Adaptive Responses of *Campylobacter jejuni* to Antibiotic and Biocide Selection Pressures in Chemostat Culture.

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by

Olivia Duignan

Microbiology Department, School of Natural Sciences,
National University of Ireland, Galway

Head of School: Professor Vincent O'Flaherty
Head of Department: Professor James P. O'Gara
Supervisors: Dr Cyril V. Carroll and Dr Gerard T. Fleming

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To my family

Michael and Gráinne

Breege and Thomas

Michelle, Seán, Lorraine, Louise, Sinéad, Leeanne, Anita, Gráinne, Aisling, Eimear, Avril and Conor

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List of Abbreviations

ABC	ATP-binding cassette
AIAO	All In All Out
AMP	Ampicillin
AMR	Antimicrobial resistance
AspA	Aspartate ammonium lysate
AspS	aspartyl tRNA synthase
ATP	Adenosine Tri Phosphate
ATR	Adaptive Tolerance Response
BKC	Benzalkonium chloride
℃	Degrees Celsius
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CCP	Critcal control point
CE	Competitive exclusion
CEF	Ceftotaxmine
CEN	Comité Européen de Normalisation
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards institute
cm	Centimetre
D	Dilution rate
Da	Dalton
DDDMAC	Didecyl-dimethyl-ammonium chloride
DNA	Deoxyribonucleic Acid
DnaK	DnaK heat shock protein
DTDMAC	Ditallowdimethylammomium chloride
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Efflux pump inhibitor
EPS	Extracellular polysaccharide
EPS	Exopolymeric substance
ERY	Erythromycin
EU	European Union
FAO	Food and Agriculture Organization
FQ	Fluoroquinolone
g	Gram
GI	Gastrointestinal
GryA	DNA Gyrase alpha subunit
GroEL	Heat shock chaperon
HACCP	Hazard Analysis Critical Control Point
h	Hour
HO·	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HtpG	Heat shock protein 90
Icd	Isocitrate dehydrogenase
IEF	Isoelectic focusing
IPG	Immobilized pH gradient
LPS	Lipopolysaccharide

M	Molar
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistant
MDR efflux pumps	Multidrug resistant
MFD	Mutant Frequency Decline
MFS	Multi Facilitator superfamily
mg l^{-1}	Milligram per liter
MH	Muller Hinton
MIC	Minimum Inhibitory Concentration
MIC_{Agar}	Minimum Inhibitory Concentration determined on Agar
MIC_{broth}	Minimum inhibitory concentration determined in broth
min	Minute
mg	Miligram
ml	Milliliter
Mm	Millimetre
MOMP	Major outer membrane protein
MQ	MilliQ water
MRSA	Methicillin resistanat Staphlyococcus Aureus
MS	Member state
MSC	Minimal selection concentration
MSMS	Materials safety data sheet
ng	Nano gram
nm	Nano meter
NaCl	Sodium Chloride
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National collection of Typed cultures
$O2^-$	Superoxide anion
OD	Optical density
P	Probability
PAβN	Phenylalanine arginine b-naphthylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMF	Proton Motor Force
ppm	Parts per million
QAC	Quaternary ammonium compound
QRDR	Quinolone resistance determining region
RNA	Ribonucleic acid
RND	Resistance-Nodulation Division
ROS	Reactive oxygen species
RpoS	Global stationary phase stress response factor
Rrc	rubrerythrin
rpm	Revolutions per minutr
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl solphate polyacrylamide gel electrophoresis
SET	Sodium chloride-EDTA-Tris buffer
SMR	Small Multidrug Resistance family

SodB	Superoxide dismutase
SucC	Succinyl-CoA synthase beta subunit,
Taq	DNA polymerase obtained from Thermus aquaticus
TCA	Tricarboxylic acid cycle
TBE	Tris/Boric acid/ EDTA buffer
TSB	Trytic soy broth
TEMED	N,N, N', N' Tetramethylethylenediamine
Tpx	Thiol peroxidase
tRNA	Transfer RNA
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloric acid
US	United States
V	Volume
v/v	Volume per volume
VBNC	Viable But Non Culturable
VH	Volt hour
w/v	Weight per volume
WGS	Whole genome sequening
WHO	World Health Organisation
WT	wild type
µg	Micro gram
µl	Micro liter
µmax	maximum specific growth rate
%	percentage

Thesis structure and Declaration of contribution

Thesis structure

Each of the three results chapters (Chapters 2-5) 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

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 exception:

Chapter 4: 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 jejuni (*C. jejuni*) is a zoonotic pathogen of major public health importance and is the leading cause of gastroenteritis woldwide. Poultry is regarded as one of the most important reservoirs for *Campylobacter* and constitutes a very significant vehicle for the transmission of *Campylobacter* to humans. Prevention measures including biosecurity and good hygiene practices (relying on the use of disinfectant) are widely used to prevent the transmission of *Campylobacter* to poultry. Benzalkonium chloride (BKC) a quaternary ammonium compound is commonly used disinfectant in the poultry industry. Fluoroquinolones are one of the main antibiotic classes used in the treatment of *Campylobacter* infections. Resistance to fluroquinolones in *Campylobacter* is rising in recent years and has been linked to their use in animal husbandry. *Campylobacter jejuni* NCTC 11168 was adapted to ciprofloxacin and BKC following long-term exposure in chemostat culture. Chemostat-adapted variants reduced susceptibility to ciprofloxacin (1,028-fold) and BKC (8-fold) were characterized. Adapted variants were less fit than their un-adapted counterpart and showed stable reduced susceptibility after prolonged culture. Strain-specific differences in efflux activity and cross-resistance profiles to other antimicrobials were observed for ciprofloxacin-adapted variants. Similarly the development of low-level cross-resistance to ciprofloxacin and cefotaxime found in BKC-adapted variants. Efficacy of the commercial disinfectant Savlon^{TM} against adapted variants was also reduced but not at recommended use concentrations. Mutations and proteomic changes in proteins involved in metabolism and the general stress response were associated with high-level ciprofloxacin-resistant variants while changes in oxidative stress proteins were found in BKC-adapted variants. This thesis provides further insights into the development of antimicrobial resistance in *Campylobacter*.

Introduction

1.1 The genus *Campylobacter*

The genus *Campylobacter* was first proposed by Sebald & Véron (1963) and contained just two taxa, *Campylobacter fetus* and *C. bubulus,* now known as *C. sputorum*. Difficulties in culturing *Campylobacter* hampered efforts to study it until the development of suitable culture media by Skirrow in 1970. At the beginning of the twentieth century much of the research on *Campylobacter spp.* focused on their role as veterinary pathogens. The first species in this genus was described in 1913 and was isolated from an aborted lamb (Wassenaar & Newell, 2001). Based on its cell morphology it was thought to be a new member of the genus *Vibrio*. Smith and Taylor (1919) had designated these spiral bacteria the name *Vibrio fetus*. By 1963, Sebald and Véron had re-assigned them to a new genus, *Campylobacter,* as they differed greatly in phenotypic respects, (GC content and growth requirement) from the typed species of the genus *V. cholerae* (Silva *et al.,* 2011). *Campylobacter spp.* were associated with septic abortions in sheep and cattle, and were also known to cause diarrhoea in cattle (Allos, 2001; Jones *et al.,* 1931; McFadyean & Stockman, 1913).

The first reports of human cases of infection with *Campylobacter spp.* began to emerge around the middle of the twentieth century. There is speculation that the spiral bacteria observed by Escherich, in 1886, in the colons of infants who had died of a ‘cholera infantum’ and in the faeces of infants suffering from diarrhoea may have been *Campylobacter* (Kist, 1985). However at the time Escherich failed to consider them to be of great clinical significance and did not study them further. Consumption of milk contaminated with *Campylobacter* led to a foodborne outbreak in humans in the US in 1946 (Levy, 1946). King (1962) carried out the first in-dept study on human isolates of *Campylobacter* in 1957. She described four human strains of a "related vibrio" (subsequently classified as *Campylobacter jejuni*) isolated from blood and was first to identify them as possible causes of gastroenteritis in humans (King, 1962). Dekeyser and co-workers (1972) succeeded in isolating *Campylobacter* from faecal samples thereby confirming King’s observation that the presence of *Campylobacter* was associated with enteritis in humans (Dekeyser *et al.,* 1972). Enteritis caused by *Campylobacter* was not considered to be a major problem until Skirrow's publication in 1977 (Koenraad *et al.,* 1997).

Currently the genus *Campylobacter* contains twenty four species and six subspecies (listed in Table 1.1; On, 2013) and its taxonomic structure has been revised over the years (Debruyne *et al.,* 2008; On, 2013). Most species of *Campylobacter* are human, animal or zoonotic pathogens (Goodwin *et al*., 1989). Together, both *Campylobacter jejuni* (*C. jeuni*) and *C. coli* account for over 95 % of *Campylobacter* infections in humans (Debruyne *et al.,* 2008; Park, 2002).

1.2 General characteristics

Members of the genus *Campylobacter* are relatively slow growing, non-spore forming bacteria and most are microaerophilic, but some species can also grow aerobically or anaerobically (Garrity *et al.,* 2005). When observed under the microscope, log-phase cells have a characteristic slender, curved, spiral shape which are 0.2-0.8 μm wide and 0.5-5 μm long (Garrity *et al.,* 2005). Cells are pleomorphic and the morphology of individual *Campylobacter* cells can change from spiral to coccid upon exposure to sub optimum growth conditions or as cultures age and eventually enter a viable but not culturable state (Rollinson & Colwell, 1986; Snelling *et al.,* 2005a). Oliver (1993) defined these viable but non-culturable cells as bacteria that are metabolically active but incapable of undergoing the cellular division required for growth**.** Filamentous forms have also been observed and Griffiths has postulated that a decoupling of cell division from the anabolic growth processes could underlie the filamentous form in *Campylobacter* (Griffiths, 1993). The bacteria are motile and move by a characteristic rotating rapid corkscrew-like motion, by unipolar or bipolar flagella (Garrity *et al.,* 2005; Snelling *et al.,* 2005a).

Campylobacters are fastidious in nature with complex growth requirements and use amino acids and intermediates of the tri-carboxylic acid cycle as an energy source. They are unable to ferment or oxidize carbohydrates as the genome lacks the gene encoding the glycolytic enzyme 6-phosphofructokinase (Parkhill, 2000; Velayudhan & Kelly, 2002). They are relatively biochemically inactive and typical biochemical reactions are the reduction of fumarate to succinate; a negative methyl red reaction, acetoin production, and indole production. Most species are catalase positive and do not hydrolyze hippurate or possess urease activity (Table 1.1).

Biochemical and growth characteristics of *Campylobacter* species									
					Optimal Growth			Growth Antibiotic Susceptibility	
Species	Catalase	H_2S	Hippurase Test	Urease activity	25ºC	37ºC	42ºC	Cephalothin Resistant	Nalidixic acid Resistant
C. avium	W	-	+	+	-	+	+	+	-
C. canadensis	V	V	-	V	-	+	+	-	V
C. coli	+	-	-	-	-	+	+	+	-
C. concisus	-	-	-	-	-	+	(+)	-	(+)
C. cuniculorum	+	-	-	-	-	+	(+)	(+)	V
C. curvus	-	(-)	(-)	-	-	V	V	-	+
C. fetus subsp fetus	+	-	-	-	+	+	(+)	-	+
C. fetus subsp veneralis	(+)	-	-	-	+	+	-	-	V
C. gracilis	V	-	-	-	-	-	V	-	V
C. helveticus	-	-	-	-	-	+	+	-	-
C. hominis	-	-	-	-	-	+	(-)	-	V
C. hyointestinalis subsp hyointestinalis	+	+	-	-	-	+	+	(-)	+
C. hyointestinalis subsp lawsorii	+	+	-	-	-	+	+	-	+
C. insulanigrae	+	-	-	-	-	+	-	+	+
C .jejuni subsp doylei	V	-	+	-	-	+	-	-	-
C. jejuni subsp jejuni	+	-	+	-	-	+	+	-	+
C. lari subsp concheus	+	N/D	-	-	-	+	+	+	-
C. lari subsp lari	+	-	-	-	-	+	+	+	(+)
C. mucosalis	-	+	-	-	-	+	+	-	(+)
C. peloridis	+	N/D	-	N/D	N/D	N/D	+	(-)	(+)
C. rectus	(-)	-	-	-	-	-	(-)	-	(+)
C. showae	+	V	-	-	-	V	V	-	-
C. sputorum	V	+	-	V	-	+	+	-	(+)
C. subantarcticus	+	-	-	N/D	-	+	+	-	+
C. upsaliensis	-	-	-	-	-	+	+	(-)	+
C. ureolyticus	+	-	-	+	-	+	+	-	-
C. volucris	+	-	-	N/D	-	+	+	+	+

Table 1.1. Biochemical and growth characteristics of *Campylobacter* species adapted from Rossi *et al.,* 2009; Debruyne *et al.,* 2009; Debruyne *et al.,* 2010a, 2010b; Vandamme *et al.,* 2010. Note: symbols denote the following:- +, 90-100 % of strains positive; -, 0-10 % of strains positive; (+), 75-89 % of strains positive; (-), 11-25 % of strains positive; V, 26-74 % of strains positive; W, weakly positive; N/A, not determined.

The optimum growth temperature for most *Campylobacter spp.* is between 30°C and 37°C. However, *C. jejuni* and *C. coli* are distinguished from most other *Campylobacter* species by their unusually high optimum growth temperature of 42°C (Corry & Atabay, 2001). *Campylobacter* are unable to grow below 30°C as they lack cold shock protein genes which play a role in low-temperature adaptation (Silva *et al.,* 2011). *Camplobacter* are able to survive below their optimum growth temperatures and are still metabolically active at 4°C (Hazeleger *et al.*, 1998; Moen *et al.,* 2005; Murphy *et al.,* 2006).

1.3 Campylobacterosis: post-infection sequalae and treatment

Campylobacter spp. are important foodborne pathogens and are the leading causative agents of bacterial gastroenteritis worldwide. *C. jejuni, C. coli,* and to a lesser extent

C. lari, are the most important species of *Campylobacter* involved in human cases of campylobacterosis (Humphrey *et al.,* 2007). Internationally, it is estimated that approximately twenty to thirty percent of all *Campylobacter* infections can be attributed to the handling, preparation and consumption of undercooked poultry (ESFA, 2010). In 2014, 2,615 notifications of campylobacteriosis were reported to the Irish health protection surveillance centre representing an incidence rate of 57.0 cases per 100,000 of the population, approximately eight times the incidence of salmonellosis (HPSC, 2014) and represented an increase of 57.4 % compared to 2010.

Symptoms of campylobacterosis include acute abdominal pain, fever, headache and profuse diarrhoea and usually occur 1–10 days post infection. *Campylobacter* have a low infectious dose with as few as 500 cells required to elicit illness (Black, 1988). Clinical presentations of campylobacterosis are similar to that caused by other bacterial enteric pathogens. Thus diagnosis is not possible on the basis of symptoms alone and must be confirmed from the presence of *Campylobacter* in stool samples (Galanis, 2007; Granato *et al.,* 2010).

Most cases of *Campylobacter* infections are self-limiting and supportive treatment particularly fluid and electrolyte replacement is usually administered (Skirrow & Blaser, 2000). Illness usually resolves within 2–5 days but may last up to several weeks (Galanis, 2007). Antibiotic treatment can be considered if the patient has severe or prolonged symptoms. Typically the broad-spectrum antibiotics erythromycin, ciprofloxacin and tetracycline are prescribed in the treatment of *Campylobacter* infections. Fluoroquinolones (FQ) are often used for the treatment of enteritis including Campylobacterosis regardless of the aetiological agent when a microbiological diagnosis is absent (Engberg *et al.,* 2001; Gibreel & Taylor, 2006). However because resistance to the FQ antibiotics rising, erythromycin is replacing ciprofloxacin as the drug of choice for *Campylobacter* infections (Engberg *et al.,* 2001). Development of FQ resistance in *Campylobacter spp*. is discussed further in section 1.10.

Campylobacter infections can trigger a number of post-infection squalae including the development of the peripheral neuropathies, Guillain–Barré and Miller Fisher syndromes, and reactive arthritis (Bremell *et al.,* 1991; Doorduyn, *et al.,* 2008;

Galanis, 2007; Humphrey *et al.,* 2007; Nachamkin *et al.,* 2002). *Campylobacter* infections are also associated with the development of inflammatory bowel disease and other autoimmune syndromes (Gradel, *et al.,* 2009; Helms *et al.,* 2006).

Guillain-Barré syndrome is an acute inflammatory demyelinating disease caused by the production of ganglioside-like epitopes in the lipo-oligosaccharide of *C. jejuni* that mimic the gangliosides of the host, causing flaccid paralysis, with an incidence of 1.3 cases per 100,000 cases of *Campylobacter* infection (Nachamkin *et al.,* 1998). Miller Fisher Syndrome is a variant of Guillain-Barré syndrome associated with anti-GQ1b antibodies and results in the absence of reflexes, paralysis of eye movement and difficulty walking (Snyder *et al.,* 2009). Reactive arthritis is a common post infection sequalae following campylobacteriosis with an incidence rate of beween 2 % to 7 %. Patients often present with pain and joint swelling within two weeks of *Campylobacter* infection and symptoms of reactive arthritis may last for as long as 12 months (Granato *et al.,* 2010).

1.4 Sources of *Campylobacter*

The main route of transmission of *Campylobacter* to humans are (i) by consumption of contaminated food, particularly undercooked poultry, (ii) direct contact with animals/pets, (iii) recreational swimming or (iv) drinking water from streams or other natural water sources (Wagenaar *et al.,* 2006). *C. jejuni* is the main species of *Campylobacter* associated with food borne illness and is primarily isolated from poultry but also from cattle, sheep, goats, pets and wildlife (rats, mice, foxes, badgers and rabbits, birds and insects) (Newell *et al.,* 2011). *C. coli* is isolated from poultry, cattle, sheep and swine. Other *Campylobacter spp*. causing illness in humans include *C. lari, C. concisus, C. curvus, C. fetus subsp fetus*, *C. hyointestinalis subsp hyointestinalis*, and *C. upsaliensis.* These have been isolated from poultry, wild birds, domestic farm animals (including cattle, sheep, pigs, and horses), deer, domestic cats, dogs and hamsters as well as from reptiles, ostriches and shellfish (On *et al.,* 2013). Carriage of *Campylobacter* is ubiquitous in the gastrointestinal tract of many of the above animal hosts, representing part of the normal microbiota (Jones, 2001).

Faecal shedding from infected hosts contributes to the environmental load of

Campylobacter on farmlands and in agricultural run-off into nearby water sources (Jones, 2001). Untreated fresh and marine water sources that have become contaminated by such waste can act as a reservoir for *Campylobacter* (Jones, 2001; Wilson *et al.,* 2008). Survival of *Campylobacter* in water sources may be due to their association with protozoans and their ability to form biofilms (Snelling *et al.*, 2005b; Robyn *et al.*, 2015). *Campylobacter* biofilms in water systems have been indirectly implicated in a limited number of outbreaks of *Campylobacter* infections in humans and livestock (Pearson *et al.,* 1993; Jakopanec *et al.,* 2008).

1.5 Transmission of *Campylobacter* in the domestic environment

Poultry is regarded as one of the most important reservoirs for *Campylobacter* and constitutes a very significant vehicle for the transmission of *Campylobacter* to humans (FSAI, 2011). Research has shown that between 20% and 30% of human cases of campylobacteriosis may be attributed to handling, preparation and cooking of raw poultry and between 50 % and 80 % may be attributed to the reservoir as a whole (EFSA, 2010). Thus control of *Campylobacter* in the poultry reservoir would have a significant impact on the microbiological safety of poultry food products and the incidence of *Campylobacter* infections in humans (Guerin *et al.,* 2010; O'Mahony *et al.,* 2011; Nauta *et al.,* 2009; Newell *et al.,* 2011). Current intervention strategies to control *Campylobacter* colonization in the poultry reservoir including biosecurity and good hygiene practices (including the use of disinfectant) are not completely effective and the majority of broiler flocks are infected within the third and fourth week of rearing (Bolton, 2015; Robyn *et al.*, 2015). The home is the final control location to prevent the transmission of *Campylobacter* in the food chain from poultry (Bolton *et al.*, 2014; Kennedy *et al.*, 2011b). So, the consumer, as the last link in the food supply chain, plays an important role in the prevention of its transmission (Azevedo *et al.*, 2014).

The high levels of contamination of retail chicken with *Campylobacter* may increase the risk of exposure to the consumer (Nauta & Havelaar, 2008). The prevalence of *Campylobacter* in poultry products in Ireland at retail level is high and raw poultry meat can have very high (10^7 cells per carcass) *Campylobacter* levels (Jørgensen *et al.,* 2002; Madden *et al.,* 2011; Whyte *et al.,* 2004). A survey of retail chicken on sale

in Ireland identified that 84.3 % were contaminated with *Campylobacter* (Madden *et al.,* 2011). *Campylobacter* have also been isolated from the external packaging of retail chicken (Burgess *et al.,* 2005; FASI, 2010). The external contamination of raw poultry packaging may expose the consumer to *Campylobacter* and increase the risk of cross-contamination of other foodstuffs (Burgess *et al.,* 2005). Product labeling practices of raw poultry produce may also increase the risk of exposure of consumers to *Campylobacter* in particular the practice of printing cooking instructions on the inside of the wrapper, which is more heavily contaminated (FASI, 2011).

Intervention strategies to control *Campylobacter* in the domestic environment can be divided into three main categories (i) decrease cross contaminations, (ii) hinder microbial growth (storage temperature), (iii) decrease microbial load (cooking, cleaning of hand, dishes, dish cloths, sponges, towels, equipment and utensil surfaces) (Taché & Carpentier, 2014). As *Campylobacter* is heat sensitive, and cannot grow at temperatures below 30◦C, contamination from raw food via utensils and hands to ready-to-eat products is the most important risk factor for *Campylobacter* (Nauta *et al.*, 2008).

Cross-contamination of hands and other foodstuffs from fresh poultry produce is believed to be the most important route of transmission of *Campylobacter* to humans and is possibly a more common cause of infection than consumption of under-cooked meat (Kennedy *et al.*, 2011a; Luber, 2009; Nauta & Havelaar 2008). The preparation of meals using raw chicken results in the widespread dissemination of *Campylobacter* around the food preparation area (Cogan *et al.*, 2002; Humphrey *et al.*, 2001; Kennedy *et al.*, 2011a). Cross-contamination from raw food via utensils and hands to ready-to-eat products and has been shown to be an important risk factor for infection (Nauta *et al.*, 2008). *Campylobacter* have been recovered from the surfaces of kitchen wood and plastic cutting boards 3 hours after the preparation of broiler chickens (Wanyenya *et al.,* 2004).

Cleaning surfaces with detergent and drying are important to control persistence of *Campylobacter* in domestic environments (Keener *et al.,* 2004). When used properly, kitchen biocides could be helpful in reducing the public health and food poisoning risks associated with kitchen cross-contamination with *Campylobacter spp.*

(Humphrey *et al.*, 2001; Azevedo *et al.*, 2014). The use of disinfectant wipes on surfaces contaminated after the preperation of raw poultry produce was found to lower the risk of *Campylobacter* infection by 2-3 orders of magnitude, reducing the risk from 2:10 to 2: 1,000 (Lopez *et al.*, 2015).

Improper handling and storage of contaminated poultry produce may increase the risk of contamination to the consumer produce (Burgess *et al.,* 2005; Sampers *et al.,* 2010). Maintenance of temperatures during storage are essential prerequisites for the quality and safety of pre- packaged meats including raw poultry (Burgess *et al.,* 2005; Sampers *et al.,* 2010). Thaw water (chicken juice) from frozen poultry produce may prolong the survival of any residual contaminating *Campylobacter* present on produce and represent a source of cross-contamination. Lower temperatures also increase viability of *C. jejuni* and after 8 weeks incubation at 5^{o}C in juice contamination levels of 2 X 10^{2} CFU ml^{-1} was found (Birk *et al.*, 2004; Park *et al.*, 2014).

Measures aimed at improving consumer education about the handing, preparation and cooking of poultry have also correlated with reduced incidence of campylobacteriosis in humans (Kennedy *et al.*, 2011b; Rosenquist *et al.*, 2003). *Campylobacter* are highly sensitive to high temperatures (>70 ^{o}C) are readily inactivated by pasteurization treatments and domestic cooking processes (Bearth *et al.*, 2014; Park, 2002). Consumers should adequately treat food to prevent outgrowth of bacteria (proper storage), to inactivate or reduce numbers of bacteria present on / in raw food (proper heating), and to prevent cross-contamination of bacteria from raw food to ready-to-eat food (De Jong *et al.*, 2008) Cook-in-the-bag technology has been shown to limit the amount of cross-contamination associated with the cooking of raw poultry produce (Bolton *et al.*, 2014).

1.6 Detection and typing methods for *Campylobacter spp.*

The accurate identification of *Campylobacter spp.*, particularly *C. jejuni* and *C. coli*, is important for the identification of the source of outbreaks and determination of routes of transmission (Wirz *et al.,* 2010; Taboada *et al.,* 2013). Efficient and reliable techniques for the isolation and identification of *Campylobacter spp.* are essential to facilitate clinical and epidemiological studies (Fredrick & Huda, 2011).

Campylobacter has a weakly clonal population due to extensive horizontal gene transfer and intra-molecular recombination (Taboada *et al.,* 2013). Subtyping of *Campylobacter* is also important for developing and testing strategies for control processes applied within the food chain. Precise genotyping and continuous monitoring of the strains have greatly improved our understanding of the genetic relationships between *Campylobacter* isolates from different sources. In addition, correct species determination is important clinically, since *C. coli* strains are reported to be resistant to different antimicrobial agents more often than *C. jejuni* strains (Engberg *et al.,* 2001; Schönberg-Norio *et al.,* 2006).

Conventional culture methods are widely used for the detection and isolation of *Campylobacter* (Granato *et al.,* 2010; Jacobs-Reitsma *et al.,* 2008). *Campylobacter spp.* have fastidious growth requirements and the detection and isolation of *Campylobacter* in complex substrates like faeces or environmental samples is difficult. Conventional methods used for isolation involve an enrichment step to promote the recovery of low numbers of sub lethally damaged *Campylobacter* cells and/or plating onto selective media (Jacobs-Reitsma *et al.,* 2008; Keramas *et al.,* 2004). Culture conditions used have to be selective enough to avoid overgrowth from competing organisms (Leblanc-Maridor *et al.,* 2011; Kelly, 2001). After detection, there are several techniques available for confirming *Campylobacter* to the genus and species level. These include biochemical, serotyping, immunological and DNA-based detection methods.

In routine clinical microbiology laboratories, species identification is mainly performed by phenotypic methods (Nakari *et al.,* 2008). Biotyping methods used in the identification of *Campylobacter spp.* are based on characterising their metabolic activities. These metabolic activities can include colonial morphology, environmental tolerances, and biochemical reactions (Eberle & Kiess, 2012). *Campylobacter* isolated from stools are typically identified by Gram stain, examination of the bacterial colony, along with the performance of an oxidase test (Granato *et al.,* 2010). A positive hippurate hydrolysis test is widely used to differentiate *C. jejuni* from other species of *Campylobacter* (Morris *et al.,* 1985). However, this activity is sometimes very weak in *C. jejuni*, and hippuricase-negative strains of this species are also well known (Debruyne *et al.,* 2008; On *et al.,* 1996). Conventional culture methods used for the

detection of *Campylobacter* are labour intensive and require 4 days to give a negative result and 6-7 days to confirm a positive result (Oliverira *et al.,* 2005). Serotyping, based on either the Penner system and Lior system, is another phenotyping method used to distinguish between strains and based on the expression of antigens on the surface of *Campylobacter* (Wassenaar & Newell, 2000). In serotyping, antibodies and antisera are used to detect these differences in the surface antigens structures (Eberle & Kiess, 2012). The discriminatory power of serotyping is dependant on the expression of surface antigens and the method is labour intestive requiring costly reagents and the limited availability of antisera (Eberle & Kiess, 2012; Pike *et al.,* 2013; Wassenaar & Newell, 2000). In addition, a high number of strains have found to be untypeable using this method (Eberle & Kiess, 2012)

The use of serotyping has decreased, and today the molecular typing methods are more commonly used in many laboratories to identify *Campylobacter* (Wassenaar & Newell, 2000). Molecular methods used for the detection of *Campylobacter* including immuno-based and DNA-based detection methods and are faster than conventional methods. Immuno-based detection methods are based on the detection of *Campylobacter* antigens by *Campylobacter* specific antibodies. Commercial kits based on antigen detection are available and include the ProSpecT *Campylobacter* kit and the Premier CAMPY EIA and the ImmunoCard STAT! CAMPY test. Both the ProSpecT *Campylobacter* kit and the Premier CAMPY EIA are based on Enzyme linked immunosorbent assay (ELISA). The ImmunoCard STAT! CAMPY test on the other hand is based on a lateral-flow immunochromatographic assay. Kits are easy to use and results are available in 2-3 hours (Granato *et al.,* 2010). The usefulness of immunogenic seperation methods for the detection of *Campylobacter spp* may be limited by the high antigenic diversity and variation of *C. jejuni* and *C. coli* making it difficult for a single antibody to recognize all strains (On *et al.,* 2013).

Several DNA-based methods have been used for genotyping of *Campylobacter*. Genotyping methods are based on the evaluation of genetic polymorphism present in the genome of *Campylobacter spp.* can be broadly divided into three categories: (a) in whole genome; (b) in a single locus of the genome; (c) in multiple loci of the genome (Ahmed *et al.,* 2012). Fluorescent in situ hybridization (FISH) techniques uses fluorescently labeled oligonucleotide DNA probes that bind specifically to unique

target sites on *Campylobacter* ribosomal RNA (Poppert *et al.,* 2008). Molecular primers and probes are often designed to detect simultaneously the thermotolerant *Campylobacter* species: *C. jejuni*, *C. coli* and *C. lari,* and often also *C. upsaliensis,* by a single assay. A list of *Campylobacter* specific primers has been published which includes those primers that can be used to detect the 16S rRNA (Bang *et al.,* 2002; Josefsen *et al.,* 2004; Moreno *et al.,* 2003) or 23S rRNA (Fermer & Engvall, 1999) ribosomal genes, or flagellin genes *flaA* and *flaB* (Moore *et al.,* 2001; Waage *et al.,* 1999) and the *hipO* gene. Primers which detect the *hipO* gene may even provide a more accurate differentiation between *C. jejuni* and *C. coli* than the phenotypic hippurate test (Abu-Halaweh *et al.*, 2005; Abulreesh *et al.,* 2006; Jensen *et al.,* 2005). Both FISH and PCR-based methods can detect *Campylobacter* in samples without the need to culture (Abubakar *et al.,* 2007). However a drawback to using FISH and PCR-based methods is that they do not differentiate between viable and non-viable cells and this must be addressed before these new techniques can replace the conventional cultivation methods (Birch *et al.,* 2001; Nogva *et al.,* 2000).

Whole genome methods for the identification of *Campylobacter spp.* include pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) (Ahmed *et al.,* 2012). Single locus genotyping methods involve PCR amplification followed by by sequencing of the locus or by restriction profiling (Amhed *et al.,* 2012). In the comparison of large numbers of strains, irrespective of their geographical or chronological origin, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and multi-locus sequence typing analysis techniques can be employed (On *et al.,* 2008). Analysis by pulsed-field gel electrophoresis (PFGE) has been used successfully for inter- and intraspecies differentiation of *Campylobacters*. PFGE is a technically demanding method, consisting of digestion of the whole genome with rare-cutting restriction enzymes and separation of fragments in a gel using a coordinated pulsed electric field (Wassenaar & Newell, 2000). By applying an electric field that periodically changes direction, PFGE enables the separation of large DNA molecules in an agarose gel matrix (Taboada *et al.,* 2013). PFGE is currently performed worldwide according to the PulseNet protocol and has exhibited a high discriminatory power (On *et al.,* 2008).
AFLP is based on the selective PCR amplification of restriction fragments generated from the whole genomic DNA and is useful for the differentiation of genetically

related strains (Amhed *et al.,* 2012). In AFLP profiling, the digestion of DNA is followed by oligonucleotide adaptor ligation, PCR amplification with a labeled primer, and finally electrophoresis (Wassenaar & Newell, 2000). The flaA–RFLP typing is widely used and it is one of the simplest and most cost- effective genotyping methods for the investigation of large number of *Campylobacter spp.* (Amhed *et al.,* 2012; Taboada *et al.,* 2013). The technique involves PCR amplification of the flagellin gene locus. PCR products are then subjected to restriction enzyme digestion to generate simple restriction fragment length polymorphic fingerprints on agarose gel electrophoresis. This method is often used in combination with other genotyping methods such as MLST when the strains need further subtyping (Amhed *et al.,* 2012).

The MLST method for *C. jejuni* was developed by Dingle et al. (2001) is based on the sequencing of a set of essential or housekeeping genes that are present in all strains of a bacterium (Amhed et al., 2012; On *et al.,* 2008). MLST indexes the neutral genetic variation in housekeeping genes, which evolve slowly and are not under selective pressure (Sails *et al.,* 2003). The sequenced PCR products of seven housekeeping gene loci (*asp, glnA, gltA, glyA, pgm, uncA, tkt*) in *Campylobacter* are assigned an allele number based on a complete match to an allele in the global PubMLST database (Amhed *et al.,* 2012; Taboada *et al.,* 2013). The array of seven allele numbers is then assigned a sequence type (ST) according to the database. Sequence types are grouped into clonal complexes and *Campylobacter spp*. that share four or more alleles belong to the same clonal complex (CC) or lineage (Dingle *et al.,* 2001; Taboada *et al.,* 2013). One major advantage of MLST over other genotyping methods such as PFGE is its very high reproducibility due to minimal variation in DNA sequencing (Taboada *et al.,* 2013). In addition, global accessibility of data transmitted electronically via the internet from publically available database allows the inter- laboratory comparison of data without the requirement of reference isolates, unlike PFGE (On *et al.,* 2008; Sails *et al.,* 2003; Taboada *et al.,* 2013). An ongoing challenge with MLST has been the high cost and time consuming nature of analysis. The risk of contamination of samples from post-PCR manipulation of amplicons is also a disadvantage of MLST (Taboada *et al.,* 2013).

1.7 Antimicrobial resistance in food borne pathogens

The current intensification of animal production and the economic loss associated with disease outbreak among production animals has resulted in an increased agricultural use of critically important therapeutic antibiotics in veterinary medicine (da Costa *et al.,* 2013; Hammerum & Heuer, 2009). The increased agricultural use of antibiotics provides favourable conditions for the selection, persistence and spread of antimicrobial resistance on the farm and in food of animal origin, and represents an important reservoir of antibiotic resistant bacteria (da Costa, 2013; Lavilla Lerma *et al.,* 2013). Antibiotic-resistant bacteria are frequently isolated in foods and food-processing environments and the spread of resistance genes to humans via food is well documented (Poole, 2005; Lavilla Lerma *et al.,* 2013). Nowadays, antibiotic resistance has become a significant and increasing public health problem, and infection with antimicrobial resistant organisms leads to treatment failures and increased morbidity, mortality, length of hospitalisation and cost of healthcare (Cosgrove, 2006; Lavilla Lerma *et al.,* 2013). Concern about bacterial resistance has led to calls for increased education of both the public and professionals on the correct use of antibiotics and more stringent infection control measures to reduce the transmission of infection (Gilbert & Bain, 2003). The prevalence of multi-antimicrobial resistance in *Campylobacter* is rising in food production environments in central and southern EU-member states (MSs) (Možina *et al.,* 2011). In Ireland, multi- antimicrobial resistance in *Campylobacter spp.* is cause for concern to the Irish poultry industry. Fallon et al., (2003), reported that thirty per cent of Irish isolates of *Campylobacter spp* from combined human and animal origins were multi-drug resistant. *Campylobacter spp.* exhibits intrinsic resistance to a variety of antibiotics, including bacitracin, novobiocin, rifampicin, streptogramin B, trimethoprim and vancomycin (Taylor *et al.,* 1988). Due to the high levels of fluoroquinolone resistance among *Campylobacter* isolates, macrolide antibiotics are currently the antibiotics of choice for campylobacterosis. However a prevalence of resistance to macrolide antibiotics (ranging from 15–80 %) has been observed in some *C. coli* isolates from chickens and swine (Luangtongkum *et al.,* 2009; Gibreel and Taylor, 2006; Kim *et al.,* 2006). The WHO, Food and Agriculture Organization (FAO) and the Organisation Internationale des Epizooties have issued guidelines for the prudent use of antibiotics in food producing animals. Good on-farm management practices will limit the need

for antimicrobial use in farm animals (Stöhr & Wegener, 2000; Gilbert & McBain, 2003).

1.7.1 Incidences of biocide resistance in food borne pathogens

Biocides are widely used in animal husbandry and food production to maintain hygiene, for the disinfection of production plants, to control microbial growth in food and drinks in order to increase the safety and quality of the product. Biocides play an essential role in limiting the spread of foodborne pathogens in the food industry (Cerf *et al.*, 2010; Condell *et al.,* 2012; Doyle & Ericson, 2006; SCENIHR, 2009). Biocides have an intergal part to play in biosecurity programs to control the entry of pathogenic microorganisms into the farm (Condell *et al.,* 2012). Gaining an understanding of how biocide tolerant bacteria can be selected from *in situ* investigations, e.g. on the poultry farm, is essential in order to perserve the role of such compounds in infective control and hygiene (SCENIHR, 2009). There is a growing concern that the use of disinfectants and preservatives in domestic and production environments may be a contributory factor to the development and selection of antibiotic resistant strains (Gilbert & McBain, 2003; Lavilla Lerma *et al.,* 2013). The selective pressure exerted by biocides has been shown to activate the expression of genes in antibiotic multidrug resistance leading to low-level cross-resistance to antibiotics in bacteria (Braoudaki & Hilton, 2004; Karatzas *et al.,* 2007; Moken *et al.*, 1997, SCENIHR, 2009). Adaptation to triclosan and a quaternary ammonium disinfectant containing formaldehyde and glutaraldehyde has been shown to lead to the overexpression of acrB gene encoding a subunit of the AcrAB multidrug efflux pump involved in multidrug resistance in *Salmonella* Typhimurium (Karatzas *et al.,* 2007). Adaptation to disinfectant pine oil also resulted in upregulation of AcrAB in *E. coli* as a result of enhanced marA expression (Moken *et al.*, 1997).

Survival of foodborne pathogens on food contact surfaces after cleaning and disinfection can increase the risk of cross-contamination of products and represents a potential food safety hazard (Langsrud *et al.,* 2003; Lavilla Lerma *et al.,* 2013; Meyer, 2006; Payrat *et al.,* 2008). Regular exposure to sub-lethal concentrations of disinfectant may contribute to increased disinfectant tolerance in foodborne pathogens and/or disinfectant failure (Lavilla Lerma *et al.,* 2013). Food-borne pathogens can be

exposed to sub-lethal concentrations of biocide as a result of stagnant water in harbourage sites (hollow parts of equipment, recesses of buildings, crevices or cracks in flooring materials) that can dilute the concentration of biocide. Disinfectant failure can also arise in situations where care is not taken to sufficiently clean and remove all organic matter on a surface prior to disinfection or where the concentration of disinfectant is too low to cope with a gradual increase in the inorganic matter load, for example in footbaths (Cerf *et al.,* 2010). In 2009, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), ranked biocides (used as disinfectants in the food industry as well as in consumer products) according to the likelihood that exposure to that biocide would induce resistance in bacteria. Quaternary ammonium compounds (QACs), bisguanides and phenolics and heavy metals obtained the highest ranking. Isothiazolones, anilides, diamidines, inorganic acids and their esters and alcohols, received an intermediate risk ranking. Highly reactive biocides such as oxidising agents and alkylating agents received a low risk ranking for the development of resistance (SCENIHR, 2009).

Disinfectants which are commonly used in the food industry can be broadly categorized into two main categories based on their mechanism of action (Chapman, 2003a). Oxidants including paracetic acid and chlorine based compounds (sodium hypochlorite and chlorine dioxide) mediate their bacteriocial effect by the generation of reactive oxygen species to induce damage. Membrane active biocides make up the remainder. These include the quaternary ammonium compounds, biguanides (chlorhexidine) and alcohol-based biocides and they act to destabilise the membrane and induce cell lysis (Chapman, 2003a; Holah *et al.,* 2002; Doyle & Ericson, 2006). The underlying genetic mechanisms of bacterial resistance to biocides is less well studied overall when compared to antibiotics. Most of what is known about biocide resistance mechanisms relates to the QACs and peroxides classes of disinfectants (Chapman, 2003a; SCENIHR, 2009).

Several reports have described isolates especially among Gram-negative species from various food processing environments that possess a reduced susceptibility to chlorine and quaternary biocides relating to practical usage (Chapman, 2003a). Chlorine based biocides are frequently used as disinfectants and sanitisers in food production. Exposure of *Salmonella enteritidis* strains to chlorine (25ppm) induced 2-8-fold

increases of MIC to the antibiotics chloramphenicol, nalidixic acid and tetracycline (Potenski *et al.,* 2003). Hypochorous acid resistant-*Salmonella* were isolated from neck and cloacae of poultry carcasses at different stages of production from a poultry abattoir. Resistant strains were able to grow in hypochorous acid concentrations up to 72ppm (Mokgatla *et al.,* 1998). Biocides may be effective against planktonic populations, however, they may be ineffective against adhearant biofilms (SCENIHR, 2009).

Decreased susceptibility of bacteria to oxidants is mainly associated with biofilm growth (Langsrud *et al.,* 2003). Interaction with the extrapolymatrix of biofilms may render some biocides, such as iodine and povidone-iodine, chlorine, and peroxygens inactive (Gilbert *et al.,* 2002). Certain degradative enzymes such as catalase can become concentrated within the glycocalyx and can lead to a decrease of biocidal activity of hydrogen peroxide (Bridier *et al.,* 2011a). Such mechanisms might lead to the formation of a sublethal gradient-concentration, which in turn might induce further resistance through mechanisms such as efflux (Gilbert & McBain, 2003).

There have been many reports of reduced susceptibility to the membrane active biocides in the scientific literature particularly for the QACs, chlorhexidine and triclosan (SCENIHR, 2009). Reduced susceptibility was also found to be associated with cross-resistance to antibioitics (Braoudaki & Hilton, 2004; Christensen *et al.,* 2011; Randall *et al.*, 2003)

QAC-based disinfectants are widely used in the food industry due to their excellent non-tainting, non-toxic and non-corrosive (to human skin as well as hard surfaces) nature (Holah *et al.,* 2002; Langsrud *et al.,* 2003). Decreased susceptibility to the QAC-based disinfectants has been reported to occur in both Gram-negative foodborne pathogens including *Salmonella spp.* and *E. coli*, as well as the Gram-positive pathogens *Listeria monocytogenes* and *S. aureus*. *In vitro* studies have indicated that the majority of disinfectants are efficacious against *Campylobacter* (Avrain *et al.,* 2003; Gutiérrez-Martín *et al.,* 2011). However others have shown that *Campylobacter* can survive and persist on surfaces after cleaning and disinfection (Kudirkiene *et al.,* 2011; Peyrat *et al.,* 2008). *Salmonella spp.* are the second largest cause of food poisoning after *Campylobacter*. Development of increased biocide tolerence of

Salmonella spp. to BKC was assessed by Condell et al., (2012) and Braoudaki and Hilton (2004b). Condell et al., (2012) found that adaptation (4-fold decrease in MIC to BKC) of *Salmonella* hvittingfoss S41 by serial passage resulted in cross-resistance to the quinolone antibiotic nalidixic acid and the cephalosproin antibiotic ceftriaxone, and intermediate resistance to ampicillin, piperacillin, cephalexin, cefpodoxime, cefpirome, tetracycline, ciprofloxacin and choramphenicol. Braoudaki and Hilton (2004b) similarly found that when *Salmonella* Virchow was adapted to BKC it too was multidrug resistant (resistant to the antibiotics amoxicillin, amoxicillin-clavulanic acid, clindamycin, imipenem, trimethoprim and biocide chlorhexidine). Both studies also showed that not all adapted *Salmonella* serotypes developed a MDR phenotype (Condell *et al.,* 2012; Braoudki & Hilton; 2004). Both Langsrud et al., (2004) and Braoudaki and Hilton (2004b) showed that *E. coli* developed a multidrug resistant (MDR) phenotype upon adaptation to BKC. Both in *E. coli* and *Salmonella spp.*, the profile of cross-resistance of BKC-adapted strains has been shown to be strain-dependent with different combinations of biocide and antibiotic resistances occurring in adapted strains (Braoudaki & Hilton 2004; Langsrud *et al.,* 2004). *E. coli* strains ATCC 11775 and DSM682 which were pre-adapted to grow in the presence of 150 mg l^{-1} BKC showed enhanced survival following exposure to 20 mg l^{-1} BKC in suspension tests. The same strains were shown to have developed low-level cross-resistance (1.5 to 3-fold higher MIC) to a number of antibiotics including ampicillin, penicillin, norfloxacin, nalidixic acid, kanamycin, gentamicin, chloramphenicol, tetracycline and erythromycin (Langsrud *et al.,* 2004). Sheridan et al., (2012) did not find that *E. coli* 0103 adapted to BKC (MIC 20 mg l^{-1} BKC) became multidrug resistant. Walsh et al., (2003) found differences in the cross–resistance profiles of *E. coli* ATCC 10536 and ATCC 8739 when adapted to the quaternary ammonium compound didecyl-dimethyl-ammonium chloride (DDDMAC). DDDMAC-adapted *E. coli* ATCC 10536 which showed ~10-fold increased resistance to DDDMAC (from 11.6 mg l^{-1} to 108 mg l^{-1}) was cross-resistant to chloramphenicol, ciprofloxacin, tetracycline carbenicillin and piperacillin. DDDMAC-adapted *E. coli* ATCC 8739 which showed ~4-fold increase in resistance (from 76.4 mg l^{-1} to 292 mg l^{-1} DDDMAC) also developed cross-resistance to the antibiotics chloramphenicol, tetracycline and cefuroximine. The same strain however was found to be more susceptible to the aminoglycoside antibiotics (gentamycin, amikacin, neomycin and kanamycin), ciprofloxacin, ceftazidine and carbenicillin (Walsh *et al.,* 2003). Buffet-

Bataillion et al., (2011) found that resistance to clotrimoxazole (a combination of the antibiotics trimethoprim and sulfmethoxazole) was found to be associated with decreased susceptibility to alkyl-dimethyl-benzylammonium chloride and DDDMAC in clinical isolates of *E. coli*.

Listeriosis is caused by the Gram-positive foodborne pathogen, *Listeria monocytogenes* (Larsen *et al.,* 2014). The bacterium is ubiquitous in the environment and may be transmitted into food processing facilities by raw materials, workers, trucks, tools, cleaning materials or machines (Larsen *et al.,* 2014). *L. monocytogenes* has been shown to persist on surfaces following cleaning and disinfection in food production environments (Aase *et al.,* 2000; Holah *et al.,* 2002). Aase et al., (2000) examined the susceptibility of persistent strains of *Listeria monocytogenes* isolated from food processing environments to BKC. Persistent strains showed reduced susceptibility with MIC values between 4 mg l^{-1} and 7 mg l^{-1} BKC. Aase et al., (2000) also found that if they adapted a BKC-sensitive *L. monocytogenes* strain to BKC then the BKC-adapted strains showed a similar level of reduced susceptibility to BKC (7 mg l^{-1} BKC) to isolates occurring in the food processing environment.

