disease caused by campylobacters. Enteric pathogens, in order to transit through the gut and cause disease, must survive the acid pH of the stomach. A survival mechanism that is activated as a response to pH stress is well-known for other Gram-negative pathogens such as *Escherichia* and *Salmonella*, but still little is known about the molecular mechanisms in *Campylobacter*.

In this study, the chemostat challenged strains to different disinfectants (citric acid, lactic acid, acidified sodium chlorite and trisodium phosphate), were collected at different time intervals from the continuous cultures while challenged to the different disinfectants, in order to investigate the proteins' expression changes during the chemostat trials. The expression of the proteins for chemostat strains was compared to the expression for their wild type counterparts. In addition, all the proteins' expression gels were compared between each other in order to investigate the similarity of the bacterial proteome response to the different disinfectant stress. Following PDQuest analysis, changes in the proteins' expression greater than 2.5-fold were considered significant. The majority of the analysed proteins were down-regulated. Mass spectrometry was carried out on 18 spots of interest (14 spots were identified from different gels run for *C. jejuni* disinfectant challenge strains and 4 spots were identified from the gels run for *C. jejuni* non-disinfectant challenge strains). The results obtained from the mass spectrometry showed that among proteins that were identified, those involved in the oxidative stress response: thiol peroxidase (Tpx), oxidoreductase and alkyl hydroperoxide reductase (AphC); transport/binding activity: molybdate ABC transporter (ModA), glutamine-binding protein and bacterioferritin (Bfr); or taking part with translation process: ribosome recycling factor (Frr) and translation elongation factor (FusA) were detected. Among the ions binding proteins involved in oxidative stress: hemerythrin family non-heme iron protein, flavohemoprotein and rubrerythrin (Rbr) were also found.

Furthermore, the investigation of protein expression alterations in a control chemostat performed for strain Ch1136DFM (run without disinfectant challenge) was carried out in order to investigate the effect of the long-term culture on the proteins' expression. Results obtained showed that long-term culture (up to 861 hours) does not have a significant effect on the proteins' expression.

5.2. INTRODUCTION

The research that has been presented in the previous chapters was carried out in order to determine if the disinfectants such as citric acid, lactic acid, acidified sodium chlorite and trisodium phosphate are suitable for *Campylobacter* decontamination in poultry farms and in processing plants in terms of producing more resistant and more pathogenic strains. Disinfectants used for decontamination purposes should effectively reduce the bacterial contamination level, should not produce more virulent phenotypes and be safe for humans and the environment. Although on the commercial scale recommended doses are much higher than the MIC level obtained for *Campylobacter*, it is important to investigate the effects of applications of sub-inhibitory doses on *Campylobacter* response. Due to a lack of proper personnel training or disinfectant inactivation caused by the presence of organic debris, bacteria may only be exposed to sub-lethal concentrations and survive and adapt after repeated exposure. In order to examine the potential risk related to disinfectant application on the farms and in the processing plants as a decontaminants, investigation of possible *Campylobacter* adaptation to disinfectants as well as their risk in terms of producing more pathogenic strains were performed. The work presented in the thesis, shows that depending on the *Campylobacter* strain and the disinfectant used, that sub-inhibitory levels of disinfectant might contribute not only in a better bacterial survival in the presence of disinfectant but also might result in producing more virulent strains.

Besides this type of study not only allows us to investigate if the proposed disinfectants will be safe and efficiently remove bacterial contamination in poultry farms and processing plants, but as well it might allow a better understanding of the response mechanism of *Campylobacter* to pH/oxidative stress and its pathogenicity. Although survival mechanisms that are activated during response to pH stress are well known for other food-borne pathogens as *Escherichia* and *Salmonella* (Audia *et al.*, 2001; Bearson *et al.*, 1998), still little is known about the molecular mechanisms in campylobacters (Shaheen, 2007). Phongsisay and co-workers investigated the role of the *htrB* gene in response to pH stress (Phongsisay *et al.*, 2007). This gene encodes a lipid A acyltransferase in *Escherichia coli* and *Salmonella typhimurium*. The *htrB* homologue was also found in *C. jejuni*, and is similar to the *htrB* gene of *Salmonella typhimurium* and *Escherichia coli* (Phongsisay *et al.*, 2007). The authors in their study used *Salmonella htrB* mutant that was not able to grow under heat, acid and osmotic stresses. They constructed a plasmid carrying the *C. jejuni htrB* gene and placed it the *S. typhimurium htrB* mutant. Their results showed that presence of plasmid

with *htrB* gene allowed the normal morphology in *Salmonella* to be restored and parts of its physiology. This indicates that *htrB* homolog in *Campylobacter* plays the same role as *htrB* gene in *Salmonella* (Phongsisay *et al.*, 2007).

One of the *Campylobacter* response mechanisms to acid stress is the induction of an adaptive tolerance response, ATR (Murphy *et al.*, 2003). A study on protein expression during activation the ATR, showed up-regulation of proteins such as DnaK (heat shock protein) and GroEL (chaperone protein). These proteins are involved in repairing proteins from damages caused by acid (Murphy *et al.*, 2006). In addition, DnaK and GroEL have been found to play important roles in other pathogens. For instance, these proteins have been up-regulated in *Salmonella* as a response to acid stress and are involved in repairing damages after acid stress (Foster, 1991).

A studies by Reid and others, investigated the *Campylobacter* proteome response for *in vivo* acid shock (Reid *et al.*, 2008a) and growth in acidic conditions, pH 5.5 (Reid *et al.*, 2008b). Following comparison of the proteins' expression results obtained from both experiments, they found that down-regulated protein were succinate dehydrogenase subunits (SdhAB), a gene encoding a Na⁺/H⁺ antiporter, genes for nitrite respiration and series genes of unknown function. Up-regulation, however was found in single-domain globin associated with nitric oxide stress defence, the peroxide regulator gene, a putative ferric-siderophore transporter, genes encoding putative oxidoreductase subunits and genes of unknown function (Reid *et al.*, 2008b).

Campylobacter jejuni NCTC 11168, in response to pH stress (20 min at pH 4.5), started up-regulating genes such as *metA* (putative homoserine O-succinyltransferase), *metY* (putative homoserine O-succinyltransferase), *Cj0414* (putative oxidoreductase subunit), *Cj0876c* (a putative periplasmic protein) and *Cj1586* (putative bacterial hemoglobin) (Padney, 2005). However, when the sample was collected for DNA microarray analysis after 2 min of exposure to acid stress, the overexpression of genes encoding chaperones protein GroEL, GrpE; heat shock proteins such as DnaK (heat shock protein) and HrcA (heat-inducible transcription repressor); transport proteins PebC (ABC-type amino-acid transporter) and periplasmic proteins p19 (periplasmic protein); oxidative stress protein such as AhpC (alkyl hydroperoxide reductase), KatA (catalase), Cft (ferritin), TrxB (thioredoxin reductase) and PerR (peroxide stress regulator) have been found (Padney, 2005).

The work presented in this chapter concentrates on the analysis of proteins changes between *C. jejuni* wild types and *C. jejuni* long-term chemostat challenged with different disinfectants strains in order to better understand the *Campylobacter* response to pH stress at the protein translation level.

5.3. Material and Methods

5.3.1. Bacterial isolates

Originally six *C. jejuni* strains, three clinical 23GC, 354DC, 301BC and three food isolates 1136DFM, 2124GF and 1140DFO were long-term challenged with various disinfectants using the chemostat culture. The effect of citric acid (CA) on strain 2124GF and 1136DFM was carried out, while strain 23GC was challenged with lactic acid (LA). Additionally, strains 1136DFM and 354DF was tested for acidified sodium chlorite (ASCh), while 301BC and 1140DFO for trisodium phosphate (TSPH). In this study, samples from the chemostat disinfectant challenged cultures were collected from the fermenter after their adaptation to each of the tested concentration of disinfectant. For the proteome study samples of bacteria challenged to sub-MIC, at MIC and supra-MIC were used.

5.3.2. Two-dimensional gel electrophoresis

The whole cells protein were separated by two dimensional gel electrophoresis (2-D gel) using a modified version of Farrell's method (1975).

A 200 ml aliquot of *Campylobacter* spp. was collected aseptically from the fermenter via the sampling port into sterile Beckman centrifuge tube. Chloramphenicol (Sigma) was added at a final concentration of 10 µg/ml to inhibit protein synthesis. After centrifugation at 21,000 × *g* at 4°C for 10 min, the supernatant was discarded and pellets were resuspended and washed with 10 ml of sonication buffer (10 mM Tris-HCl, 0.1 mM EDTA, 5 mM MgCl₂) supplemented with 10 µg/ml of chloramphenicol and centrifuged at 5,500 × *g* at 4°C for 20 min. Following this, the pellets were resuspended in 2 ml of the sonication buffer containing 10 µg/ml of chloraphenicol and 1% (v/v) of protease inhibitor cocktail (Sigma-Aldrich). The suspensions were sonicated on ice where 10 pulses were applied, with 30 s rest intervals between pulses. Soluble proteins were separated from the cell debris by centrifugation for 30 min at 13,000 × *g*. Supernatant was aliquoted into 1.5-ml centrifuge tubes and Benzonase® Nuclease (Sigma-Aldrich) was added to each sample (final concentration of 44 U/ml). Samples were subsequently incubated at 37°C for 30 min. The Benzonase® Nuclease treatment was repeated once. The protein's samples were centrifuged at 13,000 × *g* for 30 min and the supernatants were removed. Protein

concentrations were determined using the Bio-Rad D_c Protein Assay kit (CA, USA) following the manufacturer's protocol with bovine serum albumin used as a standard (Bio-Rad).