Enterotoxin-producing *Staphlococcus spp.*, are important Gram-positive food poisoning pathogens. Contamination of food usually occurs during processing and preparation (Le Loir *et al.,* 2003; Holah *et al.,* 2002). QAC resistance determinants encoding efflux pumps occur widely in *Staphococcus spp.* isolated from food products and food production environments (Heir *et al.,* 1999a). They are usually located on plasmids that carry antibiotic resistance determinants (Heir *et al.,* 1999a). When *S. aureus* strain RN4220 containing the *qac*G gene, encoding the efflux pump qacG, was adapted to BKC, it showed significantly increased survival rates in suspension tests when exposed to BKC (10 mg l^{-1}) (Heir *et al.,* 1999b).

Pseudomonas spp. are often isolated from food equipment surfaces and are important food spoilage organisms (Langsrud *et al.,* 2003). Langsrud and Sundheim (1997) found that 30 % of *Pseudomonas spp.* isolated from raw poultry carcasses were found to grow in in-use concentrations of BKC (200 mg l^{-1} BKC). The same study found that the prevelence of BKC-resistant *Pseudomonas spp.* was higher in a poultry processing plant which used QAC-based dinfectants for cleaning and disinfection than

in a poultry processing plant that used a chlorine-based disinfectant. When a *Pseudomonas* strain isolated from a 1 % solution of a QAC-based commercial disinfectant was grown overnight in TSB containing 200 mg l^{-1} BKC, its resistance to the bacteriocidal effects of BKC in suspension tests was found to be enhanced. The observed resistance however, was unstable and was lost after susequent growth in biocide free media. Long-term and short-term exposure of *P. aeruginosa* to sub-inhibitory (residual) concentration of chlorhexidine diacetate have also been shown to lead to some marked increase in resistance to this biguanide, and in some cases cross-resistance to the QAC benzalkonium chloride (Thomas *et al.,* 2000).

(Braoudaki & Hilton, 2004a) examined adaptation of *E. coli* O157 to chlorhexidine (MIC increase from 4- 512 mg l^{-1} chlorhexidine) and found they were cross-resistant to the biocide triclosan. In the same study, BKC-adapted *Salmonella* Virchow (MIC 256 mg l^{-1} BKC) showed elevated resistance to chlorhexidine, chlorhexidine-adapted *Salmonella* Virchow (MIC 128 mg l^{-1} chlorhexidine) did not demonstrate reciprocal cross-resistance to benzalkonium chloride, indicating that specific rather than generic resistance mechanisms play a role in adaptation to biocides (Braoudaki & Hilton, 2004a).

E. coli O157 was shown to rapidly develop high levels of resistance (0.25 µg/ml-1024 µg/ml) to triclosan after only two sublethal exposures (Braoudaki & Hilton, 2004b). Cross-resistance to other antibiotics was also observed and triclosan-adapted *E. coli* O157 demonstrated decreased susceptibilities to various antimicrobial agents including chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim as well as to the biocides chlorhexidine and BKC (Braoudaki & Hilton, 2004b). Randall *et al*., (2003) examined multidrug resistant *Campylobacter spp., C. jejuni* and *C. coli* strains with reduced susceptibility to triclosan (256 mg l^{-1} to > 512 mg l^{-1} triclosan; 8-16 fold greater than susceptible strains) were significantly more resistant (2-4 fold increases in MIC values) to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid and tetracycline. *C. jejuni* and *C. coli* strains also more resistant to ethidium bromide and acridine orange. MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps have all been shown to to confer high-level triclosan resistance (MIC approximately 1, 000 mg l^{-1} triclosan) in *Pseudomonas aeruginosa* (Chuanchuen *et al.,* 2001, 2003). Exposure to triclosan resulted in the selection of

strains which hyperexpressed the MexCD-OprJ efflux system in susceptible Δ(*mex*AB-*opr*M) strains of *P. aeruginosa* that were also cross-resistant to tetracycline, ciprofloxacin, trimethoprim and erythromycin (Chuanchuen *et al.,* 2001).

In *Listeria monocytogenes*, exposure to sublethal concentrations of triclosan (1 and 4 mg l^{-1}) did not alter susceptibility to the biocide. However it did induced resistance to the antibiotic gentamicin (upto 16-fold increase in MIC) and to other aminoglycoside antibiotics including kanamycin, streptomycin, and tobramycin (Christensen *et al.,* 2011). Adaptation to triclosan in *S. aureus* (MIC 1.5 and 4 mg l^{-1}; 24- to 60-fold less susceptible than unadapted strains) resulted in the formation of small colony varients. Triclosan-adapted *S. aureus* small colony varients also showed reduced susceptibility to penicillin (32-fold decrease; two strains) and gentamicin (2.5- to 10-fold decrease) but not to the biocides chlorhexidine or cetylpridinium chloride (Seaman *et al.,* 2007). The use of triclosan in food production environments is no longer permitted in the EU because of concerns about the development of antibiotic resistance in food-borne pathogens (SCCS, 2010).

Many reports on the development of bacterial resistance to biocides frequently describe phenotypic adaptation. Phenotypic adaption is not hereditary and is lost once the biocide is removed (Meyer, 2006). Growth as part of a sessile community, referred to as a biofilm, is a form of phenotypic adaptation to antimicrobial agents (SCENIHR, 2009). Physiological and structural process are responsible for the observed resistance and include modified physiological state of bacteria as a result of sessile growth, impairment of biocide penetration into the biofilm matrix, quenching by the exopolysaccharides, and enzymatic inactivation (Gilbert & McBain, 2003; SCENIHR, 2009). Biofilms represent a protected mode of growth that allows cells to survive in hostile environments. Bacteria in biofilms are able to persist in the food industry environment, because biofilms protect bacteria from heat, desiccation, other environmental stresses and antibacterial agents. Biofilm formation by bacteria on food products or food processing equipment contributes to spoilage, and contamination of food and to the spread of food-borne pathogens (Van Houdt & Michiels, 2010). Outbreaks of *L. monocytogenes*, *C. jejuni*, *Salmonella spp.*, *Staphylococcal spp.*, and *E. coli* O157:H7 have been associated with the presence of biofilms (Simões e*t al.,* 2010). In *Staphylococcus spp.,* biofilm formation was positively correlated with

resistance to QACs (Gilbert & Moore, 2005). Biofilms are difficult to erradicate and can act i) as harbourage sites or ii) as a substrate for other bacteria less prone to biofilm formation to adhere to thus increasing the pathogens survival and further dissemination during food processing (Lavilla Lerma *et al.,* 2013; Simões *et al.,* 2010). Adaptation can be avoided by enhancing the action of cleaning, and disinfection regimes will decrease the opportunity of foodborne pathogens to form biofilms (Meyer, 2006; Simões *et al.,* 2010). Effective cleaning procedures must break up or dissolve the EPS biofilm matrix so that biocides can gain access to viable cells (Simões *et al.,* 2010). Processing equipment should be designed with high standands of hygiene in mind, such that the design of equipment should facilitate easy removal of organic material and biofilms. Identification of suitable materials or incorperation of antimicrobials into products which could suppress biofilm formation on food contact surfaces may also control biofilm formation (Simões *et al.,* 2010). Pan *et al.,* (2006) found that resistance of *Listeria* biofilms against disinfectants present on stainless steel was lower than on Teflon.

1.8 Assessing the efficacy of antimicrobial agents

Antibiotics are used *in vivo* in human and animal medicines to kill or inhibit the growth of pathogenic microorganisms, while biocides (disinfectants) are used to kill pathogenic bacteria on surfaces, thus limiting their transmission in the food chain (Cerf *et al.,* 2010; Langsrud *et al.,* 2003; Russell, 2003). Antibiotics mediate their action against a single target site and affect physiological processes, which are vital for cell survival, for example protein or DNA synthesis (Gilbert & McBain, 2003; Cerf *et al.,* 2010). In contrast to antibiotics, biocides have multiple target sites within the bacterial cell and the actions of biocides are rarely pharmacologically precise (Gilbert & McBain, 2003; McDonnell & Russell, 1999). They target outer and cytoplasmic membranes of the bacteria as well as cellular components such as proteins, DNA, RNA through physicochemical interactions or chemical reactions (Cerf *et al.,* 2010; Maillard *et al.,* 2002). They are usually applied at much higher concentrations then those for antibiotics and unlike antibiotics, biocides are expected to rapidly kill the bacterial target population after short contact times, usually minutes (Gilbert & McBain, 2003; Maillard, 2005; McDonnell & Russell, 1999).

Antimicrobial properties of antibiotics and disinfectants can be evaluated by determining their bacteriostatic and/or bactericidal activities. The minimum inhibitory concentration of an antimicrobial agent is used to evaluate the bacteriostatic activity of an antimicrobial agent (Gilbert & McBain, 2003). The minimum inhibitory concentration (MIC) is defined as the minimum concentration of antimicrobial agent that can completely inhibit growth of the target pathogen after a fixed length of time (usually 24 h) under a particular set of conditions (Cerf *et al.,* 2010). Relating antibiotic MIC data to achievable antibiotic blood serum or tissue concentrations after oral or parenteral administration is useful to predict treatment outcome (Gilbert & McBain, 2003; Russell, 2003). Biocides are used for the decontamination of environmental surfaces and in practice are applied at much higher concentrations than their MICs (SCENIHR, 2009; McDonnell & Russell, 1999). The bactericidal activity of an antimicrobial agent can be evaluated by establishing a survival curve for that antimicrobial (a single concentration is applied and survivors are enumerated over time). MBC is usually defined as the biocide concentration that results in a five-fold Log_{10} reduction in viable bacteria after a given time. MBCs can also be established from MIC experiments by subculturing from growth-negative media into fresh drug-free media and monitoring for regrowth following biocide exposure (Russell, 2003).

The activity of biocides can be affected by a number of factors and understanding the factors affecting antimicrobial activity is essential to ensure that a biocidal product/formulation is used properly (Maillard, 2005). The most important factor to affect antimicrobial activity of a biocide is its concentration exponent. The concentration exponent or dilution coefficient (η) measures the effect of concentration on the antimicrobial activity of a chemical agent (Russell, 2003). Biocides with a high η-value (e.g. alcohols and phenolic compounds) are affected considerably by changes in concentration and rapidly lose their activity upon dilution (Maillard, 2002; Russell, 2003). Biocides with a low η-value (e.g. organomercurials and formaldehyde) are less affected by changes in concentration (Maillard, 2002; Russell, 2003). Poor understanding of the concentration exponent can result in incorrect application of a biocide that can lead to microbial survival on surfaces and also in products, thus leading to infection or spoilage (Maillard, 2005). Sufficient exposure/treatment time with biocide is also essential, and reduced contact time with disinfectant is often

associated with a decrease in bacteriocidal activity of the biocide (Maillard, 2005). Standard efficacy test guidelines set out by the European standardization body CEN recommend a minimal exposure time of five minutes for the testing of disinfectants and antiseptics and one minute for the testing of hygienic handwash (Meyer *et al.,* 2010a; Miallard, 2005). Factors such as pH may effect the biocide's ionization and hence its activity (Maillard, 2005). Changes in pH will change the overall surface charge of the bacteria. Increasing the pH enhances interaction between the outer membrane surface and the activity of glutaraldehyde and cationic biocides (Maillard, 2005, McDonnell & Russell, 1999; Russell, 2003). The presence of organic matter can adversely affect the efficacy of the disinfectant by either reacting chemically with the disinfectant or blocking the physical access of the disinfectant to the microbial target (Meyer *et al.,* 2010a; Russell, 2003). An increase in temperature can also augment the activity of biocides (Russell, 2003). Finally microorganisms themselves can affect the treatment outcome of biocides (Gilbert & McBain, 2003). The susceptibility of different types of microorganisms to biocides differs, as a result differences in impermeability of their cell wall and the inability of biocides to accumulate at their target site occur (Gilbert & McBain, 2003; Russell, 2003). Bacteria susceptibility to biocides may also be altered as a result of the conditions under which they were cultivated or exposed (phenotypic change as a result of nutrient depletion, physiological growth rate and growth as a biofilm growth; Gilbert & McBain, 2003).

Susceptibility testing is important to ensure appropriate and timely antibiotic treatment in cases of severe or recurrent *Campylobacter* infections (Moore *et al.,* 2005). Since 2004, the European food safety authority has been monitoring data on antimicrobial resistance in *Campylobacter spp*. from food producing animals and food from all member states (MS) of the EU under the framework of Directive 2003/99/EC (EFSA, 2012). Further technical specifications for standardization of susceptibility testing of *Campylobacter spp.* were implemented in 2007 and 2008 aimed at harmonizing the collection and reporting of antimicrobial resistance data in food-producing animals and food derived from animals in the European Union (EU). The most notable changes to the guidelines recommended the reporting of quantitative antimicrobial resistance data, harmonization of sampling schemes and antimicrobials to be tested, standardization of laboratory methodology used and the use of harmonised

epidemiological cut-off values (ECOFFs) as interpretative criteria for the identification of microbiological resistance in *Campylobacter* (EFSA, 2012).

1.8.1 Antimicrobial resistance mechanisms and definitions

Resistance is defined as the relative insusceptibility of a microorganism to a particular antimicrobial under a specific set of conditions. Antibiotic resistance can be defined as a change in susceptibility of a bacteria to an antibiotic that renders the antibiotic ineffective against an infection that had previously been treatable by that antibiotic (Gilbert & McBain, 2003). Resistance to biocides is harder to define. Russell (1991) stated that microorganisms are said to be resistant if they are 'not killed...by a disinfectant at concentrations used in practice, or if they are not killed by a concentration that kills the majority of cells in a culture or when a strain is not killed by an agent that kills similar strains at a specified concentration'. Antimicrobial resistant mechanisms of bacteria including zoonotic pathogens can fall into two main categories of intrinsic and acquired resistance mechanisms depending on their origin (Cerf *et al.,* 2010). Tolerance is a term used to describe bacteria with decreased susceptibility (raised MIC) to biocides compared to the susceptible population, but are not considered to be resistant because they can still be inactivated by the in-use concentration of biocide containing disinfectant and the MBC to biocide is unaltered as determined according to the European Committee of Normalisation or CEN (Carpentier & Cerf, 2011). Phenotype resistance or adaptative resistance is a non-inherited form of resistance where strains become transiently resistant to antimicrobial agents and is not associated with genetic alteration (Corona & Martinez, 2013). Specific growth states such as biofilm growth and anaerobiosis can transiently reduce susceptibility to biocides (Poole, 2005).

Intrinsic resistance to antimicrobials is defined as natural (innate) chromosomally encoded resistance of the bacteria to a given antimicrobial and an ability to circumvent the action of that antimicrobial. Intrinsic resistance mechanisms pre-date antibiotic use and cannot be attributed to horizontal gene transfer (Cox & Wright, 2013; Andersson & Hughes, 2012). Bacteria may also acquire resistance through genetic mutation of the genes targeted by the antibiotic or as a result of horizontal gene transfer (Alekshun & Levy, 2007). Chromosomally encoded antimicrobial

resistance determinants are inherited by daughter cells and disseminated by clonal spread (Davison *et al.,* 2000). Resistance determinants present on plasmids or other mobile genetic elements are transmitted by horizontal gene transfer between bacteria of the same or other species or genera (Davison *et al.,* 2000). The presence of these mutations or genetic elements result in a selective advantage in antibiotic environments or when bacteria are exposed to biocides (Andersson & Hughes, 2012). Mechanisms of resistance in bacteria are varied and include target protection, target substitution, antibiotic detoxification and blocking of intracellular accumulation by efflux (Bennett & Hughes, 2009). A combination of both intrinsic and acquired resistance mechanisms plays a role in the prevalence of multidrug resistant Gram-negative bacteria (Andersson & Hughes, 2012).

Antimicrobial resistance can also be classified according to type (Cerf *et al.,* 2010). Antimicrobial resistance mechanisms can confer resistance to a single antimicrobial agent (Poole, 2002). When a given resistance mechanism results in resistance to three or more structurally unrelated antimicrobials the resistance mechanism is referred to as multidrug resistant (Pumbwe *et al.,* 2005). Cross-resistance is a term used to describe resistance to agents of the same antibiotic class or to a related class of antibiotics or biocides (Cerf *et al.,* 2010). Cross-resistance can also occur when different antimicrobial agents attack the same target, initiate a common pathway to cell death, or share a common route of access to their respective targets (Chapman, 2003b). Upregulation of mediators of stress response genes has been shown to lead to cross-resistance between biocides and antibiotics (Chapman, 2003b). Co-resistance results from the co-location of biocide resistance genes on transmissible plasmids and within conserved regions of integrons, each of which has been shown to carry multiple antibiotic resistance genes (Chapman, 2003b). Resistance mechanisms that contribute to intrinsic and acquired resistance are discussed further below.

1.8.2 Permeability of the cell envelope

Bacteria may be inherently resistant to the action of antimicrobials as a result of the reduced permeability of their cell wall that affects the ability of antimicrobials to reach their target site and exert their antimicrobial effect (Delcour, 2009). Russell (1999) attributed the higher intrinsic resistance of bacterial spores, mycobacteria and

Gram-negative bacteria to biocides to the impermeability of their cell walls. Differences in the permeability of Gram-positive and Gram-negative cell wall to antimicrobials with specific reference to *Campylobacter* are discussed below.

The cell wall of Gram-positive bacteria is shown in Figure 1.1 and consists of a cytoplasmic membrane surrounded by a thick peptidoglycan layer (20-50 nm). Some types of Gram-positive bacteria also have a capsular polysaccharide, which is loosely associated with the cell wall and functions to protect the bacteria against phagocytosis during infection or engulfment by predatory cells in the environment (Lambert, 2002; Howden *et al.,* 2010).

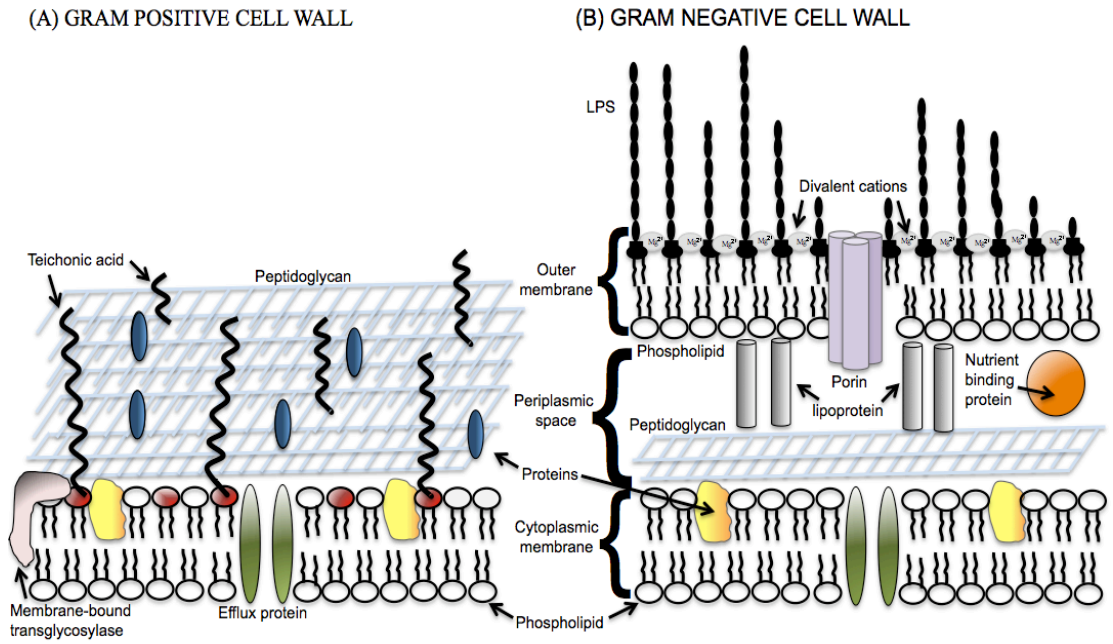

Figure 1.1 A schematic diagram of the structure of cell wall of a Gram-positive and a Gram-negative bacterium. Adapted from Lambert (2002); Denyer & Maillard (2002). Intrinsic resistance of Gram-negative bacteria to antimicrobial agents compared to Gram-positive bacteria is as a result of differences in the permeability of their cell wall.

Peptidoglycan consists of a glycan chain backbone of alternating amino sugars N-acetylglucosamine and N-acetylmuramic acid. Stem pentapeptides, with the amino acid sequence L-Alanine-D-iso-Glutamine-L-Lysine-D-Alanine-D-Alanine, are attached to the carboxy group on N-acetylmuramic acid sugars. Adjacent glycan chains are linked by interpeptide bridges formed between the terminal lysine residue on a stem pentapeptide and the penultimate alanine residue on the adjacent stem

pentapeptide. Together with glycopolymers, such as teichonic acid, peptidogylcan forms a multilayered network surrounding the cell (Howden *et al.,* 2010).

Proteins are covalently or non-covantly linked to the peptidoglycan and this mediates interactions between the cell and its environment (Lambert, 2002). Peptidoglycan is very permeable to antimicrobials and does not exclude antimicrobial agents smaller then 50 KDa (Hogan & Kolter, 2009; Lambert, 2002). Gram-positive bacteria are usually sensitive to the actions of most antibiotics and biocides unless other resistance mechanisms are present (Lambert, 2002, Ortega Morente *et al.,* 2013). The thickness and degree of cross-linking of peptidoglycan can be affected by the bacterial growth rate and any growth-limiting nutrient will affect the physiological state of the cells, that in turn can affect the sensitivity of the cell to antibiotics and biocides (Ortega Morente *et al.,* 2013). In *Staphylococcus aureus*, intermediate resistance to the glycopeptide antibiotic vancomycin occurs as a result of thickening of the cell wall at the division septum and increased production of the non-amidated glutamine component of peptidoglycan (Hogan & Kolter, 2009; Lambert, 2002). The modified peptidoglycan layer traps vancomycin, thus decreasing the amount of vancomycin access to its drug target, namely the membrane bound-transglycosylases that are located on the outer leaflet of the cytoplasmic membrane (Lambert, 2002).

In contrast to Gram-positive bacteria, the lower permeability of the Gram-negative cell wall excludes otherwise effective antibiotics. Gram-negative bacteria are surrounded by a double membrane: the cytoplasmic or inner membrane which is a phospholipid bilayer and the asymmetric outer membrane (OM) bilayer with an outer leaflet of lipopolysaccharides (LPS) and the inner phospholipid leaflet (Figure 1.1; Drlica *et al.,* 2012). The OM of the Gram-negative cell wall contains a number of proteins including general diffusion porin channels, specific porin channels, receptors for the uptake of substrates, proteins involved in OM and surface appendage biogenesis, translocases to allow the secretion of substrates, enzymes and proteins involved in LPS synthesis (Delcour, 2009). The OM also serves as the anchor for surface organelles such as pili that have a crucial role in pathogenesis (Remaut & Waksman, 2004). Between the cytoplasmic and outer membrane is the periplasmic space that contains a thin peptidoglycan layer bound to the outer membrane of the cell wall by covalent bonds to side chains of murin in lipoproteins (Denyer & Maillard,

2002). Changes in the permeability of the cell wall as a result of changes in surface hydrophobicity, outer membrane ultrastructure, outer membrane protein composition and changes in outer membrane fatty acid composition have been postulated to be responsible for acquired biocide tolerance in Gram- negative bacteria (Ortega Morente *et al.,* 2013).

Antimicrobials can cross the Gram-negative cell wall either by diffusing through general porin channels or across the lipid bilayer or by destabilization of the outer membrane structure (Denyer & Maillard, 2002; Delcour, 2009). Features of the Gram-negative cell wall that make it less permeable to the actions of antimicrobials include the low membrane fluidity of the outer membrane and presence of porins (Cox & Wright, 2013). The strong interactions between neighboring LPS molecules are as a result of the higher number fatty acids chains present in LPS and the neutralization and bridging effect of divalent cations on the phosphate groups of LPS (Nikaido, 2003). The tight packing of adjacent LPS molecules decreases the membrane fluidity of the outer membrane thus making it much a more hydrophobic barrier then a normal phospholipid bilayer (Declour, 2009; Nikaido, 2003).

The uptake of hydrophilic antimicrobials across the cell wall through general porin channels is an important route of entry for the β-lactams, tetracycline, chloramphenicol and fluoroquinolone antibiotics, whereas large molecules, such as macrolides, gain access to cells by diffusing slowly across the lipid bilayer (Delcour, 2009; Nikaido, 2003). Repression of the biosynthesis of major porins may also result in non-specific, low-level resistance to antibiotics in bacteria (Ortega Morente *et al.,* 2013). Several cationic antibiotics and biocides, positively charged host defense peptides and proteins, such as the family of mammalian defensins, selectively disrupt the ionic bridges formed between divalent cations and the negative phosphate group of the LPS molecule, resulting in loss of integrity of the outer membrane (Alexander & Rietschel, 2001). Loss of LPS results in increased permeation to antibiotics, sensitizes cells to detergents and may render the cell susceptible to the host immune response via increase complement binding (Alakomi *et al.,* 2000; O'Hara *et al.,* 2001). Alteration of LPS structure has been shown to lead to resistance to the polymixin antibiotics and QAC biocides in *P. aeruginosa* (Ortega Morente *et al.,* 2013). The mechanism of action of the cationic biocide BKC on disruption of the OM is discussed further in

section 1.10.2.

In *Campylobacter*, the presence of constitutively active efflux pumps and selective porins affects the permeabilitity of the cell wall to antimicrobials (Lin *et al.,* 2006; Labesse *et al.,* 2001; Page *et al.,* 1989). Efflux pumps in *Campylobacter* are discussed further in section 1.8.1.2 below. Cross-sections taken from the *Campylobacter* cell envelope show its physical organisation is typical of the architecture of Gram-negative bacteria with an inner membrane, a periplasmic space, and outer membrane (Smibert, 1978). The *Campylobacter* cell has been shown to have an overall relatively hydrophilic and negatively charged surface in vitro. The inner (cytoplasmic) membrane is slightly uneven but smoothly contoured and gives the cell its basic shape and is surrounded by a thick peptidoglycan layer (Graham *et al.,* 1991; Pead, 1979). The outer membrane is double layered and is loosely fitted over the cell wall with a wavy structure (Smibert, 1978). The membrane surface is organized in a regular hexagonal lattice, mainly constituted of a protein named MOMP (major outer membrane protein). MOMP is a trimeric integral membrane protein (Bolla *et al.,* 1995; Zhuang *et al.,* 1997) that not only functions as a porin permitting the diffusion of a wide variety of compounds accross the cell wall of *Campylobacter spp*. (Dé *et al.,* 2000), but is also involved in the structural organization and stabilization of the outer membrane (Amako *et al.,* 1997) and may mediate adhesion to cultured cells (Schröder & Moser, 1997). MOMP has been shown to be responsible for intrinsic resistance of *Campylobacter* to antimicrobials (Labesse *et al.,* 2001; Page *et al.,* 1989). Two porins have been characterised in *Campylobacter spp.*, the major outer membrane protein (MOMP) that is ubiquitous to all strains and a minor one (Omp50) found only in *C. jejuni* and *C. lari* (Dé *et al.,* 2000; Dedieu *et al.,* 2004). Very limited functional information is available about these porins, and thus far, no evidence has emerged that they play any role in MDR of *Campylobacter* (Pumbwe *et al.,* 2004; Payot *et al.,* 2006).

1.8.3 Efflux

Efflux pumps have been shown to underlie intrinsic and acquired antimicrobial resistance mechanisms in bacteria (Poole, 2005). Genes encoding efflux systems occur widely in the genomes of all microorganisms or extra-chromosomally on plasmids

(Piddock, 2006). Efflux systems also have physiological roles in the bacterial cell and include protecting the cell from the inhibitory activity of toxic substances such as bile salts and the exporting of molecules involved in quorum sensing which may help the bacterium to survive in their ecological niche (Alekshun & Levy, 2007; Gilbert & McBain, 2003; Piddock, 2006). Efflux mediated antibiotic resistance was first discovered in 1970s for the antibiotic tetracycline. Since then efflux proteins have been shown to transport a wide range of antibacterial agents across the bacterial cell envelope (Alekshun & Levy, 2007; Cox & Wright, 2013). Some efflux pumps have narrow substrate profiles and only export a few agents or members of the same drug class for example, the Tet efflux transporters (Alekshun & Levy, 2007). Others, namely the multidrug resistance transporters which include MexAB- OprM of *P. aeroginosa* or CmeABC of *C. jejuni* have a broad subrate profile and accommodate structurally distinct classes of molecules (Alekshun & Levy, 2007; Piddock, 2006).

The first multidrug efflux protein identified in bacteria was the MFS type efflux transporter QacA which was found on a plasmid in clinical isolates of *S. aureus* that were resistant to multiple cationic biocides. However the majority of efflux pumps carried on plasmids have a narrow substrate profile (Alekshun & Levy, 2007; Butaye *et al.,* 2003; Gilbert & McBain, 2003; Nikaido, 2009). Multidrug efflux transporters work synergistically with other mechanisms to maintain high levels of antimicrobial resistance in bacteria (Lomovskaya *et al.,* 2006; Cox & Wright, 2013).

Bacterial antimicrobial efflux transporters can be grouped into five families based on sequence homology, mechanism and supramolecular characteristics and are as follows, (i) ATP-binding cassette (ABC) transporters (traffic ATPases) (ii) Small multidrug resistance (SMR) (iii) Resistance–nodulation–division (RND) (iv) Major facilitator superfamily (MFS) and (v) Multidrug and toxic compound extrusion (MATE). Efflux of antibiotics across the bacterial cell envelope is an energy requiring process. Most efflux pumps utilise the transmembrane electrochemical gradient of protons or sodium ions as an energy source with the exception of ABC efflux tansporters, which use ATP to pump drugs out of the cell (Jeon *et al.,* 2011; Cox & Wright, 2013). Energy-dependent efflux transporters function to transport drug molecules across the cell envelope and to limit the intracellular accumulation of toxic compounds including antimicrobials (Pagès *et al.,* 2005). RND type efflux

transporters expressed by Gram-negative bacteria and expression of MFS type efflux pumps in Gram-positive bacteria are associated with clinically significant antibiotic resistance (Li & Nikaido, 2009; Piddock, 2006).

Multidrug efflux transporters are responsible for intrinsic and acquired resistance to multiple, structurally diverse antimicrobials in *Campylobacter* (Jeon *et al.,* 2011; Lin *et al.,* 2005; Payot *et al.,* 2006). In addition efflux is essential in overcoming the antimicrobial action of bile salts during intestinal colonisation in *Campylobacter spp*. (Lin & Martinez, 2006). Fourteen transporters (eleven putative efflux and three efflux transporters) are present in the sequenced genome of *Campylobacter jejuni* NCTC 11168 (Jeon *et al.,* 2011; Lin *et al.*, 2002; Parkhill *et al.,* 2000; Pumbwe *et al.,* 2005). Three have been functionally characterised, CmeABC and CmeDEF, cme for *C*ampylobacter multidrug efflux, belonging to the resistance-nodulation-cell division (RND) family of transporters and the CmeGH, a major-facilitator superfamily (MFS) type efflux transporter (Jeon *et al.,* 2011; Lin *et al.*, 2002; Pumbwe *et al.,* 2005). Together, they are capable of transporting a wide variety of substrates including antibiotics, detergents, biocides and dyes and have overlapping substrate profiles. Upregulation of energy-dependent multidrug resistance efflux pumps in *Campylobacter* promotes the emergence of mutants with specific target gene mutations. This allows *Campylobacter* to survive following exposure to high antimicrobial concentrations, which is an important cross-resistance mechanism (Jeon *et al.,* 2011; Lin *et al.,* 2005; Payot *et al.,* 2006; Yan *et al.,* 2006).

RND transporters are unique to Gram-negative bacteria and are a major and important family of drug transporters in *Campylobacter* (Poole, 2002). Structurally, RND efflux transporters consist of a transporter located in the cytoplasmic membrane, an outer membrane channel and a periplasmic 'linker' protein. This allows the extrusion of substrates from the cytoplasmic and periplasmic compartments directly into the external medium. CmeABC was the first functionally characterised RND type multidrug efflux transporter in *C. jejuni* and has also been shown to be present in the genome of *C. coli* (Corcoran *et al.,* 2005). It has been shown to be constitutively active and confers resistance to fluoroquinolone and marolide antibiotics, both of which are used as first line agents in antibiotic treatment of *Campylobacter* infection (Akiba *et al.,* 2006; Lin *et al.,* 2005; Luangtongkum *et al.,* 2009; Piddock, 2006). The

efflux pump CmeABC is encoded by an operon consisting of three genes, *cmeA*, *cmeB* and *cmeC*, which code for a periplasmic fusion protein, an inner membrane drug transporter and an outer membrane protein, respectively (Lin *et al.*, 2002). Inactivation of the CmeABC efflux pump by efflux pump inhibitors or inactivation of *cmeB* expression leads to increased susceptibility to a variety of antibiotics, bile acids and other toxic substances (Lin *et al.,* 2002). The expression of CmeABC pump is negatively regulated by the CmeR repressor. Deletion of *cmeR* gene or mutations in the DNA binding site of the repressor results in the over-expression of CmeABC (Guo *et al.,* 2008; Lin *et al.,* 2005). Upregulation of expression of Cme efflux pump has been observed in response to environmental stresses (Lin *et al.,* 2005).

The second RND type efflux transporter in *Campylobacter*, CmeDEF, was described by Pumbwe et al., (2005). It consists of a CmeF, an inner membrane drug transporter, linked to an outer membrane protein, CmeD, by a putative membrane fusion protein, CmeE. Substrates of CmeDEF include ampicillin, ethidium bromide, acridine, sodium dodecyl sulfate (SDS), deoxycholate, triclosan, and cetrimide, but not ciprofloxacin or erythromycin. Compared with CmeABC, the level of antimicrobial resistance conferred by CmeDEF is relatively moderate and is masked by the function of CmeABC in wild-type *Campylobacter* strains. Along with CmeABC, CmeDEF is important for maintaining cell viability in *C. jejuni* (Akiba *et al.,* 2006).

CmeG is the third multidrug efflux transporter and the first major facilitator superfamily type efflux transporter to be functionally characterised in *Campylobacter spp.*. Insertional inactivation of *cme*G led to increased susceptibility to various classes of antimicrobials including ciprofloxacin, erythromycin, tetracycline, gentamicin, EtBr and cholic acid as well as hydrogen peroxide. Overexpression of *cmeG* in *C. jejuni* resulted in increased resistance to fluoroquinolone antibiotics and CmeG was shown to directly extrude ciprofloxain (Jeon *et al.,* 2011). In some strains of *Campylobacter*, CmeG was co-transcribed with CmeH, a putative periplasmic protein. However, CmeH inactivation did not affect the antimicrobial resistance phenotype of *Campylobacter*. The expression of CmeGH operon appears to be regulated by the Fur regulon, although further studies are needed to confirm its role (Jeon *et al.,* 2011).

Inhibition of efflux transporters may provide an important means for preventing emerging resistance and multidrug resistance in bacteria and prevent the transmission of *Campylobacter* via the food chain (Quinn *et al.,* 2007). Efflux pump inhibitors (EPIs) act to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Lin & Martinez, 2006). Efflux pump inhibitors also decrease the intrinsic resistance of bacteria to antibiotics, reverse acquired resistance even in highly resistant strains with multiple target mutations, and reduce the frequency of emergence of resistant mutant strains (Marquez, 2005). A number of different mechanisms for efflux pump inhibitors have been described and include the following: (i) EPIs that target the expression of genes that induces MDR efflux transporters, (ii) inhibition of the assembly of membrane transporter complex involved in drug efflux by EPI, (iii) EPIs which act as proton motive force inhibitors preventing active transport and (iv) the inhibition of the flux of molecules inside the efflux channel by competition or blocking (via steric hindrances; Pagès *et al.,* 2010).

Phenylalanine arginine b-naphthylamide (PAβN) is an broad spectrum EPI which was developed by Microcide and Daiichi Pharmaceuticals and is active against RND type multidrug efflux transporters (Lomovskaya *et al.,* 2006). It targets RND type transporters directly by reversibly binding an internal binding pocket inside the transporter thereby inhibiting its abiltity to catalyse the efflux of substrates. Its mechanim of action is described in Figure 1.2.

Initally PAβN was developed as an inhibitor of the RND type transporter, Mex-AB in *Pseudomonas aeruginosa*, however it was shown to inhibit efflux activity and restore antibiotic sensitivity in a number of other Gram-negative bacteria (Lomovskaya *et al.,* 2006; Marquez, 2005). PAβN inhibits the function of CmeABC efflux pump and potentiates the activity of antibiotics against *Campylobacter* (Martinez & Lin, 2006). It was also shown that PAβN suppressed the emergence antimicrobial resistance in Gram-negative bacteria including *Campylobacter* (Piddock, 2006; Yan *et al.,* 2006).

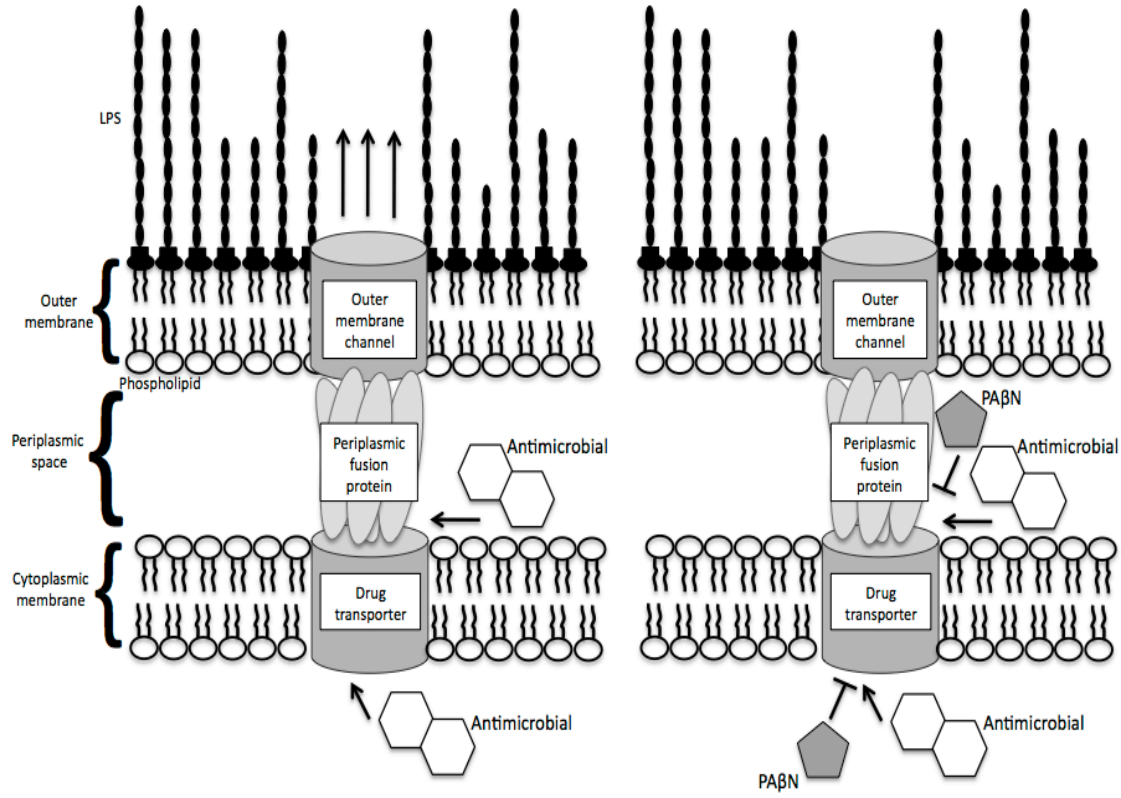

Figure 1.2 Mechanism of action of PAβN. RND efflux transporters capture their antimicrobial substrates either from the phospholipid bilayer of the inner membrane of the bacterial cell envelope or the cytoplasm and transports them into the external medium. PAβN inhibits this process by competing for binding the same binding site as the antimicrobial binding site thus blocking its transport out of the cell. Because there can be different binding sites for antimicrobial substrates of RND type transporters not all antimicrobial substrate efflux will be inhibited.

Inhibitors can also cause a depletion of energy by disturbing the proton gradient (Marquez, 2005). Proton motive force dependant efflux systems include RND, MFS, SNR and MATE types efflux transporters. Carbonyl cyanide m chlorophenyl-hydrazone (CCCP) dissipates the proton motive force, thus interrupting the main source of energy of PMF-dependant efflux. A model of the mechanism of action of proton ionophores has been described; see Figure 1.3 (Ozaki *et al.,* 2008).

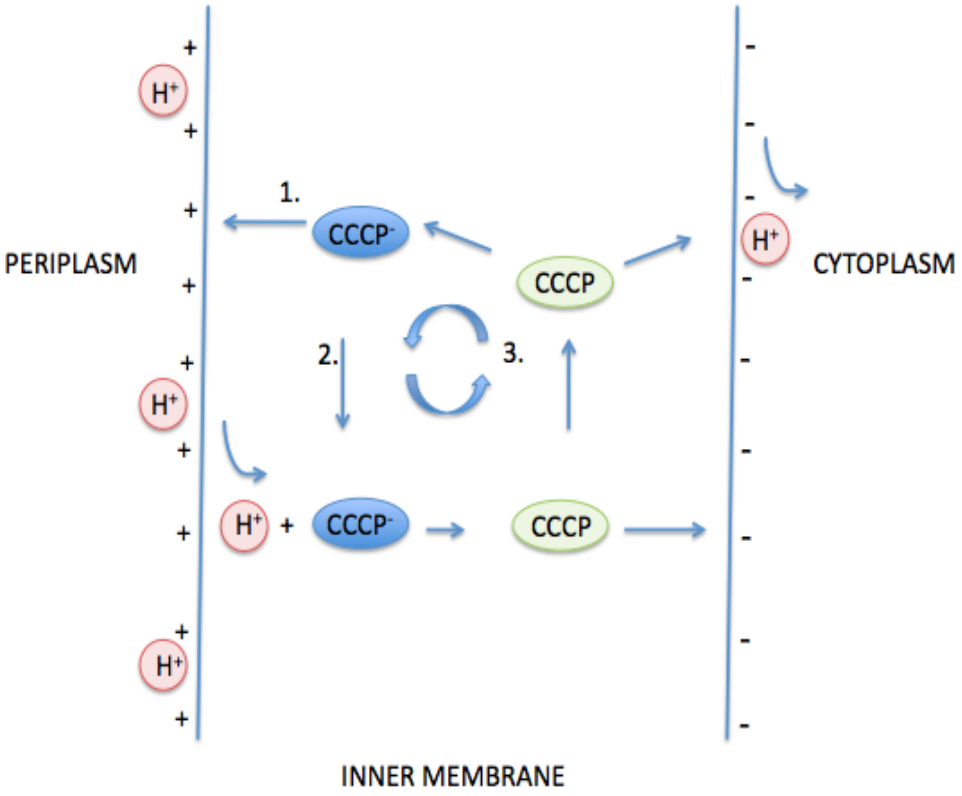

Figure 1.3 Mechanism of action of CCCP. Adapted from Ozaki et al., 2008. The CCCP anion (blue) is attracted to the positively charged periplasmic side of the inner membrane. Here it combines with a proton (red) to form the neutral form of CCCP (green). The neutral form of CCCP (green) then diffuses across the inner membrane along its concentration gradient where it ionises into the anionic form (blue) and a proton (red). The cycle repeats many times until the membrane potential across the inner membrane collapses. This collapse in membrane potential across the inner membrane inhibits transport of antimicrobial substrates of PMF-dependant efflux pumps.

Various EPIs have been identified but despite their *in vivo* activity none of them is in clinical use (Marquez, 2005). Efflux is important as a multidrug resistance mechanism in both intrinsic and acquired resistance in *Campylobacter*. Inhibitors of CmeABC may not only control antibiotic resistance but also increase the susceptibility of *Campylobacter* to bile salts *in vivo*, consequentially preventing the colonisation of the intestinal tract of the host (Piddock, 2006). Elucidating its role in adaptation to antibiotics and biocides as well as acting synergistically with other resistance mechanisms is important in developing strategies to prevent or reduce the entry of *Campylobacter* into the food chain and the prevention of *Campylobacter* infections.

1.8.4 Mutational resistance mechanisms

Mutation in the genes encoding the molecular target of an antibiotic is a common antibiotic resistance mechanism (Poole, 2002). Target site mutations reduce the affinity an antibiotic has for its molecular target and may even affect its functionality (Gilbert & McBain, 2003). Mutations in the regulators or regulatory elements of genes can similarly contribute to antibiotic or biocide resistance by leading to increased expression of either the target itself or intrinsic resistance determinates such as efflux (Hogan & Kolter, 2009). Mutations in genes have been shown to alter the antibiotic binding sites on DNA gyrase and Topoisomerase IV for the fluoroquinolone, leading to fluoroquinolone resistance in bacteria including *Campylobacter spp.* (Alekshun & Levy, 2007; Nikaido, 2009; Moore *et al.,* 2005; Luangtongkum *et al.,* 2009). Mutation in genes encoding the 23S rRNA gene (rrnB operon) and/or ribosomal proteins L4 or L22 in *Campylobacter* confers high-level resistance to the macrolide antibiotics (Luangtongkum, 2009; Wieczorek & Osek, 2013).

Vancomycin antibiotics mediate their antimicrobial action by binding to the D-ala-D-ala termini of the UDP-N-acetylmuramyl-pentapeptide precursor of peptidoglycan (Russell, 1997). Genes encoded on the VanA result in the production of an altered terminus on UDP-N-acetylmuramyl-pentapeptide precursor to confer both intrinsic and acquired resistance to the glycopeptide antibiotics. Sulfonamide and trimethoprim resistance is mediated by the sul1, sul2 and sul3 genes, that encode enzymes which are insensitive to the action of these antibiotics (Nikaido, 2009). Methicillin resistance in *S. aureus* is mediated by the production of low-affinity penicillin binding proteins PBP2a and confers resistance to virtually all β-lactam antibiotics (Russell, 1997). In *P. aeruginosa*, expression of the mexAB-oprM efflux pump is under the control of three repressors, MexR, NalD, and NalC. Mutations in genes encoding these repressors results in the loss of their ability to inhibit transcription and upregulates the expression of the mexAB-oprM efflux pump (Poole, 2012). Similarly in *C. jejuni*, mutations in the gene encoding CmeR or in the *cme*ABC promoter sequence which inhibits binding of CmeR to the promoter site on the DNA leads to enhanced production of the MDR efflux pump CmeABC in *C. jejuni* (Lin *et al.,* 2005). In contrast to antibiotics, biocides have multiple and non-specific target sites against

bacteria (Maillard, 2012). The overall damage inflicted by the biocide at these sites results in the bacteriocidal effect of biocides (Maillard, 2002).

Target site mutations are rare in biocide-resistant organisms and are unlikely to lead to high-level biocide resistance (Ortega Morente *et al.,* 2013; Russell, 2003). However, at low concentrations biocides may be bacteriostatic and may have more specific actions at a primary (unique?) site within the cell as exemplified by the biocide triclosan (Maillard, 2002; 2012). Decreased tolerance of the biocide triclosan was shown to occur as a result of a mutation in the fabI gene encoding the enoyl-acyl carrier protein reductase of fatty acid biosynthesis (Russell, 2003). Mutations in the fabI gene in *Campylobacter* resulting in triclosan resistance have not been reported in the literature.