Prior to separation by isoelectric focusing (IEF), 600 µg of protein was precipitated overnight at -20°C using ice cold acetone. The suspension was subsequently centrifuged at 13,000 × *g* for 15 min and air dry for 30 min. The dried pellets were resuspended in 230 µl of DeStreak™ Rehydration Solution (GE Healthcare), supplemented with 0.4% (v/v) IPG buffer (pH 3-10, pH 4-7 or pH 7-11; obtained from GE Healthcare, Sweden) and 2.5 mg of DTT (GE Healthcare), by vortexing for 2 min every 10 min for 1 hour at room temperature, and then incubated in a 37°C for 15 min. The resuspended material was centrifuged for 10 min at 13,000 × *g* and the insoluble material was removed. The supernatant was loaded onto the IPG strips (pH 3-10, pH 4-7 or pH 7-11) placed in rehydration tray. Strips were covered with mineral oil (Sigma-Aldrich) and the tray was left at room temperature for 18 hours, allowing sufficient rehydration. Subsequently, isoelectric focusing was performed at two steps using an Investigator™ 5000 (Genomic Solutions, USA). The first step was carried out with the following parameters; voltage of 500 V, 12,000 Volt-Hours, 125 Vhold, 50 µA/gel, 24 hours, temperature of 20°C, while the second at: 5,000 V, 14,000 V-Hours, 125 Vhold, 50 µA/gel, 3 hours, at a temperature of 20°C.

Prior to running the second dimension gel electrophoresis (SDS-PAGE, with 16% acrylamide), IPG strips were equilibrated for 20 min in 10 ml with equilibration buffer A (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg ml⁻¹ of DTT, 0.05% (w/v) bromophenol blue, 45 mM Tris base, pH 7) followed by another 20 min in 10 ml of equilibration buffer B (as buffer A, but containing 25 mg ml⁻¹ iodoacetamide instead of DTT). Gels were prepared using a modified version of the method described by Schägger (2006). To prepare 16% acrylamide buffer 10 ml of Bis-acrylamide (0.73 v/v of 40% acrylamide, 0.8 mg ml⁻¹ of N,N'-methylenebisacrylamide); 10 ml of 3X gel buffer (3 M Tris, 0.3% SDS adjusted to pH 8.45 with HCl); 3 ml of glycerol was mix and supplemented with sterile MQ water up to 30 ml. The polymerisation was carried out by adding 100 µl of 10% ammonium per sulphate (APS, Pierce) and 10 µl of N,N,N',N' tetramethylethylenediamide (TEMED, Pierce). PageRuler™ Plus Prestained Protein Ladder (Fermentas) with a range of 10-250 kDa was used as molecular weight markers. The gel was run in 1X Tris-glycine buffer (5X buffer contained: 1.5% w/v Tris; 7.2% w/v glycine; 0.5% w/v SDS) cooled down to 4 °C. The second dimension gels were run in pairs using a CRITERION™ Cell (Bio-Rad, California, USA), under a constant power, 120 V, until the bromophenol blue dye line reached the bottom of the gel.

Gels were stained overnight in Comassie[®] Brilliant Blue R-250 (Bio-Rad) containing 50% methanol and 10% acetic acid. Following this, the gel was destained with destaining solution (10% methanol and 10% acetic acid) until the dye was washed out from the background.

5.3.3. Data analysis

Gels were systematically analysed by sight and with the PDQuest-Advanced software version 8.0.1 (Bio-Rad, California, USA). Data was normalised using the Linear Regression Model. A curve was computed in the scatter plot and the normalisation factor for each spot was calculated. Protein expression differences greater than 2.5-fold were considered significant. Four replicates of acrylamide gels were carried out for each tested strain, in two separately protein isolation. However, for PDQuest analysis only two replicates were taken.

5.3.4. Proteins identification; Analysis on the TripleTOF 5600

The proteins spots were identified by the Mass Spectrometry and Proteomics Facility, Centre for Biomolecular Sciences, University of St. Andrews, Scotland. The gel band was excised and cut into 1mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols (Shevchenko, et al, 1996). Briefly the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37°C. The peptides were extracted with 10% formic acid and concentrated down to 20 µl using a SpeedVac (ThermoSavant).

The peptides were then separated using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler equipped with a nanoflex cHiPLC chip based chromatography system (Eskigent), using a ChromXP C18-CL trap and column (Eskigent). The peptides were eluted with a gradient of increasing acetonitrile, containing 0.1% formic acid (5-35% acetonitrile in 45 min, 35-50% in a further 3 min, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile). The eluent was sprayed into a TripleTOF 5600 electrospray tandem mass spectrometer (ABSciex, Foster City, CA) and analysed in Information Dependent Acquisition (IDA) mode, performing 250 ms of MS followed by 100 ms MSMS analyses on the 20 most intense peaks seen by MS. The MS/MS data file

generated was analysed using the ProteinPilot 4.1 Paragon algorithm (ABSciex) against the NCBI nr database July 2012 with no species restriction, trypsin as the cleavage enzyme and carbamidomethyl modification of cysteines.

5.4. RESULTS

Campylobacter samples collected at different time intervals during performing the long-term challenge with disinfectant cultures were used for protein expression alterations. In order to determine the changes in the proteins' expression, two dimensional gel electrophoresis (2-D gels) were carried out. The whole cell proteins were separated at first by the pI of the proteins and then by their molecular weight. Spots obtained on the gels for the different chemostat challenged strains were compared to their WT counterparts using the PDQuest software. For the mass spectrometry proteins identification those with a significant changes were chosen.

5.4.1. Protein expression changes in the disinfectant challenged chemostat cultures

In investigating the effect of four different disinfectants for six different *C. jejuni* isolates on the bacterial proteome response, different spots from different gels were chosen for analysis. Table 5.1. below presents which spot from which disinfectant challenged strain was collected for the mass spectrometry analysis. In total 14 protein spots were taken from the different strains challenged to the different disinfectants. The spot number 1 was taken from the strain Ch1140DFO challenged with TSPH, spots number 2, 3 and 4 were collected for the strain Ch23GC challenged with LA. The spots number 5 and 6 were collected from strain Ch301BC challenged with TSPH. The spots 7 and 9 were taken from strain Ch354DC challenged with ASCh, while spot number 8, 10 and 11 was taken from strain Ch2124GF challenged with CA. From strain Ch1136DFM challenged with ASCh the spots 12, 13 and 14 were taken for the mass spectrometry analysis. The number of the spots is the same as shown in Table 5.2., where their suggested function are presented.

Table 5.1. Summary of which protein spots were chosen from chemostat disinfectant challenged strain for mass spectrometry analysis.

Chemostat challenged strain	Spot no. §													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ch1140DFO challenged with TSPH	√													
Ch301BC challenged with TSPH					√	√								
Ch354DC challenged with ASCh							√		√					
Ch1136DFM challenged with ASCh												√	√	√
Ch2124GF challenged with CA								√		√	√			
Ch23GC challenged with LA		√	√	√										

§ The proposed function of the proteins are presented in Table 5.2.

Figure 5.1. below presents a summary (composite) photo of a 2-D gel of the wild type *C. jejuni* 1136DFM strain, with the 14 different protein spots depicted (arrowed) that were taken for the mass spectrometry identification. These proteins were selected as their expression levels significantly changed upon the different disinfectant challenges. The decision on which spots should be identified was based on PDQuest analysis. First of all, the PDQuest analysis was performed in order to compare of proteins' expression of a given chemostat challenged with a particular disinfectant, below, at and above the MIC value, and then gels obtained from different chemostats were compared to each other. Proteins with an expression differences greater than 2.5-fold (between chemostat challenged strains and their WT counterpart) were considered significant and were taken for the mass spectrometry identification. In addition, the all 2-D gels taken for PDQuest analysis are presented in Supplemental materials in Section 5.6. of this chapter (Figure S5.1.–Figure S5.4.).

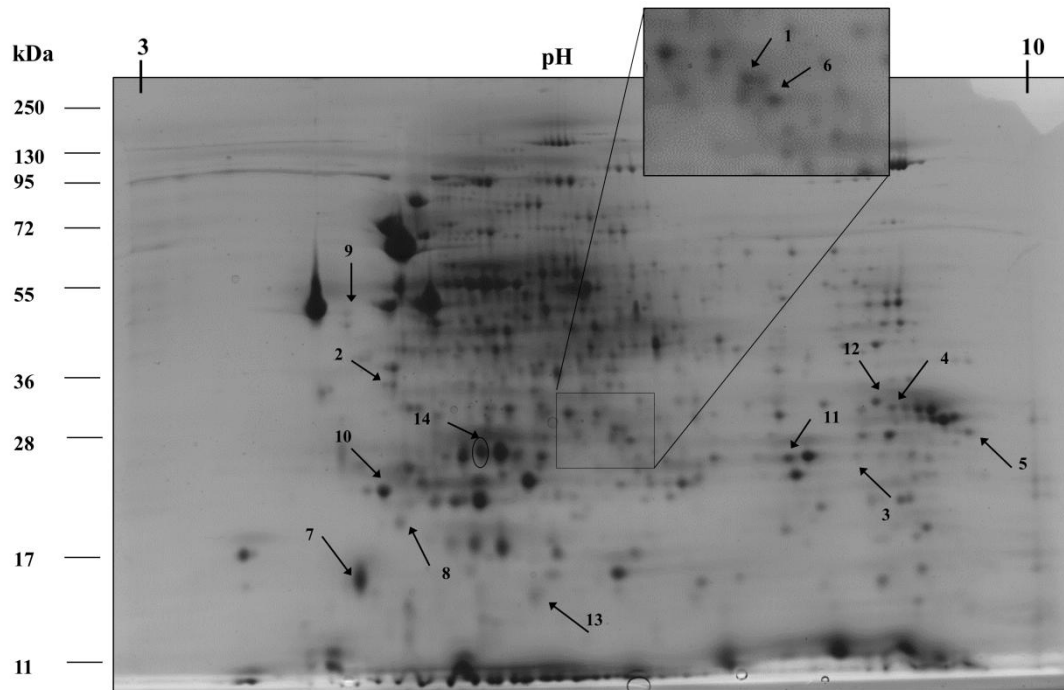


Figure 5.1. Two-dimensional gel image of *C. jejuni* 1136DFM WT used as an example to visualise protein location collected for mass spectrometry identification. pH range 3-10. Proteins indicated by arrows, were identified using the mass spectrometry (see also Table 5.1.).

An overview of the proteins identified by mass spectrometry and their suggested function in the cell biological process is presented in Table 5.2. In summary, among the proteins that were identified that re-occurred for the different disinfectant treatments, three were involved in the oxidative stress response: thiol peroxidase (Tpx), oxidoreductase and alkyl hydroperoxide reductase (AphC). Some of the identified proteins were found to be relevant for transport/binding activity: molybdate ABC transporter (ModA), glutamine-binding protein, bacterioferritin (Bfr) or taking part in protein translation process: ribosome recycling factor (Frr) and translation elongation factor (FusA). Others identified proteins related to their role in the biosynthetic process include imidazole glycerol phosphate synthase (HisH) and orotate phosphoribosyltransferase (PyrE). Among iron binding protein: hemerythrin family non-heme iron protein, flavohemoprotein and rubrerythrin (Rbr) were found.

Table 5.2. Identification of protein isolated from *C. jejuni* disinfectant challenged strains.

Spot § no.	Accession no.	Protein name	Suggested function	Mass (kDa)	pI
1	EIB48209	Hemerythrin family non-heme iron protein	Metal ion binding	23.62	6.26
2	ZP01068465	Chaperone protein DnaK (Heat shock 70 kDa protein) - fragment	Stress response	67.41	4.97
2	ZP01099471	GroEL protein (60 kDa chaperonin) - fragment	Protein refolding	57.97	5.02
3	ZP01072114	Molybdate ABC transporter, periplasmic molybdate-binding protein (ModA)	Molybdate transmembrane-transporting ATPase activity	27.66	8.80
3	ZP01099980	Conserved hypothetical protein	unkn.	26.60	8.84
4	ZP01067423	Oxidoreductase, short chain dehydrogenase (reductase family)	Oxidation-reduction process	29.34	8.24
5	ZP01100821	Major antigenic peptide PEB2	unkn.	27.32	9.46
6	ZP01100913	Aspartate ammonia-lyase (AspA) - fragment	Tricarboxylic acid cycle / aspartate metabolic process	51.80	5.50
7	ZP01068476	Thiol peroxidase (Tpx)	Cell redox homeostasis	18.44	5.13
8	EIB33327	Thiol peroxidase (Tpx)	Cell redox homeostasis	18.41	5.13
9	ZP01099471	Co-chaperonin GroEL (GroEL)	Protein refolding	57.97	5.02
10	ZP00367606	Antioxidant, AhpC/Tsa family VC0731	Antioxidant activity / peroxiredoxin activity	21.99	5.26
11	YP178307	Ribosome recycling factor (Frr)	Translation	20.75	7.79
11	ZP01071256	Imidazole glycerol phosphate synthase, glutamine amidotransferase subunit (HisH)	Glutamine metabolic process / histidine biosynthetic process	22.70	7.68
11	ZP01069541	Orotate phosphoribosyltransferase (PyrE)	'De novo' UMP biosynthetic process/ pyrimidine nucleobase biosynthetic process	22.19	8.35
12	ZP01070993	Glutamine-binding protein (belongs to the bacterial solute-binding protein 3 family)	Transporter activity	28.19	9.05
13	ZP01070477	Flavoheмоprotein, truncation (belongs to the globin family)	Iron ion binding / oxygen binding / oxygen transporter activity	16.08	5.47
13	ZP01070234	Conserved hypothetical protein	unkn.	14.63	5.76
14	ZP01070558	Rubrerhythrin (Rbr)	Iron ion binding / oxidoreductase activity	24.51	5.49

Symbols present in the table indicate: § the number of the spots is the same as presented in the Figure 5.1.; **unkn.** - unknown function. The proteins suggested functions were given based on the information comprised in www.uniprot.org

In some instances, the protein spots that had been identified, presented more than one protein. The reason for this is that peptide sequences obtained following trypsin digestion showed a high confidence within different proteins.

The gels with protein expression obtained from different strains challenged with different disinfectants were compared to each other. Table 5.3. presents the summary of protein expression ratio changes as identified by PDQuest analysis. In this study, the expression of proteins in the disinfectant challenged cultures was compared with the expression obtained for their wild type counterpart. In addition, the expression of the proteins from different experiments was compared between themselves.

Table 5.3. Summary of changes in protein expression level for chemostat disinfectant challenge strains.

Chemostat challenged strains	Spot no [§] (protein expression fold change)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Ch1140DFO 0.004%TSPH	-1.6	2.1		6.9	-1.3			1.1		-1.7	5.0			1.1		
Ch1140DFO 0.06%TSPH	-2.7	-1.2	NID	5.1	-4.8	NID	M	-1.3	P	1.0	5.9	NID	P	1.9		
Ch1140DFO 0.125%TSPH	-1.6	-5.5		M	-1.1			-1.2		-1.7	9.6			1.5		
Ch301BC 0.008%TSPH	-5.9	43.7		1.5	1.1	8.6	2.4	1.1		2.9				-2.2		
Ch301BC 0.03%TSPH	-3.7	53.8	NID	-1.2	1.7	10.1	1.9	1.0	P	2.1	P	NID	P	-2.2		
Ch301BC 0.125%TSPH	-7.0	18.4		1.3	M	10.2	1.2	1.0		5.2				1.1		
Ch354DC 6.25µg/ml ASCh		-2.4		1.0	1.3		3.8	-3.5	4.8	1.1	-4.4			-2.9		
Ch354DC 25µg/ml ASCh	NID	2.2	M	-1.1	1.2	NID	7.1	2.9	1.2	1.2	M	NID	NID	1.3		
Ch354DC 100µg/ml ASCh		M		M	M		-1.1	M	-1.9	M	M			M		
Ch1136DFM 6.25µg/ml ASCh	-1.1		3.2	1.4	-4.2	5.1	2.6	1.0	2.2	4.5	1.6	4.3	9.1	-3.7		
Ch1136DFM 25µg/ml ASCh	-3.4	P	-1.8	-2.3	-4.6	1.5	1.2	-1.4	-1.4	-1.3	-2.2	1.1	1.8	-10.8		
Ch1136DFM 50µg/ml ASCh	1.7		M	1.5	-10.4	-2.1	1.2	1.7	5.1	1.0	1.5	-2.5	-9.5	-29.3		
Ch2124GF 0.008%CA		P	NID	3.5	1.6	1.0	2.3	-1.3	4.2		M	1.2	M	3.5	-2.6	
Ch2124GF 0.25%CA				M	-1.0	1.1	-3.1	1.0	1.2			2.9	-10.1	M	NID	-1.1
Ch1136DFM 0.008%CA	-1.1		-4.0	-1.0	1.7	2.2	-1.2	1.1	-1.2	-1.3	-1.3	-1.2	-2.1	-2.6		
Ch1136DFM 0.06%CA	M	NID	M	-1.4	-1.5	M	M	M	M	M	M	M	M	M		
Ch1136DFM Finish	2.9		-2.5	-1.1	1.0	1.2	-1.2	-2.9	-1.1	-1.3	-2.1	1.1	1.0	-1.8		
Ch23GC 0.008%LA	1.4	195.1	M	-1.8	2.3	1.4	-1.7	-1.7	-2.6	-2.6	1.0			-1.7		
Ch23GC 0.125%LA	2.5	42.1	P	-2.5	1.6	2.5	-2.1	-1.5	-29.6	-2.0	1.3	M	NID	-1.1		
Ch23GC 0.14%LA	1.6	M	M	M	2.2	1.6	-5.7	-1.7	1.8	-6.7	-4.7			-3.3		

The changes indicate the expression ratio for the chemostat challenged strains to disinfectants and the wild type, expressed as a negative reciprocal for proteins that were present at reduced level in the chemostat challenged strain.

NID - not identified, P – present; M – missing; § the number of the spots are the same as presented in the Figure 5.1.