1.8.5 Enzymatic mediated resistance

Antimicrobial resistance can occur as a result of enzyme inactivation of the antimicrobial agent or indirectly as a result of enzymatic mediated neutralizing of the toxic effects of antimicrobials (Alekshun & Levy, 2007; Cloete, 2003; Nikaido, 2009). It is a common resistance mechanism for antibiotics of natural origin including the marcolides, β-lactams and the aminoglycosides (Nikaido, 2009). Enzymatic modification 23S rRNA which methylates adenine at position 2058 by methyltransferase enzyme erm confers resistance to 14- and 16- member-ring macrolide, lincosamide, and streptogramin B antibiotics (Nikaido, 2009). To date, enzyme-mediated macrolide resistance has only been reported to occur in *Campylobacter rectus* (Luangtongkum, 2009).

Inactivation of the aminoglycosides is achieved by modifications such as acetylation, phosphorylation or adenylation by aminoglycoside modifying enzymes that act to reduce the net positive charge of these polycationic antibiotics (Alekshun & Levy, 2007; Nikaido, 2009). Multiple aminoglycoside modifying enzymes are present in *Campylobacter spp.* including type I, III, IV and VII 3'-aminoglycoside phosphotransferases, 3',9-aminoglycoside adenyltransferase and 6-aminoglycoside

adenyltransferase (Luangtongkum, 2009). β-lactam antibiotics include the penicillins, cephalosporins and carbapenems and are inactivated by enzymatic hydrolysis of the β-lactam ring by β-lactamases in *Campylobacter spp*. (Luangtongkum, 2009; Wieczorek & Osek, 2013).

Bacterial enzymatic inactivation of biocides is less common although enzymatic inactivation of the aldehyde biocides has been reported in the literature as a result of increased expression of aldehyde dehydrogenase (Chapman, 2003a; Cleote, 2003). Oxidizing agents such as the peroxygens and hypochlorous acid, mediate their biocidal action via free radical production. In *E. coli*, increased expression of catalases or induction of the oxyR or soxRS regulons results in increased survival following exposure to hypochlorous acid (Chapman, 2003a). In *Campylobacte*r, tpx/bcp double mutants encoding thiol peroxidases resulted in increased sensitivity to hydrogen peroxide and organic peroxides (Atack & Kelly, 2009).

1.8.6 Transmission of Resistance genes by horizontal gene transfer

Horizontal gene transfer of resistance genes by transformation, transduction or conjugation to *Campylobacter* has been shown to occur both *in vitro* in bacterial cultures, and *in vivo* in the gastrointestinal tract of poultry (Luangtongkum *et al.,* 2009). *Campylobacter* is a naturally transformable microorganism (Wang & Taylor, 1990; Wilson *et al.,* 2003) that is capable of acquiring a diverse array of Gram-positive (Werner *et al.,* 2001) and Gram-negative (Pinto-Alphandary *et al.,* 1990) antimicrobial resistant genes (Wilson *et al.,* 2003). Wilson et al., (2003) showed that ciprofloxacin resistance could to be acquired through the uptake of ciprofloxacin-resistance-encoding chromosomal DNA by natural transformation.

Plasmids are extrachomosomal DNA elements that contain their own origin of replication allowing them to replicate autonomously. Resistance (R)-plasmids mediate horizontal gene transfer and the dissemination of resistance genes to antimicrobials between bacteria of the same species or even distantly related organisms such as Gram-positive and Gram-negative bacteria (Nikaido, 2009). Plasmids also may confer

multidrug resistance to bacteria as a result of the co-location of antibiotic and biocide resistance determinants on the same plasmid (Chapman, 2003b). Bacteria can carry multiple plasmids simultaneously, each contributing to the total genetics of the organism (Alekshun & Levy, 2007).

The dissemination of heavy metal and antibiotic resistance genes on plasmids has been widely studied (Russell, 1997). Plasmid encodes resistance to cationic biocides in antibiotic resistant *S. aureus* and *S. epidermidis*. In *S. aureus*, the plasmid pSK1 family plasmids commonly confer resistance to antiseptics and disinfectants (via qacA), as well as to aminoglycosides (via Tn4001) and trimethoprim (via dfrA). Although plasmid-mediated resistance to biocides has also been found in Gram-negative bacteria, it has been proposed that intrinsic resistance in these organisms is of greater significance (Russell, 1997). Recently resistance to the cationic biocides was found to be encoded by the qacE and qacEΔ1 genes located on the 3' conserved sequence on Class 1 integrons located on plasmids found in both Gram-positive and Gram-negative bacteria (Russell, 1997). In Gram-negative bacteria, the contribution of qacE and qacEΔ1 genes to biocide resistance was neglible when compared to plasmidless strains (Butaye *et al.,* 2003). Gaze et al., (2005) showed that aerobic Gram-negative and Gram-positive bacteria isolated from effluent and soil samples from a QAC polluted environmental were shown to harbour class 1-integrons.

Many conjugative plasmids isolated from *Campylobacter* carry genes mediating resistance to the tetracyclines and aminoglycosides and plasmids (Luangtongkum *et al.,* 2009; Moore *et al.,* 2005). Plasmid encoded tetracycline resistance is associated with the tetO gene and is present on transmissible plasmids in *C. jejuni* and *C. coli* (Luangtongkum *et al.,* 2009; Wieczorek & Osek, 2013). Kanamycin resistance in *Campylobacter* is conferred by a 3'-aminoglycoside phosphotransferase genes aphA-1, aphA-2, aphA-3 and aphA- 7 (Moore *et al.,* 2005). The 3'-aminoglycoside phosphotransferase gene aphA-3 is often co-located with tetO on large resistance plasmids in *Campylobacter* (Moore *et al.,* 2005; Wieczorek & Osek, 2013).

1.8.7 Biofilms

A biofilm is a structured consortium of bacteria that are adhered to a surface and are embedded in a complex matrix of exopolymeric substance (EPS) composed mainly of polysaccharides, proteins and nucleic acids, and exhibit an altered phenotype with respect to growth rate and gene transcription (Bridier *et al.,* 2011a; Høiby *et al.,* 2010; Russell, 2003). Adherent populations (sessile bacteria) express phenotypes that show increased tolerance to antibiotics and disinfectants (Høiby *et al.,* 2010).

Resistance of bacteria to disinfectants is frequently associated with the presence of biofilms on inanimate surfaces and biocontamination removal is a challenge for the food industry (Bridier *et al.,* 2011a; Fux *et al.,* 2005). In biofilms, both structural and physiological mechanisms play a role in biofilm resistance to disinfectants as well as physiological (the appearance of the resistant persistor cell phenotype) and genetic heterogeneity among the biofilm population (Bridier *et al.,* 2011a). The age of the biofilm is also an important factor to consider. Older biofilms take longer to eradicate and the time taken to inactivate adherent cells may be longer than planktonic cells (Cerf *et al.,* 2010). Most naturally occurring biofilms are multispecies biofilms, which are more resistant to antimicrobials than monospecies biofilms (Burmølle *et al.,* 2006).

Structural organization of the biofilm can result in decreased diffusion of antimicrobials into the deeper layers (Figure 1.4). The EPS may bind and sequester antimicrobials and quench some disinfectants and thus reduce their efficacy against biofilms (Bridier *et al.,* 2011a; Cerf *et al.,* 2010; Stewart, 2002). Inactivation enzymes present in the biofilm matrix as a result of bacterial secretion or bacterial cell lysis may also impede the progress of antimicrobials into the deeper layers (Bridier *et al.,* 2011a; Fux *et al.,* 2005). Reactive or oxidative biocides, including bleach or superoxides, can be neutralized or bound by the EPS and are effectively 'diluted' to sublethal concentrations before they can penetrate the deeper layers within the biofilm (Hall-Stoodley *et al.,* 2004). Sequestering by the EPS matrix also impedes penetration of aminogylocides and β-lactam antibiotics into biofilms and has been documented to be the result of enzymatic inactivation (Stewart, 2002).

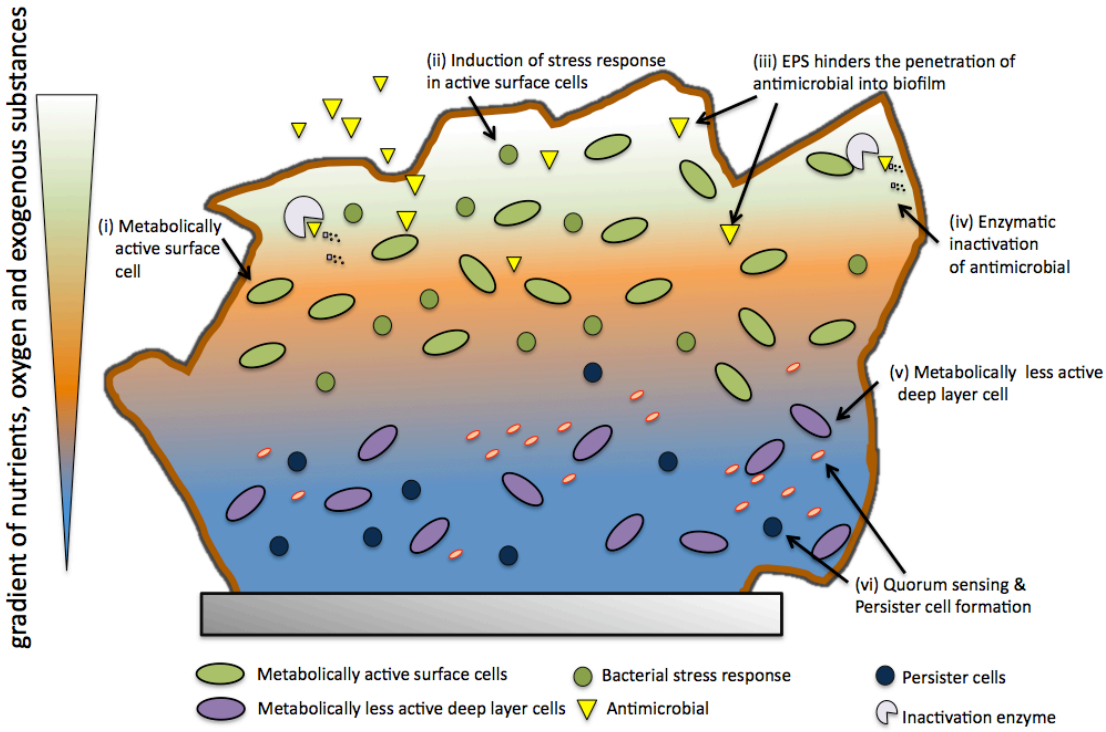

Figure 1.4 Antimicrobial resistance mechanisms in biofilms. (i) Metabolically active surface cells which are susceptible to antimicrobial action (ii) Induction of the stress response in metabolically active surface cells altering their suscepbility to antimicrobials (iii) EPS sequesters and binds antimicrobial and hinders its penetration into biofilm (iv) Inactivation of antimicrobial as a result of the presence of inactivating enzymes in biofilm matrix (v) Metabolically less active deep layer cells as a result of oxygen and nutrient gradients present in the biofilm (vi) Quorum sensing and presistor cell formation

Conventional resistance mechanisms such as increased up regulation of efflux proteins has also been found in sessile bacterial populations present in the biofilm. These mechansims may additionally contribute to the survival of biofilms (Høiby *et al.,* 2010). The inactivity of the antibiotic tobramycin against *P. aeroginosa* biofilms was shown to be as a result of sequestering glycans present in its periplasm (Fux *et al.,* 2005). However, not all antimicrobial agents are affected by decreased rate of penetration into biofilms and the decreased efficacy of antimicrobial agents against biofilms may also be as a result of physiological mechanisms (Bridier *et al.,* 2011a; Stewart, 2002).

Biofilms are characterised by oxygen and nutrients stresses and sessile growth. Phenotypic heterogeneity can exist within the biofilm population as a result of oxygen and nutrient gradients present in the biofilm and the adaptation of constituent bacteria

to the different microenvironments (Stewart, 2002). Many antibiotics target macromolecule synthesis such as DNA synthesis and most antibiotics require cells to be actively dividing to mediate their inhibitory effect (Cerf *et al.,* 2010). Decreased nutrient and oxygen availability in the interior biofilm is accompanied by reduced growth rate of cells or cells that are metabolically inactive, which in turn decreases their susceptibility to antimicrobial agents (Corona & Martinez, 2013; Parsek & Fuqua, 2004; Stewart, 2002). Many bacteria are able to sense environmental stresses such as starvation and oxidative stress and adapt to their environment by activated in the stress response may be important to increase resistance to antimicrobial agents. In *P. aeruginosa* catalase is upregulated in biofilm cells. Quorum sensing has been shown to induce a biofilm specific phenotype and formation of biofilms is regulated by quorum sensing (Parsek & Fuqua, 2004; Stewart & Costerton, 2001; Figure 1.4).

A small proportion of the biofilm population may differientiate into a highly protected phenotypic state called persistor cells or persistors (Stewart & Costerton, 2001). Presistor cells have been shown to occur in planktonic cultures and are thought to constitute a higher percentage of the population in biofilms (Bridier *et al.,* 2011a). Persistors are a phenotypic variant of the original antimicrobial susceptible bacteria that display high tolerance to high doses of antibiotic and comprise 0.001 % to 0.1 % of cells in an isogenic bacterial populations (Corona & Martinez, 2013; Kint *et al.,* 2012). They are generally non-growing and upon removal of antimicrobial selection pressure, presistor cells resume growth and give rise to a bacterial population that have an antimicrobial susceptible phenotype (Kint *et al.,* 2012). Bacteria can switch to a persistor phenotype under certain conditions such as entry into stationary phase, in response to specific environmental cues such as quorum sensing, the presence of indole produced in stationary phase as a consequence of nutrient limitation, or as a result of activation of the SOS response (Kint *et al.,* 2012; Levin & Rozen, 2006; Figure 1.4). Persistor cells can also be formed spontaneously as a result of stochastic processes. Unimodal variations in gene expression together with random fluctuations in rates of synthesis and degradation of key persistor proteins cause persistor cells to be formed spontaneously within isogenic bacterial populations (Kint *et al.,* 2012). Increased frequency of persistor subpopulation in hipA mutants of *E. coli* occur as a result of over expression of the hipA toxin that results in inhibition of translation of antibiotic targets (Kint *et al.,* 2012; Levin & Rozen, 2006). A general feature of

persistor cells is the presence of active defence systems against oxidative stress imposed by antibiotic stress through specifically induced mechanisms. Persistor subpopulations have been shown to be heterogeneous in nature with specific antibiotic tolerance profiles within a single persister subpopulation (Kint *et al.,* 2012).

Sessile growth of bacteria in biofilms can also lead to emergence of extensive genetic diversity among bacterial populations. Genetic variation as well as natural selection, generate genetic heterogeneity in biofilms and may contribute to antimicrobial resistance in biofilms. Endogenous oxidative stress has been shown to lead to genetic diversity in bacteria. Recombinant repair of oxidative stress induced DNA damage may cause the emergence of antibiotic and biocide resistant variants with enhanced genetic mutations (Bridier *et al.,* 2011a).

Biofilm physiology also enables embedded bacteria to survive antimicrobial exposure long enough to acquire specific resistance to antimicrobials (Bridier *et al.,* 2011a; Fux *et al.,* 2005). Cells may be exposed to sublethal concentrations of antimicrobial agent depending on the location in the biofilm (Fux *et al.,* 2005; Stewart & Costerton, 2001). The high cell density and presence of large quantities of DNA in EPS of biofilms may constitute an optimum environment for the efficient exchange of genetic material by horizontal gene transfer leading to antimicrobial resistance (Fux *et al.,* 2005).

1.8.8 Stress response and multi-antimicrobial resistance

Antimicrobials are growth-inhibiting stressors that often trigger physiological changes including biofilm formation and subsequently confer protective responses in bacteria (Poole, 2012). Some bactericidal antimicrobials mediate their lethal effects, in part, as a result of metabolic perturbations that generate reactive oxidants including reactive oxygen species (ROS), which contribute to cell death (Dwyer *et al.,* 2014, 2015). Evidence of oxidative stress following antimicrobial exposure stems from studies carried out by Kohanski *et al.,* (2007). Kohanski et al., (2007) showed the generation of hydroxy radicals in *E. coli* and *S. aureus* following treatment with β-lactams, fluoroquinolones, and aminoglycosides antibiotics using the fluorescent reporter dye 3′-(p-hydroxyphenyl) fluorescein (HPF), which has high *in vitro* specificity for

hydroxyl radicals. Kohanski et al., (2007) also showed that bactiocidal effects of these antibiotics was reduced using the iron chelator 2,2′-dipyridyl or the ROS quencher thiourea (Dwyer *et al.,* 2015).

Multidrug efflux systems of the Resistance-Nodulation-Division (RND) family, which are major determinants of intrinsic and acquired antimicrobial resistance in Gram-negative bacteria, are increasingly recognized as components of bacterial stress responses, including oxidative and nitrosative stress responses (Poole, 2012). The activation of either the soxRS or the marRAB system results in enhanced resistance to both superoxide-generating agents and multiple antibiotics (Pomposiello *et al.,* 2001). Furthermore, the expression of a number of multidrug efflux systems are positively impacted by agents of oxidative stress (Dwyer *et al.,* 2009; Poole, 2012). Stress-dependant recruitment of resistance mechanisms has also been shown occur following exposure of *Campylobacter* to peroxides and the RND family cmeABC multidrug efflux operon of *C. jejuni* was shown to be induced in response to paraquat (Atack & Kelly, 2009).

Campylobacter possess unique regulatory mechanisms for response to oxidative stress (Kim *et al.,* 2015). The peroxide resistance regulator regulates the transcription of genes that confer protection against peroxide and oxidative stress (Kim *et al.,* 2015; Palyada *et al.,* 2009). A second regulator, the *Campylobacter* oxidative stress regulator (CosR) also controls the transcription of genes involved in oxidative stress (Hwang *et al.,* 2011; Kim *et al.,* 2015). The expression of CosR, was upregulated in response to paraquat ($O2^-$) exposure in *Campylobacter* (Atack & Kelly, 2009). Exposure of bacteria to a stressful environment induces an adaptive tolerance response in many bacteria and provides protection towards subsequent exposures to a lethal stress. *Campylobacter* has previously also been shown to possess an adaptive tolerance response to acid and aerobic stresses (Murphy *et al.,* 2003, 2006). The effect of acid stress on antibiotic resistance was investigated by Kumar-Phillips et al., (2013) who found that antibiotic-resistant *C. jejuni* isolates were found to be more sensitive to antibiotics after stress-adaptation but in a few cases *C. jejuni* isolates showed increased resistance. Bacteria increase their rate of mutation in stressful conditions, and, subsequently, the probability of mutational changes arising that have an impact on antimicrobial susceptibility increases (Hänninen & Hannula, 2007). Acid stress

induced mutagenesis resulting in mutations in antibiotic resistance genes may be one reason for the changes in antibiotic susceptibilities observed (Kumar-Phillips *et al.*, 2013)

1.9 Ameloriating the cost of resistance

The dissemination of antimicrobial resistance in a bacterial population depends on the stability of the resistance mechanism in the absence of the antimicrobial and is a key contributor to the widespread emergence of problems in the treatment of infectious diseases (Almofti *et al.,* 2011; ESFA, 2009). The stability of the resistance mechanism is affected by a number of factors including the acquisition of additional compensatory mutations which can affect the stability of resistance traits, while retaining the original resistance in bacterial populations, the genetic linkage between different resistance genes on extra-chromosomal DNA or when development of resistance has no-cost associated with resistance development (Levin *et al.,* 2000).

Resistance mutations usually occur in genes that have essential functions in the cell such as DNA replication and protein synthesis and as a consequence growth rate is affected. Numerous studies have shown that acquisition of antibiotic resistance mechanisms impairs bacterial fitness, causing a reduction in growth rate and competitive performance between antibiotic-susceptible and resistant-isolates in the absence of the antimicrobial agent (Andersson & Levin, 1999; Andersson & Hughes, 2010; Bjorkman & Andersson, 2000). Development of additional compensatory mutation can ameliorate fitness costs of acquisition of a particular resistance mechanism. Compensatory mutations in resistant bacteria occur more frequently than mutations (by a single specific mutational change) that cause reversion to drug sensitivity. Moreover, once a compensated mutant has evolved in a population, it is highly unlikely that the mutant will reverse to a susceptible state (Andersson & Hughes, 2010). Schrag and colleagues (1997) showed this in evolved fitness-compensated rpsL strains of *E.coli* with streptomycin-resistance. They replaced the rpsL allele conferring streptomycin resistance with a wild type $rpsL^{+}$ allele. The resultant streptomycin-susceptible bacteria were less fit than wild type, uncompensated rpsL mutants or fitness-compensated mutants. They showed that the genetic background created by compensatory mutations could negatively impact on the fitness of sensitive alleles making reversion of resistance by back mutation

unfavorable. Thus because reversion of resistance is unfavorable, this makes it more stable in the population (Schrag et al., 1997).

In both experimental and real-world settings, many microbial populations alternate between a phase of population growth followed by a population bottleneck created by serial passage or transmission to a new host (Handel & Bennett, 2008). During subculture of antimicrobial resistant strains, it is more likely that the most commonly occurring genotypes, contain compensatory mutations, rather than fitter genotypes are transferred. This also contributes to the low levels of reversion of antimicrobial resistance in bacterial populations (Andersson & Levin, 1999; Andersson & Hughes, 2010; Levin *et al.,* 2000).

1.10 Ciprofloxacin and benzalkonium chloride: Mechanism of action, resistance and role in the prevention of transmission of *Campylobacter jejuni*

Campylobacter may encounter many situations throughout the food chain that may provide a selective environment for adaptive resistance to both antibiotics and disinfectants. Fluoroquinolone antibiotics are an important class of wide-spectrum antibiotics that are frequently used for empiric treatment of undefined gastroenteritis cases including campylobacterosis regardless of the aetiological agent when a microbiological diagnosis is absent (Engberg *et al.,* 2001; Gibreel &; Taylor, 2006;Wassenaar, 2011). Table 1.2 lists fluoroquinolone antibiotics that have been approved for clinical use (Lode *et al.,* 2014; Van Bambeke *et al.,* 2005).

List of floroquinolone antibiotics
First generation • Nalidixic acid
Second generation • Norfloxacin • Pefloxacin • Ciprofloxacin • Ofloxacin
Third generation • Levofloxacin
Fourth generation • Moxifloxacin • Prulifloxacin • Gemifloxacin • Sitafloxacin • Besifloxacin

Table 1.2 List of quinolone antibiotics approved for clinical use. Adapted from Van Bambeke *et al.,* 2005, Lode *et al.*, 2014.

Fluoroquinolone use in food production animals is strictly regulated in the EU and antimicrobial veterinary medicinal products may be supplied on prescription only basis (ESVAC, 2013). In poultry, fluoroquinolones are effective against a broad range of important poultry pathogens, including *Mycoplasma*, *E. coli* and *Pasteurella spp.* (Landoni & Albarellos, 2015).

QACs are widely used in the food industry for disinfectant purposes and the control of transmission of foodborne pathogens including *Campylobacter* (Holah *et al.,* 2002 Langsrud *et al.,* 2003; Peyrat *et al.,* 2008*)*. The mechanism of action of the fluoroquinolone antibiotic ciprofloxacin and the biocide benzalkonium chloride and the role of resistance mechanisms to these antimicrobials are discussed below.

1.10.1 Fluoroquinolones

Fluoroquinolone antibiotics are topoisomerase inhibitors that inhibit DNA replication by inducing double-stranded DNA break formation and cause arrest of topoisomerase function (Aldred *et al.,* 2014; Hooper, 2001). The fluoroquinolone antibiotics were derived from nalidixic acid, which was discovered as a byproduct of chloroquine (quinine) synthesis. It was found to be active against Gram-negative bacteria and was introduced in the 1960s to treat urinary tract infections (Kohanski *et al.,* 2010). Newer generations were introducted with imporoved activity against Gram-negative bacteria (second generation) and Gram-positive bacteria (third generation). The introduction of a fluorine atom at the sixth position of the quinolone core (Figure 1.5) improved antibacterial activity of the fluoroquinolone antibiotics against Gram-negative and Gram-positive bacteria (Hooper & Wolfson, 1985). The structure of the fluoroquinolone antibiotic ciprofloxacin is given in Figure 1.5.

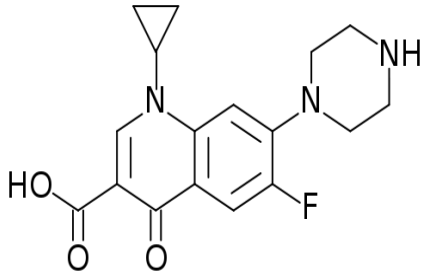

Figure 1.5 Structure of the fluoroquinolone antibiotic ciprofloxacin.

DNA gyrase (a type II topoisomerase) and topoisomerase IV (topoIV) are responsible for negative supercoiling of DNA during replication in bacteria. DNA gyrase plays an important role in DNA transcription and translation as well as cell division. DNA gyrase alter DNA topology by passing an intact double helix through a transient break (Aldred *et al.,* 2014). Fluoroquinolone antibiotics (Figure 1.6) inhibit DNA gyrase (a type II topoisomerase) and topoisomerase IV (topoIV) in bacteria. Protein synthesis-dependent or protein synthesis-independent pathways of quinolone mediated cell death are illustrated in Figure 1.6.

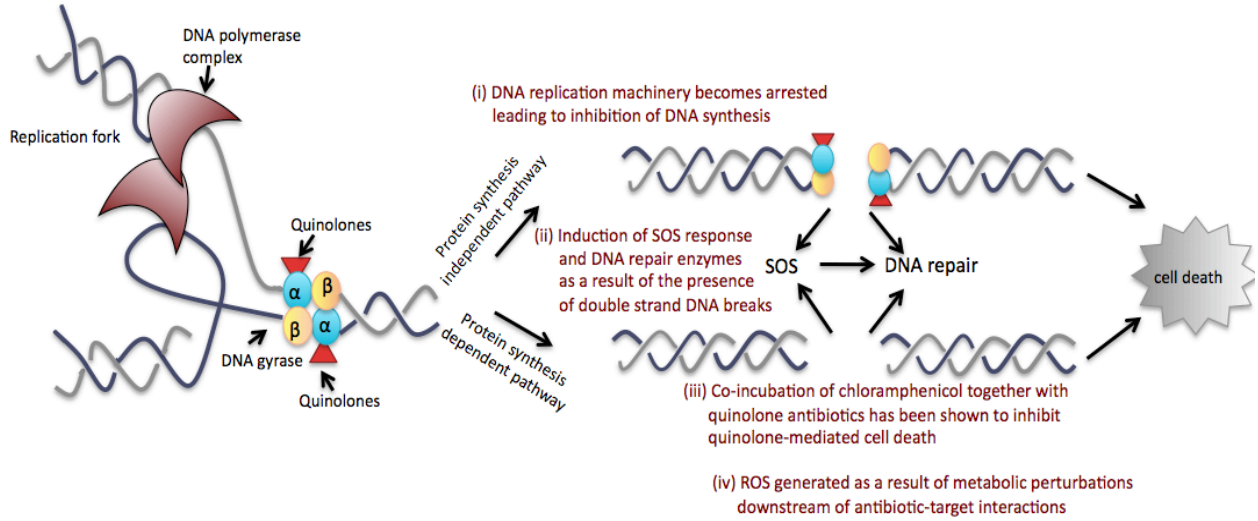

Figure 1.6 Mechanism of action of Fluoroquinolone antibiotics. The fluoroquinolone class of antibiotics are topoisomerase inhibitors that work by interfering with changes in DNA supercoiling by binding to bacterial type II and type IV topoisomerases. This induces double-stranded DNA breakage and leads to cell death in either a protein synthesis dependent or protein synthesis independent fashion (adapted from Kohanski *et al.,* 2010).

Ciprofloxacin and other fluoroquinolone antibioitics inhibit DNA replication by binding to DNA gyrase-DNA complex (after it has cleaved DNA) and inhibiting gyrase-mediated DNA re-ligation resulting in the formation of double stranded breaks in the bacterial chromosome (Aldred *et al.,* 2014b). As a result of quinolone-topoisomerase-DNA complex formation, DNA replication machinery becomes arrested at blocked replication forks, leading to inhibition of DNA synthesis, which immediately leads to bacteriostasis and eventually cell death via a protein independent pathway (Kohanski *et al.,* 2010). The introduction of double strand DNA breakes as a result of topoisomerase inhibition by quinolones has also been shown to induce the DNA stress response (SOS response) and the expression of DNA repair enzymes (Figure 1.6).

Evidence for a protein synthesis-dependent pathway for quinolone-mediated cell death in bacteria comes from studies using of the protein synthesis inhibitor chloramphenicol. Co-incubation of chloramphenicol together with quinolone antibiotics has been shown to inhibit quinolone-mediated cell death in bacteria (Kohanski *et al.,* 2010; Figure 1.6). During treatment with fluoroquinolone antibiotics, reactive oxygen species are generated as a result of metabolic perturbations downstream of antibiotic-target interactions (Kohanski *et al.,* 2007, 2010; Dwyer *et al.,* 2015; Figure 1.6). However, the exact molecular mechanism by which ROS is generated as a result of ciprofloxacin treatment in *Campylobacter* remains to be elucidated (Hwang *et al.,* 2013). Formation of ROS including the superoxide anion radical (0_2^-), hydrogen perioxide (H_2O_2) and highly reactive hydroxyl radicals (OH^-) can cause damage to proteins, nucleic acids and membranes (Atack & Kelly, 2009).

The approval and use of fluoroquinolones in poultry in Europe and the USA was followed by increases in fluoroquinolone resistance in *Campylobacter spp*. isolated from animals and human patients (Endtz, 1991; Luangtongkum *et al.,* 2009; Smith & Fratamico, 2010; Takkinen *et al.,* 2003). In *Campylobacter*, chromosomally mediated resistance involves mutations in the gyrA gene of DNA gyrase or in the regulatory genes governing bacterial permeability or efflux (Luangtongkum *et al.,* 2009; Payot *et al.,* 2006). There is growing evidence that *Campylobacter* spp. lack topoIV and that DNA gyrase is the sole target for fluoroquinolone antibiotics (Luo *et al.,* 2003; Parkhill *et al.,* 2000; Piddock *et al.,* 2003). The absence of a secondary target for fluoroquinolones in *Campylobacter* leads to a situation where a unique modification in the GyrA subunit (Thr-86-Ile, corresponding to position 83 in GyrA of *E. coli* or *Salmonella*) is sufficient to confer a resistant phenotype to fluoroquinolones in a number of *Campylobacter spp.* (Alfredson & Korolik, 2007; Payot *et al.,* 2006; Piddock *et al.,* 2003). Other mutations in the QRDR of gyrA gene have also been reported to be associated with quinolone resistance. These include mutations at positions Ala-70-Thr, Asp-90-Asn, Thr-86-Lys and Pro-104-Ser associated with moderate resistance (MIC 8-16 μg/ml) and the less frequent mutation at position Thr-86-Ala (associated with high-level resistance to nalidixic acid and low level resistance to ciprofloxacin), Thr-86-Val and Asp-90-Tyr . Double mutations of gyrA combining Thr-86-Ile and Asp-85-Tyr or Asp-90-Asn or Pro-104-Ser were also reported leading

to high levels of resistance to floroquinolones (Alfredson & Korolik, 2007; Luo *et al.,* 2003; Payot *et al.,* 2006).

Resistance-associated gyrA mutations occur spontaneously at a relatively high frequency in *Campylobacter* and FQ treatment rapidly selects for pre existing FQ-resistant mutants in bacterial populations containing greater then 10^6 to 10^9 colony-forming units (CFU) (Luangtongkum *et al.,* 2009). Emergence of mutations conferring fluoroquinolone resistance in *C. jejuni* has been associated with Mutant Frequency Decline (Mfd), a transcription-repair coupling factor involved in strand-specific DNA repair. Mfd has been shown to be upregulated in *C. jejuni* following treatment with ciprofloxain (Han *et al.,* 2008). Overexpression of the efflux pump CmeABC has been shown to increase the frequency of emergence of fluoroquinolone resistant *C. jejuni* by 17-fold (Yan *et al.,* 2006). CmeABC functions synergistically with the gyrA mutation to confer a high level of resistance to fluoroquinolones in *in vivo* selected isolates of *C. jejuni*, which can often underly differences in the susceptibility of fluoroquinolone resistant-strains with the same mutation in the topoisomerase gene (Luo *et al.,* 2003).

1.10.2 Benzalkonium chloride

Benzalkonium chloride (BKC) is widely used in the poultry industry as a disinfectant active ingredient (Peyrat *et al.,* 2008). The QACs are amphoteric surfactants generally containing one quaternary nitrogen associated with at least one major hydrophobic substituent and three methyl groups (Figure 1.7; Gilbert & Moore, 2005; Hugo, 1967). The disinfectant properties of the QACs were first described in 1916 however, commerial preperations were not available until 1935 (Jacobs, 1916; Resuggan 1952; Hugo, 1991). BKC is a member of the QACs which are cationic surfactants with low-level disinfectant activity (McDonnell & Russell, 1999).

The antimicrobial activity of QACs is a function of N-alkyl chain length, which alters the lipophilicity of QACs. QACs with chain lenghts of 12-14 alkyls are most active against Gram-positive bacteria and yeasts. Optimal activity against Gram-negative bacteria is achieved with QACs which have N-alkyl chain lenghts between fourteen and sixteen. QACs with N-alkyl chain lenghts greater then eighteen or less then four

are virtually inactive (Buffet-Bataillon *et al.,* 2012). The molecular structure of BKC is shown in Figure 1.7.

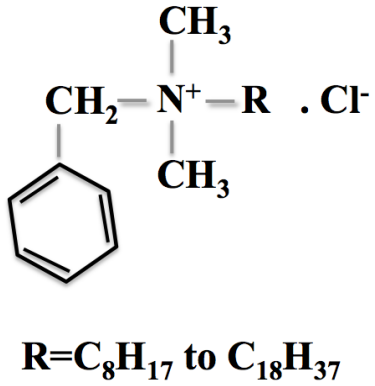

Figure 1.7 Molecular structure of BKC. The structure of benzalkonium chloride consists of a positively- charged ammonium ion where at least one hydrogen atom was substituted by a long chain alkyl radical of various chain lengths and the three remaining atoms substituted usually by methyl groups (Hugo, 1967)

The bacterial cytoplasmic membrane is considered the major target site of biocides including BKC (Maillard, 2002). The mechanism of action of BKC is similar to other cationic disinfectants through interaction with the bacterial cell envelope and subsequent leaking of the cellular contituents and is shown in Figure 1.8 (Gilbert & Moore, 2005). The outermost surface of bacterial cells universally carries a net negative charge, often stabilized by the presence of divalent cations such as Mg^{2+} and $Ca^{2+.}$ The antimicrobial effect involves interaction of the positively charged quaternary nitrogen of BKC with the negatively charged head groups of acidic phospholipids in bacterial membranes.

Cationic agents such as BKC intially interact with the wall and membrane by displacing these divalent cations (Gilbert & Moore, 2005). BKC is then adsorbed onto the cell membrane of the bacterium and the hydrophobic tail of the QAC integrates into membrane core (Russell & Path, 1986; Gilbert & Moore, 2005). BKC subsequently destablises the lipopolysaccharide layer of the outer membrane by forming mixed micellar aggregate (Buffet-Bataillon, 2012; Gilbert & Moore, 2005). This destabilises the cell wall and outer membrane of Gram-negative bacteria promoting their own uptake so that they can reach their target site(s), the cytoplasmic membrane cellular constituents within the cytoplasm (Maillard, 2002). At lower

concentrations, integration of BKC into the cytoplasmic cell membrane permeabilizes the cell membrane and results in leakage of cellular constituents, preturbed growth, and loss of membrane fluidity. At higher concentrations BKC degrades proteins and nucleic acids, and eventually lysis of the bacterial cell occurs leading to cell death (Gilbert & Moore, 2005).

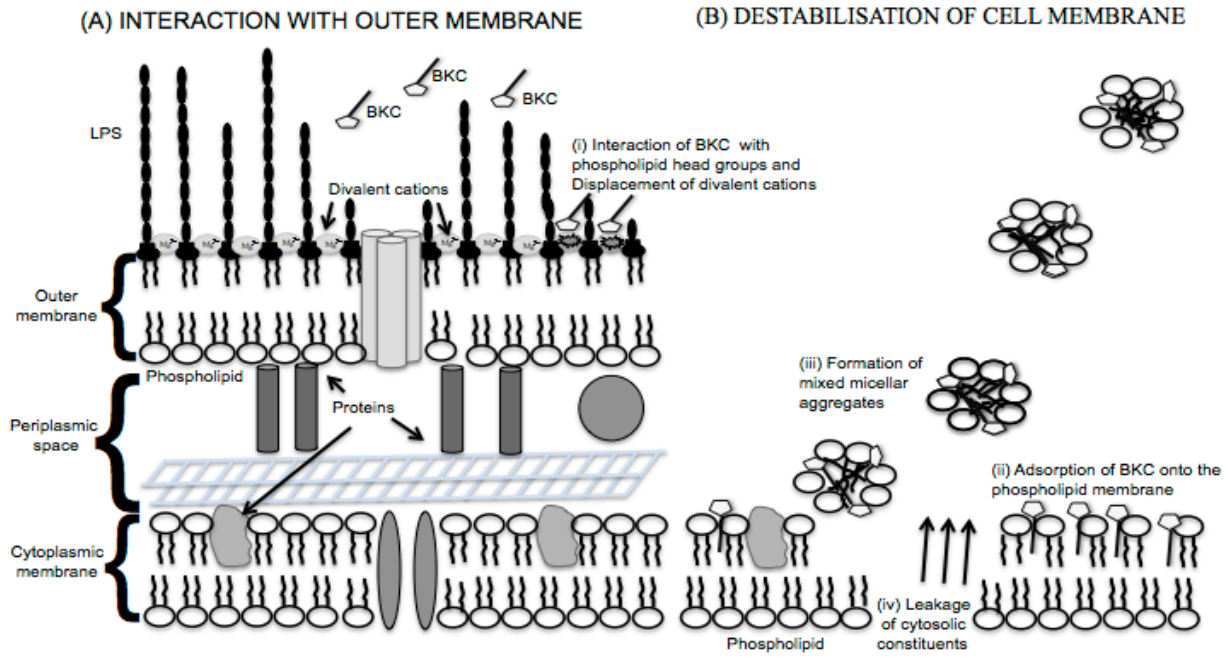

Figure 1.8 Proposed mechanism of action of the cationic biocide BKC. BKC disrupts the integrity of the outer membrane of Gram-negative cells through (i) interaction with the phospholipid head groups and interation with cationic divalent cations (removing Mg^{2+}) and destablises the lipopolysaccharide layer of the outer membrane permeabilizing the cell membrane. (ii) BKC is then adsorbed onto the cytoplasmic membrane causing formation of mixed micellar aggregates (iii) and destabilisation of the membrane. (iv) Finally leakage of the cellular contituents occurs at low concentrations of biocide. Degradation of proteins and nucleic acids occurs at higher concentrations enventually followed by cell lysis.

Recently it was shown that *C. jejuni* strains were able to survive over-night on the surface of slaughterhouse equipment after disinfection with a BKC-containing biocide (Peyrat *et al.,* 2008). Moreover, a quaternary ammonium disinfectant applied to a *Campylobacter* contaminated soil sample from poultry grow-out facilities, was ineffective in decreasing *Campylobacter* populations present in the sample (Payne *et al.,* 2005). BKC was also shown to be ineffective against *Campylobacter* biofilms (Trachoo and Frank, 2002a, 2002b). Given the widespread use of BKC in the poultry industry research into the reasons for disinfectant failure or resistance development is

warranted.

1.11 Development of adapted strains *in vitro*

Development of adapted strains *in vitro* by microbial laboratory evolution experiments can be a powerful tool in predicting antimicrobial resistance as well as understanding the underlying molecular mechanisms leading to adaptation and maintenance of antibiotic resistant traits in bacterial populations (Fernández *et al.,* 2011; Hall, 2004). Evolutionary experiments can generate clonally related strain libraries with a wide spectrum of antimicrobial resistant profiles and known evolutionary histories that can be subject to further study (Bennett & Hughes, 2009). The most common protocols used in the development of antimicrobial resistance in bacterial populations include serial transfer of antibiotic sensitive strains in broth or agar containing increasing concentrations of the antimicrobial agent, and selective enrichment of adapted strains in chemostat culture (Petrosino *et al.,* 1998; Lenski & Travisano, 1994). Adaptation of bacteria to novel environments is dependent on the rate of *de novo* mutations that create genetic variation within the bacterial population and the relative fitness (reproductive success) of both the mutant and parent cell when in competition with one another in that environment (Elena & Lenski, 2003). The stability of the adaptation in the absence of a selective agent is due to low frequency of occurrence of back mutations that reverse the effects of the beneficial mutation as described in section 1.9 (Almofti *et al.,* 2011; Schrag *et al.,* 1997).

1.11.1 Selection using serial transfer

Selection of resistance to antimicrobials using serial culture or serial passage has been a widely employed method for studying acquired antimicrobial resistance (Brazas *et al.,* 2007; Jones *et al.,* 1989; Limoncu *et al.,* 2003; Loughlin *et al.,* 2002; Martínez *et al.,* 2011; Sierra *et al.,* 2009). Bacteria are serial passaged on agar or broth containing incrementally increasing sub-lethal concentration of an antimicrobial agent referred to as the selector for extended periods of time to select for the resistant phenotype. In broth batch culture bacteria are grown in a finite volume of liquid nutrient medium and growth will continue until an essential nutrient becomes depleted (Elena & Lenski, 2003). There is no addition of fresh media or removal of spent media and this

approximates a closed system (Bull, 2010). During serial transfer in batch culture, a proportion of the population are periodically transferred into fresh medium in which it grows until it exhausts the limiting nutrient, and it is again transferred into a fresh medium (Elena & Lenski, 2003). Bacteria are subjected to alternating periods of growth and stasis upon serial transfer because of the dramatic changes in environmental culture conditions from feast to famine (Sauer, 2001). However serial passage is associated with severe population transmission bottlenecks resulting in the selection of the most frequently occurring clones of mutants of a lower fitness rather than higher fitness mutants (Levin *et al.,* 2000).

1.11.2 Periodic selection

Periodic section is the classic model for explaining bacterial evolution and adaptation in homogenous environments like chemostats (Atwood *et al.,* 1951). The basic underlying principle of the model is that as adaptive mutations arise and become fixed in the population in succession during adaptive sweeps – they result in the complete replacement of the population with an adaptive clone (Atwood *et al.,* 1951). These population takeovers occur repeatedly (periodically) as each new beneficial mutation arises (conferring a benefit to that subpopulation over the resident population) (Sauer, 2001). In large populations, beneficial mutations are expected to occur in each generation (Dykhuizen, 1990). When beneficial mutations occur that confer increased fitness (improving performance and reproductive success in a given environment) to the new population the new population will increase in frequency as a result of clonal expansion and displace the resident population (Elena & Lenski, 2003; Dykhuizen, 1990; Dykhuizen & Hertl, 1983). The dynamics of period selection have been described, and are illustrated in Figure 1.9 (Dykhuizen & Hartl 1983; Dykhuizen, 1990; Sauer, 2001).

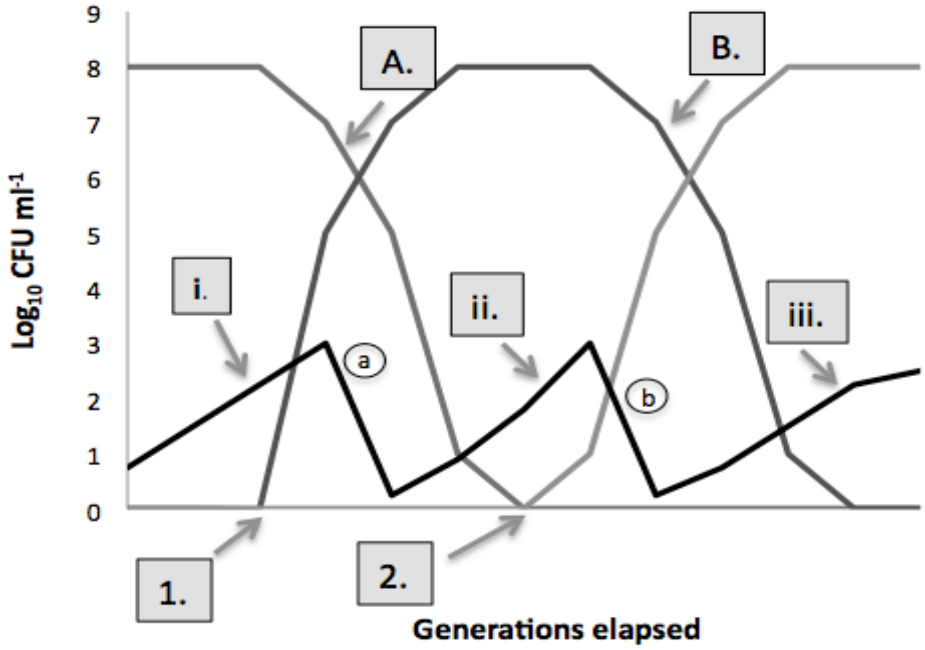

Figure 1.9 Schematic represntation of the population dynamics occurring during adaptive evolution in an asexual population. 1. First adaptive benefical mutation occurs (beneficial mutation *A*). 2. Second adaptive benefical mutation occurs (beneficial mutation *B*). Increase (i) and decrease (a, circled) in neutral mutation conferring resistance to phage T5 in original clone. Increase (ii) and decrease (b, circled) in neutral mutation conferring resistance to phage T5 in first adaptive mutant. Increase (iii) in neutral mutation conferring resistance to phage T5 in second adaptive mutant A. selection against original clone. B. selection against first adaptive mutant. Adapted from Sauer, 2001.

Periodic selection can be monitored by observing the frequency of a neutral mutation in a population (i.e. resistance to phage T5 in a phage-free environment) present in a small fraction of the population that does not confer a selective advantage to the mutant (Sauer, 2001). The proportion of cells carrying the neutral mutation should increase linearly over time in a population of a constant size, assuming the mutation rate is constant in these cells. The cells that do not carry the neutral mutation (i.e. T5 sensitive clones) comprise the majority of cells in the population (Dykhuizen & Hartl 1983). The first adaptive beneficial mutations, which arise in the population, are more likely to occur in the population of cells that do not carry the neutral mutation (T5 sensitive clones) when they occur in a greater frequency in the population. The first adaptive mutant carrying beneficial mutation *A* arises in the population (1, Figure 1.9) and it displaces the original clone. This simultaneously results in a drastic reduction in the fraction of population carrying the neutral mutation conferring T5 resistance (a, circled, Figure 1.9). However the frequency of occurrence of the neutral mutation

conferring T5 resistance in the first adaptive mutant (T5 sensitive clones carrying beneficial mutation *A*) increases linearly over time as a result of recurrent mutations (ii, Figure 1.9). When a second adaptive mutant carrying beneficial adaptive mutation *B* arises in population the frequency of the neutral mutation conferring T5 resistance (2, Figure 1.9) will again drop (b, circled, Figure 1.9). The frequency of neutral mutation in the second adaptive clone (T5 sensitive clones carrying beneficial mutation B) increases in the population (iii, Figure 1.9).