Strain Ch1140DFO challenged with 0.004%, 0.06% and 0.125% for TSPH, showed overexpression of Frr, HisH, PyrE (spot 11) at 5.0-fold, 5.9-fold and 9.6-fold. Significant down-regulation of DnaK, GroEL (spot 2), AphC (spot 10), oxidoreductase (spot 4) was observed for strain Ch1140DFO challenged with TSPH, whereas for the clinical Ch301BC strains challenged with 0.008%, 0.03%, 0.125% TSPH, up-regulation of DnaK and GroEL (spot 2) at a fold 43.7-fold, 53.8-fold, 18.4-fold, was observed. In addition, up-regulation of AspA (spot 6) at 8.6-fold, 10.1-fold, 10.2-fold, and AhpC (spot 10) at 2.9-fold, 2.1-fold, 5.2-fold was found for strains Ch301BC challenged with 0.008% TSPH, Ch301BC challenged

with 0.03% TSPH, Ch301BC challenged with 0.125% TSPH, respectively. Down-regulation however, of hemerytin family non-heme protein (spot 1) and major antigenic peptide PEB2 (spot 5) also was observed for Ch301BC TSPH challenged strains.

Initial overexpression, followed by down-regulation of Tpx (presented by spot 7 and spot 8), GroEL (spot 2) protein for Ch354DC strains challenged with ASCh was found. Initial overexpression followed by down-regulation in ModA/conserved hypothetical protein (spot 3), Tpx (spot 7 and spot 8), AspA (spot 6), AhpC (spot 10), flavohemoprotein (spot 13) for strains Ch1136DFM challenged with ASCh was also detected. Significant decreasing of Rbr (spot 14) and major antigenic peptide (spot 5) expression for Ch1136DFM challenged with $6.25 \mu\text{g ml}^{-1}$, $25 \mu\text{g ml}^{-1}$, $50 \mu\text{g ml}^{-1}$ of ASCh for was observed.

Ch1136DFM strains challenged with 0.06% CA resulted in the majority of protein not being detected. Bacterial growth however, was obtained at a higher concentration of 0.125% CA in the chemostat which was the MIC value for the parent *Campylobacter* 1136DFM WT strain. Absence of these proteins for Ch1136DFM challenged with 0.06% CA may indicate their denaturation. Additionally, no significant protein changes for Ch1136DFM were observed after completion of CA challenge and running the culture for a further 580 hours without disinfectant selective pressure. Down-regulation for most of the protein was observed for Ch2124GF challenged with CA with the exception of AhpC where 2.9-fold overexpression for Ch2125GF 0.25% CA was detected.

5.4.2. Protein expression changes in the control chemostat

The continuous culture for food isolate 1136DFM was performed in the absence of disinfectant for 1750.8 h and was carried out in order to investigate the effect of the long-term culture on proteins' expression changes.

Three bacterial samples collected from the fermenter during the long-term culture after 525, 861 and 1510 h were used for protein extraction and 2D-gel electrophoresis. The two dimensional gel electrophoresis for strips at pH range 4-7 and pH 6-11 either for 1135DFM WT and Ch1136DFM control strains were carried out. PDQuest analysis did not show significant differences between cultures taken after 525 and 861 h and the wild type strain. Initial PDQuest analysis showed the long-term culture (up to 861 hours) had no influence on proteins' expression changes when continuously cultured in Mueller-Hinton broth.

However, significant overexpression of some proteins was observed after analysis of the sample collected after 1050 h. At that time, the biofilm formation was also present in the fermenter. Figure 5.2. presents 6 spots that were analysed by mass spectrometry. Spots 16 – 19 were identified in this study, while spots number 20 and 21 were found by comparison of the spots obtained and published by Kalmokoff *et al.* (2006). Table 5.4. presents the suggested function of the proteins, their molecular mass and pI.

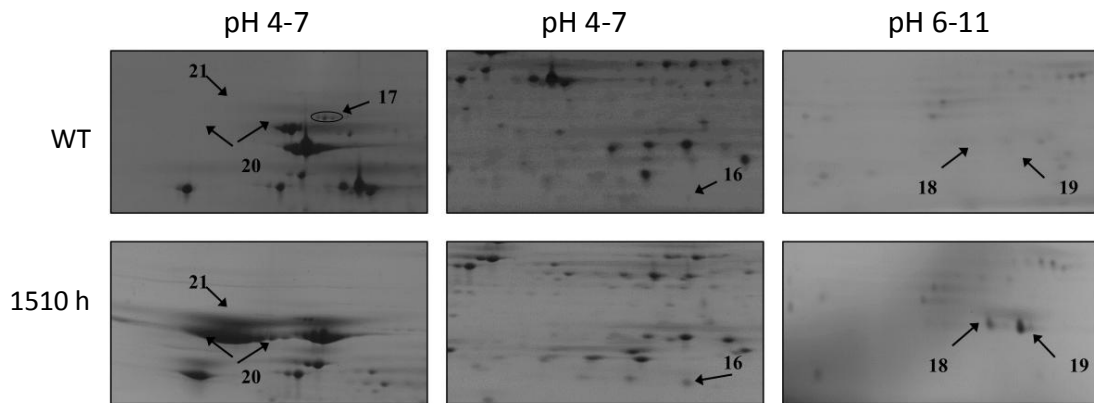


Figure 5.2. Up-regulation of the proteins in 1136DFM WT strain and Ch1136DFM control chemostat strain, taken after 1510 h of the culture.

Scanning electron microscopy results carried out for control chemostat strain Ch1136DFM sample collected after 1750.8 h in the culture showed lack of planktonic cells (see Section 3.4.7 in Chapter III). This strain grew as a biofilm which parts of the biofilm probable being shredded into the broth culture medium. Probably the sample collected via the sampling port of the fermenter after 1510 h, was shredded biofilm. This suggests that the overexpression of the proteins might be related with proteins involved in biofilm formation.

Table 5.4. Identification of protein isolated from *C. jejuni* in non-disinfectant challenge strains.

Spot no.§	Accession no.	Protein name	Suggested function	Mass (kDa)	pI
16	YP179683	Bacterioferritin (belongs to Dps family)	Cellular iron ion homeostasis	17.20	5.55
17	ZP01069972	Translation elongation factor G (FusA)	GTP catabolic process	76.75	5.07
18	ZP01071041	Putative periplasmic protein	unkn.	20.53	9.23
19	ZP01071041	Putative periplasmic protein	unkn.	20.53	9.23
20*	YP002344726	Flagellin (FlaB)	Flagellar motility	59.19	5.41
20*	YP002344727	Flagellin (FlaA)	Flagellar motility	59.04	5.59
20*	YP002344848	Flagellar hook-associated protein FlgK (FlgK)	Flagellar motility / flagellum assembly	67.09	4.70
21*	YP002343979	Flagellar capping protein (FliD)	Cell adhesion / cellular component movement / flagellum assembly	69.78	4.81

§ the number of the spots are the same as presented in the Figure 5.2.; The asterisk (*) indicates proteins that were found by comparison of the spots identified and published by Kalmokoff *et al.* (Kalmokoff *et al.*, 2006); unkn. - unknown function.

The spots comparison was carried out only by sight as the spots that were present in the gel run for strain Ch1136DFM collected after 1510 h were found to be missing in the 1136DFM WT. Bacterioferritin (spot 16) was found to be up-regulated in the sample collected after 1510 h of the continuous culture from the control chemostat 1136DFM in comparison with its wild type. In addition, translation elongation factor G (FusA), putative periplasmic proteins, flagellin proteins (FlaA, FlaB, FlgK, FliD) were found up-regulated in the sample, where in the wild type, these proteins were found to be missing.

Overexpression of the flagellar genes (*flaA flaB, flaK, fliD*) can be associated with the biofilm formation as an effect of response to limiting nutrient concentration. Additionally, overexpression of bacterioferritin was found in Ch1136DFM strains analysed after 1050 h of non-challenged culture. Bacterioferritin that belongs to Dps protein family (DNA protection during starvation) has been found to play an important role in *Campylobacter* cellular iron ion homeostasis. This protein also protects DNA from oxidative damage by chelation intracellular Fe²⁺ ion and storing it in the form of Fe³⁺. FusA (translation elongation factor G) were among the down-regulation proteins identified by mass spectrometry. FusA is responsible for catalysis of the GTP (guanosine 5'-triphosphate), dependent ribosomal translocation step during translation elongation.

5.5. DISCUSSION

In this study, in order to investigate *Campylobacter* proteome response to disinfectant treatment, 2D-gel electrophoresis was performed. The protein expression level were analysed as it has been reported that the expression of functional proteins are not always directly proportional to amount of mRNA and the genes that have been found to be down-regulated may still be required for survival under stressful condition (Reid *et al.*, 2008). In addition, the study of proteome changes may give a better understanding of bacterial response to stress conditions, indicating the expression of functional proteins.

According to Padney (2005), the genes that have been found to be highly up-regulated in *Campylobacter jejuni* immediately after a pH shift from 7.0 to 4.5, include the genes encoding chaperones or chaperonins (*groEL*), protein transport (*pebC*, *exbB2*) and heat shock regulators (*dnaK*, *hrcA*, *grpE*). In addition, the genes encoding oxidative stress defense proteins such as alkyl hydroperoxide reductase (Ahpc), the catalase (KatA), the ferritin (Cft), the thioredoxin reductase (TrxB), and the peroxide stress regulator (PerR) have been also found. The author suggests that the up-regulation of genes involved in oxidative stress during investigation of the *Campylobacter* pH stress response, indicates a strong correlation between the acid stress and oxidative stress response mechanisms.

In this study, the protein expression analysis was carried out to determine functional proteins involved in adaptation to slowly increased disinfectant concentration, that altered pH of the environment (with the exception of ASCh). In general, the majority of the proteins decreased their translation abundance with increased disinfectant concentrations. Especially, when the concentrations above the bacterial MIC were tested, only a few proteins were present in the gels. This might indicate that the proteins were denatured. However, the incubation period of *Campylobacter* to cause campylobacteriosis, from time of exposure to onset of symptoms, is between 2 and 5 days (FDA, 2012). In addition the disease typically lasts from 2 to 10 days (FDA, 2012) and during this time bacteria are able to survive the acidic conditions of the gastric juice. This suggests that *Campylobacter* possessed a different *in vivo* and *in vitro* survival mechanism or the response mechanism is dependent upon the type of compound that decreases the internal pH of the cell.

Among proteins that have been identified by mass spectrometry are those involved in oxydation stress. These proteins, belonging to the binding proteins class, are the

hemerythrin family protein, flavohemoprotein and rubrerythrin. The hemerythrin family is a non-heme iron protein that has been found to bind oxygen in some marine invertebrates (French *et al.*, 2008; Xiong *et al.*, 2000). Hemerythrin exists in deoxyhemerythrin form that contains ferrous iron (Fe^{2+}) and oxyhemerythrin form that contains a peroxo (O_2^{2-}) anion bound to two ferric iron (Fe^{3+}) atoms (Stenkamp, 1994). This indicates, that oxygen is bound at a non-heme iron centre consisting of two oxo-bridged iron atoms (French *et al.*, 2008). The hemerythrin motifs have been found in some subspecies of *Campylobacter jejuni* (French *et al.*, 2008). In *Campylobacter jejuni* genes encoding proteins with homology to the eukaryotic oxygen binding hemerythrin proteins have been found (Hofreuter *et al.*, 2006; Van Vliet *et al.*, 2002). However, the role of hemerythrin in *Campylobacter* is not fully understood. Rubrerythrin, that has been found to alter its expression in *Campylobacter* as a response to disinfectant stress and is another protein involved in oxidation stress protection (Pinto *et al.*, 2011; Van Vliet *et al.*, 2002). Rubrerythrins have been proposed to eliminate hydrogen peroxide through its reduction to water (Pinto *et al.*, 2011). Mutants with rubrerythrin-deletion showed increased sensitivity to oxygen and hydrogen peroxide (Pinto *et al.*, 2011; Santosa *et al.*, 2012). In addition, rubrerythrins have been found to complement catalase null strains. The study of *C. jejuni* NCTC 11168 genome showed the presence of the gene encoding a rubrerythrin-like protein. This *cj0012c* gene has been suggested to be involved in the hydrogen peroxide stress response (Pinto *et al.*, 2011). Besides, a 26 kDa polypeptide, alkyl hydroperoxide reductase (AhpC), has been also found to play an important role in oxidative stress, as in *C. jejuni* *ahpC* mutants increased sensitivity to oxidative stresses caused by cumene hydroperoxide and exposure to atmospheric oxygen was observed (Baillon *et al.*, 1999). However, changes in hydrogen peroxide resistance in these mutants was not detected (Baillon *et al.*, 1999). Expression of the proteins involved in oxidative stress response during the study on bacterial adaptation to disinfectants suggest that the low pH may increase the toxicity of oxygen radicals (Padney, 2005).

A GroEL, chaperones protein responsible for preventing the protein misfolding under stressful conditions, and DnaK, a chaperone protein responsible for heat shock, have been found to be up-regulated for strain 301BC challenged with TSPH and strain 23GC challenged with LA. This suggests that these disinfectants had a destructive role on proteins. The elevated overexpression of these chaperone proteins have been found in other studies as a response to bile salts and low pH (Okoli *et al.*, 2007; Padney, 2005).

Molybdate is transported by an ABC-type transporter, encoded by the *modABC* operon, comprising three proteins, ModA encodes the periplasmic binding protein, ModB encodes the membrane protein and ModC encodes the ATP-binding protein (Taveirne *et al.*, 2009). In addition, Mod A in *C. jejuni* as a component of ModABC transporter accumulate molybdate and tungstate (Smart *et al.*, 2009). There is significant diversity in this gene cluster across the different *Campylobacter* spp. strains. Although the *C. jejuni* 81-176 modABCD cluster is nearly identical to the equivalent gene clusters in *C. jejuni* RM1221 and *Campylobacter coli* RM2228, it is significantly divergent from the modABCD genes in *C. jejuni* reference strain NCTC 11168 (Hofreuter *et al.*, 2006). In our study, although the ModA was found to be initially up-regulated for strain Ch1136 challenged with ASCh and strain Ch2124GF challenged with CA, the increased concentration of disinfectant resulted in the absence of the protein in the analysed gel. In general, in the chemostat disinfectant challenged cultures this protein was in the majority of cases down-regulated. The results might suggest a degenerative role of the tested antimicrobial agents on bacterial binding protein.

The analysis of the protein expression in the control chemostat for strain 1136DFM after 1510 h of culture showed an increased abundance of proteins involved in flagella expression. The overexpression of the flagellar proteins is explained by the presence of strong biofilm that has formed during the chemostat trail. This proves the flagella role in biofilm formation. In addition, the expression of bacterioferritin that belongs to the Dps family, might suggest the presence of limited nutrient concentration in the later stages of continuous culture.

To conclude, with the exception of Frr/HisH/PyrE in the case of strain Ch1140DFO challenged with TSPH and DnaK/GroEL and AspA in the case of Ch301BC challenged with TSPH where these proteins have been found overexpressed for each PDQuest analysed disinfectant dose, the majority of the proteins were found to be down-regulated. Even after initial up-regulation, the proteins were down-regulated with application of higher dose of disinfectant. Based on the results obtained, it is difficult to find in *Campylobacter* a common proteome response to stress to a particular class of disinfectant (i.e. weak organic acid).

Acknowledgments

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5.6. SUPPLEMENTAL MATERIALS

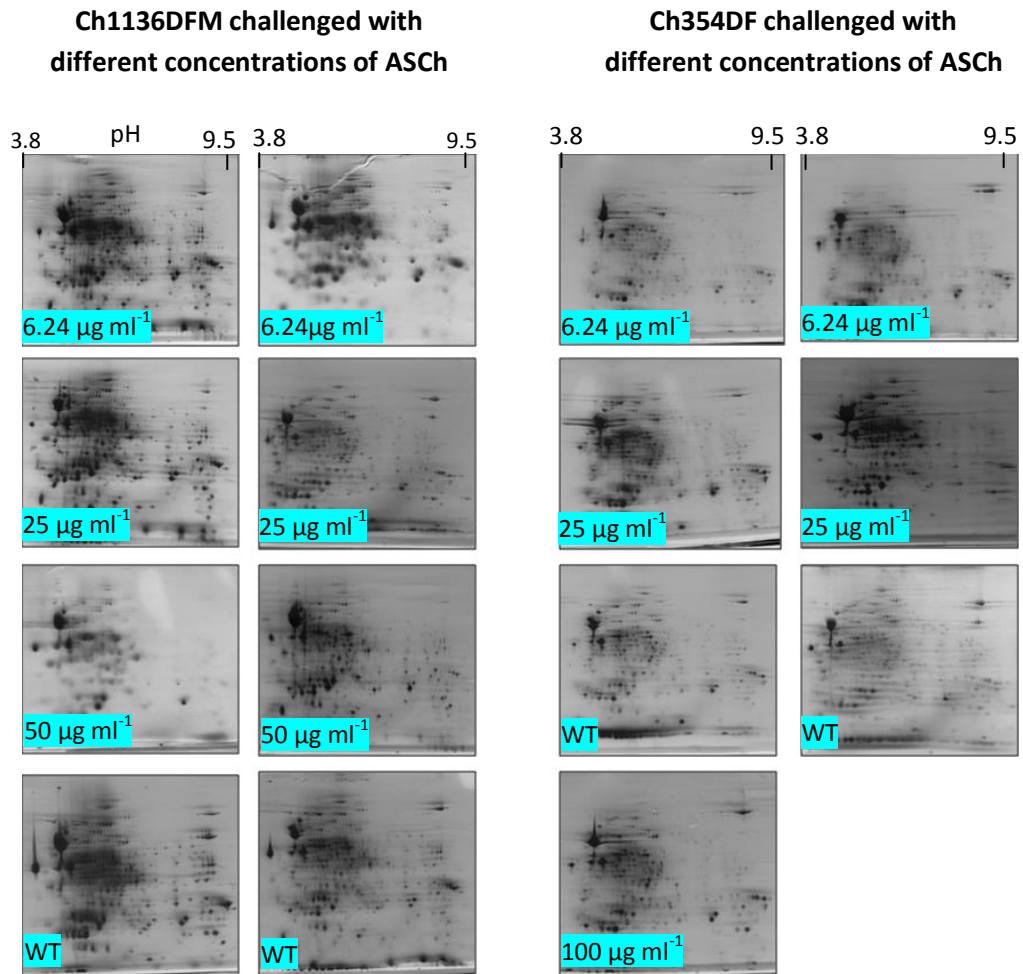


Figure S5.1. Two dimensional acrylamide gels presenting protein expression in chemostat challenged with ASCh strains. These gels were taken for PDQuest analysis, where the protein expression in a chemostat ASCh challenged strains were compared with the protein expression in their WT counterparts. The concentrations present in the footnote indicate the concentration of ASCh that the strain was challenged with.