As each adaptive beneficial mutation arises in the population the cycle is repeated. A saw-tooth pattern of the oscillations in frequency of T5 resistant clones over time will emerge and is indicative of a population undergoing periodic selection (Dykhuizen and Hartl, 1983; Dykhuizen, 1990). Periodic selection is non-specific in nature and results in a hitchhiking effect (linkage of neutral mutation and beneficial mutation on the same bacterial chromosome) on all genes initially present in the favored clone. If the neutral mutation resulting in a T5 resistance clone occurs in a clone that carries the beneficial mutation its frequency will increase as a result of genetic hitchhiking.

The time between population turnovers is dependent on two variables. The time required for the beneficial mutation to appear in the population and the time required for the number of clones carrying the beneficial mutation to increase in frequency by selection so that they replace the original population (Dykhuizen, 1990). Bacterial fitness is commonly measured in competition experiments, where the evolved strain and the parent strain are co-cultured for several generations. The change in ratio of the two strains relative to one another over the course of the competition experiment can be monitored using a genetic marker such as antibiotic resistance (Lenski, 1990).
Population fitness or reproductive success in a given environment increases in a stepwise manner (Elena & Lenski, 2003). The effect of the presence of a beneficial mutation will not affect the overall population fitness until it comprises a significant percentage of the population. The short periods of rapid fitness gains followed by longer periods of evolutionary stasis observed in adaptation experiments can be explained by the amount of time required for the generation of beneficial mutants in the population and for such clones carrying the beneficial mutation to comprise a substantial proportion of the population (Elena & Lenski, 2003). Relative fitness of the adapted and parent strain can be measured by allowing them to compete against

one another.

The selection co-efficient is a quantitative measure of the differences in fitness of two clones in a particular environment (Lenski, 1991). It can be expressed mathematically as

$$\ln[X_1(t)/X_2(t)]=\ln[X_1(0)/X_2(0)]-S(t)$$

where $X_1(0)$ and $X_1(t)$ and represent the relative fraction of the evolved phenotype at time 0 and time t. $X_2(0)$ and $X_2(t)$ represent the relative fraction of the original strain at time 0 and time t. S is a measure of the differential growth rate per unit time (selection coefficient). If $S > 0$ then selection of the evolved phenotype is favoured. Similarly if $S < 0$, prevailing environmental conditions select against the evolved phenotype and if $S = 0$ there is no selection of the evolved phenotype. Selection experiments are carried out as follows. Two clones are mixed together at the same initial ratio (1:1) in the environment of interest. After certain time intervals have elapsed, samples are obtained and the ratio of the two clones is determined for each sample. If the ratios of the two clones relative to one another increases or decreases in a systematic fashion it can be stated that one clone is fitter then the other in that environment (Lenski, 1991). The natural logarithm of the ratio of the two clones are plotted and should exibit a linear relationship with time. The slope of the line (derived from regression analysis of the natural logarithm of the ratio of the two clones) is equivalent to the selection co-efficient and has units of inverse time (Lenski, 1991).

The minimal selection concentration (MSC), is the lowest concentration of an antimicrobial that gives the resistant strain a selective advantage when competed against the parent strain (O' Reilly & Smith, 1999). The value of an antibiotic's MSC is affected by the fitness cost of the resistant mutation to the antimicrobial agent and reducing this cost through the acquisition of compensatory mutations can decrease the MSC (Gullberg *et al.,* 2011). Competition studies carried out in chemostats can be used to test the evolved phenotype compared to the original strain under a variety of conditions. McCay et al. (2010) used competition experiments to determine the MSC of BKC-adapted and sensitive *P. aeruginosa* strain to BKC in nutient broth and in minimal media under conditions of glucose-limitation and magnesium-limitation.

Glucose was used to represent a low-energy environment and was used to test if adaptation of the strain was associated with a metabolic burden. Magnesium is integral to the structure of LPS and is important for outer membrane barrier function (McCay *et al.,* 2010). McCay et al. (2010) found in the absence of sub-inhibitory concentrations of BKC the original *P. aeroginosa* strain outcompeted its BKC-adapted counterpart irrespective of nutrient limitation. When the strains were competed against each other under magnesium-limitation the MSC for the BKC-adapted *P. aeruginosa* strain was determined to lie somewhere between 0 and 1 mg l^{-1} BKC (between 0 and 5 % of the MIC of the original BKC-sensitive strain). In complex medium the MSC lay between 1 mg BKC l^{-1} and 5 mg l^{-1} BKC (between 5 and 20 % of the MIC of the original BKC-sensitive strain). However MSC concentrations could not be determined for the BKC-adapted *P. aeruginosa* strain under glucose-limitation suggesting that the metabolic burden associated with reduced susceptibility to BKC was selectively disadvantaging cells in the low energy environment (McCay *et al.,* 2010). Gullburg *et al*. (2011) showed that ciprofloxacin applied at its MSC (1/10 of its MIC) could be used to select for ciprofloxacin resistance (8-fold increase MIC) after 600 generations using serial passage in *E. coli* MG1655. The same group showed that streptomycin resistance (16-fold increase MIC) could arise in *Salmonella enterica* serovar Typhimurium LT2 after exposure to MSC of streptomycin of the strain for 600 generations.

1.11.3 Specific selection in continuous culture

Directed evolution is a general term used to describe various techniques used for the isolation of genetic variants with the desired characteristics from a large diverse population using screening or selection protocols (Arensdorf *et al.,* 2002; Sauer, 2001). Exposure to sub-lethal concentrations of antimicrobial agents can select for bacterial populations with pre-existing antimicrobial resistance traits and is a particularly effective method in driving the selection of resistant strains *in vitro* (Fernández *et al.,* 2011; Meyer *et al.,* 2010b; Silbergeld *et al.,* 2008).

Chemostat selection can be designed to enrich for a desired phenotype without knowledge of the genotype or underlying molecular mechanisms, and thus it can be used in directed-evolution strategies with samples ranging from well-characterized

cloned genes to uncharacterized microorganisms and can allow for the manifestation of rare mutants within a large population (Dykhuizen & Hartl, 1983; Sauer, 2001). Furthermore, the cost of resistance to antimicrobial agents is reduced to negligible proportions through sufficient evolution (Cowen *et al*., 2001). A chemostat is a culturing vessel for microorganisms that is supplied with fresh sterile media at a constant flow rate. The removal of culture and spent media from the vessel at the same rate maintains a fixed culture volume in the chemostat. The specific growth rate is controlled by the supply of limiting nutrient substrate(s) in the growth media. The limiting nutrient is the nutrient that is essential for growth of the organism but present in the lowest concentration in the media. All other essential nutrients present in the growth media are in excess. Monod (1942) derived an equation to determine the relationship between growth rate and substrate concentration of the limiting nutrient.

$$\mu=\mu_{max}\left(s/K_s+s\right)$$

Where μ is the growth rate, s is the nutrient concentration and K_s is the nutrient concentration at which a growth rate of half the maximum specific growth rate is achieved. The rate of medium flow into the vessel is related to its volume, and is defined by the dilution rate, D

$$D=F/V$$

when steady state conditions are established, growth rate of the organism becomes equivalent to D. As D approaches the maximum specific growth rate of the organism the system becomes unstable and if D exceeds a critical value D_{crit} washout of the culture occurs (Hoskisson & Hobbs, 2005; Bull, 2010).

The chemostat allows a bacterial culture to be grown at a reduced growth rate for an indefinite period (Winder *&* Lanthaler, 2011). The advantages of using chemostats include: (i) Control of the growth rate of the organism can be achieved by varying the supply of growth limiting nutrient through altering the dilution rate without changing the medium composition or operating conditions (ii) The effect of environmental parameters at fixed growth rates can be determined or at defined transitional states of culture which is useful for investigating metabolic regulation (iii) The nature of the growth limiting substrate can be changed e.g. carbon-, nitrogen- phosphorus- or

magnesium-limiting (Gresham & Hong, 2014; Hoskisson & Hobbs, 2005). In practice, steady state in continuous culture is rarely maintained for long, as nutrient limited growth induces strong selectional pressures for mutational change that rapidly sweep chemostat populations. The residual concentration of limiting nutrient continues to drop over hundreds of hours of continuous culture as a result of this selection pressure (Bull, 2010). In a chemostat, continuous cell division ensures that new cells are continuously generated and thus, even at low spontaneous mutation rates, reasonably large numbers of random mutants can be generated (Dykhuizen & Hartl, 1983; Sauer, 2001). Any random mutant that is better adapted than the original strain to grow under the selective pressures established within the chemostat will eventually establish itself as the dominant population (Dykhuizen & Hartl, 1983).

1.12 Elucidating antimicrobial resistance mechanisms through proteomic investigations

Proteomic technologies can be utilised for the global analysis of protein changes in *C. jejuni* and have been applied to investigate differences in the protein expression profiles of cells grown under a broad spectrum of growth conditions and with different stress factors including some antibiotics inhibiting protein synthesis or gyrase function (Bandow *et al.,* 2003). The array of protein species linked to antimicrobial resistance has been explored in a large diversity of microorganisms and with different antimicrobial agents (Scott & Cordwell, 2009). The elucidation of the molecular details of drug resistance by comparative proteomic analyses is helpful not only to further new insights into antimicrobial-resistant mechanism(s), but also to lead the improvements in extending the efficacy of the current antimicrobials to the control of bacteria (Radhouani *et al.,* 2012).

In most *Campylobacter* proteome studies, two-dimensional gel electrophoresis has been the method of choice, coupled with mass spectrometric (MS) or tandem MS (MS/MS) to identify changes in protein production in adapted strains of *C. jejuni* (Scott & Cordwell, 2009). The technique was first described by O'Farrell (1975). Proteins are separated according to their isoelectric point using isoelectric focusing and then according to their molecular weight by sodium dodecyl polyacrylamide gel electrophoresis. Tandem mass spectroscopy enables the identification of proteins by

effectively providing the sequence of peptides derived from proteolytic digestion of whole proteins. Fragment ion spectra are matched against the predicted ion fragmentation predicted from within a database using search tools such as Mascot or Sequest. The principal strength of 2-DE consists of its particularly high-resolution power compared to other separation approaches and a good visualization of the obtained results. With the introduction of immobilized pH gradients (IPG) resolution, reproducibility and protein load capacity have been greatly improved (Görg *et al.,* 2004).

1.13 Aims and objectives

Campylobacter may encounter many situations throughout the food chain that may provide a selective environment for adaptive resistance to both antibiotics and disinfectants. The aims of this study was to investigate the effects of exposure of *Campylobacter jejuni* NCTC 11168 to sub inhibitory concentrations of antibiotics and disinfectants on cell adaptation and the generation of resistance / cross resistance.

The objectives of this project were:

(i) To characterize of the development of resistance to ciprofloxacin and tolerance to BKC in adapted variants in chemostat culture (Chapter 2).
(ii) To characterize phenotypic changes (changes in fitness and stability) following adaptation to ciprofloxacin and BKC (Chapter 3).
(iii) To determine if development of resistance to antibiotics leads to cross-resistance to biocides and *vice versa*, and/or alters the efficacy of disinfectant (namely Savlon^{TM} and its active biocidal ingredient BKC) against adapted variants (Chapter 3).
(iv) To investigate if efflux mechanisms were involved in conferred resistance to ciprofloxacin and/or cross-resistance to other antimicrobials in ciprofloxacin resistant variants (Chapter 3).
(v) Finally, to evaluate the mechanisms of resistance to both these antimicrobials at the molecular and genetic level (Chapter 4 & Chapter 5).

1.14 Bibliography

Aase, B., Sundheim, G., Langsrud, S., & Rørvik, L. M. (2000). Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. *Int. J. Food Microbiol., 62*(1), 57-63.

Abu-Halaweh, M., Bates, J., & Patel, B. K. (2005). Rapid detection and differentiation of pathogenic *Campylobacter jejuni* and *Campylobacter coli* by real-time PCR. *Res. Microbiol., 156*(1), 107-114.

Abubakar, I., Irvine, L., Aldus, C., Wyatt, G., Fordham, R., Schelenz, S., & Hunter, P. (2007). A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. *Health Technol. Assess., 11*(36).

Abulreesh, H. H., Paget, T. A., & Goulder, R. (2006). *Campylobacter* in waterfowl and aquatic environments: incidence and methods of detection. *Environ. Sci. Technol., 40*(23), 7122-7131.

Ahmed, M. U., Dunn, L., & Ivanova, E. P. (2012). Evaluation of current molecular approaches for genotyping of *Campylobacter jejuni* strains. *Foodborne Pathog. Dis., 9*(5), 375-385.

Akiba, M., Lin, J., Barton, Y.-W., & Zhang, Q. (2006). Interaction of CmeABC and CmeDEF in conferring antimicrobial resistance and maintaining cell viability in *Campylobacter jejuni*. *J. Antimicrob. Chemother., 57*(1), 52-60.

Alakomi, H.-L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Appl. Environ. Microbiol., 66*(5), 2001-2005.

Aldred, K. J., Breland, E. J., Vlčková, V., Strub, M.-P., Neuman, K. C., Kerns, R. J., & Osheroff, N. (2014a). Role of the water–metal ion bridge in mediating interactions between quinolones and *Escherichia coli* topoisomerase IV. *Biochemistry, 53*(34), 5558-5567.

Aldred, K. J., Kerns, R. J., & Osheroff, N. (2014b). Mechanism of quinolone action and resistance. *Biochemistry, 53*(10), 1565-1574.

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell, 128*(6), 1037-1050.

Alexander, C., & Rietschel, E. T. (2001). Invited review: bacterial lipopolysaccharides and innate immunity. *J. Endotoxin Res., 7*(3), 167-202.

Alfredson, D. A., & Korolik, V. (2007). Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol. Lett., 277*(2), 123-132.

Acheson, D., & Allos, B. M. (2001). *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Dis., 32*(8), 1201-1206.

Allos B. M. (2001). *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Dis., 32*(8), 1201–1206.

Almofti, Y. A., Dai, M., Sun, Y., Haihong, H., & Yuan, Z. (2011). Impact of erythromycin resistance on the virulence properties and fitness of *Campylobacter jejuni*. *Microb. Pathog., 50*(6), 336-342.

Amako, K., Baba, N., Suzuki, N., Wai, S. N., & Umeda, A. (1997). A structural analysis of the regularly arranged porin on the outer membrane of *Campylobacter jejuni* based on correlation averaging. *Microbiol. Immunol., 41*(11), 855-859.

Andersson, D. I., & Levin, B. R. (1999). The biological cost of antibiotic resistance. *Curr. Opin. Microbiol., 2*(5), 489-493.

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol., 8*(4), 260-271.

Andersson, D. I., & Hughes, D. (2012). Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resist. Updat., 15*(3), 162-172.

Arensdorf, J. J., Loomis, A. K., DiGrazia, P. M., Monticello, D. J., & Pienkos, P. T. (2002). Chemostat approach for the directed evolution of biodesulfurization gain-of-function mutants. *Appl. Environ. Microbiol., 68*(2), 691-698.

Atack, J. M., & Kelly, D. J. (2009). Oxidative stress in *Campylobacter jejuni*: responses, resistance and regulation. *Future Microbiol., 4*(6), 677-690.

Atwood, K., Schneider, L. K., & Ryan, F. J. (1951). Periodic selection in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A., 37*(3), 146.

Avrain, L., Allain, L., Vernozy-Rozand, C., & Kempf, I. (2003). Disinfectant susceptibility testing of avian and swine *Campylobacter* isolates by a filtration method. *Vet. Microbiol., 96*(1), 35-40.

Azevedo, I., Albano, H., Silva, J. & Teixeira, P., (2014). Food safety in the domestic environment. *Food Control*, *37*, 272-276.

Bandow, J. E., Brötz, H., Leichert, L. I. O., Labischinski, H., & Hecker, M. (2003). Proteomic approach to understanding antibiotic action. *Antimicrob. Agents Chemother., 47*(3), 948-955.

Bang, D. D., Wedderkopp, A., Pedersen, K., & Madsen, M. (2002). Rapid PCR using nested primers of the 16S rRNA and the hippuricase (hip O) genes to detect *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. *Mol. Cell. Probes, 16*(5), 359-369.

Bearth, A., Cousin, M.E. & Siegrist, M., (2014). Poultry consumers' behaviour, risk perception and knowledge related to campylobacteriosis and domestic food safety. *Food Control*, *44*, 166-176.

Bennett, A. F., & Hughes, B. S. (2009). Microbial experimental evolution. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, *297*(1), R17-R25.

Birch, L., Dawson, C., Cornett, J., & Keer, J. (2001). A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Lett. Appl. Microbiol., 33*(4), 296-301.

Birk, T., Ingmer, H., Andersen, M.T., Jørgensen, K. and Brøndsted, L., 2004. Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. *Lett. Appl. Microbiol., 38*(1), pp.66-71.

Björkman, J., & Andersson, D. I. (2000). The cost of antibiotic resistance from a bacterial perspective. *Drug Resist. Updat., 3*(4), 237-245.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P., & Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis., 157*(3), 472-479.

Bolla, J.-M., Loret, E., Zalewski, M., & Pagés, J.-M. (1995). Conformational analysis of the *Campylobacter jejuni* porin. *J. Bacteriol., 177*(15), 4266-4271.

Bolton, D.J. (2015). *Campylobacter* virulence and survival factors. *Food Microbiol.*, *48*, 99-108.

Bolton, D., Meredith, H., Walsh, D. and McDowell, D., (2014). Poultry food safety control interventions in the domestic kitchen. *J Food Saf, 34*(1), 34-41.

Boysen, L., Knøchel, S., & Rosenquist, H. (2007). Survival of *Campylobacter jejuni* in different gas mixtures. *FEMS Microbiol. Lett., 266*(2), 152-157.

Boysen, L., & Rosenquist, H. (2009). Reduction of thermotolerant *Campylobacter* species on broiler carcasses following physical decontamination at slaughter. *J. Food Protect., 72*(3), 497-502.

Braoudaki, M. & Hilton, A.C., (2004a). Adaptive resistance to biocides in *Salmonella* enterica and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. *J. Clin. Microbiol*., *42*(1), 73-78.

Braoudaki, M., & Hilton, A. C. (2004b). Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and E. *coli* O55 compared to E. *coli* O157. *FEMS Microbiol. Lett., 235*(2), 305-309.

Brazas, M. D., Breidenstein, E. B., Overhage, J., & Hancock, R. E. (2007). Role of lon, an ATP-dependent protease homolog, in resistance of *Pseudomonas aeruginosa* to ciprofloxacin. *Antimicrob. Agents Chemother., 51*(12), 4276-4283.

Bremell, T., Bjelle, A., & Svedhem, A. (1991). Rheumatic symptoms following an outbreak of *Campylobacter* enteritis: a five year follow up. *Ann. Rheum. Dis., 50*(12), 934-938.

Bridier, A., Briandet, R., Thomas, V., & Dubois-Brissonnet, F. (2011a). Resistance of bacterial biofilms to disinfectants: a review. *Biofouling, 27*(9), 1017-1032.

Bridier, A., Briandet, R., Thomas, V., & Dubois-Brissonnet, F. (2011b). Comparative biocidal activity of peracetic acid, benzalkonium chloride and ortho-phthalaldehyde on 77 bacterial strains. *J. Hosp. Infect., 78*(3), 208-213.

Buffet-Bataillon, S., Branger, B., Cormier, M., Bonnaure-Mallet, M., & Jolivet-Gougeon, A. (2011). Effect of higher minimum inhibitory concentrations of quaternary ammonium compounds in clinical E. *coli* isolates on antibiotic susceptibilities and clinical outcomes. *J. Hosp. Infect., 79*(2), 141-146.

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M., & Jolivet-Gougeon, A. (2012). Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds—a critical review. *Int. J. Antimicrob. Agents, 39*(5), 381-389.

Bull, A. T. (2010). The renaissance of continuous culture in the post-genomics age. *J. Ind. Microbiol. Biotechnol., 37*(10), 993-1021.

Burgess, F., Little, C., Allen, G., Williamson, K., & Mitchell, R. (2005). Prevalence of *Campylobacter*, *Salmonella*, and *Escherichia coli* on the external packaging of raw meat. *J. Food Protect., 68*(3), 469-475.

Burmølle, M., Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J., & Kjelleberg, S. (2006). Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl. Environ. Microbiol., 72*(6), 3916-3923.

Butaye, P., Cloeckaert, A., & Schwarz, S. (2003). Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. *Int. J. Antimicrob. Agents, 22*(3), 205-210.

Carpentier, B., & Cerf, O. (2011). Review—Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol., 145*(1), 1-8.

Cerf, O., Carpentier, B., & Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts:"resistance" has different meanings. *Int. J. Food Microbiol., 136*(3), 247-254.

Chapman, J. S. (2003a). Biocide resistance mechanisms. *Int. Biodeterior. Biodegradation, 51*(2), 133-138.

Chapman, J. S. (2003b). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation, 51*(4), 271-276.

Christensen, E. G., Gram, L., & Kastbjerg, V. G. (2011). Sublethal Triclosan Exposure Decreases Susceptibility to Gentamicin and Other Aminoglycosides in *Listeria monocytogenes*. *Antimicrob. Agents Chemother., 55*(9), 4064-4071.

Chuanchuen, R., Karkhoff-Schweizer, R.R. and Schweizer, H.P., 2003. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. *Am J Infect Control, 31*(2), 124-127.

Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R., & Schweizer, H. P. (2001). Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a

susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. *Antimicrob. Agents Chemother., 45*(2), 428-432.

Cloete, T. E. (2003). Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeterior. Biodegradation, 51*(4), 277-282.

Cogan, T.A., Slader, J., Bloomfield, S.F. and Humphrey, T.J., (2002). Achieving hygiene in the domestic kitchen: the effectiveness of commonly used cleaning procedures. *J. Appl. Microbiol*., *92*(5), 885-892.

Condell, O., Iversen, C., Cooney, S., Power, K. A., Walsh, C., Burgess, C., & Fanning, S. (2012). Efficacy of biocides used in the modern food industry to control *Salmonella*-links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. *Appl. Environ. Microbiol., 78*(9), 3087-3097.

Corcoran, D., Quinn, T., Cotter, L., O'Halloran, F., & Fanning, S. (2005). Characterization of a cmeABC operon in a quinolone-resistant *Campylobacter coli* isolate of Irish origin. *Microb. Drug Resist., 11*(4), 303-308.

Corona, F., & Martinez, J. L. (2013). Phenotypic resistance to antibiotics. *Antibiotics, 2*(2), 237-255.

Corry, J., & Atabay, H. (2001). Poultry as a source of *Campylobacter* and related organisms. *J. Appl. Microbiol., 90*(S6), 96S-114S.

Cosgrove, S. E. (2006). The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clin. Infect. Dis., 42*(Supplement 2), S82-S89.

Cowen, L. E., Kohn, L. M., & Anderson, J. B. (2001). Divergence in fitness and evolution of drug resistance in experimental populations of Candida albicans. *J. Bacteriol., 183*(10), 2971-2978.

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol., 303*(6), 287-292.

Da Costa, P. M., Loureiro, L., & Matos, A. J. (2013). Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. *Int. J. Environ. Res. Public Health, 10*(1), 278-294.

Davison, H. C., Woolhouse, M. E., & Low, J. C. (2000). What is antibiotic resistance and how can we measure it? *Trends Microbiol*., 8(12), 554-559.

Dé, E., Jullien, M., Labesse, G., Pagès, J. M., Molle, G., & Bolla, J. M. (2000). MOMP (major outer membrane protein) of *Campylobacter jejuni*; a versatile pore-forming protein. *FEBS Lett., 469*(1), 93-97.

De Los Santos, F. S., Donoghue, A., Venkitanarayanan, K., Reyes-Herrera, I., Metcalf, J., Dirain, M., Donoghue, D. (2008). Therapeutic supplementation of caprylic acid in feed reduces *Campylobacter jejuni* colonization in broiler chicks. *Appl. Environ. Microbiol., 74*(14), 4564-4566.

Debruyne, L., Samyn, E., De Brandt, E., Vandenberg, O., Heyndrickx, M., & Vandamme, P. (2008). Comparative performance of different PCR assays for the identification of *Campylobacter jejuni* and *Campylobacter coli*. *Res. Microbiol., 159*(2), 88-93.

Debruyne, L., On, S. L., De Brandt, E., & Vandamme, P. (2009). Novel *Campylobacter* lari-like bacteria from humans and molluscs: description of *Campylobacter* peloridis sp. nov., *Campylobacter* lari subsp. concheus subsp. nov. and *Campylobacter* lari subsp. lari subsp. nov. *Int. J. Syst. Evol. Microbiol., 59*(5), 1126-1132.

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S. L., & Vandamme, P. (2010a). *Campylobacter* subantarcticus sp. nov., isolated from birds in the sub-Antarctic region. *Int. J. Syst. Evol. Microbiol., 60*(4), 815-819.

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S. L., & Vandamme, P. (2010b). *Campylobacter* volucris sp. nov., isolated from black-headed gulls (Larus ridibundus). *Int. J. Syst. Evol. Microbiol., 60*(8), 1870-1875.

Dedieu, L., Pagès, J.-M., & Bolla, J.-M. (2004). Use of the omp50 gene for identification of *Campylobacter* species by PCR. *J. Clin. Microbiol., 42*(5), 2301-2305.

De Jong, A.E.I., Verhoeff‐Bakkenes, L., Nauta, M.J. and De Jonge, R., (2008). Cross‐contamination in the kitchen: effect of hygiene measures. *J. Appl. Microbiol., 105*(2), 615-624.

Dekeyser, P., Gossuin-Detrain, M., Butzler, J., & Sternon, J. (1972). Acute enteritis due to related *Vibrio*: first positive stool cultures. *J. Infect. Dis., 125*(4), 390-392.

Delcour, A. H. (2009). Outer membrane permeability and antibiotic resistance. *BBA-Proteins Proteom., 1794*(5), 808-816.

Denyer, S., & Maillard, J. Y. (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram‐negative bacteria. *J. Appl. Microbiol., 92*(s1), 35S-45S.

Dingle, K., Colles, F., Wareing, D., Ure, R., Fox, A., Bolton, F., Maiden, M. (2001). Multilocus sequence typing system for*Campylobacter jejuni*. *J. Clin. Microbiol., 39*(1), 14-23.

Doorduyn, Y., Van Pelt, W., Siezen, C., Van Der Horst, F., Van Duynhoven, Y., Hoebee, B., & Janssen, R. (2008). Novel insight in the association between salmonellosis or *Campylobacter*iosis and chronic illness, and the role of host genetics in susceptibility to these diseases. *Epidemiol. Infect., 136*(09), 1225-1234.

Doyle, M., & Erickson, M. (2006). Reducing the carriage of foodborne pathogens in livestock and poultry. *Poult. Sci., 85*(6), 960-973.

Drlica, K., Zhao, X., Malik, M., Salz, T., & Kerns, R. (2012). Fluoroquinolone resistance: mechanisms, restrictive dosing, and anti-mutant screening strategies for new compounds. *Antibiotic Discovery and Development* (pp. 485-514): Springer.

Dwyer, D. J., Kohanski, M. A., & Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr. Opin. Microbiol., 12*(5), 482-489.

Dwyer, D. J., Belenky, P. A., Yang, J. H., MacDonald, I. C., Martell, J. D., Takahashi, N., Collins, J. J. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci., 111*(20), E2100-E2109.

Dwyer, D. J., Collins, J. J., & Walker, G. C. (2015). Unraveling the physiological complexities of antibiotic lethality. *Annu. Rev. Pharmacol. Toxicol., 55*, 313-332.

Dykhuizen, D. E., & Hartl, D. L. (1983). Selection in chemostats. *Microbiol. Rev., 47*(2), 150.

Dykhuizen, D. E. (1990). Experimental studies of natural selection in bacteria. *Annu. Rev. Ecol. Syst.*, 373-398.

Eberle, K., & Kiess, A. (2012). Phenotypic and genotypic methods for typing *Campylobacter jejuni* and *Campylobacter coli* in poultry. *Poult. Sci., 91*(1), 255-264.

EFSA. (2009). Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic infections. *EFSA Journal, 7*(11):1372.

EFSA. (2010). Scientific Opinion on Quantification of the risk posed by broiler meat to human *Campylobacter*iosis in the EU. *EFSA Journal, 8*(1):1437.

EFSA (2011). Scientific opinion on *Campylobacter* in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA Journal.,* 9(4):2105.

EFSA. (2012). Technical specifications for the analysis and reporting of data on antimicrobial resistance (AMR) in the European Union Summary Report *EFSA Journal,* 10(2);2587.

El-Shibiny, A., Connerton, P., & Connerton, I. (2009). Survival at refrigeration and freezing temperatures of *Campylobacter coli* and *Campylobacter jejuni* on chicken skin applied as axenic and mixed inoculums. *Int. J. Food Microbiol., 131*(2), 197-202.

Elena, S. F., & Lenski, R. E. (2003). Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet., 4*(6), 457-469.

Endtz, H. P., Ruijs, G. J., van Klingeren, B., Jansen, W. H., van der Reyden, T., & Mouton, R. P. (1991). Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. *J. Antimicrob. Chemother., 27*(2), 199-208.

Engberg, J., Aarestrup, F. M., Taylor, D. E., Gerner-Smidt, P., & Nachamkin, I. (2001). Quinolone and macrolide resistance in *Campylobacter jejuni* and C. *coli*: resistance mechanisms and trends in human isolates. *Emerging Infect. Dis., 7*(1), 24.

ESVAC (2013). *Sales of Veterinary Antimicrobial Agents in 25 EU/EEA Countries in 2011 (EMA/236501/2013)*, London, UK: ESVAC.

Fallon, R., O'sullivan, N., Maher, M., & Carroll, C. (2003). Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolates from broiler chickens isolated at an Irish poultry processing plant. *Lett. Appl. Microbiol., 36*(5), 277-281.

Farkas, J. (1998). Irradiation as a method for decontaminating food: a review. *Int. J. Food Microbiol., 44*(3), 189-204.

Fermér, C., & Engvall, E. O. (1999). Specific PCR identification and differentiation of the thermophilic *Campylobacters*, *Campylobacter jejuni*, C. *coli*, C. lari, and C. upsaliensis. *J. Clin. Microbiol., 37*(10), 3370-3373.

Fernández, L., Breidenstein, E. B., & Hancock, R. E. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resist. Updat., 14*(1), 1-21.

Frederick, A., & Huda, N. (2011). *Campylobacter* in Poultry: Incidences and Possible Control Measures. *Res. J. Microbiol., 6*(2), 182-192.

FASI. (2011). *Recommendations for a practical control programme for Campylobacter in the poultry production and slaughter chain*. Food Safety Authority of Ireland. Dublin, Ireland: Retrieved from www.fsai.ie.

FSAI. (2010). *How to Minimise the Risk of Leakage of Potentially Contaminated Juices from Fresh Poultry Products.* Food Safety Authority of Ireland. Dublin, Ireland: Retrieved from www.fsai.ie.

Fux, C., Costerton, J., Stewart, P., & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends Microbiol., 13*(1), 34-40.

Galanis, E. (2007). *Campylobacter* and bacterial gastroenteritis. *Can Med Assoc J, 177*(6), 570-571.

Garrity, G. M., Bell, J. A., & Lilburn, T. (2005). Class V. Epsilonproteobacteria. . In D. J. Brenner, N. R. Krieg, J. T. Staley, & G. M. Garrity. (Eds.), *Bergey's Manual of Systematic Bacteriology*. New York: Springer.

Gaze, W., Abdouslam, N., Hawkey, P., & Wellington, E. (2005). Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob. Agents Chemother., 49*(5), 1802-1807.

Gibreel, A., & Taylor, D. E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 58*(2), 243-255.

Gilbert, P., Allison, D., & McBain, A. (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *J. Appl. Microbiol., 92*(s1), 98S-110S.

Gilbert, P., & McBain, A. J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin. Microbiol. Rev., 16*(2), 189-208.

Gilbert, P., & Moore, L. (2005). Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol., 99*(4), 703-715.

Goodwin C. S., Armstrong J. A., Chilvers T., Peters M., Collins M. D., Sly L., Harper W. E. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. *nov.* and *Helicobacter mustelae comb.* nov., respectively. *Int. J. Syst. Evol. Microbiol., 39*(4), 397-405.

Görg, A., Weiss, W., & Dunn, M. J. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics, 4*(12), 3665-3685.

Gradel, K. O., Nielsen, H. L., Schønheyder, H. C., Ejlertsen, T., Kristensen, B., & Nielsen, H. (2009). Increased short-and long-term risk of inflammatory bowel disease after *Salmonella* or *Campylobacter* gastroenteritis. *Gastroenterology, 137*(2), 495-501.

Graham, L., Harris, R., Villiger, W., & Beveridge, T. (1991). Freeze-substitution of Gram-negative eubacteria: general cell morphology and envelope profiles. *J. Bacteriol., 173*(5), 1623-1633.

Granato, P. A., Chen, L., Holiday, I., Rawling, R. A., Novak-Weekley, S. M., Quinlan, T., & Musser, K. A. (2010). Comparison of premier CAMPY enzyme immunoassay (EIA), ProSpecT *Campylobacter* EIA, and ImmunoCard STAT! CAMPY tests with culture for laboratory diagnosis of *Campylobacter* enteric infections. *J. Clin. Microbiol., 48*(11), 4022-4027.

Gresham, D. and Hong, J. (2015). The functional basis of adaptive evolution in chemostats. *FEMS Microbiol. Rev., 39*(1), 2-16.

Griffiths, P. (1993). Morphological changes of *Campylobacter jejuni* growing in liquid culture. *Lett. Appl. Microbiol., 17*(4), 152-155.

Guerin, M., Sir, C., Sargeant, J., Waddell, L., O'Connor, A., Wills, R., Byrd, J. (2010). The change in prevalence of *Campylobacter* on chicken carcasses during processing: A systematic review. *Poult. Sci., 89*(5), 1070-1084.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., & Andersson, D. I. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog, 7*(7), e1002158.

Guo, B., Wang, Y., Shi, F., Barton, Y.-W., Plummer, P., Reynolds, D. L., Zhang, Q. (2008). CmeR functions as a pleiotropic regulator and is required for optimal colonization of *Campylobacter jejuni in vivo*. *J. Bacteriol., 190*(6), 1879-1890.

Gutiérrez-Martín, C. B., Yubero, S., Martínez, S., Frandoloso, R., & Rodríguez-Ferri, E. F. (2011). Evaluation of efficacy of several disinfectants against *Campylobacter jejuni* strains by a suspension test. *Res. Vet. Sci., 91*(3), e44-e47.

Hall, B. G. (2004). Predicting the evolution of antibiotic resistance genes. *Nat. Rev. Microbiol., 2*(5), 430-435.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol., 2*(2), 95-108.

Hammerum, A. M., & Heuer, O. E. (2009). Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clin. Infect. Dis., 48*(7), 916-921.

Han, J., Sahin, O., Barton, Y.-W., & Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog, 4*(6), e1000083.

Handel, A., & Bennett, M. R. (2008). Surviving the Bottleneck: Transmission Mutants and the Evolution of Microbial Populations. *Genetics, 180*(4), 2193-2200.

Hänninen, M.L. and Hannula, M. (2007). Spontaneous mutation frequency and emergence of ciprofloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 60*(6), 1251-1257.

Hazeleger, W.C., Wouters, J.A., Rombouts, F.M. and Abee, T., (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. *Appl. Environ. Microbiol., 64*(10), pp.3917-3922.

Heir, E., Sundheim, G., & Holck, A. (1999a). The qacG gene on plasmid pST94 confers resistance to quaternary ammonium compounds in *Staphylococci* isolated from the food industry. *J. Appl. Microbiol., 86*(3), 378-388.

Heir, E., Sundheim, G., & Holck, A. L. (1999b). Identification and characterization of quaternary ammonium compound resistant *Staphylococci* from the food industry. *Int. J. Food Microbiol., 48*(3), 211-219.

Helms, M., Simonsen, J., & Mølbak, K. (2006). Foodborne bacterial infection and hospitalization: a registry-based study. *Clin. Infect. Dis., 42*(4), 498-506.

Hogan, D., & Kolter, R. (2002). Why are bacteria refractory to antimicrobials? *Curr. Opin. Microbiol., 5*(5), 472-477.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents, 35*(4), 322-332.

Holah, J., Taylor, J., Dawson, D., & Hall, K. (2002). Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *J. Appl. Microbiol., 92*(s1), 111S-120S.

Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infect. Dis., 7*(2), 337.

Hoskisson, P. A., & Hobbs, G. (2005). Continuous culture–making a comeback? *Microbiology, 151*(10), 3153-3159.

Howden, B. P., Davies, J. K., Johnson, P. D., Stinear, T. P., & Grayson, M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin. Microbiol. Rev., 23*(1), 99-139.

HPSC. (2014). *Annual Report 2014 Health Protection Surveillance Centre*. Health Protection Surveillance Centre. Dublin Ireland: Retrieved from http://www.hpsc.ie/AboutHPSC/ AnnualReports/File,14421,en.pdf

Hugo, W. (1967). The mode of action of antibacterial agents. *J. Appl. Bacteriol., 30*(1), 17-50.

Hugo, W. (1991). A brief history of heat and chemical preservation and disinfection. *J. Appl. Bacteriol., 71*(1), 9-18.

Humphrey, T.J., Jørgensen, F., Frost, J.A., Wadda, H., Domingue, G., Elviss, N.C., Griggs, D.J. & Piddock, L.J., (2005). Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter spp.* in commercial poultry flocks before, during, and after treatment with fluoroquinolones. *Antimicrob. Agents Chemother., 49*(2), 690-698.

Humphrey, T., O'Brien, S., & Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol., 117*(3), 237-257.

Humphrey, T.J., Martin, K.W., Slader, J. & Durham, K., (2001). *Campylobacter spp.* in the kitchen: spread and persistence. *J. Appl. Microbiol.*,90(S6).

Hwang, S., Kim, M., Ryu, S., & Jeon, B. (2011). Regulation of oxidative stress response by CosR, an essential response regulator in *Campylobacter jejuni*. *PLoS One, 6*(7), e22300.

Hwang, S., Ryu, S., & Jeon, B. (2013). Roles of the superoxide dismutase SodB and the catalase KatA in the antibiotic resistance of *Campylobacter jejuni*. *J Antibiot, 66*, 351-353.

Jacobs, W. A. (1916). The bactericidal properties of the quaternary salts of hexamethylenetetramine I. The problem of the chemotherapy of experimental bacterial infections. *J. Exp. Med., 23*(5), 563-568.

Jacobs-Reitsma, W., Lyhs, U., & Wagenaar, J. (2008). *Campylobacter* in the food supply. In I. Nachamkin, C. Szymanski, & M. Blaser (Eds.), *Campylobacter* (pp. 627-644).

Jakopanec, I., Borgen, K., Vold, L., Lund, H., Forseth, T., Hannula, R., & Nygård, K. (2008). A large waterborne outbreak of *Campylobacter*iosis in Norway: the need to focus on distribution system safety. *BMC Infect. Dis., 8*(1), 128.

James C., James S. J., Hannay N., Purnell G., Barbedo-Pinto C., Yaman H., Araujo M., Gonzalez M. L., Calvo J., Howell M., & Corry J. E. (2007). Decontamination of poultry carcasses using steam or hot water in combination with rapid cooling, chilling or freezing of carcass surfaces. *Int. J. Food Microbiol. 114*, 195-203.

Jensen, A. N., Andersen, M., Dalsgaard, A., Baggesen, D. L., & Nielsen, E. (2005). Development of real-time PCR and hybridization methods for detection and identification of thermophilic *Campylobacter spp.* in pig faecal samples. *J. Appl. Microbiol., 99*(2), 292-300.

Jeon, B., Muraoka, W. T., & Zhang, Q. (2010). Advances in *Campylobacter* biology and implications for biotechnological applications. *Microb. Biotechnol., 3*(3), 242-258.

Jeon, B., Wang, Y., Hao, H., Barton, Y.W. and Zhang, Q., 2011. Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni*. *J. Antimicrob. Chemother.*, *66*(1), 79-85.

Jones, F., & Little, R. B. (1931). The etiology of infectious diarrhea (winter scours) in cattle. *J. Exp. Med., 53*(6), 835-843.

Jones, M., Herd, T., & Christie, H. (1988). Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. *Microbios, 58*(234), 49-61.

Jones, K. (2001). *Campylobacters* in water, sewage and the environment. *J. Appl. Microbiol., 90*(S6), 68S-79S.

Jørgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D., Bolton, F., Humphrey, T. (2002). Prevalence and numbers of *Salmonella* and *Campylobacter spp.* on raw, whole chickens in relation to sampling methods. *Int. J. Food Microbiol., 76*(1), 151-164.

Josefsen, M. H., Jacobsen, N. R., Hoorfar, J. (2004). Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of food-borne thermotolerant *Campylobacters*. *Appl. Environ. Microbiol.,* 70(6) 3588-3592.

Karatzas, K. A., Webber, M. A., Jorgensen, F., Woodward, M. J., Piddock, L. J., & Humphrey, T. J. (2007). Prolonged treatment of *Salmonella enterica* serovar *Typhimurium* with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J. Antimicrob. Chemother., 60*(5), 947-955.

Kashmir, Z., & Mankr, S. (2014). Free radicals and oxidative stress in bacteria. *Int. J. Curr. Microbiol. Appl. Sci., 3*, 9-40.

Keener, K., Bashor, M., Curtis, P., Sheldon, B., & Kathariou, S. (2004). Comprehensive review of *Campylobacter* and poultry processing. *Compr. Rev. Food Sci. Food Saf, 3*(2), 105-116.

Kelly, D. (2001). The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. *J. Appl. Microbiol., 90*(S6), 16S-24S.

Kennedy, J., Nolan, A., Gibney, S., O'Brien, S., McMahon, M.A.S., McKenzie, K., Healy, B., McDowell, D., Fanning, S. and Wall, P.G., (2011a). Deteminants of cross-contamination during home food preparation. *British Food Journal*, *113*(2), 280-297.

Kennedy, J., Gibney, S., Nolan, A., O'Brien, S., McMahon, M.A.S., McDowell, D., Fanning, S. and Wall, P.G., (2011b). Identification of critical points during domestic food preparation: an observational study. *British Food Journal*, *113*(6), 766-783.

Keramas, G., Bang, D. D., Lund, M., Madsen, M., Bunkenborg, H., Telleman, P., & Christensen, C. B. V. (2004). Use of culture, PCR analysis, and DNA microarrays for detection of *Campylobacter jejuni* and *Campylobacter coli* from chicken feces. *J. Clin. Microbiol., 42*(9), 3985-3991.

Kim, J.-C., Oh, E., Kim, J., & Jeon, B. (2015). Regulation of oxidative stress resistance in *Campylobacter jejuni*, a microaerophilic foodborne pathogen. *Front. Microbiol., 6*.

Kim, J.-S., Carver, D. K., & Kathariou, S. (2006). Natural transformation-mediated transfer of erythromycin resistance in *Campylobacter coli* strains from turkeys and swine. *Appl. Environ. Microbiol., 72*(2), 1316-1321.

King, E. O. (1962). The laboratory recognition of *Vibrio fetus* and a closely related *Vibrio* isolated from cases of human *Vibrio*sis. *Ann. N.Y. Acad. Sci., 98*(3), 700-711.

Kint, C. I., Verstraeten, N., Fauvart, M., & Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends Microbiol., 20*(12), 577-585.

Kist, M. (1985). The historical background of *Campylobacter* infection: new aspects. In A. D. Pearson (Ed.), *Proceedings of the Third International Workshop on Campylobacter Infections, Ottawa, 7 - 10 July* (pp. 23-27).

Koenraad, P., Rombouts, F., & Notermans, S. (1997). Epidemiological aspects of thermophilic *Campylobacter* in water-related environments: a review. *Water Environ. Res., 69*(1), 52-63.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell, 130*(5), 797-810.

Kohanski, M. A., Dwyer, D. J., Wierzbowski, J., Cottarel, G., & Collins, J. J. (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell, 135*(4), 679-690.

Kohanski, M. A., Dwyer, D. J., & Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol., 8*(6), 423-435.

Kudirkienė, E., Bunevičienė, J., Brøndsted, L., Ingmer, H., Olsen, J. E., & Malakauskas, M. (2011). Evidence of broiler meat contamination with post-disinfection strains of *Campylobacter jejuni* from slaughterhouse. *Int. J. Food Microbiol., 145*, S116-S120.

Kumar-Phillips, G. S., Hanning, I., & Slavik, M. (2013). Effect of Stress-Adaptation on Antibiotic Sensitivity Profiles of *Campylobacter jejuni*. *Adv. Microbiol., 3*(1), 76-82.

Labesse, G., Garnotel, E., Bonnel, S., Dumas, C., Pages, J. M., & Bolla, J. M. (2001). MOMP, a divergent porin from *Campylobacter*: cloning and primary structural characterization. *Biochem. Biophys. Res. Commun., 280*(1), 380-387.

Lambert, P. (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. *J. Appl. Microbiol., 92*(s1), 46S-54S.

Landoni, M.F. and Albarellos, G., 2015. The use of antimicrobial agents in broiler chickens. *Vet. J., 205*(1), 21-27.

Langsrud, S., & Sundheim, G. (1997). Factors contributing to the survival of poultry associated *Pseudomonas spp.* exposed to a quaternary ammonium compound. *J. Appl. Microbiol., 82*(6), 705-712.

Langsrud, S., Sidhu, M. S., Heir, E., & Holck, A. L. (2003). Bacterial disinfectant resistance—a challenge for the food industry. *Int. Biodeterior. Biodegradation, 51*(4), 283-290.

Langsrud, S., Sundheim, G., & Holck, A. (2004). Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. *J. Appl. Microbiol., 96*(1), 201-208.

Larsen, M. H., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., Wagner, M. (2014). Persistence of foodborne pathogens and their control in primary and secondary food production chains. *Food Control, 44*, 92-109.

Lavilla Lerma L., Benomar, N., Gálvez, A., & Abriouel, H. (2013). Prevalence of bacteria resistant to antibiotics and/or biocides on meat processing plant surfaces throughout meat chain production. *Int. J. Food Microbiol., 161*(2), 97-106.

Le Loir, Y., Baron, F., & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genet Mol Res, 2*(1), 63-76.

Leblanc-Maridor, M., Beaudeau, F., Seegers, H., Denis, M., & Belloc, C. (2011). Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiol., 11*(1), 113.

Lee, A., Smith, S. C., & Coloe, P. J. (1998). Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions. *J. Food Protect., 61*(12), 1609-1614.

Lenski, R. (1990). Quantifying fitness and gene stability in microorganisms. *Biotechnology (Reading, Mass.), 15*, 173-192.

Lenski, R. E., & Travisano, M. (1994). Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl. Acad. Sci., 91*(15), 6808-6814.

Levin, B. R., Perrot, V., & Walker, N. (2000). Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics, 154*(3), 985-997.

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat. Rev. Microbiol., 4*(7), 556-562.

Levy, A. (1946). A gastro-enteritis outbreak probably due to a bovine strain of *Vibrio*. *Yale J. Biol. Med., 18*(4), 243.

Li, X.-Z., & Nikaido, H. (2009). Efflux-mediated drug resistance in bacteria. *Drugs, 69*(12), 1555-1623.

Limoncu, M. H., Ermertcan, S., Çetin, Ç. B., Cosar, G., & Dinç, G. (2003). Emergence of phenotypic resistance to ciprofloxacin and levofloxacin in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains. *Int. J. Antimicrob. Agents, 21*(5), 420-424.

Lin, J., Michel, L. O., & Zhang, Q. (2002). CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob. Agents Chemother., 46*(7), 2124-2131.