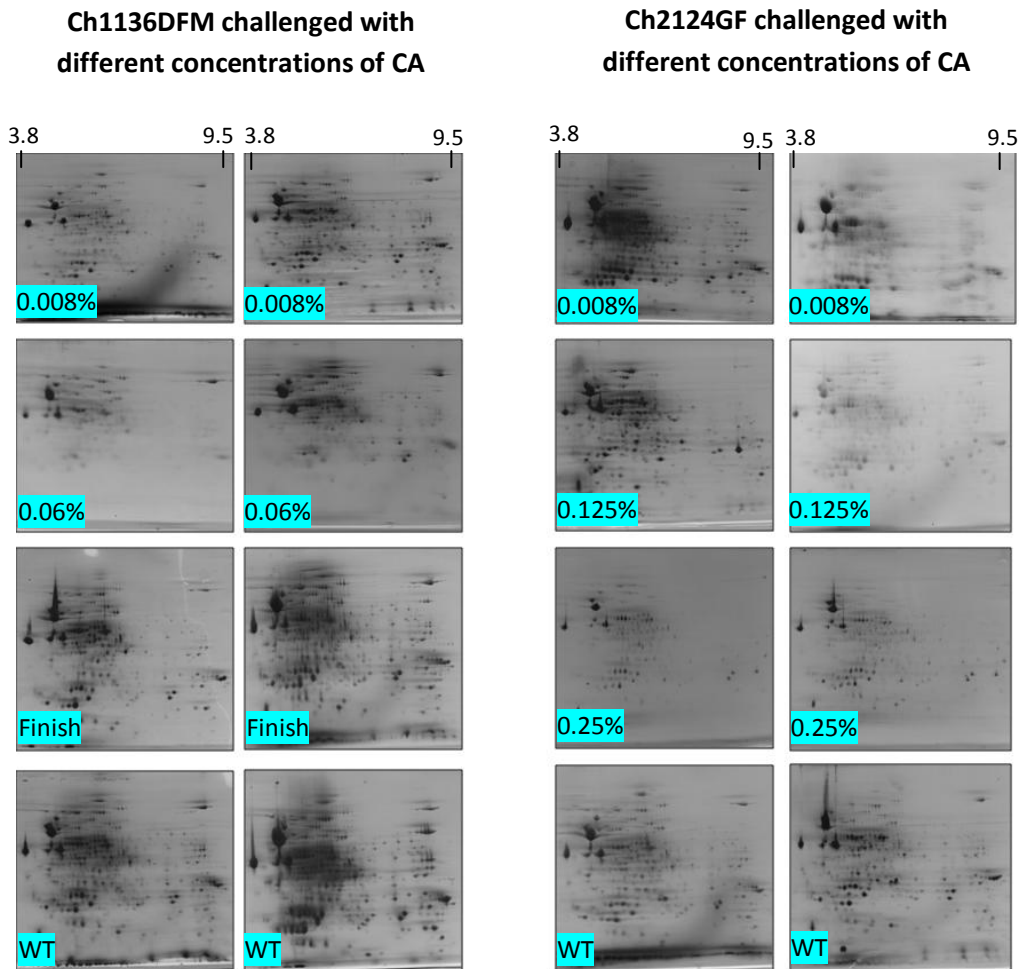


Figure S5.2. Two dimensional acrylamide gels presenting protein expression in chemostat challenged with CA strains. In addition, the protein expression in the WT are also present. These gels were taken for PDQuest analysis, where the protein expression in a chemostat CA challenged strains were compared with the protein expression in their WT counterparts. The concentrations present in the footnote indicate the concentration of CA that the strain was challenged with.

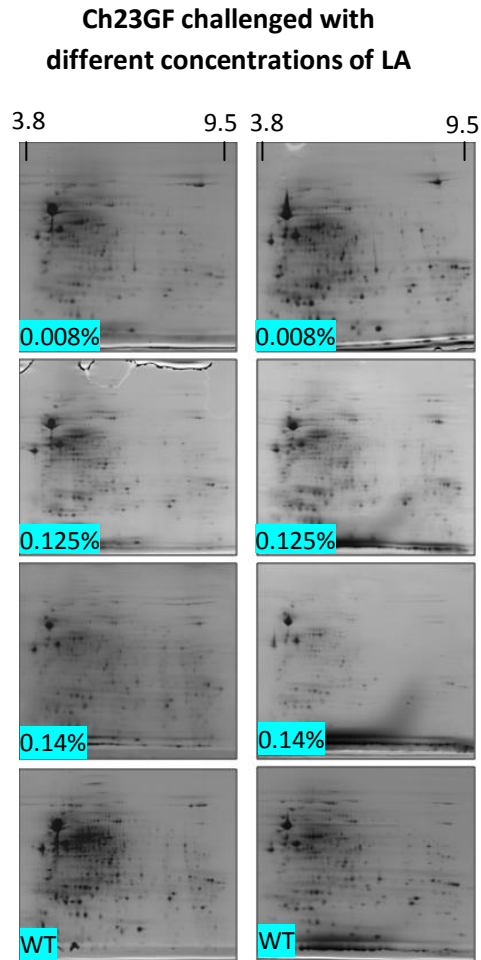


Figure S5.3. Two dimensional acrylamide gels presenting protein expression in chemostat challenged with LA strain. In addition, the protein expression in the WT are also present. These gels were taken for PDQuest analysis, where the protein expression in a chemostat LA challenged strain was compared with the proteins' expression in its WT counterparts. The concentrations present in the footnote indicate the concentration of LA that the strain was challenged with.

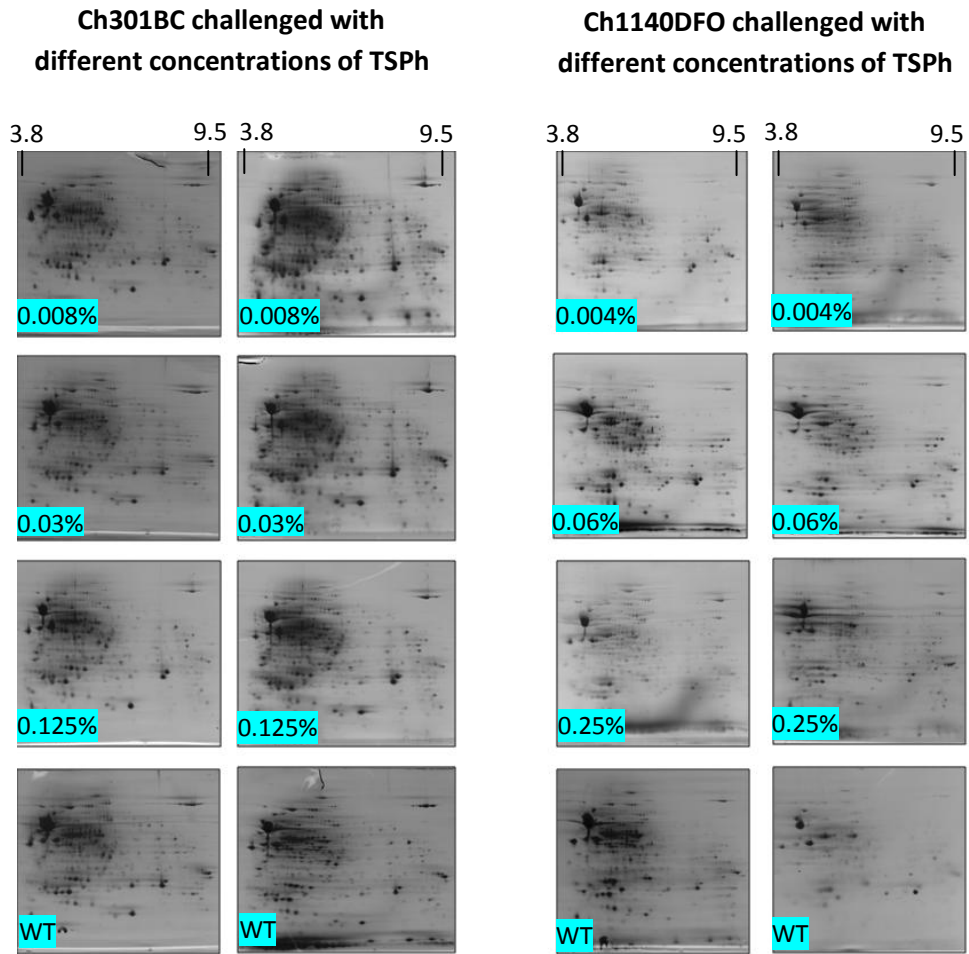


Figure S5.3. Two dimensional acrylamide gels presenting protein expression in chemostat challenged with TSPH strains. In addition, the protein expression in the WT are also present. These gels were taken for PDQuest analysis, where the protein expression in a chemostat TSPH challenged strains were compared with the protein expression in their WT counterparts. The concentrations present in the footnote indicate the concentration of TSPH that the strain was challenged with.

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CHAPTER VI

General discussion

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6.1. Overview

In the EU, diseases caused by *Campylobacter*, a food-borne pathogen, have remained the most reported zoonotic infection in humans since 2005 (EFSA, 2012). The most common mode of *Campylobacter* transmission to humans is through the consumption of improperly prepared food, particularly undercooked poultry products or raw contaminated dairy products such as unpasteurized milk (MacKichan *et al.*, 2004). To reduce the number of illness cases, different intervention strategies at poultry farms and at processing plants have been investigated. Among them, the application of biological, physical and chemical methods are being considered. Before the approval of any method for commercial use, the efficiency of the method and the safety of product, in relation to both humans and environment, have to be investigated. At the moment, in the EU, besides potable water and lactic acid (lactic acid has been recently officially approved by the European Commission <http://europa.eu/rapid/midday-express-04-02-2013.htm>), no chemicals are permitted for bacterial decontamination of meat products (Loretz *et al.*, 2010). The EU Regulation (EC) No. 853/2004, laying down specific hygiene rules for food of animal origin, highlights disinfectants that might be used for bacterial decontamination of meat products but recommends withholding their use until a risk assessment is provided by the EFSA (Loretz *et al.*, 2010). Although the recommended disinfectants' doses are much higher than the MIC for bacteria and higher than the concentrations used in this study, often these disinfectants become inactive or have reduced potency as a result of organic debris contamination. These lower concentrations may be at sub-inhibitory concentration or at concentrations close to the MIC for bacteria, whereby after repeat exposure to the antimicrobial agent bacteria may adapt to them and/or become more resistant.