Lin, J., Akiba, M., Sahin, O., & Zhang, Q. (2005). CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. *Antimicrob. Agents Chemother., 49*(3), 1067-1075.

Lin, J., & Martinez, A. L. (2006). Effect of efflux pump inhibitors on bile resistance and *in vivo* colonization of *Campylobacter jejuni*. *J. Antimicrob. Chemother., 58*(5), 966-972.

Lode, H. M. (2014). Preserving the efficacy of front-line fluoroquinolones through selective use to optimise clinical outcomes. *Int. J. Antimicrob. Agents, 43*(6), 497-507.

Lomovskaya, O., & Bostian, K. A. (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic—a vision for applied use. *Biochem. Pharmacol., 71*(7), 910-918.

Lopez, G.U., Kitajima, M., Sherchan, S.P., Sexton, J.D., Sifuentes, L.Y., Gerba, C.P. and Reynolds, K.A., (2015). Impact of disinfectant wipes on the risk of *Campylobacter jejuni* infection during raw chicken preparation in domestic kitchens. *J. Appl. Microbiol*., 119(1), 245-252.

Loughlin, M., Jones, M., & Lambert, P. (2002). *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *J. Antimicrob. Chemother., 49*(4), 631-639.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C. M., & Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol., 4*(2), 189-200.

Luber, P. (2009). Cross-contamination versus undercooking of poultry meat or eggs—which risks need to be managed first? *Int. J. Food Microbiol., 134*(1), 21-28.

Luo, N., Sahin, O., Lin, J., Michel, L. O., & Zhang, Q. (2003). *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother., 47*(1), 390-394.

Madden, R. H., Moran, L., Scates, P., McBride, J., & Kelly, C. (2011). Prevalence of *Campylobacter* and *Salmonella* in raw chicken on retail sale in the republic of Ireland. *J. Food Protect., 74*(11), 1912-1916.

Maillard, J. Y. (2002). Bacterial target sites for biocide action. *J. Appl. Microbiol., 92*(s1), 16S-27S.

Maillard, J.-Y. (2005). Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Ther. Clin. Risk Manag., 1*(4), 307.

Maillard, J. Y. (2012). Mechanisms of bacterial resistance to microbicides. *Russell, Hugo & Ayliffe's: Principles and Practice of Disinfection, Preservation and Sterilization, 5th Edition*, 108-120.

Marquez, B. (2005). Bacterial efflux systems and efflux pumps inhibitors. *Biochimie, 87*(12), 1137-1147.

Martinez, A. l., & Lin, J. (2006). Effect of an efflux pump inhibitor on the function of the multidrug efflux pump CmeABC and antimicrobial resistance in *Campylobacter*. *Foodborne Pathog. Dis., 3*(4), 393-402.

Martínez J. L., Baquero F., Andersson D. I. (2011). Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. *Curr. Opin. Pharmacol*. 11, 439-445.

McCay, P. H., Ocampo-Sosa, A. A., & Fleming, G. T. (2010). Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. *Microbiology, 156*(1), 30-38.

McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev., 12*(1), 147-179.

McFadyean, J., & Stockman, S. (1913). *Abortion in sheep: Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to enquire into epizootic abortion*.

McGill, K., Cowley, D., Moran, L., Scates, P., O'leary, A., Madden, R., Fanning, S. (2006). Antibiotic resistance of retail food and human *Campylobacter* isolates on the island of Ireland from 2001–2002. *Epidemiol. Infect., 134*(06), 1282-1291.

Meyer, B. (2006). Does microbial resistance to biocides create a hazard to food hygiene? *Int. J. Food Microbiol., 112*(3), 275-279.

Meyer, B., Morin, V., Rödger, H. J., Holah, J., & Bird, C. (2010a). Do European Standard Disinfectant tests truly simulate in-use microbial and organic soiling conditions on food preparation surfaces? *J. Appl. Microbiol., 108*(4), 1344-1351.

Meyer, J. R., Agrawal, A. A., Quick, R. T., Dobias, D. T., Schneider, D., & Lenski, R. E. (2010b). Parallel changes in host resistance to viral infection during 45,000 generations of relaxed selection. *Evolution, 64*(10), 3024-3034.

Moen, B., Oust, A., Langsrud, Ø., Dorrell, N., Marsden, G.L., Hinds, J., Kohler, A., Wren, B.W. and Rudi, K., (2005). Explorative multifactor approach for investigating global survival mechanisms of *Campylobacter jejuni* under environmental conditions. *Appl. Environ. Microbiol.*, *71*(4), 2086-2094.

Moken, M.C., McMurry, L.M. and Levy, S.B., 1997. Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and acrAB loci. *Antimicrob. Agents* Chemother*., 41*(12), 2770-2772.

Mokgatla, R., Brözel, V., & Gouws, P. (1998). Isolation of *Salmonella* resistant to hypochlorous acid from a poultry abattoir. *Lett. Appl. Microbiol., 27*(6), 379-382.

Monod, J. (1942). *Recherches sur la croissance des cultures bactériennes*. Paris: Hermann.

Moore, J., Caldwell, P., & Millar, B. (2001). Molecular detection of *Campylobacter spp.* in drinking, recreational and environmental water supplies. *Int. J. Hyg. Environ. Health, 204*(2), 185-189.

Moore, J. E., Corcoran, D., Dooley, J. S., Fanning, S., Lucey, B., Matsuda, M., O'Mahony, R. (2005). *Campylobacter*. *Vet. Res., 36*(3), 351-382.

Moreno, Y., Botella, S., Alonso, J. L., Ferrús, M. A., Hernández, M., & Hernández, J. (2003). Specific detection of Arcobacter and *Campylobacter* strains in water and sewage by PCR and fluorescent *in situ* hybridization. *Appl. Environ. Microbiol., 69*(2), 1181-1186.

Morris, G., El Sherbeeny, M., Patton, C., Kodaka, H., Lombard, G., Edmonds, P., Brenner, D. (1985). Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter spp. J. Clin. Microbiol., 22*(5), 714-718.

Možina, S. S., Kurinčič, M., Klančnik, A., & Mavri, A. (2011). *Campylobacter* and its multi-resistance in the food chain. *Trends Food Sci. Tech., 22*(2), 91-98.

Murphy, C., Carroll, C., & Jordan, K. N. (2003). Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. *FEMS Microbiol. Lett., 223*(1), 89-93.

Murphy, C., Carroll, C., & Jordan, K. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol., 100*(4), 623-632.

Nachamkin, I., Allos, B. M., & Ho, T. (1998). *Campylobacter* species and Guillain-Barre syndrome. *Clin. Microbiol. Rev., 11*(3), 555-567.

Nachamkin, I., Liu, J., Li, M., Ung, H., Moran, A. P., Prendergast, M. M., & Sheikh, K. (2002). *Campylobacter jejuni* from patients with Guillain-Barré syndrome preferentially expresses a GD1a-like epitope. *Infect. Immun., 70*(9), 5299-5303.

Nakari, U.-M., Puhakka, A., & Siitonen, A. (2008). Correct identification and discrimination between *Campylobacter jejuni* and C. *coli* by a standardized hippurate test and species-specific polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis., 27*(7), 513-518.

Nauta, M., Hill, A., Rosenquist, H., Brynestad, S., Fetsch, A., van der Logt, P., Borck, B. (2009). A comparison of risk assessments on *Campylobacter* in broiler meat. *Int. J. Food Microbiol., 129*(2), 107-123.

Nauta, M.J., Fischer, A.R., Van Asselt, E.D., De Jong, A.E., Frewer, L.J. and De Jonge, R., (2008). Food safety in the domestic environment: the effect of consumer risk information on human disease risks. *Risk Anal., 28*(1), 179-192.

Nauta, M. J., & Havelaar, A. H. (2008). Risk-based standards for *Campylobacter* in the broiler meat chain. *Food Control, 19*(4), 372-381.

Newell, D., Elvers, K., Dopfer, D., Hansson, I., Jones, P., James, S., Connerton, I. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter spp.* on poultry farms. *Appl. Environ. Microbiol., 77*(24), 8605-8614.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev., 67*(4), 593-656.

Nikaido, H. (2009). Multidrug resistance in bacteria. *Annual review of biochemistry, 78*, 119.

Nikaido, H., & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev., 49*(1), 1.

Nogva, H. K., Bergh, A., Holck, A., & Rudi, K. (2000). Application of the 5′-Nuclease PCR Assay in Evaluation and Development of Methods for Quantitative Detection of *Campylobacter jejuni*. *Appl. Environ. Microbiol., 66*(9), 4029-4036.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem., 250*(10), 4007-4021.

O'Mahony, E., Buckley, J. F., Bolton, D., Whyte, P., & Fanning, S. (2011). Molecular epidemiology of *Campylobacter* isolates from poultry production units in southern Ireland. *PLoS One, 6*(12), e28490.

O'Reilly, A., & Smith, P. (1999). Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. *Aquaculture, 180*(1), 1-11.

Oliveira, T. C., Barbut, S., & Griffiths, M. W. (2005). Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. *Int. J. Food Microbiol., 104*(1), 105-111.

Oliver, J. D. (1993). Formation of viable but nonculturable cells. In S. Kjelleberg (Ed.), *Starvation in Bacteria* (pp. 239-272): Springer US.

On, S. (1996). Identification methods for *Campylobacters*, *Helicobacter*s, and related organisms. *Clin. Microbiol. Rev., 9*(3), 405-422.

On, S. L.W. (2013). Isolation, identification and subtyping of *Campylobacter*: where to from here?. *J. Microbiol. Methods, 95*(1), 3-7.

Ortega Morente, E., Fernández-Fuentes, M. A., Burgos, M. J. G., Abriouel, H., Pulido, R. P., & Gálvez, A. (2013). Biocide tolerance in bacteria. *Int. J. Food Microbiol., 162*(1), 13-25.

Ozaki, S., Kano, K., & Shirai, O. (2008). Electrochemical elucidation on the mechanism of uncoupling caused by hydrophobic weak acids. *Phys. Chem. Chem. Phys., 10*(30), 4449-4455.

Page, W. J., Huyer, G., Huyer, M., & Worobec, E. (1989). Characterization of the porins of *Campylobacter jejuni* and *Campylobacter coli* and implications for antibiotic susceptibility. *Antimicrob. Agents Chemother., 33*(3), 297-303.

Pagès, J.-M., Masi, M., & Barbe, J. (2005). Inhibitors of efflux pumps in Gram-negative bacteria. *Trends Mol. Med., 11*(8), 382-389.

Pagès J.M., Sandrine A.F., Mahamoud A., Bolla J.M., Davin-Regli A., Chevalier J., Garnotel E. (2010). Efflux pumps of Gram-negative bacteria, a new target for new molecules. *Curr Top Med Chem.* 10(18), 1848-1857.

Palyada, K., Sun, Y.-Q., Flint, A., Butcher, J., Naikare, H., & Stintzi, A. (2009). Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics, 10*(1), 481.

Pan, Y., Breidt, F., & Kathariou, S. (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Appl. Environ. Microbiol., 72*(12), 7711-7717.

Park, N.Y., Ro, E.Y., Jo, H.J., Park, K.S. and Yoon, K.S., (2014). Effect of Packaging and Temperature on Survival Kinetics of *Campylobacter jejuni* on Precooked Chicken Breast. *J Food Saf, 34*(4), 371-379.

Park, S. F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol., 74*(3), 177-188.

Parkhill, J., Wren, B., Mungall, K., Ketley, J., Churcher, C., Basham, D., Holroyd, S. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature, 403*(6770), 665-668.

Parsek, M. R., & Fuqua, C. (2004). Biofilms 2003: emerging themes and challenges in studies of surface-associated microbial life. *J. Bacteriol., 186*(14), 4427-4440.

Patterson, M. (1995). Sensitivity of *Campylobacter spp.* to irradiation in poultry meat. *Lett. Appl. Microbiol., 20*(6), 338-340.

Payne, J., Kroger, E., & Watkins, S. (2005). Evaluation of disinfectant efficacy when applied to the floor of poultry grow-out facilities. *J. Appl. Poult. Res., 14*(2), 322-329.

Payot, S., Bolla, J.-M., Corcoran, D., Fanning, S., Mégraud, F., & Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in *Campylobacter spp. Microbes Infect., 8*(7), 1967-1971.

Pead, P. (1979). Electron microscopy of *Campylobacter jejuni*. *J. Med. Microbiol., 12*(3), 383-385.

Pearson, A., Greenwood, M., Healing, T., Rollins, D., Shahamat, M., Donaldson, J., & Colwell, R. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol., 59*(4), 987-996.

Petrosino, J., Cantu, C., & Palzkill, T. (1998). β-Lactamases: protein evolution in real time. *Trends Microbiol., 6*(8), 323-327.

Peyrat, M., Soumet, C., Maris, P., & Sanders, P. (2008). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol., 124*(2), 188-194.

Piddock, L. J., Ricci, V., Pumbwe, L., Everett, M. J., & Griggs, D. J. (2003). Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J. Antimicrob. Chemother., 51*(1), 19-26.

Piddock, L. J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev., 19*(2), 382-402.

Pike, B.L., Guerry, P. and Poly, F., (2013). Global distribution of *Campylobacter jejuni* Penner serotypes: a systematic review. *PloS one, 8*(6), e67375.

Pinto-Alphandary, H., Mabilat, C., & Courvalin, P. (1990). Emergence of aminoglycoside resistance genes aadA and aadE in the genus *Campylobacter*. *Antimicrob. Agents Chemother., 34*(6), 1294-1296.

Pomposiello, P. J., Bennik, M. H. J., & Demple, B. (2001). Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J. Bacteriol., 183*(13), 3890-3902.

Poole, K. (2002). Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. *Curr. Pharm. Biotechnol., 3*(2), 77-98.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother., 56*(1), 20-51.

Poole, K. (2012). Bacterial stress responses as determinants of antimicrobial resistance. *J. Antimicrob. Chemother.*, dks196.

Poppert, S., Haas, M., Yildiz, T., Alter, T., Bartel, E., Fricke, U., & Essig, A. (2008). Identification of thermotolerant *Campylobacter* species by fluorescence *in situ* hybridization. *J. Clin. Microbiol., 46*(6), 2133-2136.

Potenski, C. J., Gandhi, M., & Matthews, K. R. (2003). Exposure of *Salmonella Enteritidis* to chlorine or food preservatives increases susceptibility to antibiotics. *FEMS Microbiol. Lett., 220*(2), 181-186.

Pumbwe, L., Randall, L. P., Woodward, M. J., & Piddock, L. J. (2004). Expression of the efflux pump genes cmeB, cmeF and the porin gene porA in multiple-antibiotic-resistant *Campylobacter jejuni*. *J. Antimicrob. Chemother., 54*(2), 341-347.

Pumbwe, L., Randall, L. P., Woodward, M. J., & Piddock, L. J. (2005). Evidence for multiple-antibiotic resistance in *Campylobacter jejuni* not mediated by CmeB or CmeF. *Antimicrob. Agents Chemother., 49*(4), 1289-1293.

Quinn, T., Bolla, J.-M., Pagès, J.-M., & Fanning, S. (2007). Antibiotic-resistant *Campylobacter*: could efflux pump inhibitors control infection? *J. Antimicrob. Chemother., 59*(6), 1230-1236.

Radhouani, H., Pinto, L., Poeta, P., & Igrejas, G. (2012). After genomics, what proteomics tools could help us understand the antimicrobial resistance of *Escherichia coli*? *J. Proteomics, 75*(10), 2773-2789.

Rajkovic, A., Tomic, N., Smigic, N., Uyttendaele, M., Ragaert, P., & Devlieghere, F. (2010). Survival of *Campylobacter jejuni* on raw chicken legs packed in high-oxygen or high-carbon dioxide atmosphere after the decontamination with lactic acid/sodium lactate buffer. *Int. J. Food Microbiol., 140*(2), 201-206.

Randall, L., Ridley, A., Cooles, S., Sharma, M., Sayers, A., Pumbwe, L., & Woodward, M. J. (2003). Prevalence of multiple antibiotic resistance in 443

Campylobacter spp. isolated from humans and animals. *J. Antimicrob. Chemother., 52*(3), 507-510.

Remaut, H., & Waksman, G. (2004). Structural biology of bacterial pathogenesis. *Curr. Opin. Struct. Biol., 14*(2), 161-170.

Resuggan, J. (1952). The antibacterial activity of quaternary ammonium compounds. *J. Appl. Microbiol., 15*(1), 166-171.

Robyn, J., Rasschaert, G., Pasmans, F. and Heyndrickx, M., (2015). Thermotolerant *Campylobacter* during broiler rearing: Risk factors and intervention. *Compr Rev Food Sci Food Saf., 14*(2), pp.81-105.

Rollins, D., & Colwell, R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol., 52*(3), 531-538.

Rosenquist, H., Nielsen, N. L., Sommer, H. M., Nørrung, B., & Christensen, B. B. (2003). Quantitative risk assessment of human *Campylobacter*iosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol., 83*(1), 87-103.

Rossi, M., Debruyne, L., Zanoni, R. G., Manfreda, G., Revez, J., & Vandamme, P. (2009). *Campylobacter* avium sp. nov., a hippurate-positive species isolated from poultry. *Int. J. Syst. Evol. Microbiol., 59*(9), 2364-2369.

Russell, A., & Path, F. (1986). Chlorhexidine: antibacterial action and bacterial resistance. *Infection, 14*(5), 212-215.

Russell, A. D. (1991). Principles of antimicrobial activity. . In S. S. Block (Ed.), *Disinfection, Sterilization, and Preservation* (pp. 27-58.): Lea & Febiger.

Russell, A. (1997). Plasmids and bacterial resistance to biocides. *J. Appl. Microbiol., 83*(2), 155-165.

Russell, A. (2003). Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect. Dis., 3*(12), 794-803.

Sails, A. D., Swaminathan, B., & Fields, P. I. (2003). Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by *Campylobacter jejuni*. *J. Clin. Microbiol., 41*(10), 4733-4739.

Sampers, I., Habib, I., De Zutter, L., Dumoulin, A., & Uyttendaele, M. (2010). Survival of *Campylobacter spp.* in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *Int. J. Food Microbiol., 137*(2), 147-153.

Sauer, U. (2001). Evolutionary engineering of industrially important microbial phenotypes *Metabolic Engineering* (pp. 129-169): Springer.

SCENIHR. (2009). *Assessment of the Antibiotic Resistance Effects of Biocides*. Scientific Committee on Emerging and Newly Identified Health Risks. Brussels, Belgium: Retrieved from http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf

SCCS (2010). *Opinion on triclosan (antimicrobial resistance)* 22 June 2010. Scientific Committee on Consumer Safety. Brussels, Belgium: Retrieved from http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_023.pdf

Scherer, K., Bartelt, E., Sommerfeld, C., & Hildebrandt, G. (2006). Comparison of different sampling techniques and enumeration methods for the isolation and quantification of *Campylobacter spp.* in raw retail chicken legs. *Int. J. Food Microbiol., 108*(1), 115-119.

Schönberg-Norio, D., Hänninen, M.-L., Katila, M.-L., Kaukoranta, S.-S., Koskela, M., Eerola, E., Rautelin, H. (2006). Activities of telithromycin, erythromycin, fluoroquinolones, and doxycycline against *Campylobacter* strains isolated from Finnish subjects. *Antimicrob. Agents Chemother., 50*(3), 1086-1088.

Schrag, S. J., Perrot, V., & Levin, B. R. (1997). Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. *Proceedings of the Royal Society of London B: Biological Sciences, 264*(1386), 1287-1291.

Schröder, W., & Moser, I. (1997). Primary structure analysis and adhesion studies on the major outer membrane protein of *Campylobacter jejuni*. *FEMS Microbiol. Lett., 150*(1), 141-147.

Scott, N. E., & Cordwell, S. J. (2009). *Campylobacter* proteomics: guidelines, challenges and future perspectives. *Expert Rev. Proteomics, 6*(1), 61-74.

Seaman, P. F., Ochs, D., & Day, M. J. (2007). Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother., 59*(1), 43-50.

Sebald, M. V., M. (1963). Teneur en bases de 1'ADN et classification des *Vibrio*ns. *Ann. Inst. Pasteur, 105*(5), 897-910.

Sheridan, À., Lenahan, M., Duffy, G., Fanning, S., & Burgess, C. (2012). The potential for biocide tolerance in *Escherichia coli* and its impact on the response to food processing stresses. *Food Control, 26*(1), 98-106.

Sierra, J., Ortega, M., Tarrago, C., Albet, C., Vila, J., Terencio, J., & Guglietta, A. (2009). Decreased linezolid uptake in an *in vitro*-selected linezolid-resistant *Staphylococcus epidermidis* mutant. *J. Antimicrob. Chemother., 64*(5), 990-992.

Silbergeld, E. K., Graham, J., & Price, L. B. (2008). Industrial food animal production, antimicrobial resistance, and human health. *Annu. Rev. Public Health, 29*, 151-169.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., & Teixeira, P. (2011). *Campylobacter spp.* as a foodborne pathogen: a review. *Front. Microbiol., 2*.

Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT-Food Sci. Technol., 43*(4), 573-583.

Skirrow, M., & Blaser, M. (2000). Clinical aspects of *Campylobacter* infection. In I. Nachamkin & M. J. Blaser (Eds.), *Campylobacter* (pp. 68-88): ASM Press.

Smibert, R. M. (1978). The genus *Campylobacter*. *Annu. Rev. Microbiol., 32*(1), 673-709.

Smith, J. L., & Fratamico, P. M. (2010). Fluoroquinolone resistance in *Campylobacter*. *J. Food Protect., 73*(6), 1141-1152.

Smith, T., & Taylor, M. S. (1919). Some morphological and biological characters of the spirilla (*Vibrio fetus, n. sp*.) associated with disease of the fetal membranes in cattle. *J. Exp. Med., 30*(4), 299-311.

Smith J. L., Fratamico P. M. (2010). Fluoroquinolone resistance in *Campylobacter*. *J. Food Prot.* 73(6), 1141-1152.

Snelling, W., Matsuda, M., Moore, J., & Dooley, J. (2005a). *Campylobacter jejuni*. *Lett. Appl. Microbiol., 41*(4), 297-302.

Snelling, W.J., McKenna, J.P., Lecky, D.M. and Dooley, J.S.G., (2005b). Survival of *Campylobacter jejuni* in waterborne protozoa. *Appl. Environ. Microbiol., 71*(9), 5560-5571.

Snyder, L. A., Rismondo, V., & Miller, N. R. (2009). The Fisher variant of Guillain-Barré syndrome (Fisher syndrome). *J. Neuroophthalmol., 29*(4), 312-324.

Sterzo, E., Paiva, J., Penha Filho, R., & Berchieri Junior, A. (2005). Time required to protect the intestinal tract of chicks against *Salmonella enterica* serovar *Enteritidis* using competitive exclusion. *Rev. Bras. Cienc. Avic., 7*(2), 119-122.

Stewart, P. S., & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet, 358*(9276), 135-138.

Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol., 292*(2), 107-113.

Stöhr, K., & Wegener, H. C. (2000). Animal use of antimicrobials: impact on resistance. *Drug Resist. Updat., 3*(4), 207-209.

Taboada, E. N., Clark, C. G., Sproston, E. L., & Carrillo, C. D. (2013). Current methods for molecular typing of *Campylobacter* species. *J. Microbiol. Methods, 95*(1), 24-31.

Taché, J. and Carpentier, B., (2014). Hygiene in the home kitchen: Changes in behaviour and impact of key microbiological hazard control measures. *Food Control, 35*(1), pp.392-400.

Takkinen, J., Ammon, A., Robstad, O., & Breuer, T. (2003). European survey on *Campylobacter* surveillance and diagnosis 2001. *Euro surveillance: bulletin Europeen sur les maladies transmissibles-European communicable disease bulletin, 8*(11), 207-213.

Taylor, D. E., & Courvalin, P. (1988). Mechanisms of antibiotic resistance in *Campylobacter* species. *Antimicrob. Agents Chemother., 32*(8), 1107.

Thomas, L., Maillard, J.-Y., Lambert, R., & Russell, A. (2000). Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a'residual'concentration. *J. Hosp. Infect., 46*(4), 297-303.

Trachoo, N., Frank, J., & Stern, N. (2002a). Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. *J. Food Protect., 65*(7), 1110-1116.

Trachoo, N., & Frank, J. F. (2002b). Effectiveness of chemical sanitizers against *Campylobacter jejuni*-containing biofilms. *J. Food Protect., 65*(7), 1117-1121.

Travers, K., & Michael, B. (2002). Morbidity of infections caused by antimicrobial-resistant bacteria. *Clin. Infect. Dis., 34*(Supplement 3), S131-S134.

Van Bambeke, F., Michot, J. M., Van Eldere, J., & Tulkens, P. M. (2005). Quinolones in 2005: an update. *Clin. Microbiol. Infect., 11*(4), 256-280.

Van Houdt, R., & Michiels, C. W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *J. Appl. Microbiol., 109*(4), 1117-1131.

Vandamme, P., Debruyne, L., De Brandt, E., & Falsen, E. (2010). Reclassification of Bacteroides ureolyticus as *Campylobacter* ureolyticus comb. nov., and emended description of the genus *Campylobacter*. *Int. J. Syst. Evol. Microbiol., 60*(9), 2016-2022.

Velayudhan, J., & Kelly, D. J. (2002). Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology, 148*(3), 685-694.

Waage, A. S., Vardund, T., Lund, V., & Kapperud, G. (1999). Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. *Appl. Environ. Microbiol., 65*(4), 1636-1643.

Wagenaar, J., Mevius, D., & Havelaar, A. (2006). *Campylobacter* in primary animal production and control strategies to reduce the burden of human *Campylobacter*iosis. *Rev Sci Tech, 25*(2), 581-594.

Walsh, S. E., Maillard, J.-Y., Russell, A., Catrenich, C., Charbonneau, D., & Bartolo, R. (2003). Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. *J. Hosp. Infect., 55*(2), 98-107.

Wang, P., Lv, C., & Zhu, G. (2015). Novel Type II and Monomeric NAD+ Specific Isocitrate Dehydrogenases: Phylogenetic Affinity, Enzymatic Characterization, and Evolutionary Implication. *Sci. Rep., 5*. doi:10.1038/srep09150

Wang, X., & Zhao, X. (2009). Contribution of oxidative damage to antimicrobial lethality. *Antimicrob. Agents Chemother., 53*(4), 1395-1402.

Wang, X., Zhao, X., Malik, M., & Drlica, K. (2010). Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. *J. Antimicrob. Chemother., 65*(3), 520-524.

Wang, Y., & Taylor, D. (1990). Natural transformation in *Campylobacter* species. *J. Bacteriol., 172*(2), 949-955.

Wanyenya, I., Muyanja, C., & Nasinyama, G. W. (2004). Kitchen practices used in handling broiler chickens and survival of *Campylobacter spp.* on cutting surfaces in Kampala, Uganda. *J. Food Protect., 67*(9), 1957-1960.

Wassenaar, T. M., & Newell, D. G. (1999). The genus *Campylobacter*. In M. Dworkin (Ed.), *The prokaryotes an evolving electronic resource for the microbiological community* (3rd ed. ed.). New York :: Springer-Verlag.

Wassenaar, T. M., & Newell, D. G. (2000). Genotyping of *Campylobacterspp. Appl. Environ. Microbiol., 66*(1), 1-9.

Wassenaar, T. M. (2011). Following an imaginary *Campylobacter* population from farm to fork and beyond: a bacterial perspective. *Lett. Appl. Microbiol., 53*(3), 253-263.

Wegener, H. C. (2010). Danish initiatives to improve the safety of meat products. *Meat Sci., 84*(2), 276-283.

Weidenmaier, C., & Peschel, A. (2008). Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat. Rev. Microbiol., 6*(4), 276-287.

Werner, G., Hildebrandt, B., & Witte, W. (2001). Aminoglycoside-Streptothricin resistance gene cluster aadE–sat4–aphA-3 disseminated among multiresistant isolates of *Enterococcus faecium*. *Antimicrob. Agents Chemother., 45*(11), 3267-3269.

White, P., Baker, A., & James, W. (1997). Strategies to control *Salmonella* and *Campylobacter* in raw poultry products. *Rev. Sci. Tech. O.I.E., 16*, 525-541.

Whyte, P., McGill, K., Cowley, D., Madden, R., Moran, L., Scates, P., Collins, J. (2004). Occurrence of *Campylobacter* in retail foods in Ireland. *Int. J. Food Microbiol., 95*(2), 111-118.

Wieczorek, K., & Osek, J. (2013). Antimicrobial resistance mechanisms among *Campylobacter*. *Biomed Res. Int., 2013*.

Wilson, D. L., Bell, J. A., Young, V. B., Wilder, S. R., Mansfield, L. S., & Linz, J. E. (2003). Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. *Microbiology, 149*(12), 3603-3615.

Wilson D. J, Gabriel E., Leatherbarrow A. J., Cheesbrough J., Gee S., Bolton E., Fox A., Fearnhead P., Hart C. A., Diggle P. J. (2008). Tracing the source of campylobacteriosis. *PLoS Genet.* 4(9), e1000203.

Winder, C. L., & Lanthaler, K. (2011). The use of continuous culture in systems biology investigations. *Methods Enzymol, 500*, 261-275.

Wirz, S. E., Overesch, G., Kuhnert, P., & Korczak, B. M. (2010). Genotype and antibiotic resistance analyses of *Campylobacter* isolates from ceca and carcasses of slaughtered broiler flocks. *Appl. Environ. Microbiol., 76*(19), 6377-6386.

Yan, M., Sahin, O., Lin, J., & Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J. Antimicrob. Chemother., 58*(6), 1154-1159.

Zhuang, J., Engel, A., Pages, J.-M., & Bolla, J.-M. (1997). The *Campylobacter jejuni* porin trimers pack into different lattice types when reconstituted in the presence of lipid. *Eur. J. Biochem., 244*(2), 575-579.

Adaptation of *Campylobacter jejuni* NCTC 11168 to antibiotic and biocide by means of chemostat enrichment

Olivia Duignan[1], Gerard Fleming[2] and Cyril Carroll[1].

1. Enteric Pathogen Research Laboratory, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland. 2. Microbial Oceanography Research Unit, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland.

2.1 Abstract

Campylobacter spp. remain highly important zoonotic pathogens and are recognized as major causes of human gastroenteritis worldwide. This work used an *in vitro* chemostat model to characterise changes in antimicrobial susceptiblity of *Campylobacter jejuni* NCTC 11168 (*C. jejuni*) following long-term exposure to the antibiotic ciprofloxacin (MIC_{Broth}: 0.06 mg l^{-1}, MIC_{Agar}: 0.125mg l^{-1}) and the biocide benzalkonium chloride (BKC; MIC_{Broth}: 0.2 mg l^{-1}, MIC_{Agar}: 8 mg l^{-1}). Cross-resistance between BKC-adapted chemostat populations and the antibiotics ciprofloxacin and cefotaxime was also assessed. Pulse field gel electrophoresis was used to determine the identity of the adapted variants as *C. jejuni* NCTC 11168. Ciprofloxacin-adapted variants had MICs 1024-fold greater than the parent strain. The largest increase in MIC_{Agar} for ciprofloxacin (0.125 to 4 mg l^{-1} ciprofloxacin: 32-fold increase compared to original strain) occurred after concentrations of applied selective pressure exceeded the MIC_{broth} of the parent strain to ciprofloxacin (>0.06 mg l^{-1}). In contrast, exposure to BKC induced a slow stepwise decrease in susceptibility to BKC. BKC-adapted variants of *C. jejuni* had MIC_{Agar} values of 64 mg l^{-1} (8-fold higher then the original strain) and adapted variants were stable (no decrease in MIC_{Agar} after 19 generations growth in absence of selection pressure). Sub-MIC_{broth} concentrations of antimicrobial selective pressure did not alter the chemostat population MIC_{Agar} to BKC or ciprofloxacin. Adaptation to BKC resulted in low-level cross-resistance to ciprofloxacin but not to cefotaxime. These results confirm that *Campylobacter* readily acquires resistance to ciprofloxacin and suggests that exposure to concentrations of antimicrobial agent, above MIC concentrations are required, for adaptation of *C. jejuni* to BKC and ciprofloxacin.

Keywords: *Campylobacter*, Ciprofloxacin, Benzalkonium chloride, Chemostat.

2.2 Introduction

Campylobacter spp. are the leading cause of foodborne illness in the world and poultry produce constitutes a very significant vehicle for the transmission of *Campylobacter* to humans (FSAI, 2011). Internationally, it is estimated that thirty percent of all *Campylobacter* infections can be attributed to the handling, preparation and consumption of undercooked poultry (ESFA, 2010). In 2014, 2,615 notifications of campylobacteriosis were reported to the Irish health protection surveillance centre representing an incidence rate of 57.0 cases per 100,000 of the population, approximately eight times the incidence of salmonellosis (HPSC, 2014) and represents an increase of 57.4 % compared to 2010. Antibiotics are important to limit the duration of illness caused by infection with *Campylobacter spp.*. Antibiotic resistance has become a significant and increasing public health problem, and infection with antimicrobial resistant organisms leads to treatment failures and increased morbidity, mortality, length of hospitalisation and cost of healthcare (Cosgrove, 2006; Lavilla Lerma *et al.,* 2013). The use of antibiotics in food production animals is strictly regulated in the EU and antimicrobial veterinary medicinal products may be supplied on prescription only basis (ESVAC, 2013). The use of fluoroquinolone antibiotics in poultry flocks, demonstrated unequivocally to select for ciprofloxacin-resistant *Campylobacter* in commercially- reared poultry, potentially compromises treatment of infection by antibiotic therapy (Humphrey *et al.,* 2005; McGill *et al.,* 2006).

Good hygiene practices and biosecurity constitute the most effective and economically viable strategy available for controlling the transmission of *Campylobacter* in the poultry industry. Disinfectants (biocides) are an integral part of good hygiene practices for the production of safe and wholesome food produce including poultry. The improper use of disinfectants may result in regular exposure of food borne pathogens to residual (sub-lethal) concentrations of disinfectants for prolonged periods. This may play a role in the development of reduced susceptibility to disinfectants and/or disinfectant failure (Langsrud *et al.,* 2003; Lavilla Lerma *et al.,* 2013). Benzalkonium chloride (BKC) belongs to the quaternary ammonium compound class of disinfectants and is widely used in the poultry industry in disinfectant formulations (Peyrat *et al.,* 2008). Studies have shown that *Campylobacter* can survive and persist on surfaces after cleaning and disinfection

with BKC-based disinfectants (Kudirkiene *et al.,* 2011; Peyrat *et al.,* 2008). Survival of antibiotic resistant foodborne pathogens on food contact surfaces after cleaning and disinfection can increase the risk of cross-contamination of products and represents a potential food safety hazard (Langsrud *et al.,* 2003; Lavilla Lerma *et al.,* 2013; Meyer, 2006; Peyrat *et al.,* 2008). Furthermore, the development of bacteria with reduced susceptibility to disinfectants may also co-select for antibiotic-resistant strains particularly in cases where the two agents share a common resistance mechanism (Chapman, 2003).

The aim of this project was to study the potential for adaptive resistance in *C. jejuni* to the antibiotic ciprofloxacin and the biocide BKC using a chemostat model. This system allowed the susceptible strain *C. jejuni* NCTC 11168 to evolve and adapt to ciprofloxacin or BKC following prolonged culture (> 1000 h). Two additional chemostats were operated without applied selection pressures and were used as controls. Chemostat populations were monitored (OD, total viablity counts, antimicrobial susceptibility) to observe changes in the evolving chemostat population as a result of adaptation. Cross-resistance to the antibiotics ciprofloxacin and cefotaxamine in the BKC-adapted chemostat population was also assessed.

2.3 Materials and methods

2.3.1 Bacterial Strains

C. jejuni NCTC 11168 was the original strain used in this study and was obtained from Dr. Majella Mahar, the National Diagnositic Center, National University of Ireland, Galway. All other strains were derivatives of *C. jejuni* NCTC 11168 and were generated by means of selective/enrichment continuous cultures supplemented with either BKC or ciprofloxacin. All stocks were maintained in cryovials at -20°C for short term storage up to 1 month and at -70°C for long term storage. Stocks was resusitated on Campylobacter blood-free agar base without addition of the selective supplement (CCDA; CM0739, Oxoid Ltd., UK) under microaerobic conditions using an anerobic gas generating kit (BR0038B, Oxoid Ltd., UK) at 42^{o}C for 48 h.

2.3.2 Culture Media and Antimicrobials

Mueller Hinton broth (MHB; Lab114, LabM, UK), Brucella broth (BBL 211088, Becton Dickinson, Sparks, MD, USA) Mueller Hinton agar (MHA; Lab039, LabM, UK) and were prepared according to the manufacturers instructions. Unless otherwise stated, MHB and brucella broth were supplemented with 0.35% (v/v) IsoVitaleX™ (211875, Becton Dickinson Sparks, MD, USA) and MHA was supplemented with 5 % lysed horse blood (Charles River Laboratories, Ireland). Antimicrobials used in this study included Ciprofloxacin (Ciproxin®, supplied as a 2 mg ml^{-1} solution, Bayer, UK), cefotaxime (Sigma-Aldrich, Steinheim, Germany) and BKC (Sigma-Aldrich, Steinheim, Germany). Except for ciprofloxacin, all other antimicrobials were diluted to appropriate working concentrations in sterile deionized water and were filter-sterilized through a 0.2 μm Millipore^{TM} membrane filter before use.

2.3.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was carried out to determine the minimum inhibitory concentration of *C. jejuni* to ciprofloxacin and BKC prior to selection using chemostat enrichment, following the agar and broth microdilution methods as outlined by the Clinical Laboratory Standards Institute (CLSI, 2006) with some minor modifications. MIC_{Agar} values to both ciprofloxacin and BKC were determined from

samples taken from chemostat culture to monitor development of resistance in the adapted strains. Cross-resistance between biocides and antibiotics was also assessed in BKC-adapted populations to antibiotics cefotaxime and ciprofloxacin.

(i) Broth MIC determinations were carried out in duplicate on 96-U bottom well plates (Orange scientific) using 2-fold serial dilutions of antimicrobial agent in MHB or brucella broth. No difference in MIC_{broth} determinations were found when using either MHB or brucella broth. Brucella broth was better able to support growth of *Campylobacter* (as seen by growth of the positive control) and as a result brucella broth was used as the medium of choice for MIC_{broth} determinations in this study. Wells contained 180 μl of MHB or brucella broth and were supplemented with 0.35 % (v/v) IsoVitaleX™ and the appropriate concentration of antimicrobial agent (either ciprofloxacin or BKC). The concentrations of antimicrobial agents tested were 0.015-0.125 mg l^{-1} ciprofloxacin and 0.05-6.4 mg l^{-1} BKC. Wells were innoculated with 20 μl of overnight MHB or brucella broth cultures of *Campylobacter*. A sterile plastic adhesive cover was aseptically placed on top of the microtiter plate. The 96-well plate was then placed into a 96-well plate reader (Genios, Tecan) and incubated at 40 °C. The plate was shaken every 2 h for 30 sec and OD_{595nm} measurements were automatically recorded in triplicate for each well at two hourly intervals over a 48 h period. For the purposes of this study the minimum inhibitory concentration (MIC) of an antimicrobial agent was defined as being the minimum concentration of antimicrobial agent required to completely inhibit growth (no increase in OD_{595nm}) after 48 h of incubation.

(ii) Agar MIC determinations were carried out in duplicate using 2-fold serial dilutions of antimicrobial agent on MHA plates (supplemented with 5 % lysed horse blood). A 0.5 Mcfarland bacterial suspension of the strains was prepared in sterile phosphate buffered saline (PBS, Oxoid, UK) from 48 h-old plate culture of test strain. A 0.1 ml aliquot of the resultant suspension was spread-plated onto MHA plates (supplemented with 5 % lysed horse blood) containing 2- fold dilutions of the antimicrobial agent (either ciprofloxacin or BKC). The concentrations of antimicrobial agents tested were 0.015-0.25 mg l^{-1} for ciprofloxacin and 1-16 mg l^{-1} for BKC. A control plate of MHA supplemented with 5 % lysed horse blood without

antimicrobial agent was also included. All plates were incubated under microaerobic conditions using an anerobic gas generating kit at 42 ^{o}C for 48 h. For the purposes of this study the minimum inhibitory concentration (MIC) of an antimicrobial agent was defined as being the minimum concentration of antimicrobial agent required to completely inhibit growth of the strain after 48 h of incubation at 42 ^{o}C under microaerobic conditions.

The above method was slightly modified to determine the MIC_{Agar} of the chemostat population. Tenfold serial dilutions of a chemostat culture were made in sterile PBS. A 0.1 ml aliquot from each dilution were then plated onto MHA plates supplemented with 5 % lysed horse blood containing two-fold increasing concentrations of the apppropiate concentration of antimicrobial agent (either ciprofloxacin or BKC). The number of viable colonies at 48 h for each antibiotic/biocide concentration were counted and plotted against the antimicrobial concentration on a semi logarithmic plot. The MIC_{Agar} of the chemostat population was then derived from the plot and was taken as the concentration of antimicrobial agent that resulted in a reduction of less then 3 $\log_{10}$ (99.9%) of viable cell counts of the population after 48 h of incubation at 42 ^{o}C under microaerobic conditions (Petersen *et al.,* 2007).

(iii) Replica plating procedure was carried out to estimate the frequencies of emergence and stability of cross-resistance to ciprofloxacin/cefotaxime in the BKC-adapted chemostat populations (CR4 and CR5). To obtain colonies for replica plating, culture samples were taken from chemostat and were serially diluted in PBS (down to 10^{-6}). A 0.1 ml aliquot from each dilution was spreadplated onto antibiotic-free MHA plates containing 5 % lysed horse blood. All incubated at 42 ^{o}C for 48 h under micro aerobic conditions. Individual colonies (n=100) were spot inoculated in a grid pattern (50 per plate) onto ciprofloxacin- or cefotaxime containing MHA plates supplemented with 5 % lysed horse blood using a sterile toothpick. A control plate of MHA plate supplemented with 5 % lysed horse blood without antimicrobial agent was also included. The concentrations of ciprofloxacin and cefotaxime used were 0.125 mg l^{-1} ciprofloxacin or 16 mg l^{-1} cefotaxime. The percentage of the population showing cross-resistance was determined by dividing the number of colonies on agar containing antibiotic by the number of colonies on antibiotic-free medium and multiplying by 100.

2.3.4 Maximum specific growth rate (μmax) determinations

Maximum specific growth rate determinations were carried out in MHB (Lab M) supplemented with 0.35% (v/v) Isovitalex™ on a 96-U bottom well plates (Orange scientific) using a multi-well plate reader (Genios, Tecan). Wells were inoculated with 20 µl an overnight culture to an OD_{595nm} of 0.05. A sterile plastic adhesive cover was aseptically placed on top of the microtiter plate. The plate was shaken every 2 h for 30 sec and OD_{595nm} measurements were automatically recorded in triplicate for each well at two hourly intervals over a 48 h period. (40 °C). Maximum specific growth rates (μmax) values were calculated from the portion of the plot showing exponential growth using Graphpad prism v 6.0. (GraphPad software, La Jolla California USA). The exponential growth equation used to calculate μmax in this study was $Y=Y_o*e^{(K*x)}$ where K is the specific growth rate expressed in reciprocal of h^{-1}, X is time, Y is the OD_{595nm} and Y_o is the initial OD_{595nm} when time is zero.

2.3.5 Adaptation of *C. jejuni* to ciprofloxacin/ BKC by enrichment chemostat culture

Chemostats were operated with/without ciprofloxacin or BKC selection pressure (see Table 2.1 for operating conditions of chemostats used in this project). The enrichment chemostat vessel used in this study (Figure 2.1.) was based on the design described by Fleming *et al.* (1988) and consisted of a modified 1 Liter Quickfit fermenter vessel (FV1L: Bibby, UK) and MAF/52 fermenter head (Quickfit). A silicone ring and butterfly clamp was used to seal the vessel. The vessels had a working volume of 520 ml ± 10 ml controlled by a central overflow tube in the fermenter. The reactor was stirred using a magnetic follower on a Stuart stirrer (Associated Glass Blowers, Dublin). Temperature was held at 42 °C ± 0.3 °C using an internal stainless steel heating coil fed with water from a bath controlled by a water thermostat (Radiometer VTS13, Copenhagen). The medium flow-rate from the reservoir was controlled by a peristaltic pump (Watson-Marlow 205U, USA). The flow-rate was monitored at regular intervals by monitoring the volume of the media reservoir and the flow-rate was adjusted, as required to maintain a steady dilution rate. The glass surfaces were thoroughly washed before use. In addition, to minimise the risk of contamination during operation, both the chemostat and peristaltic pump were housed inside a Class

II Biological safety cabinet (SterilGARD® III Advance°: Baker Company, Maine, USA). Continuous cultures were established by introducing a 6 ml bacterial suspension of *C. jejuni* in MHB previously grown overnight on CCDA (18 h at 42°C) under microaerobic conditions. The chemostat culture was allowed to grow under batch conditions until an optical density OD_{625nm} of 0.1 was reached, after which the pump was switched on and continuous culture was commenced at a dilution rate (D) of 0.025 h^{-1}. The cultures were allowed to equilibrate for approximately 5 generations (240 h at a D of 0.025 h^{-1}), prior to the addition of antibiotic or biocide selection pressures. Antimicrobial agents introduced into chemostat culture were added directly to the MHB into the feed reservoir though an injection port. Doubling concentrations of antimicrobial agent were added to the reservoir every 2 generations and OD_{625nm} returned to approximately 70% of the OD_{625nm} value recorded at the previous addition. Optical densities of the cultures were recorded at an absorbance of 625 nm using a Helios Epsilon Thermo Spectronic spectrophotometer. Deionised water was used as a blank for the spectrophotometer. Samples were routinely taken from the chemostat culture.

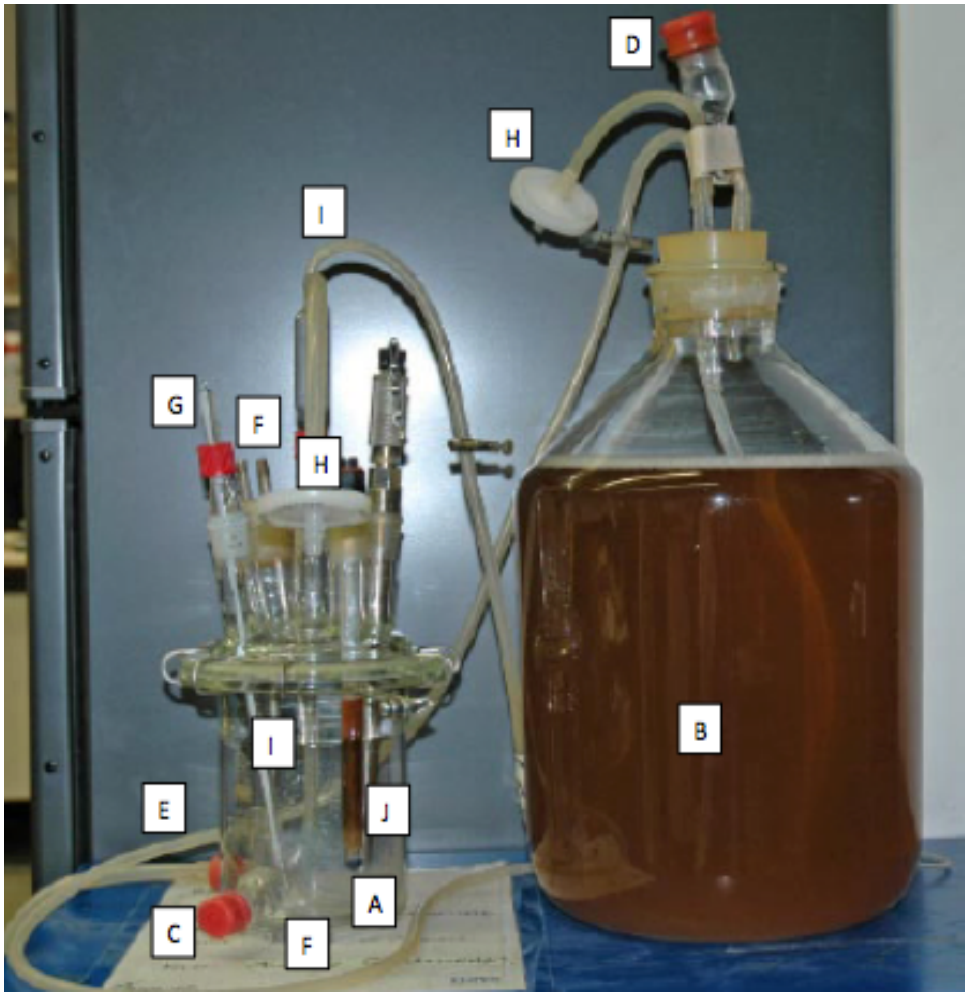

Figure 2.1 The Chemostat used in this study. The chemostat consisted of a 520 ml chemostat vessel (A), 10 litre media reservoir (B), sampling port (C), injection port (D), effluent line (E), constant temperature coil visible inside chemostat (F), Thermometer (G), filtered air supply on chemostat and media reservior (H) medium feed with medium break (visible inside chemostat; I), pH probe visible inside chemostat and oxygen electrodes were not connected during operation of chemostat (J) peristaltic pump (Watson -Marlow, 202U/1) and 20 litre waste reservoir (not shown).

OD_{625nm} measurements and total viable counts and MIC analysis were preformed.

1.5 ml 50% glycerol stock cultures were made and kept at -70^{o}C for further analysis. The ciprofloxacin concentration used in this study ranged from an initial concentration of 0.01 mg l^{-1} up to 12 mg l^{-1} and the BKC concentrations used ranged from 0.1-25.6 mg l^{-1}. To assess the stability of resistance in the adapted population the selection pressure was removed and the chemostat was operated without selective pressure for a further 500-850 h in two of the three chemostats run under antibiotic selection (CR2, CR3) and for one of the two chemostats run under biocide selection (CR5).

2.3.6 Pulsed field gel electrophoresis (PFGE)

PFGE was used to confirm the identity of the chemostat adapted strains and carried out using the protocol described by O'Leary et al., (2011) with some minor modifications. *Campylobacter* isolates were resuscitated from storage at -70 ^{o}C and subcultured twice (on CCDA at 42 ^{o}C for 48 h under microaerobic conditions) prior to analysis by PFGE. Cells were harvested from CCDA plate cultures and added to 2 ml PETT IV buffer (1M NaCl; 10 mM EDTA; 10 mM tris). The turbidity of the cell suspension was adjusted to McFarland 6. A 300 µl aliquot was mixed with 700 µl of molten 1 % (w/v) chromosomal grade agarose (Seakem Gold agarose, Lonza, Rockland, ME, US) and the mixture was immediately pipetted into plug moulds. Plug moulds were then placed in the fridge at 4 ^{o}C for approximately 10 min to allow plugs to set. Plugs were transferred into freshly prepared ESP buffer (0.5 M EDTA; 1 % (w/v) sarkosyl salt; 1 mg ml^{-1} proteinase K) and incubated overnight at 56 ^{o}C to lyse cells. Following overnight incubation, ESP buffer was decanted from the samples using a plug screen (Bio-rad). Plugs were washed for a total of 6 times in 10 ml of TE buffer (10 mM tris; 1 mM EDTA). After the final wash step, TE buffer was decanted and 5 ml TE buffer was added. Plugs in TE buffer were then kept at 4 ^{o}C until needed. A 2 mm agar slice was made from the agarose bound DNA and sample DNA was restricted by the rare cutting enzyme *Sma* I (FastDigest, Fermentas), containing 100 µl of 1X NE buffer with 20 U of *Sma* I, overnight at 25 ^{o}C.

Agarose for PFGE gels was prepared by mixing 100 ml of 0.5 X TBE buffer (45 mM tris-HCl; 45 mM boric acid; 1 mM EDTA) and 1 g of Seakem gold agarose in a 250 ml conical flask. ProMega Marker 1 ladder G301A was used as a molecular weight marker. Molecular weight marker and samples were placed onto the ends of the well combs and were affixed using a small amount of the 1 % Seakem gold agarose. Electrophoresis was performed on the CHEF-DRII pulsed field electrophoresis system (Bio-Rad). The gel was then placed in the electrophoresis chamber and left to equilibrate for 20 min. Electrophoretic separation of *Sma* I generated fragments was at 200 V (6 V/cm) using an initial switch time of 0.5 s, final switch time of 40 s, over 22.5 h at 14 ^{o}C in 0.5 X TBE buffer (45 mM tris, 45 mM borate; 1mM EDTA, pH 8.0). After the electrophoresis run was completed, gels were removed and covered with a 1 mg ml^{-1} stock concentration of ethidium bromide. Gels were placed on an

orbital shaker (Heidolph unimax 1010) and shaken for 20 min. The ethidium bromide solution was then removed and gels were distained in MQ water for 20 min. Gels were imaged using ultraviolet light (G:box, Syngene) and their images were captured using Genesnap software (Syngene).

2.4 Results

2.4.1 Colony and cell morphology of *C. jejuni* on agar and in chemostat culture

C. jejuni (original strain) and chemostat derived isolates were routinely grown on CCDA and MH agar throughout the duration of this project. *Campylobacter* colonies grown on CCDA were moist, flat and sometimes had a metallic sheen. On MHA, *Campylobacter* colonies and chemostat derived isolates were round, convex, smooth and shiny, with a regular edge and had a slightly pink colour. Agar and broth cultures were routinely Gram-stained to check for purity. When visualized microscopically, cells were Gram-negative curved rods. Aged agar cultures were observed to be coccoid. Some filamentation of cells was observed in chemostat culture under selection.

2.4.2 Minimum Inhibitory Concentration

The broth and agar minimum inhibitory concentrations **(**MICs) of the *C. jejuni* original strain were determined in accordance with CLSI (2006) with some minor modifications as described in section 2.3.3. The original strain had a broth MIC of 0.06 mg l^{-1} and agar MIC of 0.125 mg l^{-1} to ciprofloxacin. Broth and agar MIC values were 0.2 mg l^{-1} and 8 mg l^{-1} to BKC respectively. These parameters established breakpoint concentrations for the detection of adapted variants in the chemostat-derived population.

2.4.3 Maximum specific growth rate (μ_{max}) determination of *C. jejuni*

The growth characteristics of the strain were determined in MHB. It was necessary to determine the growth rate (μ_{max}) of the strain before a continuous culture of *C. jejuni* NCTC 11168 to determine the operational dilution rate (D). The specific growth rate of *C. jejuni* NCTC 11168 was determined in MHB using the microtiter plate method (as described in 2.3.4). Logarithmic readings of optical density values were plotted against time in Figure 2.2. Data was then analyzed using Graphpad prism 6.0.

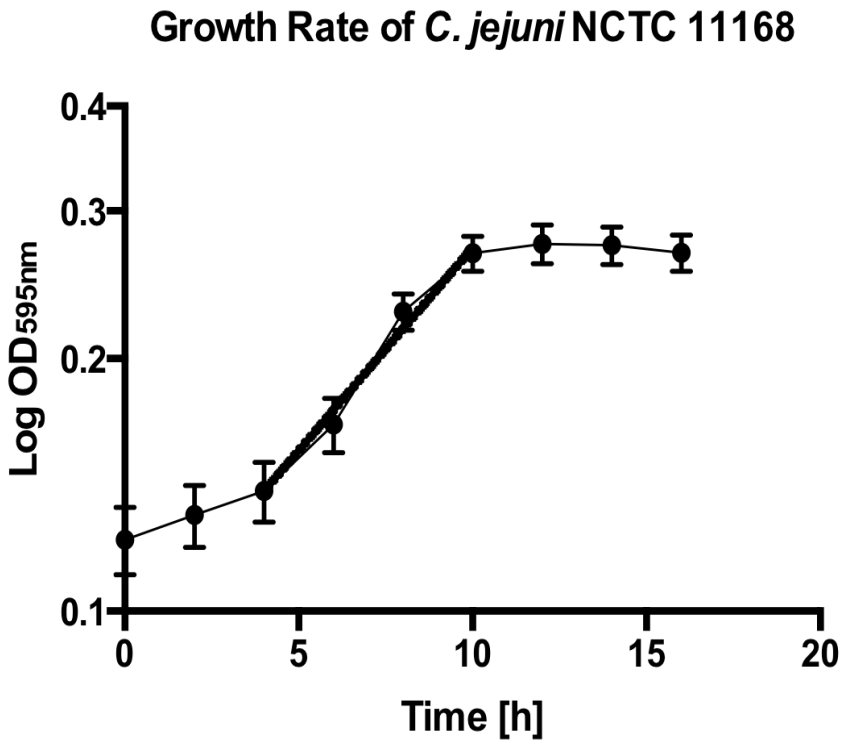

Figure 2.2 Plot of logarithmic optical density (595 nm) versus time (hours) for *C. jejuni* growth in MHB. The growth rate of *C. jejuni* in Mueller Hinton broth was determined using Graphpad prism 6.0. This figure shows mean ± SEM of OD_{595nm} versus time (n=8). Thick black line in bold indicates logarithmic growth phase from which maximum specific growth rate (μmax) was calculated. The maximum specific growth rate ± standard error of *C. jejuni* was determined to be 0.11± 0.01 h^{-1} and doubling time of 6.2 hours (C.I. 5.0 -8.3 h).

2.4.4 Adaptation of *C. jejuni* to ciprofloxacin and BKC in chemostat culture

Seven long-term continuous cultures of *C. jejuni* (original strain) were used to enrich for ciprofloxacin-adapted or BKC-adapted variants of *C. jejuni* NCTC 11168 (Table 2.1). Chemostat cultures (with and without antibiotic and biocide selection pressure) were operated for 944 – 2328 h at a dilution rate of 0.025 h^{-1}. Chemostats operated using ciprofloxacin selection pressure were designated CR1 (58 generations; 2328 h), CR2 (36 generations; 1422 h) and CR3 (49 generations; 1975 h). Chemostats operated using BKC as a selective agent were designated CR4 (39 generations; 1565 h) and CR5 (44 generations; 1760 h). CR6 (22 generations; 944 h) and CR7 (28 generations; 1100 h) were operated without added selection pressure (controls). The selection pressure applied to chemostat cultures ranged from 0.01 mg l^{-1} to 12 mg l^{-1} ciprofloxacin and 0.05 mg l^{-1} to 25.6 mg l^{-1} BKC. The selection pressure was removed after 936 h (CR2), 1160 h (CR3), 1015 h (CR5), to determine the stability of the evolved phenotypes. A summary of operating conditions, culture optical density, viable cell number, and MIC_{Agar} values, for all chemostats carried out in this study is presented in Table 2.1.

Operating conditions of chemostats							**Chemostat population parameters measured**				
Chemostat Run	**Selective agent**	**Total Run time [h]**	**Total generations Elapsed**	**Concentration range in the chemostat (mg l^{-1})**		**Removal of selection pressure§**	**Antimicrobial susceptibility (MIC_{Agar} mg l^{-1})**			**Total viable counts range (cfu ml^{-1})**	**Optical density range (625nm)**
				Initial	**Final**		**Initial**	**Highest**	**Final**		
CR1	Cip	2328	58	0.006[a]	8[a]	No	0.125[a]	128[a]	128[a]	4×10^7 - 7×10^8	0.054 - 0.113
CR2	Cip	1422	36	0.06[a]	12[a]	Yes [936 h; after 24 gen]	0.125[a]	128[a]	32[a]	8×10^6 - 3×10^8	0.070 - 0.211
CR3	Cip	1975	49	0.01[a]	12[a]	Yes [1160 h; after 29 gen]	0.125[a]	256[a]	64[a]	4×10^7 - 3×10^8	0.085 - 0.206
CR4	BKC	1565	39	0.05[b]	20[b]	No	8[b]	64[b]	64[b]	2×10^7 - 3×10^8	0.048 - 0.184
CR5	BKC	1760	44	0.2[b]	25.6[b]	Yes [1015 h; after 26 gen]	8[b]	64[b]	64[b]	1×10^4 - 3×10^8	0.040 - 0.211
CR6	-	944	22	-	-	N/A	0.125[a], 8[b]	0.125[a], 16[b]	0.125[a], 16[b]	1×10^5 - 6×10^8	0.028 - 0.201
CR7	-	1100	28	-	-	N/A	0.125[a], 8[b]	0.125[a], 8[b]	0.125[a], 8[b]	3×10^7 - 2×10^8	0.028 - 0.131

Table 2.1 Summary of operating conditions of chemostats carried out in this study and corresponding chemostat population parameters measured. Note:- § Numbers in parentheses indicates when the selection pressure was removed from the chemostat (in generation and h). Abbreviations: Cip, ciprofloxacin; BKC, benzalkonium chloride; gen, generations; a, antimicrobial susceptibility to ciprofloxacin; b, antimicrobial susceptibility to BKC, N/D, not determined.

2.4.4.1 Chemostats operated using ciprofloxacin as a selection pressure

The MIC_{Agar} of the chemostat population of CR1 to ciprofloxacin increased 1,024-fold overall (from 0.125 mg l^{-1} to 128 mg l^{-1}) over the course of 2328 h (58 generations; D=0.0025 h^{-1}, Figure 2.3). Culture optical densities, viable cell number profiles and MIC_{Agar} values to ciprofloxacin are summarised in table 2.1. Initially, MIC_{Agar} value to ciprofloxacin was determined after 26 generations had elapsed and the MIC_{Agar} for the chemostat population had increased 32-fold from 0.125 mg l^{-1} to 4 mg l^{-1}. The population MIC_{Agar} for ciprofloxacin remained stable at 16 mg l^{-1} when the applied selective pressure in the chemostat feed was between 0.5 – 2 mg l^{-1} ciprofloxacin. The MIC_{Agar} values for ciprofloxacin increased to 32 mg l^{-1} and 128 mg l^{-1} when the applied selection pressure in the chemostat feed was increased to 4 mg l^{-1} and 8 mg l^{-1} ciprofloxacin respectively. An attempt was made to further increase the levels of ciprofloxacin in the feed to 12 mg l^{-1} ciprofloxacin however the OD_{625nm} was observed to fall (data not shown) and washout of the chemostat population occurred.

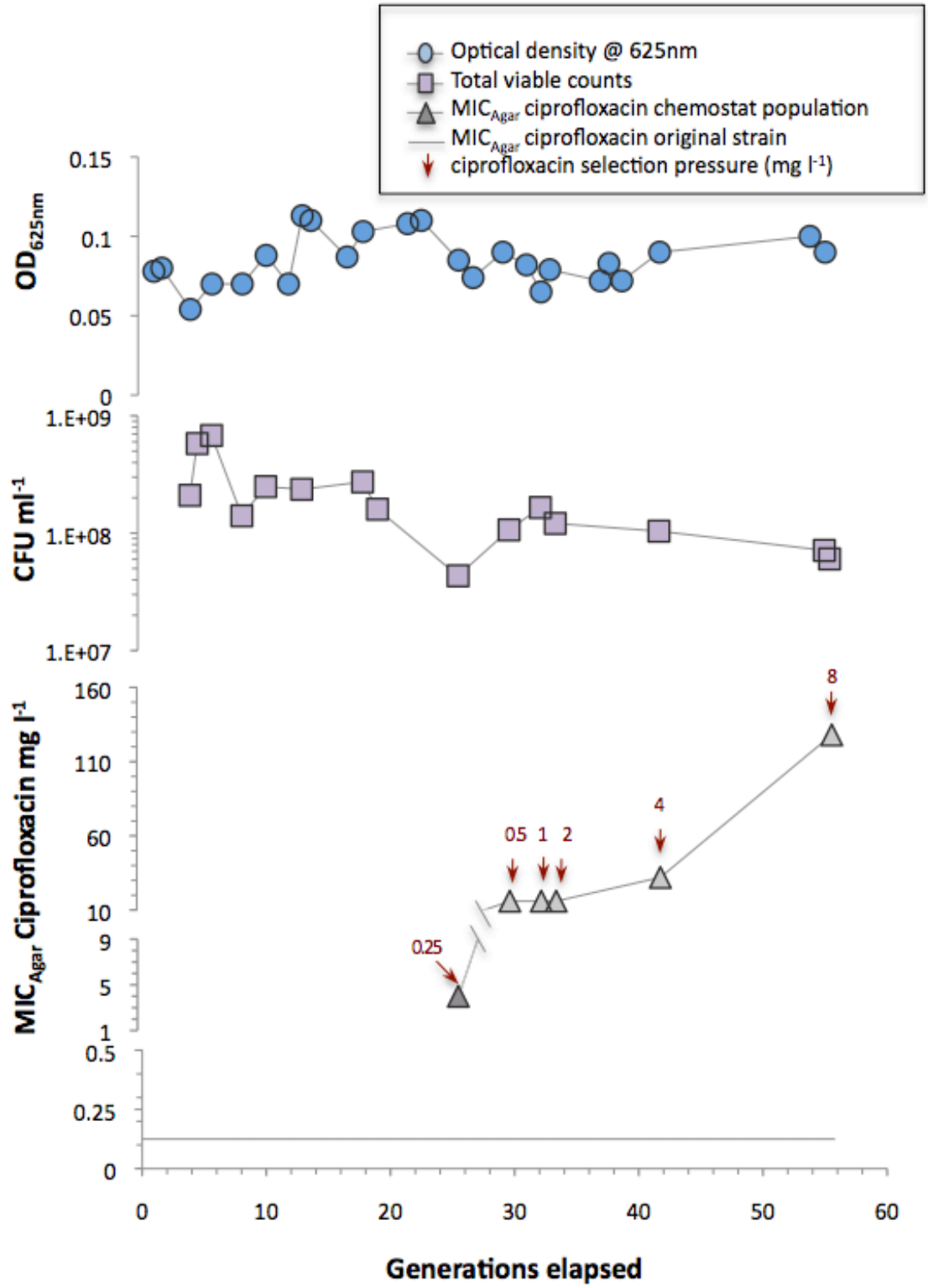

Figure 2.3 Chemostat 1: Enrichment culture of *C. jejuni* with ciprofloxacin. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values of ciprofloxacin of the chemostat population with corresponding concentrations of selection pressure in mg l^{-1}(arrowed, red) are shown. The MIC_{Agar} value of ciprofloxacin of *C. jejuni* (original strain) is also included to show the degree of adaptation of the chemostat population.

CR2 was in operation using ciprofloxacin as a selection pressure for 1422 h (36 generations; D=0.025 h^{-1}; Figure 2.4). Culture optical densities, viable cell number profiles and MIC_{Agar} values to ciprofloxacin are summarised in table 2.1.The initial concentration of ciprofloxacin selective pressure applied at 0.06 mg l^{-1} was introduced into the media feed and resulted in a corresponding 2-fold increase in resistance of the chemostat population to ciprofloxacin (from 0.125 mg l^{-1} to 0.25 mg l^{-1}). Increasing the selection pressure 2-fold to 0.12 mg l^{-1} resulted in a 16-fold rise in population resistance (as measured by plate MIC) from 0.25 mg l^{-1} to 4 mg l^{-1} ciprofloxacin. The population MIC was stable at 16 mg l^{-1} when the applied selective pressure was between 0.48 – 2 mg l^{-1} ciprofloxacin. The applied section pressure in the chemostat was incrementally increased until the section pressure in the media feed was at 12 mg l^{-1} ciprofloxacin. This resulted in a corresponding 8-fold increase in resistance in the chemostat population to ciprofloxacin (from 16 mg l^{-1} to 128 ciprofloxacin) and represented a 1,024-fold increase in resistance overall. After 23 generations (936 h) of continuous culture the selection pressure was removed from CR2 and the chemostat was operated for a further 500 h (12 generations; shaded area in Figure 2.4.). Resistance to ciprofloxacin in the chemostat population decreased 4-fold and stabilized at 32 mg l^{-1} ciprofloxacin after 48 h (after 1 generation).

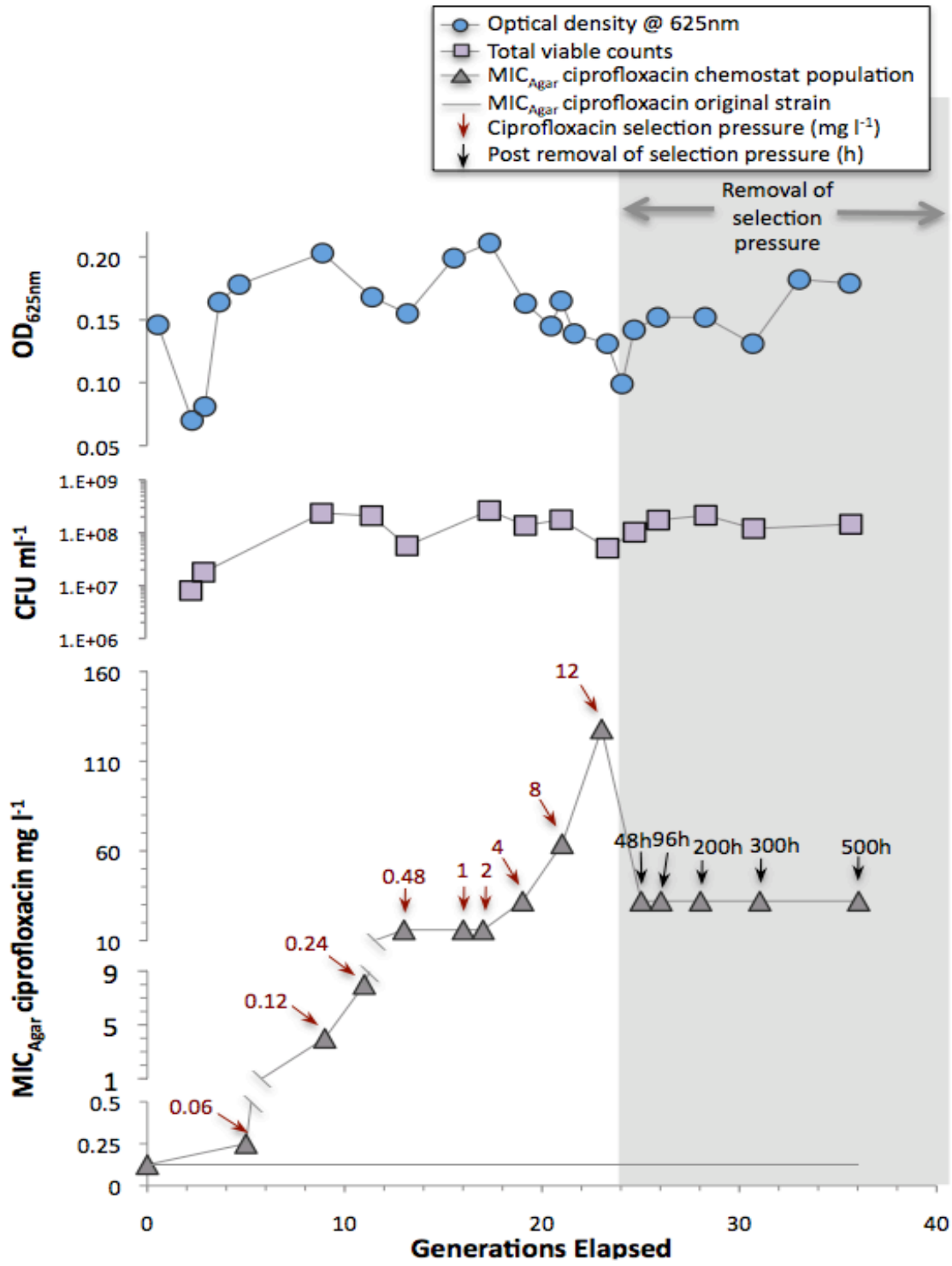

Figure 2.4 Chemostat 2: Enrichment culture of *C. jejuni* with ciprofloxacin. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values of ciprofloxacin of the chemostat population with corresponding concentrations of selection pressure in mg l^{-1} (arrowed; red) and post removal of selection pressure in hours (h; arrowed; black) are shown. The MIC_{Agar} value of ciprofloxacin of the original *C. jejuni* strain is also included to show the degree of adaptation of the chemostat population. Note after 24 generations the selective pressure was removed from the chemostat (shaded area). The population MIC_{Agar} to ciprofloxacin stabilized at 32 mg l^{-1} from generation 25 onwards.

CR3 was in operation using ciprofloxacin as a selection pressure for 49 generations (1975 h; D=0.025 h^{-1}; Figure 2.5). Culture optical densities, viable cell number profiles and MIC_{Agar} values to ciprofloxacin are shown in table 2.1. Initially the resistance of the chemostat population to ciprofloxacin in CR3 did not change following the addition of ciprofloxacin selection pressure at 0.01 mg l^{-1} to the media feed. This trend continued until the selection pressure in the media feed increased from 0.04 mg l^{-1} to 0.08 mg l^{-1} and the population resistance to ciprofloxacin increased 32 –fold (from 0.125 mg l^{-1} to 4 mg l^{-1} ciprofloxacin). The applied section pressure in the chemostat was incrementally increased until the section pressure in the media feed was at 0.64 mg l^{-1} ciprofloxacin and resistance in the population rose 4-fold to 32 mg l^{-1} ciprofloxacin. The population MIC_{Agar} was stable at 32 mg l^{-1} when the applied selective pressure in the media feed was between 0.64 – 2.56 mg l^{-1} ciprofloxacin. The applied section pressure in the chemostat was increased until the section pressure in the media feed was at 12 mg l^{-1} ciprofloxacin. This resulted in a corresponding 4-fold increase in resistance in the chemostat population to ciprofloxacin (from 32 mg l^{-1} to 128 ciprofloxacin) and represented a 1,024-fold increase in resistance overall. After 29 generations (1160 h) of continuous culture the selection pressure was removed from CR3 and the chemostat culture was operated for a further 850 h (20 generations; shaded area Figure 2.5). The population MIC_{Agar} to ciprofloxacin decreased 4-fold, to 64 mg l^{-1} ciprofloxacin, after 4 generations (200 h).

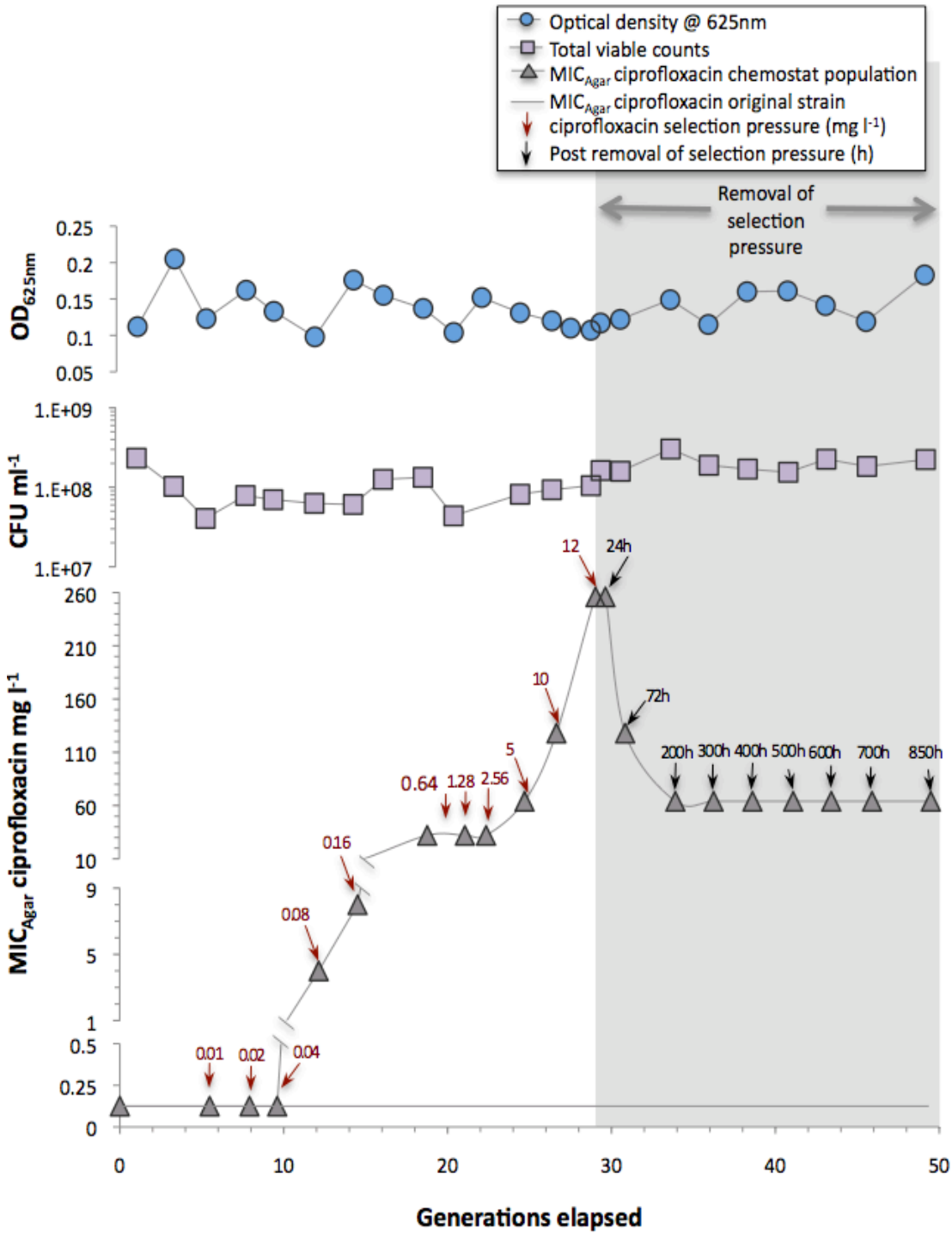

Figure 2.5 Chemostat 3: Enrichment culture of *C. jejuni* with ciprofloxacin. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values of ciprofloxacin of the chemostat population with corresponding concentrations of selection pressure in mg l^{-1} (arrowed, red) and post removal of selection pressure in hours (h; arrowed, black) are shown. The MIC_{Agar} value of ciprofloxacin of the original *C. jejuni* strain is also included to show the degree of adaptation of the chemostat population. Note after 24 generations the selective pressure was removed from the chemostat (shaded area). The population MIC_{Agar} to ciprofloxacin stabilized at 32 mg l^{-1} from generation 29 onwards.

2.4.4.2 Chemostats operated using BKC as a selection pressure

CR4 was operated for a total of 1565 h (39 generations; D=0.025 h^{-1}) and adaptation of the chemostat population to BKC occurred in a slow stepwise fashion (Figure 2.6). Culture optical densities, viable cell number profiles and MIC_{Agar} values to BKC are shown in table 2.1. The initial concentration of BKC selective pressure applied at 0.2 mg l^{-1} was introduced into the media feed and resulted in a corresponding 2-fold increase resistance of the chemostat population to ciprofloxacin (from 8 mg l^{-1} to 16 mg l^{-1}). The susceptibility of the chemostat population to BKC in CR4 did not change when the applied selective pressure in the media feed was between 0.2 and 3.2 mg l^{-1} BKC. The concentration of 10 mg l^{-1} BKC was added into the media feed at 21 generations (847 h). During this time a corresponding decrease in OD_{625nm} to 0.05 was observed and the pump was stopped at generation 23 (915 h) for 6.7 generations (268 h). The chemostat was batch fed with 100 mls of MHB containing 10 mg l^{-1} BKC at 9 h (generation 23.23) and 1063 h (generation 26.7). A new medium feed containing 10 mg l^{-1} BKC was connected and the pump was restarted at (1183 h) the population was shown have recovered (OD_{625nm} had risen to 0.183). In this time a second increase in population MIC_{Agar} to BKC to 32 mg l^{-1} BKC (2-fold) was observed. The concentration was then increased to 20 mg l^{-1} BKC via the medium feed and by generation 39 (1565 h) the population MIC_{Agar} increased to 64 mg l^{-1} BKC.

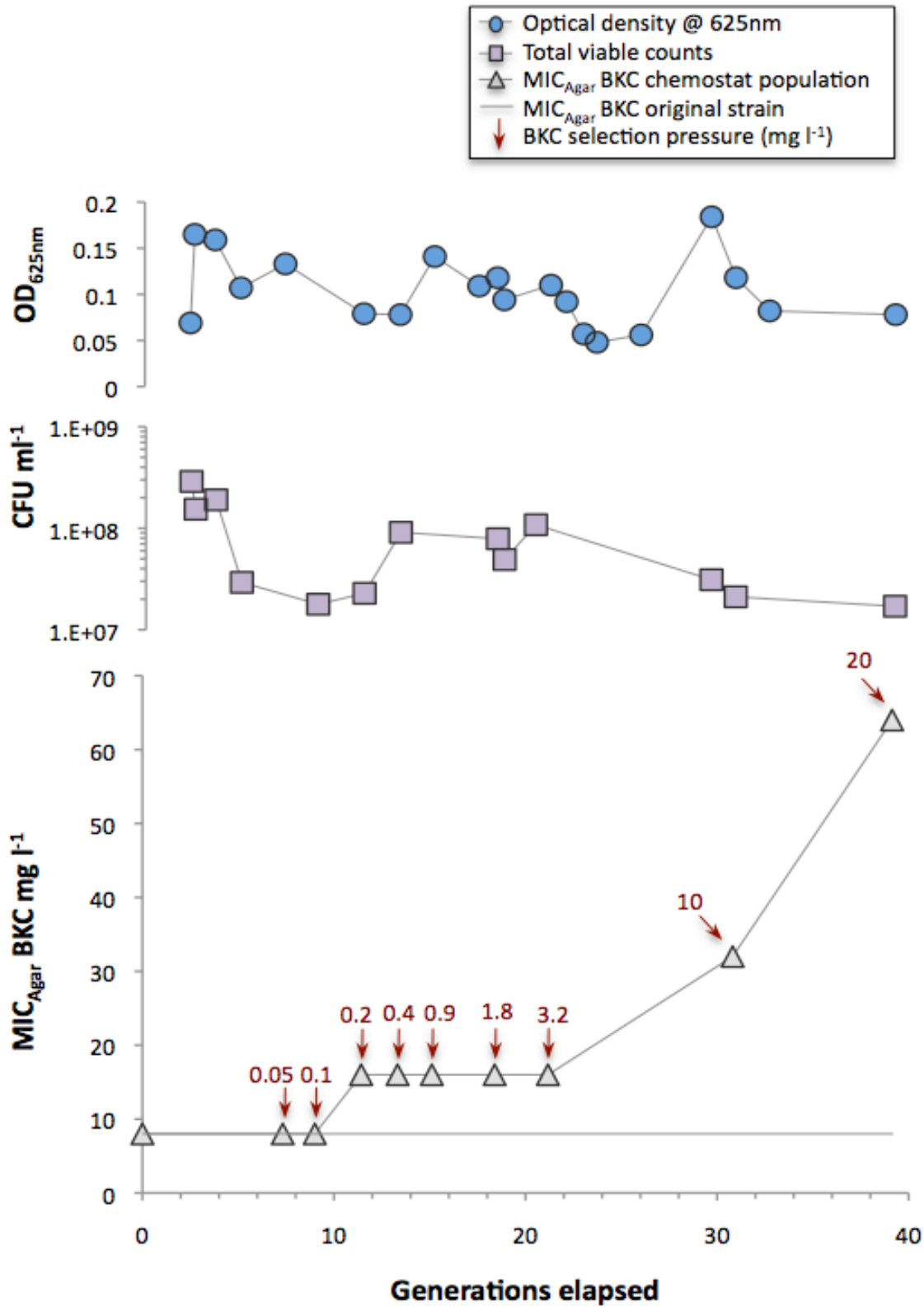

Figure 2.6 Chemostat 4: Enrichment culture of *C. jejuni* with BKC. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values of BKC of the chemostat population with corresponding concentrations of BKC selection pressure in mg l^{-1} (arrowed, red) are shown. The MIC_{Agar} value of BKC of original *C. jejuni* strain is also included to show the degree of adaptation of the chemostat population.

CR5 was operated for 1760 h (44 generations; D=0.025 h^{-1}) under selection to the biocide BKC (Figure 2.7). Culture optical densities, viable cell number profiles and MIC_{Agar} values to BKC are summarised in table 2.1. The MIC_{Agar} to BKC increased 2-fold to 16 mg l^{-1} after the initial concentration of BKC applied at 0.2 mg l^{-1} and the population MIC_{Agar} to BKC. A second increase (2-fold) in MIC_{Agar} to 32 mg l^{-1} BKC was observed when the concentration of BKC in the chemostat culture was between 0.8 and 9.6 mg l^{-1} BKC. The last increase in population MIC_{Agar} values to BKC occurred when the concentration of applied BKC selective pressure was between 12.8 and 25.6 mg l^{-1} BKC. When 25.6 mg l^{-1} BKC was added into the media feed at 21 generations (847 h) the OD_{625nm} was observed to drop. The selection pressure was removed at generation 26 (1014 h) and the chemostat was batched fed with biocide-free media for 4.6 generations (184 h). The chemostat was batch fed with 200 mls MHB at 1014 (generation 26) and 100 mls of MHB at 1077 h (generation 27), 1106 h (generation 27.65), 1132 h (generation 28.3), 1152 h (generation 28.8) and at 1176 h (generation 29.4). The population was shown have recovered (the OD_{625nm} had risen to 0.137) by generation 30. The pump was then turned on and continuous culture was allowed to continue until generation 44 when the chemostat was taken down. The population MIC_{Agar} to BKC was unaltered by the end of this time period (Figure 2.7).

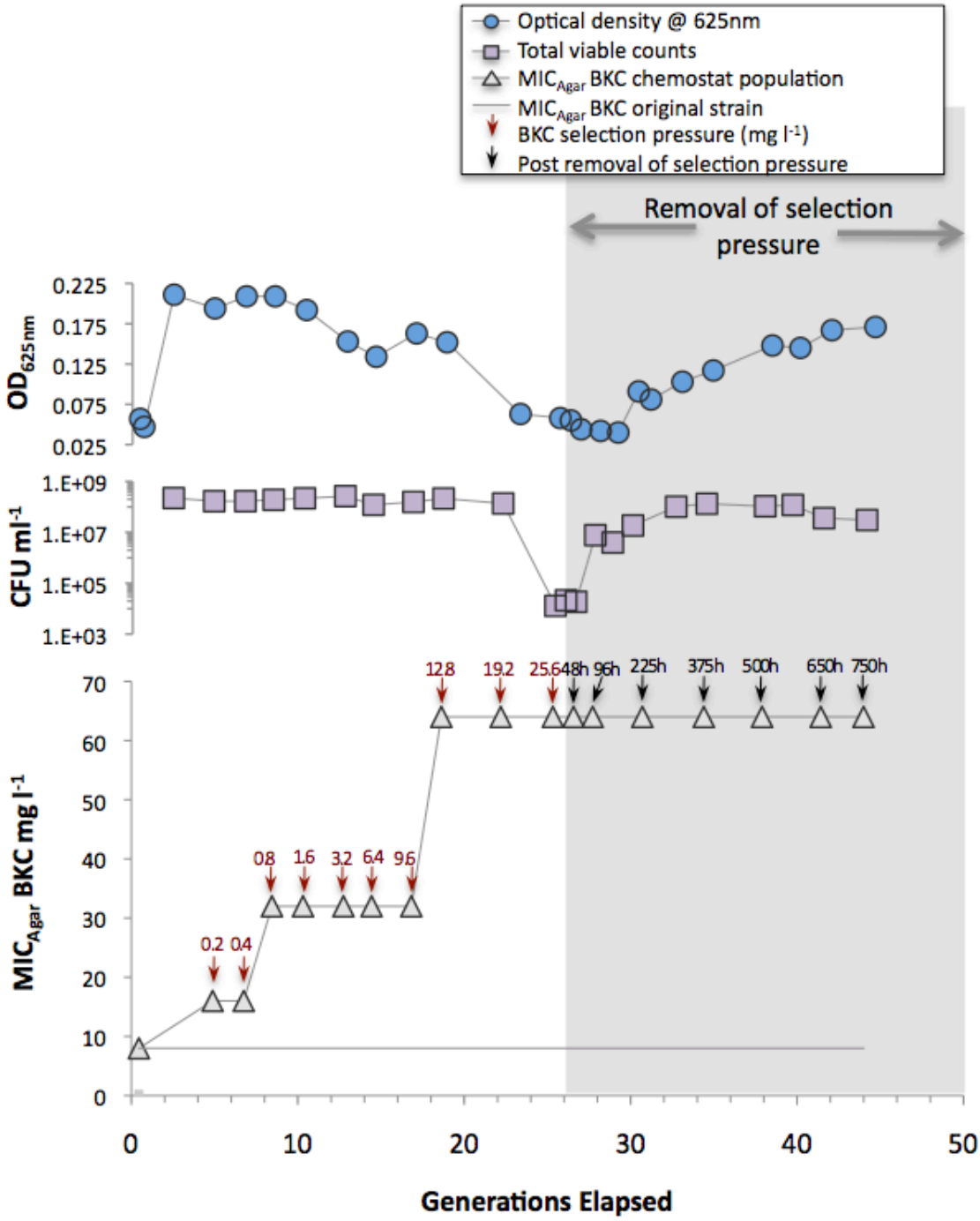

Figure 2.7 Chemostat 5: Enrichment culture of *C. jejuni* with BKC. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values of BKC of the chemostat population with corresponding concentrations of BKC selection pressure in mg l^{-1} (arrowed) and time post removal of selection pressure in hours (h; arrowed) are shown. The MIC_{Agar} value of BKC of original *C. jejuni* strain is also included to show the degree of adaptation of the chemostat population. Note after 26 generations (1016 h) the selective pressure was removed from the chemostat (shaded area). The population MIC_{Agar} to BKC stabilized at 64 mg l^{-1} (no decrease observed for 19 generations).

2.4.4.3 Chemostats operated without selection pressure

Both CR6 and CR7 were operated without selection pressure (antibiotic and biocide free) for 944 h (22 generations; D=0.0025 h^{-1}) and 1100 h respectfully (28 generations; D=0.0025 h^{-1}; Figure 2.8 & Figure 2.9). The MIC_{Agar} of the chemostat population of CR6 to BKC was 16 mg l^{-1} which was 2-fold higher the original strain (8 mg l^{-1} BKC). The MIC_{Agar} of the chemostat population to ciprofloxacin was the same (0.125 mg l^{-1}) as the original *C. jejuni* strain after 22 generations (944 h) of culture (Figure 2.8). CR6 was terminated due to contamination, which was observed after generation 22 (data after this not shown). The population MIC_{Agar} of CR7 for both ciprofloxacin and BKC was unaltered when compared with those of the original after 28 generations (1100 h) of culture (Figure 2.9). A leak in the media feed line was observed at 1204 h (generation 29). By 1226 h (generation 30) contamination was seen in the chemostat and the chemostat was terminated. Data collected from generation 29 and 30 is not shown as a result.

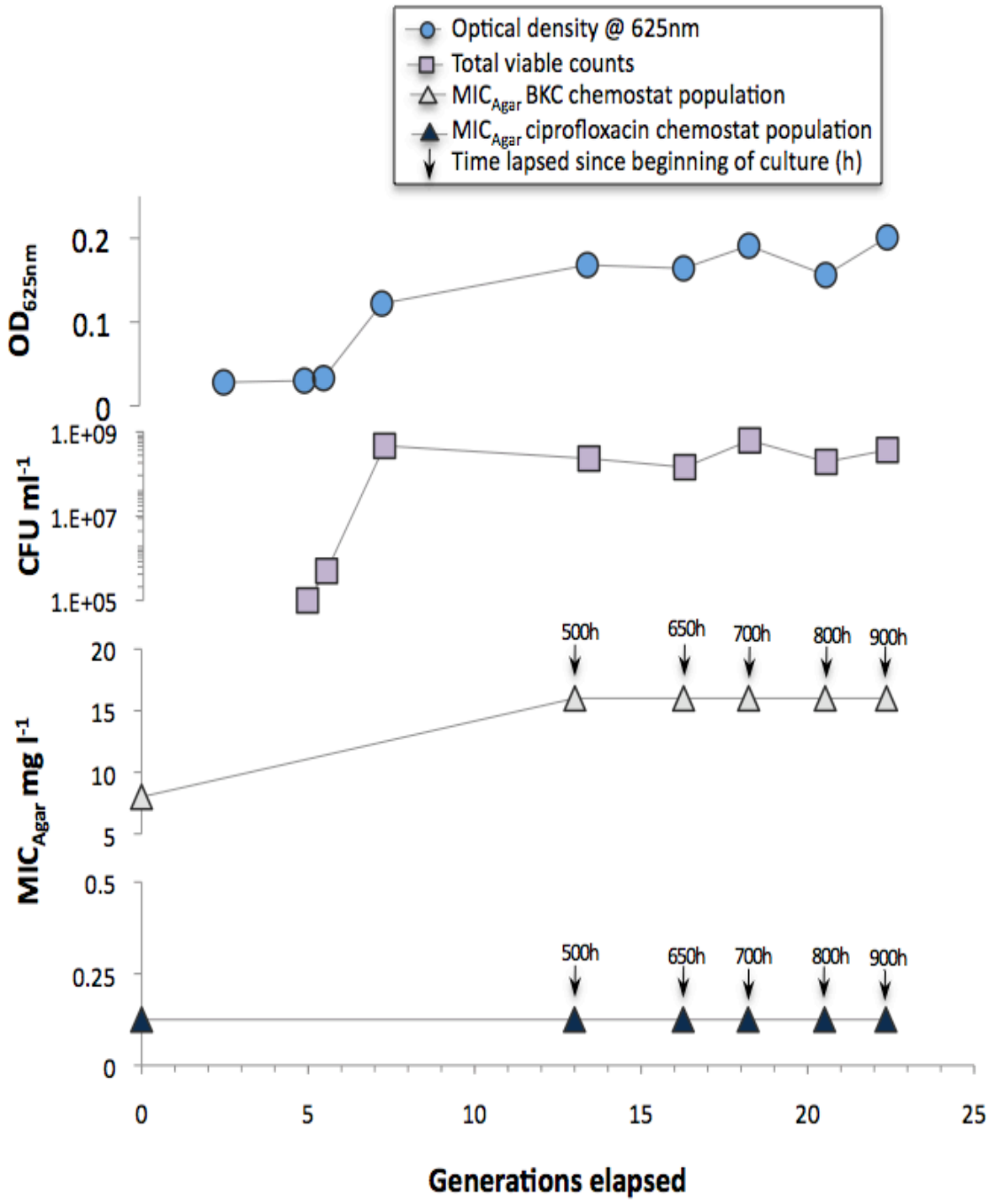

Figure 2.8 Chemostat 6: *C. jejuni* in chemostat culture without selection. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values to both ciprofloxacin and BKC of the chemostat population with corresponding chemostat culture time (h; arrowed, black) are shown.