Our focus on *Campylobacter* adaptation to disinfectants was undertaken to assess potential disinfectants suitable for decontamination on poultry farms and at processing plants. This thesis set out to investigate *Campylobacter* adaptation and its proteome and genome response to stress caused by exposure to disinfectants. The work was performed for four disinfectants: citric acid, lactic acid, acidified sodium chlorite and trisodium phosphate, highlighted in the EU Regulation (EC) No. 853/2004. To achieve the aims of the project, the disinfectant doses were incrementally increased in a chemostat fermenter, where long-term challenged studies investigating possible adaptation of *Campylobacter jejuni* were carried out. The expression of virulence genes was also examined to investigate if exposure to disinfectants had an effect on *Campylobacter* pathogenicity.

6.2. The long-term exposure of campylobacters to disinfectants revealed alterations in their resistance and possibly pathogenicity

Before official approval of CA, LA, ASCh or TSPH for commercial use as decontaminants on poultry farms and at processing plants, a study on *Campylobacter* adaptation to these disinfectants and its pathogenicity is important. This type of physiological study is crucial as there is evidence, based on other studies carried out for different pathogens, that bacterium might adapt to these compounds and may acquire cross-resistance to other antimicrobial agents. For example, *Salmonella enterica* serovar Enteritidis, following exposure to 1.5% TSPH showed a significant increase in thermotolerance, resistance to 2.5% TSPH and an increase in sensitivity to acid and hydrogen peroxide (Sampathkumar *et al.*, 2004). *Listeria monocytogenes*, following an hour of exposure to lactic acid at pH 5.5, showed an adaptive acid tolerance (ATR) response that protected the bacterial cells from further acid stress at pH 3.5 (O'Driscoll *et al.*, 1996). In addition, *L. monocytogenes* with ATR showed increased tolerance to thermal stress, osmotic stress, crystal violet, and ethanol and demonstrated increased virulence in mice. In another study *Escherichia coli* O157:H7 following exposure to lactic acid, demonstrated cross protection against increased concentrations of sodium chloride and sodium lactate (Garren *et al.*, 1998). Furthermore, based on other research carried out for other pathogens, it is quite likely that bacterium adapted to antimicrobial agents become more pathogenic (Humphrey *et al.*, 1996; Kastbjerg *et al.*, 2010; O'Driscoll *et al.*, 1996). In a study by O'Driscoll and co-workers, *Listeria monocytogenes* with the adaptive tolerance response (ATR), induced by acid stress, were more virulent to mice than the WT (O'Driscoll *et al.*, 1996). Mice injected with acid tolerant mutant strains of *Listeria monocytogenes* did not survive longer than 3 days, while a control group that was inoculated with the WT strains did not show symptoms of disease. Humphrey and co-workers reported that acid-tolerant *Salmonella enterica* are more virulent in mice and more invasive in laying hens than the unadapted strain (Humphrey *et al.*, 1996). Kastbjerg *et al.* investigated virulence genes expression in *L. monocytogenes* treated with a sub-lethal concentration of disinfectants from different groups: peroxy, chlorine and quaternary ammonium compounds (Kastbjerg *et al.*, 2010). The virulence genes were down-regulated when peroxy and chlorine compounds were used. However, in the case of quaternary ammonium compounds the abundance of expression of the genes increased. In addition, they observed that disinfectants with the same active ingredients had a similar effect on gene expression.

The results from the long-term disinfectant challenge studies showed that *Campylobacter jejuni* was able to grow up to 8-fold above the original MIC in the case of ASCh and up to 2-fold above the MIC in the case of CA. Although the growth above 2-fold can be interpreted as not significant, chemostat derived strains were able to survive better and longer exposure to CA at MIC value and at supra-MIC value than their WT counterpart. Although the *Campylobacter* growth at 8-fold above the MIC value might indicate a bacterial physiology response related to adaptation to disinfectant, no major differences in survival between chemostat derived strains and their WT were observed. Neither ASCh challenged strains nor CA challenged strains revealed cross-resistance to antibiotics (with the exception of *C. jejuni* 354DC challenged with ASCh, where a 4-fold increase in MIC for Tc was observed). In the case of *Campylobacter* strains challenged with TSPH, although long-term continuous challenge with sub-MIC concentrations showed no bacterial growth at MIC value, the chemostat derived strains were able survive up to 3 times longer exposure to TSPH at MIC level than their WT counterpart. In addition, this clinical strain showed cross-resistance to all test antibiotics. When the adaptation study was performed with LA, although *Campylobacter* grew 2-fold above the MIC in a continuous culture, increased susceptibility to re-application of LA and increase sensitivity to all test antibiotics was found. These findings suggest that the risk assessment of each of the test disinfectants should be performed separately and no overall conclusions regarding each type of disinfectants (i.e. decreasing pH of the environment) should be given.

In the case of chemostat *C. jejuni*, strains challenged either with CA, ASCh or TSPH, had a better ability to survive under disinfectant selective pressure than their WT counterpart and some of these strains developed cross-resistance to antibiotics (however, developing a cross-resistance to antibiotics were strain specific). This might also imply some changes in virulence genes expression and thereby *Campylobacter* pathogenicity. In order to confirm or reject the hypothesis that *Campylobacter* disinfectant challenged strains became more pathogenic, a study on expression of virulence genes was carried out. For the study, genes involved in adherence and invasion of a host (*flgS*, *ciaB*), a gene involved in toxin production (*cdtB*) and a gene coding efflux pump (*cmeA*) were chosen. The *flgS* and *ciaB* genes play an important role during adherence and invasion of the host (Carrillo *et al.*, 2004). The *flgS* gene, that is a part of the FlgSR two-component system, is necessary for flagella (*fla* gene) to be expressed. Besides the importance of the *fla* gene in adherence and invasion, it is also essential for the secretion of *Campylobacter* invasion antigen (CiaB) proteins. Secretion of CiaB proteins have been found to be important in the invasion of a

host's intestine (Konkel *et al.*, 2004). As a significant reduction in virulence of *Campylobacter fla* and *ciaB* mutants have been reported (Konkel *et al.*, 2004; Yao *et al.*, 1994), the overexpression of these genes in *C. jejuni* might indicate increased bacterial pathogenicity. *Campylobacter*, during invasion of a host, may produce a toxin namely CDT, that may induce host cell apoptosis (Mortensen *et al.*, 2011; Young *et al.*, 2007). However, the role of CDT production during host colonisation remains controversial as the production of CDT was found in invasive and non-invasive cells (Müller *et al.*, 2006). A study on *cmeA* expression may also give a better understanding of *Campylobacter* pathogenicity and its ability to better survive stress conditions related to pH changes. It is believed that overexpression of efflux pump might be a first step in bacteria becoming fully resistant (Piddock, 2006).

In this study, *Campylobacter* strains challenged with CA and TSPH in a chemostat model, revealed overexpression of all four virulence genes (*flgS*, *ciaB*, *cmeA* and *cdtB*). The two strains challenged with ASCh strains revealed a high overexpression of the *ciaB* gene. A high level of overexpression of the *cdtB* gene, but only in the case of strain Ch1136DFM adapted to 25 µg ml⁻¹ ASCh, was also observed. In the case of LA challenged strains, no statistically significant differences for all four genes were observed with the exception of the *ciaB* gene for Ch23GC challenged with 0.125% LA (2.5-fold overexpression in comparison with the WT counterpart). The overexpression of these genes might indicate a possible increased level of pathogenicity among chemostat derived strains. However, during analysis of the results, the effect of the long-term culture (in the absence of a disinfectant challenge) on gene expression has also to be considered. Continuous culture up to 861 h in the absence of a disinfectant challenge revealed no statistically significant effect on virulence genes expression. After that time, overexpression occurred and might be an effect of *Campylobacter's* response to stress related to long-term culture conditions. However, only a few samples where the genes were found to be up-regulated were collected after 861 h, suggesting that chemostat derived strains might be more pathogenic to humans than their WT. In order to prove the hypothesis, however, additional studies would need to be carried out investigating the adherence and invasion of epithelial cells (i.e. Coco-2, INT407 cell lines). This type of study, with the application of human epithelial cell lines, is necessary as *Campylobacter* that have colonised poultry can be harmless to them and no symptoms of disease might be observed, while consumption of this contaminated poultry by humans can cause illness (Wassenaar & Blaser, 1999).

In general, based on the results presented in this thesis, it is reasonable to conclude that a gradual incremental application of a sub-inhibitory level of disinfectant may have an effect on *Campylobacter jejuni* tolerance to disinfectants. In addition, the continuous exposure of bacteria to the disinfectants resulted in the creation of stable adapted *C. jejuni* strains that were better able to survive and tolerate a re-application of the disinfectant challenge. In addition, CA, TSPH, and ASCh challenged strains might become more pathogenic to humans as their virulence gene expression was found to be up-regulated. An exception is exposure to LA, where the long-term challenge with LA resulted in increased sensitivity of the bacteria to the disinfectant, lack of cross-resistance to antibiotics and no significant changes in all the virulence genes expression.