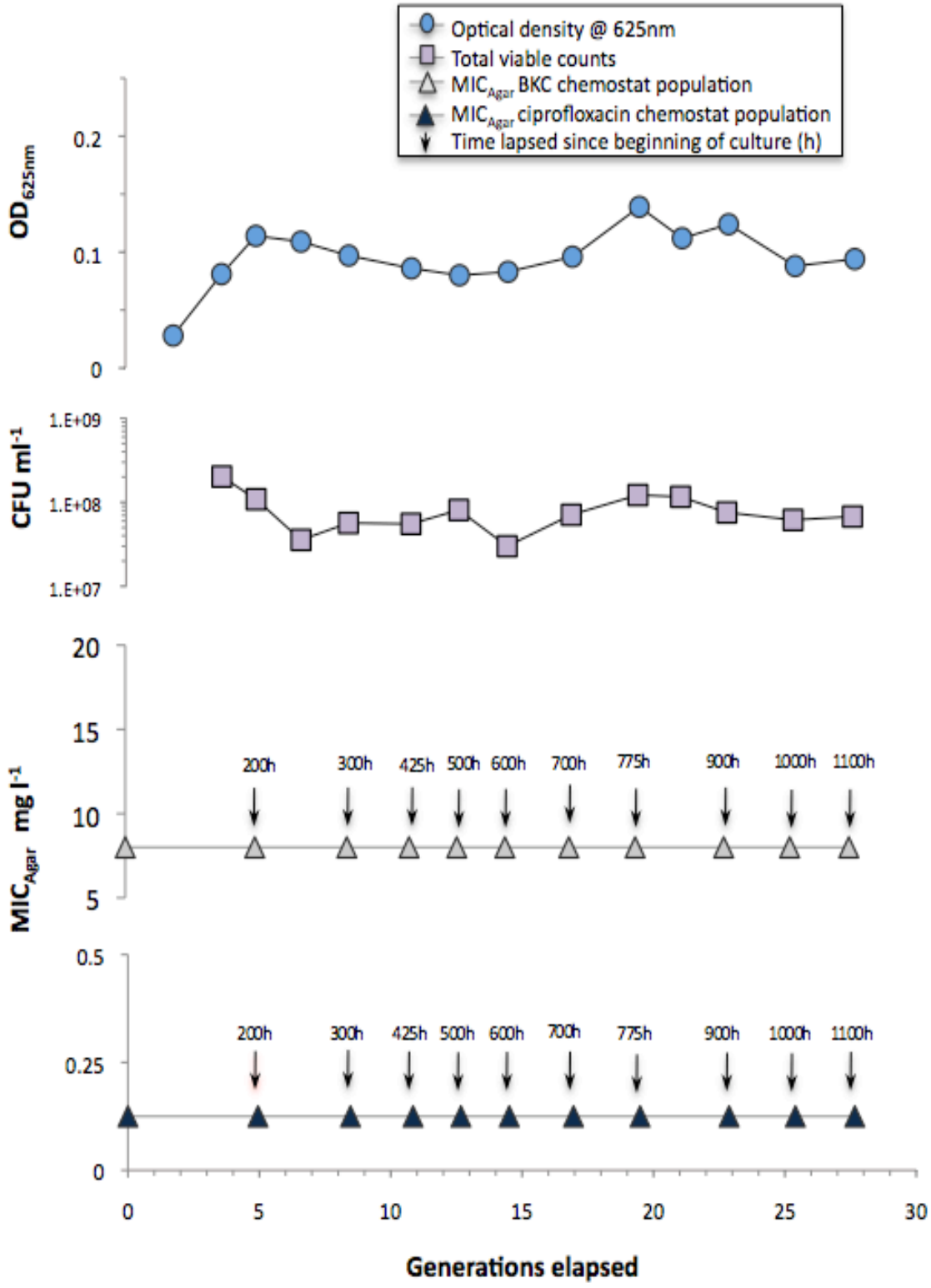

Figure 2.9 CR7: *C. jejuni* in chemostat culture without selection. Optical density readings taken at 625nm, total viable counts, MIC_{Agar} values to both ciprofloxacin and BKC of the chemostat population with corresponding chemostat culture time (h; arrowed, black) are shown.

2.4.5 Cross-resistance to antibiotics in chemostat cultures undergoing adaptation to BKC

Cross-resistance to ciprofloxacin in *C. jejuni* populations undergoing adaptation to BKC was evaluated. Cross-resistance to cefotaxime was also evaluated *C. jejuni* populations undergoing adaptation to BKC in CR5 (Figure 2.10 & Figure 2.11).

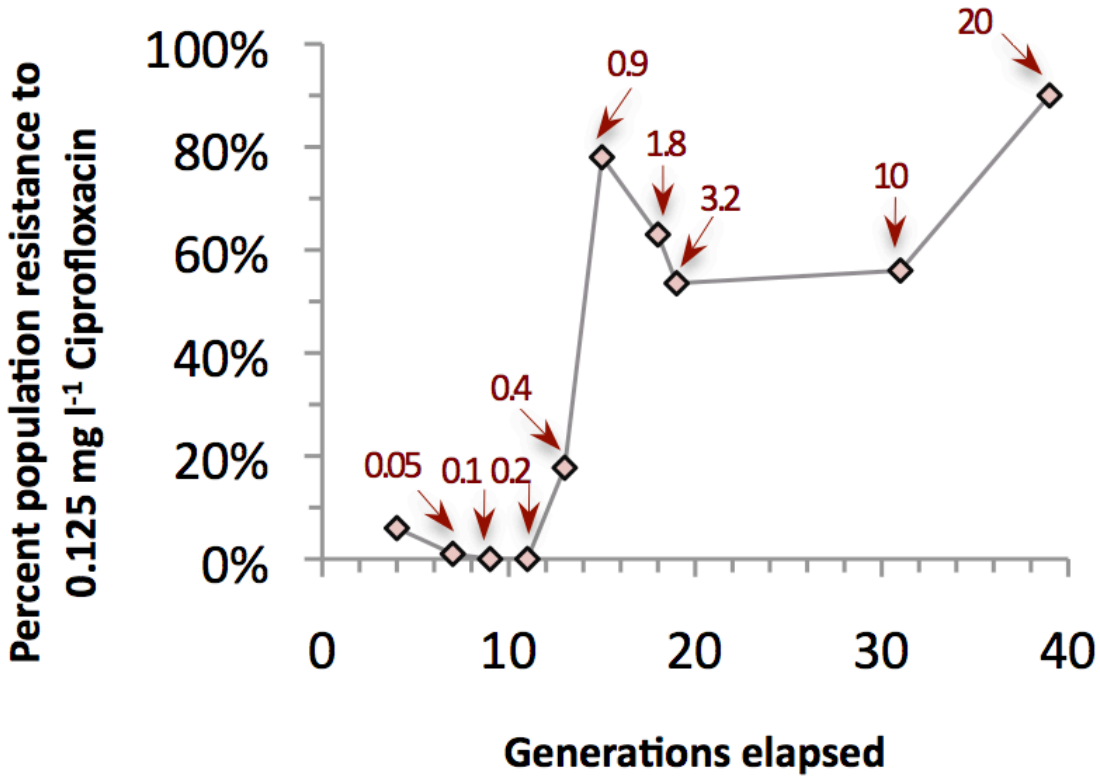

Figure 2.10 Percentage of the chemostat population undergoing selection to BKC and also showing cross-resistance to ciprofloxacin. Concentration of ciprofloxacin tested was 0.125 mg l^{-1} (2X MIC_{Agar} of original strain used to inoculate chemostat). Concentrations of BKC selection pressure in media feed in mg l^{-1} (arrowed, red). Data derived from n=100 tested at each sampling period

The percentage of population of CR4 showing cross-resistance to 0.125 mg l^{-1} ciprofloxacin (2XMIC_{Agar} of the original strain to ciprofloxacin) was 90% by the time the selection pressure had increased from 0.05 to 20mg l^{-1} BKC (Figure 2.10). In CR5, the percentage of the BKC-adapted population that showed a 2-fold increase in MIC_{Agar} to ciprofloxacin (0.125 mg l^{-1} ciprofloxacin) reached 100% by the time the applied selective pressure of BKC was 25.6 mg l^{-1} (Figure 2.11). In CR5, when the selective pressure was removed after 26 generations (1016 h). The chemostat was

operated for a further 19 generations in the absence of selection pressure. The observed increase in resistance to ciprofloxacin was unchanged (Figure 2.11).

The percentage of the BKC-adapted population in CR5 exhibiting a 2-fold increase to cefotaxime also increased over the course of adaptation to BKC (from generation 5 to generation 22) and was 100% when the selection pressure was 19.2 mg l^{-1} BKC. It dropped to 70% when the selection pressure was at 25.6 mg l^{-1} BKC and increased to 100% again 100 h post removal of section pressure. However the observed cross-resistance was unstable and was lost at the end of the culture period (650 h after selection pressure was removed; see Figure 2.11).

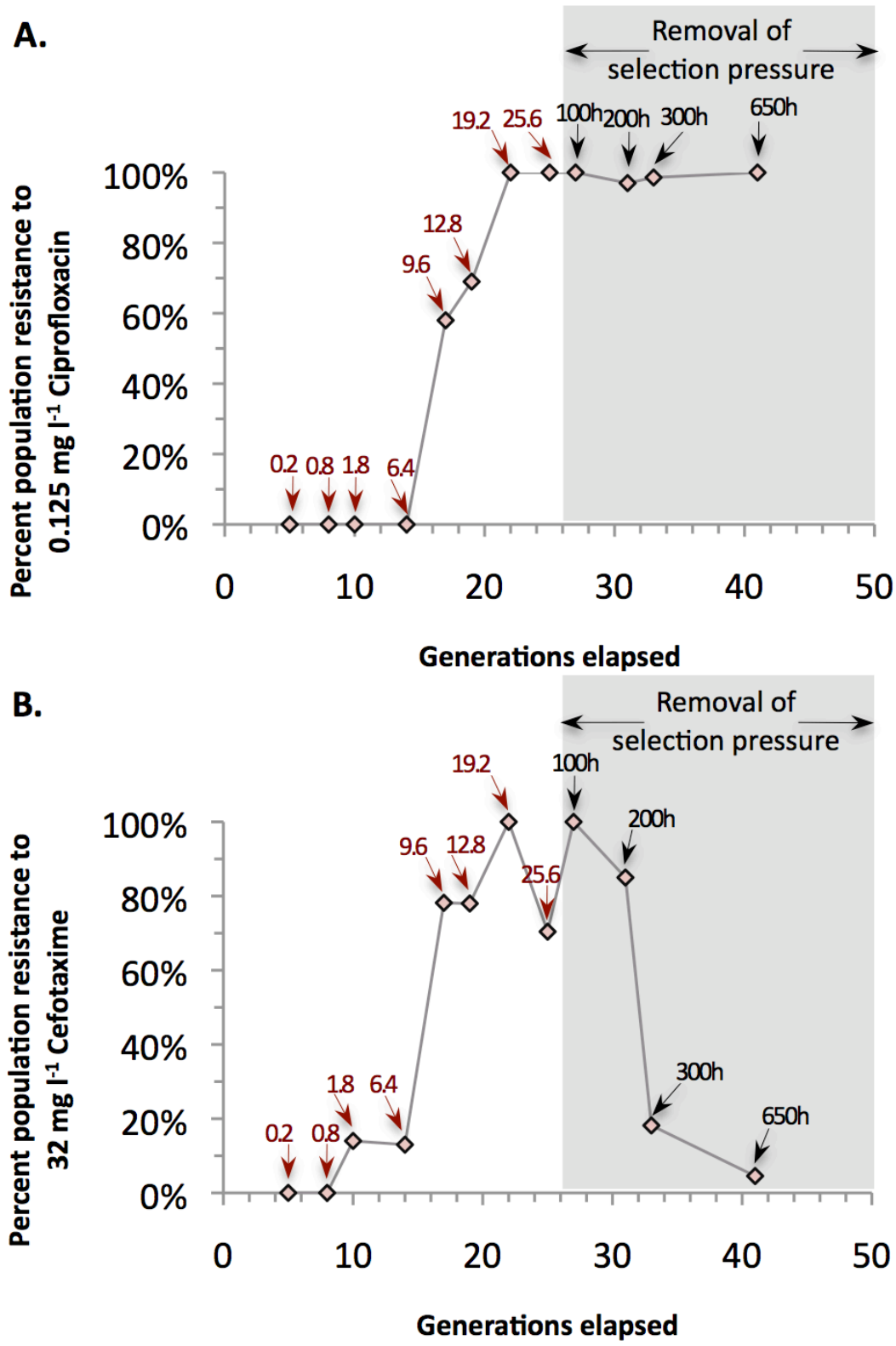

Figure 2.11 Percent population cross-resistance to the antibiotic ciprofloxacin (A) and cefotaxime (B) in CR5. Concentration of ciprofloxacin and cefotaxime used was 0.125 mg l^{-1} and 32 mg l^{-1} respectively (2X MIC_{Agar} of original strain used to inoculate chemostat to ciprofloxacin and cefotaxime). Concentrations of BKC selection pressure in media feed in mg l^{-1} (arrowed, red) and time post removal of selection pressure in hours (h; arrowed, black). Data derived from n=100 tested at each sampling period.

2.4.6 PFGE of *C. jejuni* (original strain) and adapted variants

Pulse field gel electrophoresis of the genomic DNA of the original *C. jejuni* strain and chemostat- adapted variants was carried out. The resultant *Sma I* generated PFGE profile of the original *C. jejuni* strain is shown (Figure 2.12). *Sma I* generated PFGE profiles for the adapted variants were indistinguishable from that of the original *C. jejuni* strain (Table 2.2).

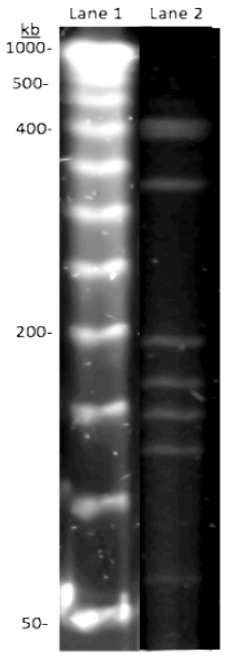

Figure 2.12 PFGE profile of *C. jejuni*. Lane **1:** Molecular marker-ProMega-Markers® Lambda Ladders. Lane **2:** *C. jejuni* (original strain).

Source	Chemostat	PFGE profile similar to original
Ciprofloxacin-adapted variant	CR2	yes
Ciprofloxacin-adapted variant	CR3	yes
BKC-adapted variant	CR5	yes

Table 2.2 Results from SmaI generated PFGE profiles comparisons of antimicrobial-adapted variants generated in this study.

2.5 Discussion

This chapter describes the initial characterization of *C. jejuni* NCTC 11168 and the development of ciprofloxacin-adapted or BKC-adapted *C. jejuni* derived variants following exposure to these agents in long term chemostat cultures. Cross-resistance between BKC-adapted chemostat populations to the antibiotics ciprofloxacin and cefotaxime was also evaluated. *C. jejuni* (original strain) was shown to have MIC values of 8 mg l^{-1} BKC and 0.125 mg l^{-1} ciprofloxacin on Mueller Hinton agar supplemented with 5% lysed horse blood and MIC values of 0.2 mg l^{-1} BKC and 0.06 mg l^{-1} ciprofloxacin in Mueller Hinton broth supplemented with 0.35% (v/v) Isovitalex™. These parameters established breakpoint concentrations for the detection of adapted variants in the chemostat-derived population. MIC_{Agar} and MIC_{Broth} values of *C. jejuni* to ciprofloxacin reported here are in agreement with those of Yan *et al.,* (2006) and McDermott *et al.,* (2005). A limited amount of information is available on the susceptibility of *Campylobacter spp.* to disinfectants in the published literature.

Two studies were identified that examined the antimicrobial susceptibility of *C. jejuni* to BKC. Peyrat *et al.,* (2008b) found that *C. jejuni* strains had MIC_{Agar} values to BKC in the range of 8-16 mg l^{-1} BKC, which is in agreement with the MIC_{Agar} value (8 mg l^{-1} BKC) of *C. jejuni* measured in this study. However the MIC_{Broth} value of *C. jejuni* to BKC at 0.2 mg l^{-1} BKC that was found in this study was lower then those published by Mavri *et al.,* (2013a, b) who reported that *C. jejuni* NCTC 11168 had an MIC_{Broth} to BKC of 0.5-1 mg l^{-1} BKC and a MIC_{Broth} to ciprofloxacin of 0.25 mg l^{-1} which was two-four fold higher than the MIC_{Broth} values obtained in this study. However, Mavri *et al.,* (2013a, b) used a slightly different methodology than the one used in this study and did not supplement media with Isovitalex™ (which is shown to support the growth of *Campylobacter*) and could be one possible explanation for the lower MIC_{Broth} values obtained in *C. jejuni* in this study.

Continuous culture enrichments were performed to obtain *C. jejuni* adapted variants exhibiting resistance to BKC or ciprofloxacin. During selection, the chemostat was fed with fresh medium containing the antimicrobial at a constant flow rate. Simultaneously, an equal volume containing both cells and spent medium was removed from the culture vessel to maintain a fixed culture volume (Gresham & Hong, 2015; Toprak *et al.,* 2011).

During continuous culture in the absence of selection, a stable equilibrium is achieved through the continuous addition of medium containing a single growth-limiting nutrient and simultaneous removal of culture (Gresham & Hong, 2015). At this steady state, the growth rate is equal to the dilution rate and the population size remains constant (Toprak *et al.,* 2011). However, during selection, the selection pressure also has an effect on growth rate. If too high a selection pressure is added to the medium feed, this may depress the growth rate of the populations so much that there would be insufficient growth to replace the chemostat population being removed by addition of new media to the chemostat. This could potentially lead to washout of the chemostat culture (Chopra *et al.,* 1990; Smith *et al.,* 1996). Determination of the maximum specific growth rate (μ_{max}) of the strain prior to commencement of continuous culture of *C. jejuni* in chemostats was necessary to prevent this from happening. This information was used to determine a suitable dilution rate for operation of the chemostat. The growth characteristics of *C. jejuni* were determined in Mueller Hinton broth. The μ_{max} of *C. jejuni* was derived from the linear portion of the exponential growth phase of the plot of OD versus time (Figure 2.2). The doubling time of *C. jejuni* was determined to be 6.2 hours, which corresponded to a μ_{max} of 0.11 h^{-1}. A dilution rate of 0.025 h^{-1}, i.e. lower than that of the growth rate of *C. jejuni*, was subsequently chosen for operation of chemostats.

In order to select for spontaneous mutants with elevated MICs for BKC or ciprofloxacin, *C. jejuni* (original strain) was grown in a continuous culture (D=0.025 h^{-1}) using Mueller Hinton broth. Viable cell counts on MHA plates (supplemented with 5% lysed horse blood) containing 2- fold dilutions of the antimicrobial agent (either ciprofloxacin or BKC) were performed to allow a more detailed analysis of the adaptation to selection pressure. The MIC of antimicrobial agent was the concentration of that agent that resulted in a maintenance of or a reduction of less then 3 $\log_{10}$ (99.9 %) of viable cell counts. This criteria, (< 3 $\log_{10}$ reduction) has been previously used to determine bacteriostatic activity in time kill kinetic studies of antimicrobial agents (Petersen *et al.,* 2007). The reversal of ciprofloxacin resistance and reduced antimicrobial susceptibility to BKC in the chemostat population was also evaluated. The selection pressure was removed from two of the chemostats (CR2; CR3) operated with ciprofloxacin selection pressure and one of the chemostats operated with BKC selection pressure (CR5). These chemostats were operated for a further 500-850 h while monitoring antimicrobial resistance. The operating conditions of all chemostats are shown in Table 2.1.

Ciprofloxacin is one of the most common antimicrobial agents used in the treatment of *Campylobacter* infections (Engberg *et al.,* 2001; Gibreel & Taylor, 2006). Its mechanism of action involves the inhibition of DNA synthesis, which results in cell death (Kohanski *et al.,* 2010). Studies have shown that a single treatment with a fluoroquinolone antibiotic in poultry is enough to lead to the emergence of ciprofloxacin resistant in *C. jejuni* strains (Humphrey *et al.,* 2005; McGill *et al.,* 2006).

Chemostat enrichment using ciprofloxacin as a selective pressure induced the greatest magnitude of adaptation in chemostat culture populations of the two types of antimicrobial agents tested. Developed variants had MIC_{Agar} values of 256 mg l^{-1} ciprofloxacin (2048-fold greater than the original parent strain; Figure 2.3-2.5). The largest rise in MIC_{Agar} from 0.125 to 4 mg l^{-1} ciprofloxacin (32-fold increase compared to original strain; Figure 2.5) occurred after concentrations of applied selective pressure exceeded the MIC_{Broth} of *C. jejuni* to ciprofloxacin (>0.06 mg l^{-1}). In CR2, a 16-fold increase in MIC_{Agar} (from 0.25 mg l^{-1} to 4 mg l^{-1}) was observed when the concentration was increased from 0.06 mg l^{-1} to 0.12 mg l^{-1} (see Figure 2.4).

Interestingly, the presence of sub-MIC_{broth} concentrations in chemostat culture did not alter the chemostat population MIC_{Agar} to ciprofloxacin. At a certain concentration of ciprofloxacin, however, termed the minimal selection concentration (MSC), *C. jejuni* strains carrying mutations should become growth rate advantaged compared to their sensitive counterpart (McCay et al., 2010; O'Reilly & Smith, 1999). In chemostat competition experiments operated by Fleming *et al*., (2002), exposure to 0.03 mg l^{-1} levofloxacin (close to 50 % of the MIC_{broth} of the sensitive strain) resulted in a levofloxacin resistant *E. coli* 35218 outcompeting its sensitive counterpart. It may be that the MSC for ciprofloxacin-adapted mutants generated in this study was also close to the MIC_{broth} of the original strain to ciprofloxacin and resulted in a population exhibited a decreased susceptibility to ciprofloxacin. Rapid development of resistance to another fluoroquinolone, levofloxacin, has been previously reported in chemostat enrichment studies using *E. coli* (Fleming *et al.,* 2002).

The stability of ciprofloxacin resistance in the chemostat population of CR2 and CR3 was also evaluated to ascertain if the evolved populations would be maintained or lost to

the population. The selective pressure was removed from both CR2 and CR3 72 h after 12 mg l^{-1} ciprofloxacin had been introduced into the medium feed. Selection pressure was removed from CR2 after 24 generations and the chemostat was operated for a further 500 h. Similarly in CR3, selection pressure was removed after 30 generations and the chemostat was operated for a further 850 h. The population MIC_{Agar} was observed to decrease (2-4 fold) following removal of selection pressure before stabilizing at 32-64 mg l^{-1} ciprofloxacin.

The molecular mechanisms underlying resistance to ciprofloxacin include, among others, mutations in the gene encoding the molecular target of ciprofloxacin DNA gyrase or as a result of efflux (Luo *et al.,* 2003). Emergence of mutations conferring fluoroquinolone resistance in *C. jejuni* has also been associated with Mfd (Mutant Frequency Decline), a transcription-repair coupling factor involved in strand-specific DNA repair. Mfd has been shown to be up-regulated in *C. jejuni* following treatment with ciprofloxain (Han *et al.,* 2008).

Campylobacters are generally considered sensitive to disinfectant although they have been shown to survive cleaning and disinfection regimes (Peyrat *et al.,* 2008a, 2008b). Peyrat et al., (2008b) observed that *Campylobacters* were routinely detected in floor surface swabs of commercial transport cages after cleaning and disinfection. The biocide BKC is widely used in the poultry industry as an active ingredient in commercial disinfectants (Peyrat *et al.,* 2008a; 2008b). Its mechanism of action has been described and involves the disruption of the bacterial cell membrane which results in leakage of intracellular constituents eventually leading to cell death (Gilbert & Moore, 2005).

In the present study, adaptation to BKC was monitored following prolonged exposure to incrementally increasing concentrations of BKC. Chemostat-based adaptation of *C. jejuni* to BKC has not been reported previously. Adaptation of the chemostat population to BKC was gradual and resistance was observed to increase in a slow stepwise manner following exposure to BKC (Figure 2.6 & 2.7). In contrast to adaptation to ciprofloxacin, the rate of adaptation of *C. jejuni* to BKC was different for both chemostat populations and did not depend on a specific concentration of BKC being attained in the chemostat. However the same degree of adaptation to BKC was achieved in both chemostat populations (BKC-adapted variants of *C. jejuni* had MIC_{Agar} values of 64 mg l^{-1} BKC, 8-

fold higher than the original strain). Similar to ciprofloxacin, presence of sub-MIC_{broth} concentrations in chemostat culture did not alter the chemostat population MIC_{Agar} to BKC. This suggests that the MSC for BKC-adapted mutants was also close to the MIC_{broth} of the original strain to BKC, which was also found for ciprofloxacin-adapted chemsotat populations. McCay *et al.,* (2010) similarly reported a slow stepwise adaption of *Pseudomonas aeruginosa* to BKC during chemostat enrichment studies.

In order to assess the stability of BKC-adaptation in the chemostat population, selective pressure was removed from the media feed in CR5 after 26 generations (1016 h; Figure 2.7) and the chemostat was operated for a further 750h (19 generations). After 750h, following removal of selection pressure, no decrease in population MIC_{Agar} to BKC in CR5 was observed (Figure 2.7), indicating that increased resistance to BKC was stable.

Molecular mechanisms leading to increased resistance to BKC include biofilm formation, blebbing of the bacterial cell wall and efflux (Joynson *et al.,* 2002; SCENHIR, 2009). Growth as part of a sessile community referred to as a biofilm is a form of phenotypic adaptation to antimicrobial agents such as BKC (Fernández *et al.,* 2011; SCENIHR, 2009). Physiological and structural processes including impairment of biocide penetration into the biofilm matrix, quenching by the exopolysaccharides, enzymatic inactivation and modified physiological state of bacteria as a result of sessile growth are responsible for the observed resistance (Gilbert & McBain, 2003; SCENIHR, 2009). Blebbing of the outer cell membrane of the cell wall of Gram- negative bacteria can also act to neutralize some of the action of BKC in the vicinity of the cell and aid its survival (Joynson *et al.,* 2002; Fernández *et al.,* 2011). Induced expression of efflux pumps as a result of exposure to bile salts and sodium deoxycholate have been shown to lead to increased resistance to biocides including BKC in *Campylobacter spp.* (Mavri & Možina, 2013). Stable inheritance of reduced susceptibility to BKC may indicate that genotypic (acquired resistance) rather than transient phenotypic (adaptive resistance) mechanisms are responsible for the adaptation of *C. jejuni* to BKC seen here (Corona & Martinez, 2013). Stable inheritance of reduced susceptibility to BKC in *Pseudomonas aeruginosa* was also noted following adaptation to BKC using chemostat enrichment (McCay *et al.,* 2010).

A further two control chemostats were operated without antimicrobial selection pressure. Samples derived from chemostat populations without antimicrobial selection pressure did not show any increase in resistance on MHA to the antibiotic ciprofloxacin after 28 generations (Figure 2.8 & 2.9). An increase (2-fold) in MIC_{Agar} to BKC was noted in CR6 after 12 generations. No further increases were noted for the remainder of the chemostat duration (up to generation 22). No increase in the population MIC_{Agar} to BKC was observed in CR7 and antimicrobial susceptibility to BKC remained unchanged when compared to that of the original parent strain. In addition the OD_{625nm} values from control chemostats did not fluctuate greatly (Figure 2.8 & 2.9) when compared to chemostats undergoing selection to antimicrobial agents BKC and ciprofloxacin (Figure 2.3-2.7). This result is in agreement with those reported by Fleming *et al.,* (2002) who also found that chemostat cultures of *E. coli* operated without levofloxacin selection showed little fluctuations in OD_{540nm} measurements. Fluctuations are more commonly associated with the generation and periodic selection of mutants (adapted strains) in long-term chemostat culture (Fleming *et al.,* 2002). In the absence of a specific selection pressure, selection in the chemostat acts on those parameters that affect genetic traits which control growth and substrate utilization, the maximal growth rate (μmax) and the nutrient concentration required for half-maximal growth rate (K_s) (Gresham & Hong, 2015). Consequently, this also leads to selection of unknown traits present in the genetically favoured variants of *C. jejuni* (Dykhuizen & Hartl, 1983). However it can be seen here that this did not result in evolution toward resistance/increased susceptibility to ciprofloxacin/BKC.

Biocides have been shown to increase low-level cross-resistance to antibiotics in bacteria (Braoudaki & Hilton, 2004; Randall *et al.,* 2003). Furthermore, low-level resistance mechanisms can facilitate the acquisition of high-level resistance mutations to antibiotics (Jacoby, 2005; Goldstein, 2007). Molecular mechanisms leading to cross-resistance between antibiotics and biocides have been described and include reduced expression of porins, overexpression of efflux pumps and acquisition of mobile genetic elements (Buffet-Bataillon *et al.,* 2012). Replica plating was preformed to assess the emergence of low-level cross-resistance to the antibiotic ciprofloxacin in CR4 and to ciprofloxacin and the cephalosporin antibiotic cefotaxime in the BKC-adapting chemostat populations CR4 and CR5 (Figure. 2.10 & 2.11). Cross-resistance between BKC adapted populations and ciprofloxacin was observed to increase (2-fold) over the course of adaptation to BKC in both chemostats (Figure. 2.10 & 2.11). In addition, cross-resistance to cefotaxime was

found to increase (2-fold) over the course of adaptation to BKC in CR5. Stability of cross-resistance to ciprofloxacin and cefotaxime was assessed in the BKC-adapted chemostat population (CR5) after selection pressure was removed from the media feed (Figure 2.11). It was observed that the developed cross-resistance to ciprofloxacin was stable (650 h after selection pressure was removed; Figure 2.11). However developed cross-resistance to cefotaxime was found to be unstable in BKC-adapted chemostat population and was lost in the same time period (Figure 2.11).

Pulsed-field gel electrophoresis (PFGE) is widely used in molecular typing studies of *Campylobacter spp.* because of its high discriminatory power (O'Leary *et al.,* 2011; On *et al.,* 2008; Sarhin *et al.,* 2010). PFGE and other molecular typing methods can provide information on the genetic subtypes in circulation and have been instrumental in enhancing epidemiological investigations aimed at tracking sources of sporadic infections with *Campylobacter spp.* (Taboada *et al.,* 2013). PFGE is based on the restriction digestion of the *Campylobacter* chromosome with rare cutting restriction enzymes, typically KpnI or SmaI, into a small number of large fragments that are then separated electrophoretically based on size in a coordinated pulsed electric field (Taboada *et al.,* 2013; Wassenaar & Newell, 2000). Together with multi-locus sequence typing, PFGE has become one of the most extensively used molecular typing methods for *Campylobacter spp.* (Sarhin *et al.,* 2010; Taboada *et al.,* 2013). A PFGE protocol had been established in our laboratory and was carried out as a quality control to show that the original strain and ciprofloxacin- and BKC- adapted variants were the same strain. Both original strain and chemostat-adapted variants isolated from CR2, CR3 and CR5 showed similar PFGE profiles (Figure 2.12 & Table 2.2) Furthermore all strains had a similar profile to a published PFGE profile of *C. jejuni* NCTC 11168 (Taylor *et al.,* 1992).

These results confirm that *Campylobacter* readily acquires stable resistance to ciprofloxacin and BKC and suggests that exposure to concentrations of antimicrobial agent, above MIC concentrations are required for the adaptation of *C. jejuni* to these antimicrobials. In addition, adaptation to BKC may also lead to low-level cross-resistance to ciprofloxacin.

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2.6 Bibliography

Braoudaki, M. and Hilton, A.C. (2004). Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. *J. Clin. Microbiol., 42*(1), 73-78.

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M. and Jolivet-Gougeon, A. (2012). Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds--a critical review. *Int. J. Antimicrob. Agents, 39*(5), 381-389.

Chapman, J.S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation, 51*(4), 271–276.

Chopra, I., Hacker, K., Misulovin, Z. and Rothstein, D.M. (1990). Sensitive biological detection method for tetracyclines using a tetA-lacZ fusion system., *Antimicrob. Agents Chemother., 34*(1), 111-116.

Clinical and Laboratory Standards Institute (2006). *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guidelines. CLSI document M45-A2*, Wayne, PA: Clinical and Laboratory Standards Institute.

Cosgrove, S.E. (2006). The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clin. Infect. Dis., 42*(Suppl 2), S82-S89.

EFSA (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA Journal, 8*(3), 1503.

Engberg, J., Aarestrup, F.M., Taylor, D.E., Gerner-Smidt, P. and Nachamkin, I. (2001). Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. *Emerging Infect. Dis., 7*(1), 24-34.

ESVAC (2013). *Sales of Veterinary Antimicrobial Agents in 25 EU/EEA Countries in 2011 (EMA/236501/2013)*, London, UK: ESVAC.

Fernández, L., Breidenstein, E.B. and Hancock, R.E. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resist. Updat., 14*(1), 1-21.

Fleming, G., Dawson, M.T. and Patching, J.W. (1988). The isolation of strains of *Bacillus subtilis* showing improved plasmid stability characteristics by means of selective chemostat culture. *J. Gen. Microbiol., 134*(8), 2095-2101.

Fleming, G.T., McCarthy, D.M., Colombet, N. and Patching, J.W. (2002). The effect of levofloxacin concentration on the development and maintenance of antibiotic-resistant clones of *Escherichia coli* in chemostat culture. *J. Ind. Microbiol. Biotechnol., 29*(4), 155-162.

FSAI (2011). *Recommendations for a Practical Control Programme for Campylobacter in the Poultry Production and Slaughter Chain.* Dublin, Ireland: Food Safety Authority of Ireland. Available at www.fsai.ie.

Gibreel, A. and Taylor, D.E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 58*(2), 243-255.

Gilbert, P. and Moore, L.E. (2005). Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol., 99*(4), 703-715.

Goldstein, F. (2007). The potential clinical impact of low-level antibiotic resistance in *Staphylococcus aureus*. *J. Antimicrob. Chemother.*, 59(1), 1-4.

Gresham, D. and Hong, J. (2015). The functional basis of adaptive evolution in chemostats. *FEMS Microbiol. Rev., 39*(1), 2-16.

Han, J., Sahin, O., Barton, Y.W. and Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog.*, *4*(6), e1000083.

HPSC (2014). *Annual Report 2014 Health Protection Surveillance Centre*, Dublin, Ireland: Health Protection Surveillance Centre. 52-54.

Jacoby, G.A. (2005). Mechanisms of resistance to quinolones. *Clin. Infect. Dis., 41* (Suppl 2), S120-S126.

Joynson, J.A., Forbes, B. and Lambert, R.J. (2002). Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. *J. Appl. Microbiol., 93*(1), 96-107.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. (2010). How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol., 8*(6), 423-435.

Kudirkienė, E., Bunevičienė, J., Brøndsted, L., Ingmer, H., Olsen, J.E. and Malakauskas, M. (2011). Evidence of broiler meat contamination with post-disinfection strains of *Campylobacter jejuni* from slaughterhouse. *Int. J. Food Microbiol., 145*(Suppl 1), S116-S120.

Langsrud, S., Sidhu, M.S., Heir, E. and Holck, A.L. (2003). Bacterial disinfectant resistance—a challenge for the food industry. *Int. Biodeterior. Biodegradation, 51*(4), 283-290.

Lavilla Lerma, L., Benomar, N., Gálvez, A. and Abriouel, H. (2013). Prevalence of bacteria resistant to antibiotics and/or biocides on meat processing plant surfaces throughout meat chain production. *Int. J. Food Microbiol., 161*(2), 97-106.

Luo, N., Sahin, O., Lin, J., Michel, L.O. and Zhang, Q. (2003). *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother., 47*(1), 390-394.

Mavri, A. and Smole Možina, S. (2013). Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol., 160*(3), 304-312.

Mavri, A. and Smole Možina, S. (2013). Effects of efflux-pump inducers and genetic variation of the multidrug transporter cmeB in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. *J. Med. Microbiol., 62*(Pt 3), 400-411.

McCay, P.H., Ocampo-Sosa, A.A. and Fleming, G.T. (2010). Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. *Microbiology, 156*(Pt 1), 30-38.

McDermott, P.F., Bodeis-Jones, S.M., Fritsche, T.R., Jones, R.N. and Walker, R.D. (2005). Broth microdilution susceptibility testing of *Campylobacter jejuni* and the determination of quality control ranges for fourteen antimicrobial agents. *J. Clin. Microbiol., 43*(12), 6136-6138.

McGill, K., Cowley, D., Moran, L., Scates, P., O'Leary, A., Madden, R.H., Carroll, C., McNamara, E., Moore, J.E., Fanning, S., Collins, J.D. and Whyte, P. (2006). Antibiotic resistance of retail food and human *Campylobacter* isolates on the island of Ireland from 2001-2002. *Epidemiol. Infect., 134*(6), 1282-1291.

Meyer, B. (2006). Does microbial resistance to biocides create a hazard to food hygiene? *Int. J. Food Microbiol., 112*(3), 275-279.

Miles, A.A., Misra, S.S. and Irwin, J.O. (1938). The estimation of the bactericidal power of the blood. *J Hyg (Lond), 38*(6), 732-749.

O'Leary, A.M., Whyte, P., Madden, R.H., Cormican, M., Moore, J.E., Mc Namara, E., Mc Gill, K., Kelly, L., Cowley, D., Moran, L., Scates, P., Collins, J.D. and Carroll, C.V. (2011). Pulsed field gel electrophoresis typing of human and retail foodstuff *Campylobacters*: an Irish perspective. *Food Microbiol., 28*(3), 426-433.

O'Reilly, A. and Smith, P. (1999). Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. *Aquaculture, 180*(1-2), 1-11.

Petersen, P.J., Jones, C.H. and Bradford, P.A. (2007). In vitro antibacterial activities of tigecycline and comparative agents by time-kill kinetic studies in fresh Mueller-Hinton broth. *Diagn. Microbiol. Infect. Dis., 59*(3), 347-349.

Peyrat, M.B., Soumet, C., Maris, P. and Sanders, P. (2008). Phenotypes and genotypes of *Campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses. *Vet. Microbiol., 128*(3-4), 313-326.

Peyrat, M.B., Soumet, C., Maris, P. and Sanders, P. (2008). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol., 124*(2), 188-194.

Randall, L.P., Ridley, A.M., Cooles, S.W., Sharma, M., Sayers, A.R., Pumbwe, L., Newell, D.G., Piddock, L.J. and Woodward, M.J. (2003). Prevalence of multiple antibiotic resistance in 443 *Campylobacter spp.* isolated from humans and animals. *J. Antimicrob. Chemother., 52*(3), 507-510.

Riedel, C.T., Brøndsted, L., Rosenquist, H., Haxgart, S.N. and Christensen, B.B. (2009). Chemical decontamination of *Campylobacter jejuni* on chicken skin and meat. *J. Food Prot., 72*(6), 1173-1180.

Sauer, U. (2001). Evolutionary engineering of industrially important microbial phenotypes. *Adv. Biochem. Eng. Biotechnol., 73*, 129-169.

SCENIHR (2009) *Assessment of the antibiotic resistance effects of biocides*, Brussels, Belgium: Scientific Committee on Emerging and Newly Identified Health Risks. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf

Smith, P., Niland, T., O'Domhnaill, F., O' Tuathaigh, G. and Hiney, M. (1996). Influence of marine sediment and divalent cations on the activity of oxytetracycline against *Listonella anguillarum*. *Bull. Eur. Ass. Fish Pathol., 16*(2), 54-57.

Taylor, D.E., Eaton, M., Yan, W. and Chang, N. (1992). Genome maps of *Campylobacter jejuni* and *Campylobacter coli*. *J. Bacteriol., 174*(7), 2332-2337.

Toprak, E., Veres, A., Michel, J.B., Chait, R., Hartl, D.L. and Kishony, R. (2011). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet., 44*(1), 101-105.

Yan, M., Sahin, O., Lin, J. and Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J. Antimicrob. Chemother., 58*(6), 1154-1159.

Phenotypic characterization of chemostat-derived variants of *C. jejuni* NCTC 11168 adapted to ciprofloxacin or benzalkonium chloride.

Olivia Duignan[1], Gerard Fleming[2] and Cyril Carroll[1].

[1]Enteric pathogen Research Laboratory, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland; [2]Microbial Oceanography Research Unit, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland.

3.1 Abstract

Phenotypic changes in ciprofloxacin-adapted and benzalkonium chloride (BKC) adapted variants of *Campylobacter jejuni* NCTC 11168 (*C. jejuni*) were examined following long-term exposure to these agents in chemostat culture. Fitness and phenotypic stability of resistance in antimicrobial-adapted isolates was determined. The efficacy of the commercial disinfectant Savlon^{TM} and its main biocidal ingredient BKC against adapted isolates was assessed. Cross-resistance and the role of efflux in ciprofloxacin-adapted isolates was also investigated against ampicillin, cefotaxime, erythromycin, BKC, Savlon^{TM}, Dettol® and TCP®. Adapted variants showed a fitness burden upon adaptation to BKC or ciprofloxacin. Developed resistance was stable in BKC-adapted isolates (100%, n=22) and in the majority of ciprofloxacin-adapted isolates (79.5%, n=39). Savlon^{TM} was efficacious against antimicrobial-adapted variants however, dilution was found to affect efficacy. When compared to the original strain, all BKC-adapted variants and some (4/8) ciprofloxacin-adapted variants showed enhanced survival at lower (1:16, 1:32) strength dilutions of Savlon^{TM}. Furthermore, some (4/8) BKC-adapted variants showed enhanced survival at one-eighth strength dilutions of Savlon^{TM}. Ciprofloxacin-adapted variants resistance showed cross-resistance to cefotaxime, erythromycin, BKC, Savlon^{TM}, Dettol® and TCP® with two to four-fold increases in MIC_{Agar} recorded to three or more of these antimicrobial agents. Efflux pump inhibitors (EPIs) carbonyl cyanide m-chlorophenylhydrazone (CCCP) and phenylalanine arginine b-naphthylamide (PAβN) were unable to reverse cross-resistance in ciprofloxacin-adapted variants to antibiotics and to BKC. However, they were able to restore susceptibility to Savlon^{TM} and Dettol® in 67% (6/9) and 70% (7/10) of variants respectively. These data suggest that adaptation to ciprofloxacin/BKC can be stable and lead to development of cross-resistance to other antibiotics/disinfectants. Also the risk of disinfectant failure due to

dilution may be augmented following adaptation to BKC or development of resistance to ciprofloxacin in *C. jejuni.*

Keywords: *Campylobacter,* ciprofloxacin, benzalkonium chloride, biocide adaptation, efflux, fitness, stability, antimicrobial susceptibility, chemostat

3.2 Introduction

The prevalence of fluoroquinolone (FQ) -resistant *Campylobacter* is increasing worldwide and has become a concern for public health (Price, 2007). Poultry is regarded as one of the most important reservoirs for *Campylobacter* and constitutes a very significant vehicle for the transmission of *Campylobacter* to humans (FSAI, 2011). FQ-resistant *Campylobacters* have been shown to persist on poultry farms up to 4 years after FQ antibiotics usage had ceased (Luangtongkum *et al.,* 2009).

Research has shown that 30 % of human cases of campylobacteriosis have been associated with the consumption of raw or undercooked poultry and between 50% and 80% of cases may be attributed to poultry reservoir as a whole (EFSA, 2009). Effective use of intervention strategies, at both the farm level and slaughter house/production house level, is paramount for the prevention of transmission of *Campylobacters* particularly FQ-resistant strains from poultry to humans. However, control measures in the domestic environment, aimed at prevention the transmission of *Campylobacter* to the consumer, are also important in prevention of Campylobacterosis (Bolton *et al.,* 2014)

Hygiene is recognized as a cost effective means to reduce infectious disease. Good hygiene practices constitute the most effective and economically viable intervention strategies at controlling the transmission of *Campylobacter* in the food chain. Control of *Campylobacter* through the use of chemical disinfectants is an important measure to prevent the transmission of *Campylobacter spp*. to humans from food sources (Bloomfield & Scott, 2013; Hermans *et al.,* 2011; Heres *et al.,* 2004; Mangen *et al.,* 2005; Newell *et al.,* 2011; Saleha *et al.,* 1998). Benzalkonium chloride (BKC) a quaternary ammonium compound is widely used in the poultry industry in disinfectant formulations (Mc Donnell & Russell, 1999; Peyrat *et al.,* 2008a,b; Tezel & Pavlostathis, 2015).

The dissemination of antimicrobial-resistant bacteria is a key contributor to the widespread emergence of problems in the treatment of infectious diseases (ESFA, 2009). The stability of the resistance mechanism in the absence of antimicrobials is an important factor to consider when discussing the emergence and dissemination of

antimicrobial resistance in bacterial population (Andersson & Hughes, 2010). All antimicrobials target vital physiological processes that are essential for bacterial growth such as DNA, protein and cell wall synthesis. Mutations in genes that are associated with antibiotic resistance may impose a biological cost to *Campylobacter* in the absence of antimicrobial selection pressure (Andersson, 2003; Zhang *et al.,* 2006). The biological cost of antimicrobial resistance may result in a reduction in growth rate compared to the un-adapted strain (McCay *et al.,* 2010). However, the cost can be offset by the acquisition of compensatory mutations that can potentially restore fitness without loss of resistance, and thus contribute to the stability of resistance (persistence of a resistance trait) within the bacterial population (Andersson & Hughes, 2010, 2011).

Campylobacter has evolved multiple mechanisms to resist the action of antimicrobial agents (Luangtongkum *et al.,* 2009). Efflux is a common resistance mechanism that can confer resistance to a broad range of antimicrobial compounds including ciprofloxacin resistance (Lin *et al.,* 2002). The possibility exists that resistance to disinfectants may co-select for drug-resistant strains particularly in cases where the two agents share a common resistance mechanism and vice versa (Chapman, 2003; McCay *et al.,* 2010). Multidrug efflux pumps are responsible for intrinsic and acquired resistance to multiple, structurally diverse antimicrobials in *Campylobacter* (Jeon *et al.,* 2011; Lin *et al.,* 2005; Payot *et al.,* 2006). Development of multidrug resistance in *Campylobacter* may have implications for successful treatment outcomes (Fallon *et al.,* 2003).

The aim of this study was to determine the phenotypic changes following adaptive resistance of *C. jejuni* to the antibiotic ciprofloxacin and the biocide BKC which arose during chemostat enrichment. Changes in fitness and the stability of resistance following adaptation will be evaluated. Determination of the efficacy of the disinfectant Savlon^{TM} and the biocidal ingredient BKC against adapted strains will also be described. Finally, development of cross-resistance to a number of antibiotics, biocides, antiseptics and disinfectants and the role of efflux pump inhibitors in reversing resistance in ciprofloxacin-adapted variants will be examined

3.3 Materials and methods

3.3.1 Bacterial Strains

C. jejuni NCTC 11168 was the original strain used in this study and was obtained from Dr. Majella Mahar, the National Diagnositic Center, National University of Ireland, Galway. Ciprofloxacin and BKC-adapted isolates, derivatives of *C. jejuni* NCTC 11168, were obtained from culture samples taken from selective/enrichment in chemostats supplemented with either BKC or ciprofloxacin (chapter 2; Table 3.1). Ciprofloxacin-adapted variants 400-02, 400-06, 500-03, 500-04, 700-03, 700-05, 700-06 and 850-03 and BKC-adapted variants 27d-10, 27d-08, 24d-05, 24d-03 and 15d-01 were also used to evaluate the effect of dilution on efficacy of Savlon™.