6.3. Assessment of the potential implication of the chemical treatments on *Campylobacter* proteome alteration

Besides carrying out the risk assessment conducted on the disinfectant application for *Campylobacter* adaptation and pathogenicity, this work also concentrated on the bacterial response to stress at a translation level. Finding the functional proteins involved during pH/oxidative stress might give a wider overview on the implications of chemical treatments and as well as giving a better understanding of *Campylobacter* adaptation/survival mechanisms.

Despite the fact that a lot of studies have been carried out investigating *Campylobacter* response mechanisms to pH/oxidative stress, many gaps in our knowledge remain. Due to the lack of common stress response factors in *Campylobacter* such as SoxRS (oxidative stress response factor) which have been found in other food-borne pathogens i.e. *Escherichia coli* (Amábile-Cuevas & Demple, 1991), identifying the bacteria's response mechanism is a challenge. Nevertheless, some response mechanisms to stress such as adaptive tolerance response (Murphy *et al.*, 2003b) or overexpression of the multidrug efflux pump, CmeABC (Lin *et al.*, 2002; Lin *et al.*, 2003) have been investigated. However, the majority of these studies investigated the response to acid shock over short exposure times (Murphy *et al.*, 2003; Padney, 2005; Reid *et al.*, 2008; Shaheen, 2007). There is a lack of studies where the effects of long-term pH stress on *Campylobacter* response and survival are investigated.

Among the proteins that have been identified in this present study by mass spectrometry are binding-proteins involved in oxidative stress. In addition, chaperone proteins and those involved in biosynthetic processes have been found. However, the majority of them were found to be down-regulated. With increased concentration of disinfectant the amount of protein spots on the gel decreased. When the gels were run for the samples collected from the fermenter above the MIC value, only a few spots were present, even after increasing the concentration of proteins during the 2-D preparation stage. This indicates that proteins have been denatured by the low/high pH value caused by CA, LA and TSPH or due to oxidative stress caused by ASCh. Overexpression of the chaperone protein (GroEL) responsible for preventing misfolding and promoting the refolding and proper assembly of unfolded polypeptides generated under stress conditions, might suggest activation of the protective mechanism against the destructive role of the disinfectant.

Based on the results obtained in this thesis, it is difficult to clearly conclude if down-regulation is only caused by protein denaturation or by the bacterial global response. Reid and co workers who investigated campylobacters' response to pH stress, reported that down-regulation of the genes could be a result of reshuffling of energy toward the expression of components required for survival (Reid *et al.*, 2008). Reid and co-workers study investigated *Campylobacter* response to acid shock and did not involve long-term culturing. Long-term exposure to pH stress (over 850 h) might involve different response mechanisms. The use of mass spectrometry on those few protein spots that were present in 2-D gels carried out for chemostat strains challenged with a high concentration of disinfectant (above the MIC value) might give an insight into why so many proteins were down-regulated.

6.4. The role of biofilm

The biofilm formation that occurred during the chemostat cultures may have had an effect on the results obtained. While the long-term non-challenged culture had no significant effect on virulence genes expression up to 861 h, the first significant transcription abundance for the genes was observed following 1054 h of the culture. At that time biofilm formation was also observed in the chemostat fermenter. However, biofilm formation was also observed in the challenged chemostat trails. Biofilm formation was more defined in trials involving CA. From when the first addition of CA was added to the vessel (after 290 h in the case of strain Ch2124GF and 192 h in the case of strain Ch1136DFM), bacteria started

to form clumps, developing to a clearly visible biofilm. Biofilm formation was also observed when LA, ASCh and TSPH were examined but only during the later stages of the culture and at lower quantities. However it is difficult to explicitly assess if the biofilm formation in these chemostats were due to stress caused by the disinfectants or to another stress factor such as limited nutrient concentration. It has been reported that *Campylobacter* under different stress conditions are able to form a biofilm as a response mechanism to ambient environmental stressors (Guerry, 2007; Reuter *et al.*, 2010). This might suggest that these stress conditions have an effect of decreasing the growth rate in *Campylobacter*. However, it has been reported that a slow growth rate in biofilm is not associated with nutrient limitation but with a general stress response initiated by biofilm formation (Trachoo *et al.*, 2002). Taking this into consideration and considering the protective role of biofilm formation under stress conditions, the bacteria in this study were exposed to different stressors. On one side there was stress connected with the addition of disinfectants to the culture medium which may induce biofilm formation, and on the other side there was stress connected with the biofilm formation itself. It is also possible that the limited nutrient concentration had an influence on the bacterial growth rate in the challenged culture. Probably all these factors influenced the bacteria's growth rate. In our study, the stress was possibly related to disinfectant addition at an early stage of the cultures, and at a later stage of the continuous cultures (after 850 h) to disinfectant, biofilm formation and limited nutrient concentration.

Although biofilms can have a protective role in unfavourable conditions and have an influence on decreasing bacterial growth rate, the bacteria that were long-term challenged with disinfectants could not grow above a certain concentration of the disinfectants. In the case of TSPH, both strains tested were not able to grow at 0.6%. In the case of ASCh, both strains were not able to grow at 200 $\mu\text{g ml}^{-1}$. Similar results were also obtained when CA was tested. This suggests that the disinfectant addition could sufficiently inhibit *Campylobacter* growth when an appropriate dose of disinfectant is reached.

This study set out to investigate a multifaceted problem related to *Campylobacter* adaptation to disinfectant, possible changes in its pathogenicity following long-term disinfectant stress and its global response mechanism. On the basis of the results obtained, it can be concluded that among the test disinfectants, only LA was shown to be a suitable disinfectant for decontamination on poultry farms and at processing plants.

6.5. Assessment of aims and future prospects

The aims of the project were successfully completed, these included: 1) genotypically and phenotypically characterising a set of *Campylobacter* spp. strains as a part of an initial screening to define their wild type phenotype, prior to exposing them to disinfectants for bacterial adaptation studies; 2) conducting long-term continuous culture studies on selected *Campylobacter* strains exposed to a gradually increased concentration of disinfectant in order to investigate bacterial adaptation to disinfectants and investigate acquired cross-resistance to antibiotics; 3) investigating if continuous exposure to sub-lethal concentration of disinfectants resulted in more pathogenic phenotypes by examining the expression of four virulence genes; *flgS* (two component regulator of the flagellum), *ciaB* (*Campylobacter* invasion antigens), *cmeA* (multidrug efflux pump) and *cdtB* (cytolethal distending toxin); 4) studying the proteome changes of disinfectant challenges and adapted *Campylobacter* strains to better understand their bacterial response to stress at the translation level.

Although the work presented here gives primary overview of a possible risk related to the application of disinfectants at sub-inhibitory level, the results obtained during this project are not exhaustive. To achieve a broader knowledge of *Campylobacter* adaptation to the disinfectants in question the study has to be ongoing. However, to achieve this is both challenging and time-consuming. It is difficult to maintain long-term continuous cultures without contamination and the fastidious growth conditions required by slow growing *Campylobacter* are also a constraint. With a better system for investigating the effect of disinfectants on *Campylobacter* growth, adaptation and biofilm formation, morphological differences in the bacteria could be better observed during the disinfectant challenge and its response to stress. Recently, NUI Galway procured the BioFlux System for running biologically relevant cellular assays under shear flow. This system could allow an investigation into the real-time changes to cell morphology caused by antimicrobial agents. In addition, the inclusion of inversion microscopy connected to the system could allow for the observation, not only of the physiological changes in the bacteria, but by adding a fluorescent compound, the ratio between live and dead cells could be distinguished. Furthermore, it would be interesting to perform the experiments in the BioFlux System for the chemostat derived strains obtained during this study, in order to investigate if there are any further phenotype differences between the strains during disinfectant re-treatment. It could also visually record the process of biofilm formation in *Campylobacter* and might

give more details about its protective role in bacterial survival under stress conditions. Furthermore, this work emphasizes the need to determine if the overexpression of the virulence genes in *Campylobacter* chemostat challenged strains have an influence on adherence and invasion to epithelial cells. This could be achieved by an *in vitro* study with the use of human intestinal epithelial INT407 and/or human colon Caco-2 cell lines. An advanced study on biofilm formation during disinfectant treatment may allow a better understanding of its protection role with regard to stress, and as well as bacterial response mechanisms to unfavourable conditions.

All research projects have their limitations. In the work presented, one limitation is related to the temperature in which *Campylobacter* was cultured. All the studies were carried out at 42 °C, the body temperature for poultry, the optimal temperature for *Campylobacter* growth under laboratory *in vitro* culture. However, many farms and processing plants are contaminated with campylobacters where the ambient temperature is below the optimal range for *Campylobacter* growth, between 37-42 °C (Gaynor *et al.*, 2005). It would be important, therefore, to investigate the effectiveness of disinfectant treatment on *Campylobacter* reduction and its response to this stress both on a poultry farm and in a processing plant environment. By its nature, it would be challenging to perform the experiments at lower temperatures as such a change would have physiological consequences. Campylobacters may not grow or may enter to a viable but non culturable coccoid form at these lower temperatures (Alter & Scherer, 2006; Lázaro *et al.*, 1999). These physiological changes make such a study difficult.

6.6. REFERENCES

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