Chemostat	Selective agent	Total Run time [h]	Total generations Elapsed	Concentration range in the chemostat (mg l^{-1})		Removal of selection pressure [h]
				Initial	Final	
CR1	**Cipro**	**2328**	**58**	**0.006**	**8**	**No**
CR2	**Cipro**	**1422**	**36**	**0.06**	**12**	**Yes [936h; after 24 generations]**
CR3	**Cipro**	**1975**	**49**	**0.01**	**12**	**Yes [1160h; after 29 generations]**
CR4	BKC	1565	39	0.05	20	No
CR5	**BKC**	**1760**	**44**	**0.2**	**25.6**	**Yes [1015h; after 26 generations]**
CR6	None	944	22	-	-	N/A
CR7	None	1100	28	-	-	N/A

Table 3.1 Summary of operating conditions of chemostats carried out in this study. Strains derived from chemostats from which selective pressure was removed from the media feed (highlighted in dark gray) were evaluated further. Note:- Chemostats CR1, CR2 and CR3 underwent ciprofloxacin selection (CIPRO) and CR5 underwent BKC selection.

A 100 µl aliquot of stock from a chemostat culture sample was spreadplated on campylobacter blood-free agar base (CCDA base; CM0739, Oxoid Ltd., UK) and incubated at 42 ^{o}C for 48 h under microaerobic conditions using an anerobic gas generating kit (BR0038B, Oxoid Ltd., UK). After incubation, individual colonies chosen at random were isolated for further analysis. Individual isolates were cultured on CCDA base from which stocks of isolates (50 % (v/v) glycerol and trypic soya

broth; TSB, 214889, Becton Dickinson, Sparks, MD) were made. Stocks were then maintained in cryovials at -20 ^{o}C for short term storage up to 1 month and at -70 ^{o}C for long term storage. Prior to analysis, stocks of adapted variants were resuscitated on CCDA base at 42 ^{o}C for 48 h under microaerobic conditions.

3.3.2 Culture Media and Antimicrobials

Mueller Hinton broth (MHB; Lab114, LabM, UK), Brucella broth (BBL 211088, Becton Dickinson, Sparks, MD, USA) Mueller Hinton agar (MHA; Lab039, LabM, UK) and were prepared according to the manufacturers instructions. Unless otherwise stated, MHB and brucella broth were supplemented with 0.35% (v/v) IsoVitaleX™ (211875, Becton Dickinson Sparks, MD, USA) and MHA was supplemented with 5 % lysed horse blood (Charles River Laboratories, Ireland). Antimicrobials used in this study included Ciprofloxacin (Ciproxin®, supplied as a 2 mg ml-1 solution, Bayer, UK), erythromycin, ampicillin, cefotaxime and biocide BKC purchased from Sigma-Aldrich (Steinheim, Germany). Except for ciprofloxacin, all other antimicrobials were diluted to appropriate working concentrations in sterile deionized water and were filter-sterilized through a 0.2 μm Millipore™ membrane filter before use. Savlon™ disinfectant liquid (benzalkonium chloride 1.2-1.4 % (w/w)), here after referred to as Savlon™, Dettol® antiseptic disinfectant (Chloroxylenol 4.8 % (w/v)), here after referred to as Dettol®, and TCP® liquid antiseptic (Halogenated phenols 0.68 % (w/v); phenol 0.175 % (w/v), here after referred to as TCP®, were off-the-shelf disinfectants purchased from a local supermarket and diluted as directed by manufacturer in sterile deionized water and were filter-sterilized through a 0.2 μm Millipore™ membrane filter before use.

3.3.3 Bacterial fitness

The effect of adaptation to ciprofloxacin and BKC on bacterial fitness of *C. jejuni*, was assessed by measuring its maximum specific growth rate (μmax). in brucella broth supplemented with 0.35 % (v/v) Isovitalex™ in 96-U bottom well plates (Orange scientific) using a multi-well plate reader (Genios, Tecan). Wells were inoculated with 20 μl of an overnight culture to an OD_{595nm} of 0.05. Bacterial growth

at 40 ^{o}C was measured as a function of optical density at OD_{595nm} and measurements were recorded in triplicate at 2 h intervals for up to 48 h. A sterile plastic adhesive cover was applied to the top of the microtiter plate before incubation. Maximum specific growth rates (μMax) were calculated from the portion of the plot showing exponential growth using Graphpad prism 6.0. using the exponential growth equation $Y=Y_o*e^{(K*x)}$ where K is the specific growth rate expressed in reciprocal of h^{-1}, X is time in h, Y is the OD_{595nm} and Y_o is the initial OD_{595nm} when time is zero. Specific growth rate ± standard deviation was expressed as a percentage increase or decrease in growth rate compared to that of the original *C. jejuni* strain. Experiments were repeated twice in triplicate. One-way ANOVA-Dunnett's tests were performed to assess if differences in μMax of adapted variants and the original strain were statistically significant.

3.3.4 Stability of the resistance phenotype

The stability of the resistance phenotype after non-selective growth was examined to identify strains suitable for further analysis. The adapted variants were serial sub-cultured (on CCDA base) in the absence of the primary selection pressure (either ciprofloxacin or BKC) for between five and fifteen subcultures. Susceptibility to BKC or ciprofloxacin was measured after 5, 10, 15 serial passages using the agar dilution method (section 2.3.3, Chapter 2).

3.3.5 Determining the effect of dilution on the efficacy of Savlon^{TM} against chemostat-dervived strains

The bactericidal activity of BKC-based disinfectant Savlon^{TM} and its main active ingredient BKC were assessed against the chemostat-dervived strains (BKC- and ciprofloxacin-adapted strains). Antimicrobial susceptibility to Savlon^{TM} and BKC was measured using the broth micro dilution method (section 2.3.3, Chapter 2).

A loopful of test strain taken from an overnight culture was used to inoculate 6.5 ml of MH broth (Lab M, UK) supplemented with 0.35 % (v/v) IsoVitaleX^{TM} (Becton Dickinson, UK and was incubated at 42 ^{o}C for 6-8 h. After incubation, the culture was vortexed and 1 ml of culture was transferred into a fresh MH broth containing 0.35 %

(v/v) IsoVitaleX^{TM} and incubated for a further 18 h at 42 ^{o}C. Prior to addition of disinfectant solution a 1 ml aliquot of culture was removed and transferred into a sterile 1.5 ml eppendorf and from this a 0.1 ml aliquot was removed. Ten-fold serial dilutions (10^0 to 10^6) of culture were made in sterile PBS and 0.1 ml from each dilution was spread plated onto CCDA base in triplicate. A 1 ml aliquot of disinfectant at the appropriate concentration was then added to the culture. After 15 and 30 min intervals 1 ml aliquots were removed and samples were subjected to further ten-fold serial dilutions (up to 10^7) in sterile PBS and a 0.1 ml aliquot from each dilution was spread plated onto CCDA base in triplicate. Plates were incubated for 48 h at 42 ^{o}C under microaerobic conditions and colonies enumerated. Disinfectant activity was determined by calculating the $\log_{10}$ reduction in viable counts achieved after 15 and 30 min. Results were reported as the mean±standard deviation $\log_{10}$ reduction in CFU per ml. Efficacy of each dilution of disinfectant was tested for its capacity to cause up to, or greater than, a 5 $\log_{10}$ fold reduction (99.999%) in CFU per ml of *C. jejuni* strains after 15 and 30 min exposure to disinfectant.

3.3.6 Efflux inhibition assay and antimicrobial susceptibility testing

The efflux pump inhibitors Phe-Arg β-naphthylamide dihydrochloride (PAβN) and carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) were used to assess the role of efflux pumps as a molecular mechanism underlying resistance to cross-resistance to antibiotics and biocides in ciprofloxacin-adapted *C. jejuni* variants. MIC_{Agar} values of antimicrobial agents were determined using the agar dilution method (section 2.3.3, chapter 2).

The efflux inhibition assay was preformed on ciprofloxacin-adapted variants and MICs of antimicrobial agents (ciprofloxacin, ampicillin, erythromycin, cefotaxime, BKC, Dettol®, TCP® and Savlon^{TM}) in the presence or absence of PAβN (32 mg l^{-1}; Sigma-Aldrich, UK) and CCCP (16 mg l^{-1}, Fluka, UK) were determined using a Miles and Misra modification of agar dilution method (section 2.3.3, chapter 2; Miles *et al.,* 1938; CLSI, 2006, M45-2A). Briefly, a sterile swab was used to remove bacterial cells from a 48 h plate culture of the test strain and was added to a bijioux

containing 3 mls of sterile PBS until turbidity equalling that of a 0.5 Mcfarland had been achieved. A 20 µl aliquot was spot inoculated onto the surface of the agar plates and allowed to dry. Plates were incubated under microaerobic conditions using an anerobic gas generating kit (BR0038B, Oxoid) at 42 ^{o}C for 48 h. The presence or absence of growth was recorded and used to determine MIC_{Agar} values. Results were recorded as changes in MIC_{Agar} values to antimicrobial agent in the presence or absence of EPI were determined in tripliacte and fold difference in MIC_{Agar} values for test strains compared to the original strain. For the purposes of this study the minimum inhibitory concentration (MIC) of an antimicrobial agent was defined as being the minimum concentration of antimicrobial agent required to completely inhibit growth of the strain as determined after 48 hours of incubation at 42 ^{o}C under microaerobic conditions.

3.4 Results

3.4.1 Growth characteristics and stability of adapted phenotypes

3.4.1.1 Maximum specific growth rates (μ_{max}) of chemostat-derived isolates compared with the original strain

Antimicrobial resistance in bacteria is often associated with a metabolic burden, resulting in decreased fitness compared to their susceptible counterparts in the absence of the antimicrobial agent (Fleming *et al.,* 1988). Maximum specific growth rates (μ_{max}) of individual isolates derived from the chemostat were compared to the original strain to ascertain if chemostat experience and the evolution of resistance phenotypes resulted in increased or decreased fitness compared with the original strain.

Variants of chemostat-derived isolates of *C. jejuni* were examined from CR3 (undergoing adaption to ciprofloxacin; D=0.025) and CR5 (undergoing adaption to BKC; D=0.025). Single colonies were isolated from samples taken during periods where selection pressure was added to the medium feed (between generation 8 and 29, CR3) and after the removal of the selection pressure from the feed (at generation 46 and 49, CR3; at generation 34, 40 and 41, CR5). Growth characteristics were determined in triplicate and from two independent experiments using the Tecan GENios multi-well plate reader (Section 3.3.3). The μ_{max} values of individual strains were then calculated using GraphPad prism v6.0 (GraphPad Software, USA). Growth rates ± standard deviation were expressed as a percentage increase or decrease in growth rate compared to that of the original *C. jejuni* strain. A negative value indicated a decrease in growth rate compared to the original strain. A positive value indicated an increase in growth rate compared to the original strain. One-way ANOVA-Dunnett's tests were preformed to assess if differences in maximum specific growth rates of adapted variants compared to the original strain was statistically significant.

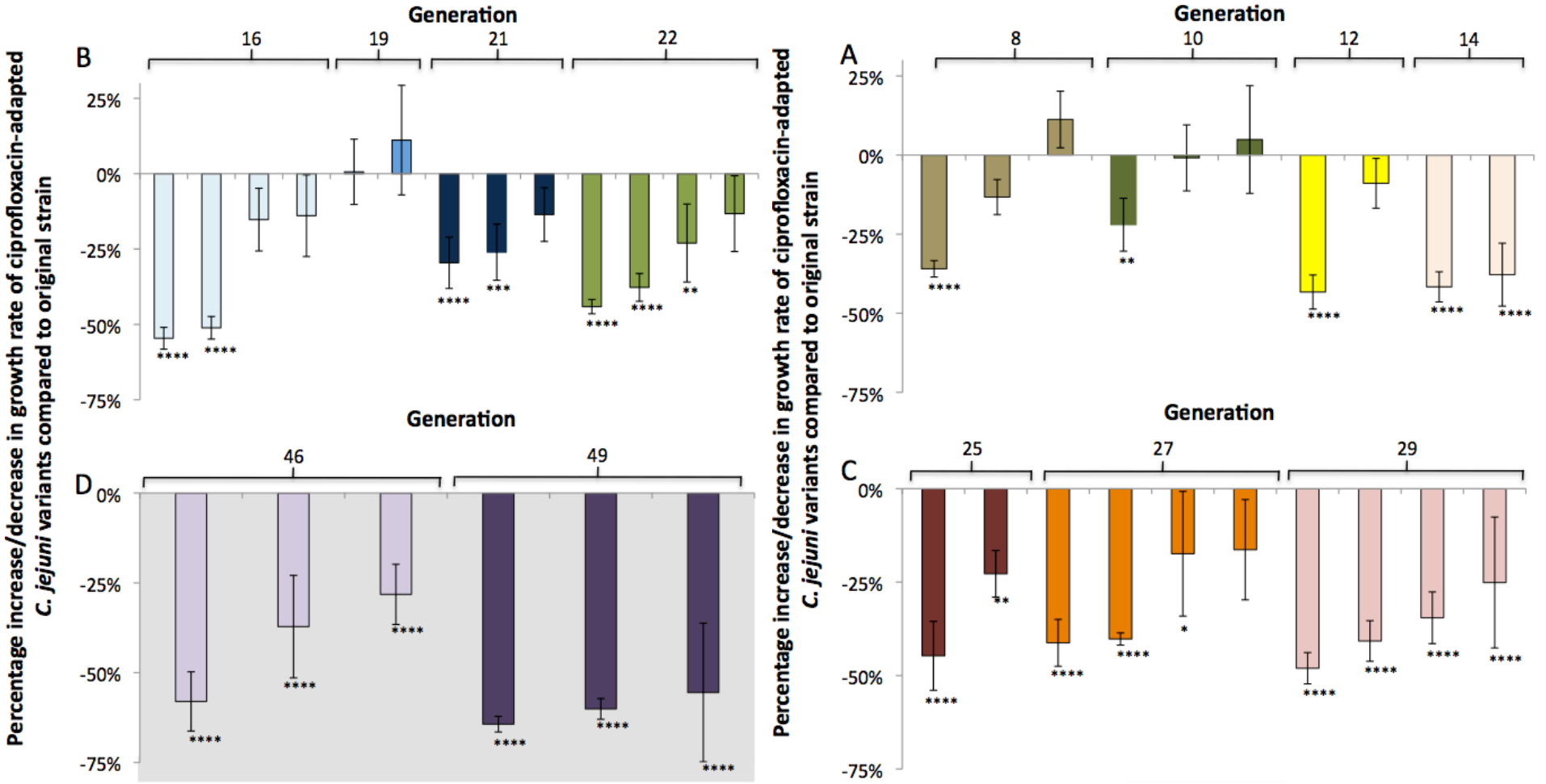

Figure 3.1 Maximum specific growth rates of populations derived from CR3 undergoing adaptation to ciprofloxacin. Note graph shows growth rates ± standard deviation of ciprofloxacin-adapted variants in order of increasing fitness by generation. Removal of ciprofloxacin selection pressure from the media feed of chemostat occurred after generation 29 (shaded gray). The generation during which variants were isolated from are indicated. Note: The level of statistical significance in growth rate differences when compared to original strain as measured by one way ANOVA-Dunnett's tests are indicated by P values *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001.

Maximum specific growth rates ± standard deviation were determined for ciprofloxacin-adapted variants derived from culture samples taken from CR3 undergoing adaptation to ciprofloxacin and are shown order of decreasing fitness in Figure 3.1. Growth rates of adapted variants ranged from 11.2 % ± 8.9 % higher to 64.3 % ± 2.2 % lower then the growth rate for the original strain. A statistically significant decrease in growth rate was recorded in twenty-seven out of thirty nine ciprofloxacin-adapted variants (Figure 3.1). The number of adapted variants exhibiting decreased growth rates was observed to increase when isolated from the latter stages of chemostat culture. The percentage of adapted variants exhibiting statistically significant decreased growth rates between generation 8 and generation 22 was 52.2% (12/23). Between generation 25 and generation 49 the percentage of adapted variants showing decreased growth rates rose to 93.7 % (15/16). Decreased growth rates (compared to the original strain) indicate that adaptation to ciprofloxacin imposed a biological cost to the ciprofloxacin adapted-variants. Variation in growth rates was also seen in isolates derived from the same culture sample (Figure 3.1).

Maximum specific growth rates ± standard deviation for BKC-adapted variants are shown in Figure. 3.2.

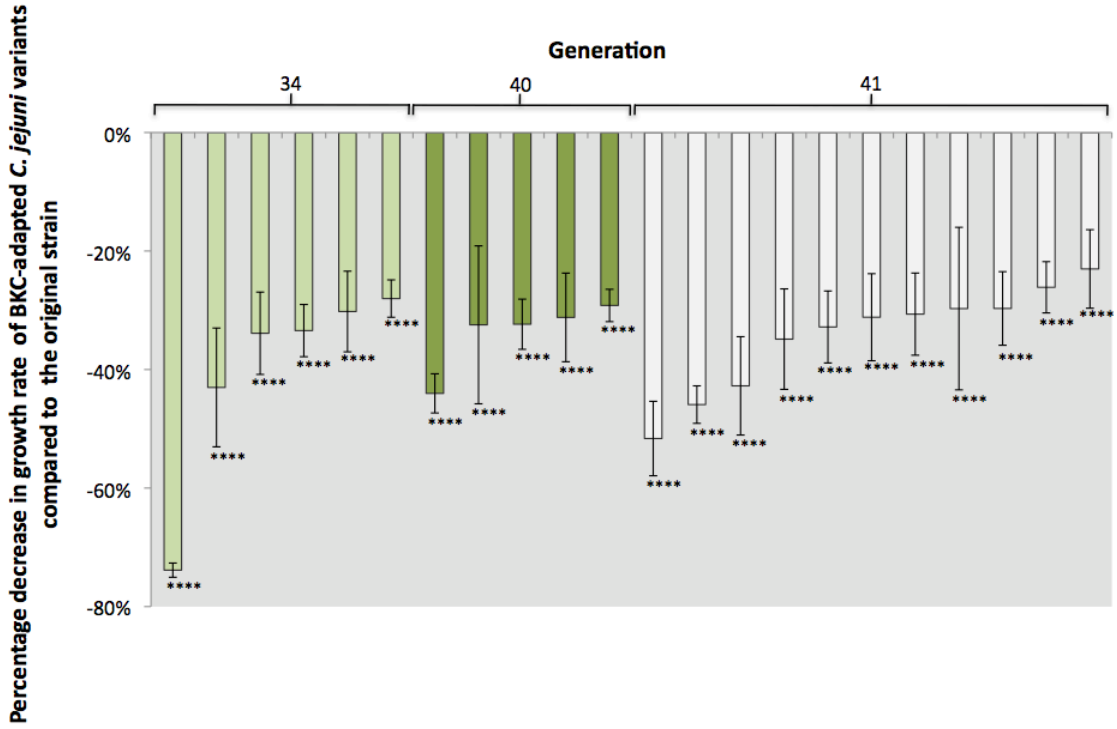

Figure 3.2 Maximum specific growth rates of BKC-adapted variants derived from CR5 after selective pressure was removed from the media feed. Note graph shows growth rates ± standard deviation of BKC-adapted variants in order of increasing fitness between generation 34-41; n=22; P< 0.0001: ****, indicated statistically significant differences in growth rate when compared to original strain as measured by one way ANOVA-Dunnett's test.

Adapted variants were obtained from culture samples of CR5 after selection pressure was removed from the media feed (generation 34-41; n=22). Growth rate of all BKC-adapted variants (22/22) were between 23.0% ± 6.6% and 73.9% ± 1.2% lower than the growth rate for the original strain. Similarly to the ciprofloxacin-adapted variants, variation in growth rates was seen in BKC-adapted variants derived from the same culture sample (Figure 3.2). Decreases in growth rate were statistically significant (P <0.0001) as measured by one-way ANOVA-Dunnett's test.

3.4.1.2 Stability of developed resistance in chemostat isolates

The stability of developed resistance to ciprofloxacin and BKC in chemostat derived isolates of *C. jejuni* was examined. Adapted variants from CR2 (n=14; ciprofloxacin concentration 0.06 – 12 mg l^{-1}; 1422 h; D=0.025 h^{-1}), CR3 (n=25;

ciprofloxacin concentration 0.01 -12 mg l^{-1}; 1422 h; D=0.025 h^{-1}) and CR5 (n=26; BKC concentration 0.2- 25.6 mg l^{-1}; 1760 h; D=0.025 h^{-1}) were sub-cultured in the absence of antimicrobial agent on CCDA base for between 5 and 15 serial transfers. Antimicrobial susceptibility testing to BKC or ciprofloxacin was measured at the start of the experiment and after 5, 10, 15 serial passages undertaken on CCDA base in the absence of antimicrobial agent using the agar dilution method described previously in section 2.3.3 (Chapter 2).

Generation	Number of isolates tested	Range of MIC_{Agar} ciprofloxacin [mg l^{-1}]	Magnitude fold increase(↑)/decrease(↓) in resistance when compared to original strain.	Developed resistance stable ♋
CR2				
*Samples taken after selection pressure removed**				
33	6	64	512 ↑	Yes (6/6)
36	8	64	512 ↑	Yes (8/8)
CR3				
Samples taken during selection§				
8	3	0.06-0.125	2 ↓	No (1/3, 2-fold ↓); Yes (2/3)
10	1	0.06	2 ↓	Yes (1/1)
12	2	1-4	8-32 ↑	No (1/2, 16-fold ↓); Yes (1/2)
16	3	4-16	32-128 ↑	No (1/4, 2-fold ↓; Yes (3/4)
19	1	4	32 ↑	No (1/1, 2-fold ↓)
21	1	8	64 ↑	Yes (1/1)
22	2	16-64	128-512 ↑	No (1/2, 2-fold ↓); Yes (1/2)
25	2	64-256	512-2,048 ↑	No (1/2, 2-fold ↓); Yes (1/2)
27	2	64-256	512-2,048 ↑	No (1/2, 2-fold ↓); Yes (1/2)
29	3	64-256	512-2,048 ↑	No (1/3, 4-fold ↓); Yes (2/3)
*Samples taken after selection pressure removed**				
46	2	128	1,024↑	Yes (2/2)
49	3	128	1,024↑	Yes (3/3)

Table 3.2 Strain characteristics of ciprofloxacin-adapted variants isolated from CR2 and CR3. Note:- Ciprofloxacin-adapted variants were derived from chemostat samples taken during selection and after selection pressure was removed from the media feed. *C. jejuni* NCTC 11168 (original strain) was included as a control and its initial MIC to ciprofloxacin was of determined to be 0.125mg l^{-1} on MHA. Symbol ↓ denotes a decrease in MIC_{Agar} value when compared to the original strain or after serial subculture; Antimicrobial susceptibility testing on isolates was determined § after 5 serial passages, *after 5, 10 and 15 serial passages on CCDA base. ♋ Numbers in parentheses represent the number of isolates exhibiting stable resistance compared to the total number of isolates tested + magnitude of decrease in resistance.

The strain characteristics of variants (antimicrobial susceptibility to ciprofloxacin/BKC and stability of developed resistance) are shown in Table 3.2-

3.3. All isolates derived from CR2 (n=14) were derived from samples taken after ciprofloxacin selection pressure was removed from the chemostat. Ciprofloxacin-adapted variants isolated from CR3 (n=25) were derived from samples taken during selection (n=20) and after selection pressure was removed from the media feed (n=5). It was also observed that approximately 20.5% (8/39) of isolates were found to be unstable after 5 serial transfers on CCDA base, however the majority of these (6/8) did not revert to susceptibility of the original strain.

Generation	Number of isolates tested	MIC_{Agar} BKC [mg l^{-1}]	Magnitude fold increase(↑)/decrease(↓) in resistance when compared to original strain.	Developed resistance stable ℑ
C. jejuni NCTC 11168	N/A	8	-	-
CR5				
*Samples taken after selection pressure removed**				
34	6	64	8-fold	Yes (6/6)
40	6	64	8-fold	Yes (6/6)
41	10	64	8-fold	Yes (10/10)

Table 3.3 Strain characteristics of BKC-adapted variants isolated from CR5. Note:- Isolates were derived from samples taken after selection pressure was removed from the media feed. *C. jejuni* (original strain) was included as a control and its initial MIC to BKC was of determined to be 8 mg l^{-1} on MHA. Symbol ↓ denotes a decrease in MIC_{Agar} value when compared to the original strain or after serial subculture. ℑ Antimicrobial susceptibility testing on isolates was determined *after 5, 10 and 15 serial passages on CCDA base.Numbers in parentheses represent the number of isolates exhibiting stable resistance compared to the total number of isolates tested + magnitude of decrease in resistance.

Isolates from CR5 were derived from culture samples taken after BKC selection pressure was removed from the chemostat (after 34, 40 and 41 generations). All isolates had the same degree of adaptation to BKC (MIC_{Agar} of 64 mg l^{-1} compared to 8 mg l^{-1} for the original *C. jejuni* strain). Adaptation was found to be stable in all isolates tested.

3.4.2 Efficacy of Savlon™ against BKC-adapted and ciprofloxacin-adapted *C. jejuni* variants

The efficacy of the commercial disinfectant Savlon™ was evaluated against BKC-adapted and ciprofloxacin-adapted strains as described in section 3.3.4. The efficacy of BKC (the main biocidal ingredient of Savlon™) was also evaluated to assess the differences of formulation on survival of BKC-adapted strains. The results were recorded as mean ± standard deviation of reduction in CFU per ml. Prior to determining the efficacy of Savlon™ against the original strain and ciprofloxacin-adapted and BKC-adapted variants the MIC_{broth} values were determined according to the method described in section 2.3.3 and results are shown in Table 3.4. Note: The recommended use concentration of Salvon™ was a 1:50 dilution (2 % v/v) of Savlon™, which for the purposes of this assay, was diluted with MHB.

Strain name	Generation	MIC_{broth} Savlon™ (BKC 1.2-1.4% w/w)
C. jejuni NCTC 11168		0.0039% v/v
CR5 (BKC)		
15d-01	34	0.0156% v/v
24d-03	40	0.0156% v/v
24d-05	40	0.0156% v/v
27d-08	41	0.0156% v/v
27d-10	41	0.03% v/v
CR3 (ciprofloxacin)		
700-03	46	0.0078% v/v
700-05	46	0.0078% v/v
700-06	46	0.0078% v/v
850-03	49	0.0078% v/v
CR2 (ciprofloxacin)		
400-02	33	0.0078% v/v
400-06	33	0.0078% v/v
500-03	36	0.0156% v/v
500-04	36	0.0078% v/v

Table 3.4 MIC_{broth} values for BKC and ciprofloxacin-adapted variants to the commercial disinfectant Savlon™

Ciprofloxacin-adapted strains had MIC_{broth} values that were 2-4 fold greater than the original strain to Savlon™. Broth MICs for BKC-adapted *C. jejuni* variants were between 4-fold and 8-fold greater (Table 3.4). Broth MIC concentrations were then used to determine the lowest concentration of Savlon™ to be used in the assay based on the assumption that concentrations below the MIC_{broth} concentrations were

not bactericidal. As a result concentrations of Savlon™ below 1:32 strength of recommended use concentration of Salvon™ were excluded from further analysis. Broth cultures of *C. jejuni* (original strain) and chemostat derived BKC- and ciprofloxacin-adapted *C. jejuni* variants were grown to approximately $\log_{10}$ 8 CFU per ml and exposed to "use" concentrations (2 % v/v) and 2-fold serial dilutions ranging from (1 % v/v- 0.06% v/v) of use concentration of Savlon™ in MHB. Log_{10} percent survival was determined following 15 and 30 minutes following exposure to Savlon™ at 42 ^{o}C. Results were recorded as (mean Log_{10} percent survival ±standard deviation) versus time and are shown in Figure 3.3-3.4.

All adapted variants exhibited variability in survival (as measured by $\log_{10}$ percent survival) to Savlon™. Dilutions of recommended use concentration of Savlon™ after 30 minutes exposure to 1:32 strength dilution (0.06 % v/v) of use concentration Savlon™, the lowest dilution, were not bactericidal (did not result in 5 $\log_{10}$-fold reduction in bacterial counts; Figure 3.3-3.4). However, adaptation of *C. jejuni* to ciprofloxacin increased the bactericidal activity of 1:32 strength dilution of Savlon™ against two of the ciprofloxacin-adapted *C. jejuni* variants after 30 minutes exposure (Shaded area; Figure 3.3). Savlon™ applied at a concentration of 1:16 strength dilution of recommended use concentration showed efficacy after 15 minutes exposure against both the original strain and half (4/8) of the ciprofloxacin-adapted variants tested. Efficacious reductions in bacterial counts (> 5 $\log_{10}$-fold) for all (8/8) of the ciprofloxacin-adapted variants was achieved after 30 minutes exposure at 42 ^{o}C (Figure 3.3). The same strength dilution of recommended use concentration of Savlon™ was not efficacious against the majority (3/4) of BKC-adapted strains tested (Figure 3.4). After 15 minutes exposure to 1:8 dilution of recommended use concentration of Savlon™ bacterial counts (CFU per ml) of BKC adapted *C. jejuni* variants had been reduced by greater than 5 $\log_{10}$ fold (see Figure 3.4). Above these concentrations, at recommended use concentration and ½ and ¼ dilutions of Savlon™, a greater than 5 $\log_{10}$-fold reduction in CFU ml^{-1} bacterial counts were observed after 15 minutes for all strains tested (data not shown).

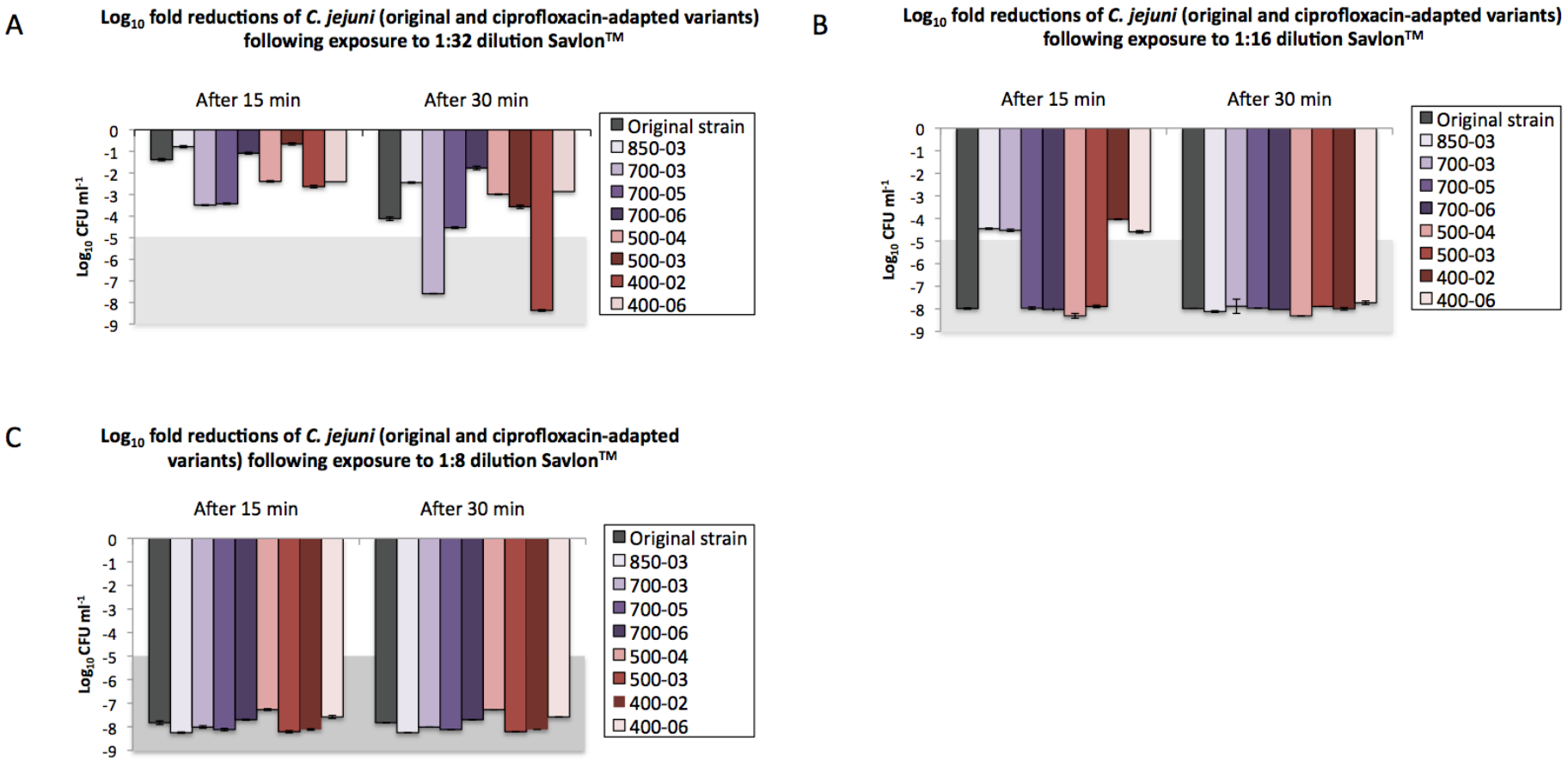

Figure 3.3 Log-fold reduction in CFU per ml in bacterial counts of the original *C. jejuni* strain and chemostat derived ciprofloxacin-adapted variants following exposure to Savlon™. Results were recorded as mean $\log_{10}$ CFU ml^{-1} ±standard deviation versus time. In some cases standard deviations are present but cannot be seen. Shaded area (gray) indicates bactericidal reductions in bacterial counts and efficacy. Chemostat-derived ciprofloxacin-adapted variants 400-02, 400-06, 500-03, 500-04 were isolated from CR2 and chemostat-derived ciprofloxacin-adapted variants 700-03, 700-05, 700-06 and 850-03 were isolated from CR3 (Chapter 2).

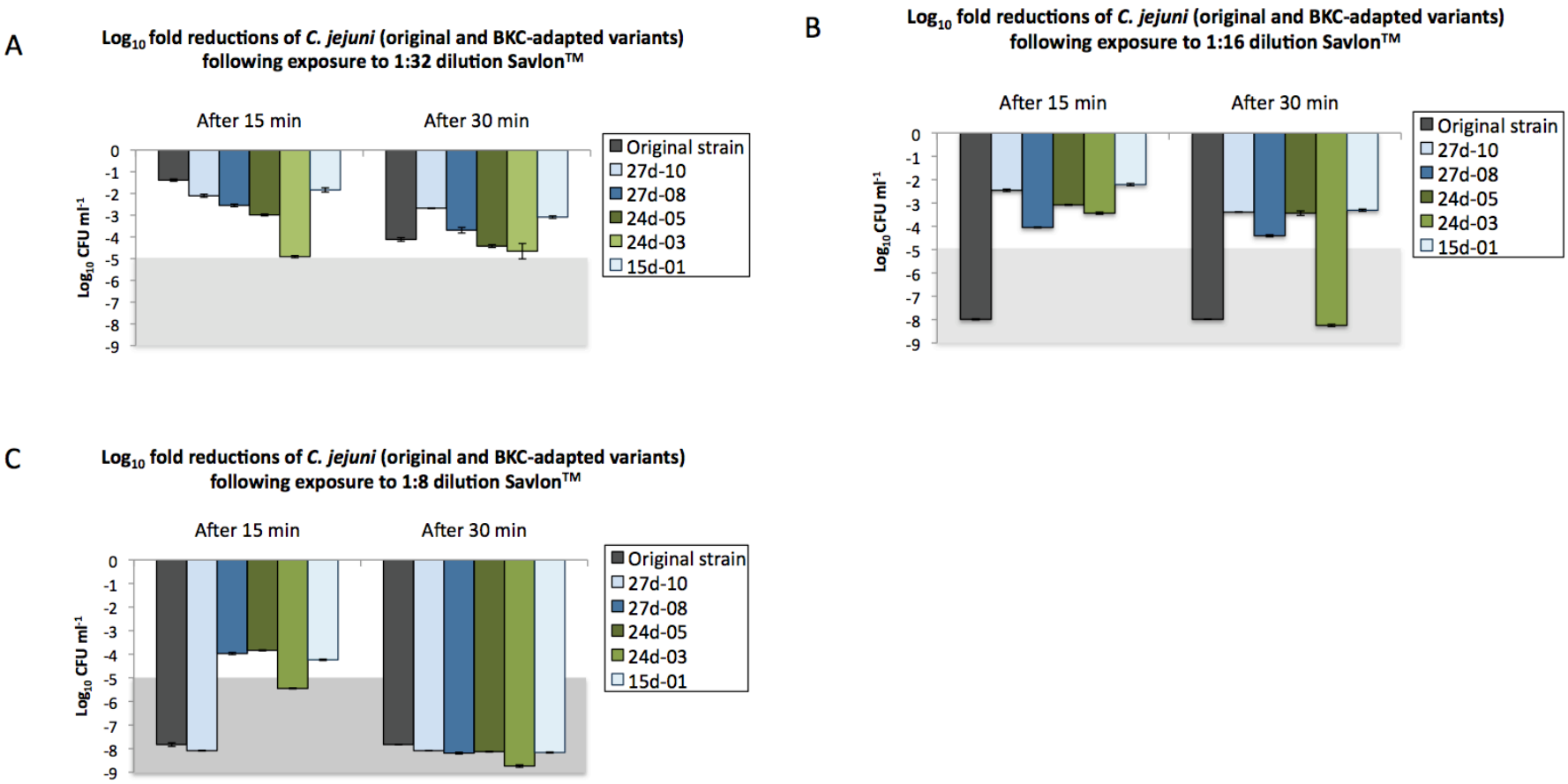

Figure 3.4 Log-fold reduction in CFU per ml in bacterial counts of the original *C. jejuni* strain and chemostat derived BKC-adapted variants of *C. jejuni* variants following exposure to Savlon™. Results were recorded as mean $\log_{10}$ CFU ml^{-1} ±standard deviation versus time. In some cases standard deviations are present but cannot be seen. Shaded area (gray) indicates bactericidal reductions in bacterial counts and efficacy. Chemostat-derived BKC-adapted variants 27d-10, 27d-08, 24d-05, 24d-03 and 15d-01 were isolated from CR5 (Chapter 2).

Prior to determining the bactericidal of BKC against *C. jejuni* (original strain) and BKC-adapted *C. jejuni* variants the MIC_{broth} values were determined (Section 2.3.3, Table 3.5). The MIC_{Broth} values for selected BKC-adapted strains to BKC were between 0.8 mg l^{-1} and 1.6 mg l^{-1} BKC, 2-4 fold higher than the original strain (Table 3.5). It was assumed that concentrations below the MIC_{broth} were also not bactericidal so the bactericidal activity of BKC was tested at concentrations above 1.6 mg l^{-1} BKC (the highest recorded MIC_{broth} value).

Strain name	Generation	MIC_{broth} BKC mg l^{-1}§
C. jejuni NCTC 11168		0.2
CR5 (BKC)		
15d-01	34	0.8
24d-05	40	1.6
27d-08	41	0.8
27d-10	41	1.6

Table 3.5 MIC_{broth} values of the original strain and BKC-adapted variants to BKC. Note: BKC is the main biocidal ingredient of the commercial disinfectant Savlon™.

The concentration of BKC tested ranged from 3.2 mg l^{-1} to 102 mg l^{-1} BKC. Exposure to 12.6 mg l^{-1} BKC was efficacious against the original strain after 30 minutes exposure. Doubling the concentration of BKC to 25.6 mg l^{-1} BKC reduced the amount of time to achieve a greater than 5 Log_{10} fold by half (Figure 3.5).

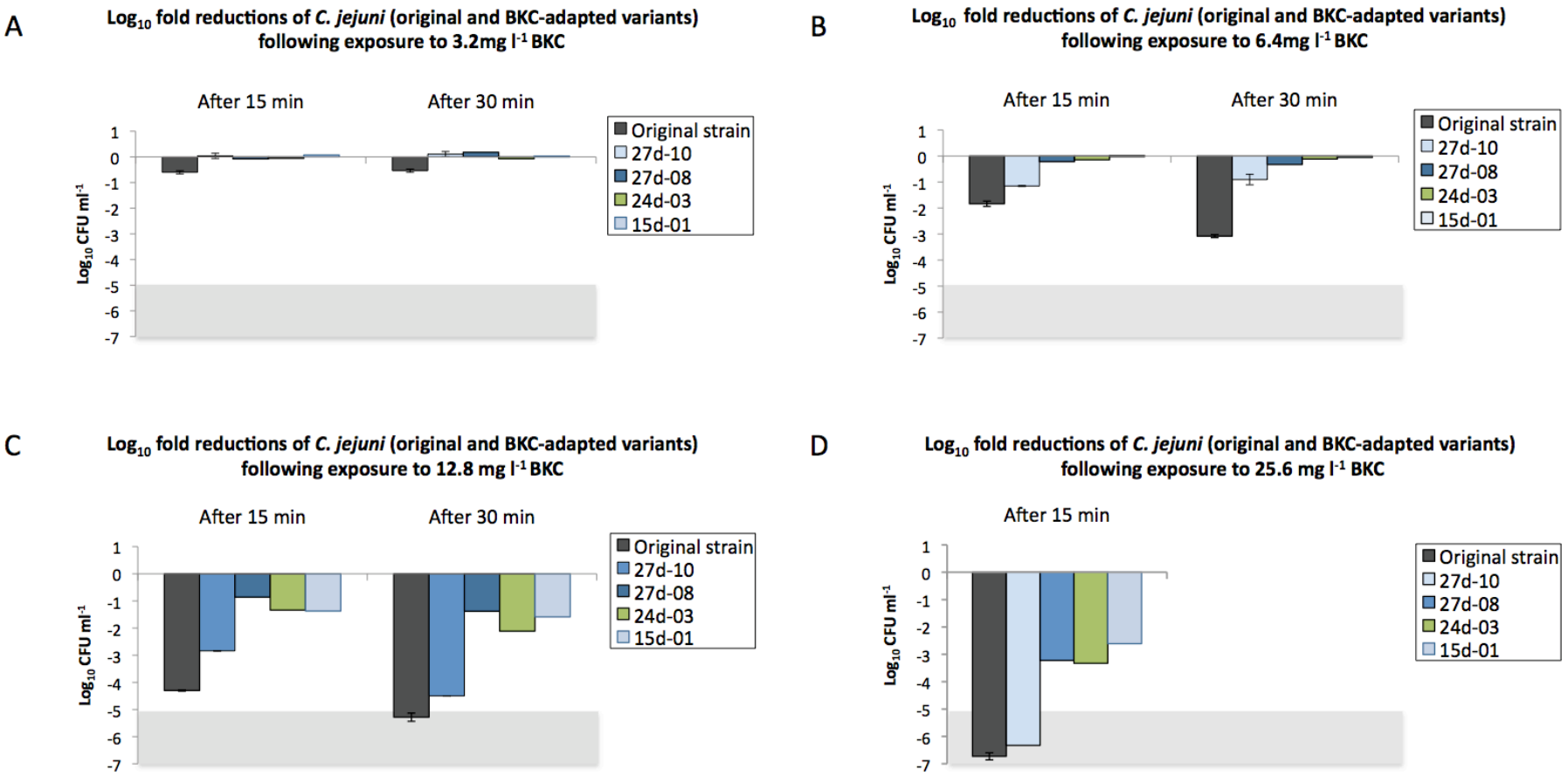

Figure 3.5 Log-fold reduction in CFU per ml in bacterial counts of the original *C. jejuni* strain and chemostat derived BKC-adapted variants following exposure to BKC. Results were recorded as mean $\log_{10}$ CFU ml^{-1}±standard deviation. In some cases they are present but cannot be seen. Shaded area (gray) indicates bactericidal reductions in bacterial counts and efficacy. Chemostat-derived BKC-adapted variants 27d-10, 27d-08, 24d-05, 24d-03 and 15d-01 were isolated from CR5 (Chapter 2).

3.4.3 Cross-resistance and the ability of two different EPIs to reverse cross-resistance in ciprofloxacin-adapted *C. jejuni* variants

Cross-resistance of antibiotic resistant strains was evaluated in chemostat derived ciprofloxacin adapted *C. jejuni* variants to three antibiotics (ampicillin, erythromycin and cefotaxime), two commercial disinfectants (Savlon™ and Dettol®; chloroxylenol 4.8% w/v), the commercial antiseptic (TCP®; halogenated phenols 0.68% w/v; phenol 0.175% w/v**)** and the biocide BKC **-** section 3.3.6. Ten ciprofloxacin adapted isolates were derived from samples taken from two chemostat cultures, CR1 (n=5) and CR3 (n=5). The ability of efflux pump inhibitors carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) and Phe-Arg β-naphthylamide dihydrochloride (PAβN) were used to examine if efflux played a role in conferring resistance to ciprofloxacin and cross-resistances and range of antibiotics and/or disinfectants seen in ciprofloxacin adapted variants. Results are displayed in Table 3.6.

Cross resistance and the ability of different EPIs to alter cross-resistance in ciprofloxacin-adapted *C. jejuni* variants

	CIP	*AMP*	*ERY*	*CEFO*	*BKC*	*SAV*	*DET*	*TCP*
Number of isolates/Total number of isolates showing cross-resistance†								
	-	0/10	8/10	10/10	10/10	9/10	5/10	2/10
Range	256-2,048 fold	-	(2-4 fold)	(2-4 fold)	(2-4 fold)	(2-fold)	(2-fold)	(2-fold)
Efflux inhibition (2-fold decrease)* in isolates showing cross-resistance								
+ CCCP	4/10	1/10ς	2/8	1/10	1/10	6/9	7/10ς	2/10ς
+ PAβN	1/10	1/10ς	1/8	3/10	1/10	0/9	6/10ς	2/10ς

Table 3.6 Cross-resistance in ciprofloxacin (CIP) adapted *C. jejuni* variants to the antibiotics - ampicillin (AMP), erythromycin (ERY) and cefotaxime (CEF), commercial disinfectants Savlon™ (SAV) and Dettol® (DET), the antiseptic TCP® and the biocide benzalkonium chloride (BKC). † Compared to the level of resistance of the original strain to antimicrobials (CIP 0.125 mg l^{-1}, AMP 32 mg l^{-1}, ERY 1 mg l^{-1}, CEFO 8 mg l^{-1}, BKC 8 mg l^{-1}, SAV 0.078%v/v, DET 0.00078%v/v and TCP 0.625%v/v). * Max decrease in resistance seen was 2-fold. ς Some isolates showed a 2-fold increase in susceptibility, in the presence of EPI, when compared to the original strain.

All ciprofloxacin-adapted *C. jejuni* variants (n= 10) were resistant to ciprofloxacin, with MIC_{Agar} values ranging from between 32 and 256 mg l^{-1}. Ciprofloxacin adapted variants also exhibited different cross-resistance profiles to the range of antimicrobial

agents tested with two to four fold increases in MIC_{Agar} recorded to three or more of the antimicrobial agents. Only one isolate showed a two-fold decrease in resistance to the antibiotic ampicillin.

Prior to performing the efflux inhibition assay, the concentrations of EPI that would not be growth inhibitory and could be used in the assay were determined. MIC_{Agar} for CCCP and PAβN to the original *C. jejuni* strain was determined by the agar dilution method described previously in section 2.3.3 (Chapter 2). The concentrations of EPIs used in the efflux inhibition assay were 16 mg l^{-1} for CCCP and 32 mg l^{-1} for PAβN and represented a concentration that was 50 % of their respective MIC_{Agar} concentration.

Resistance to ciprofloxacin was partially reversed (2-fold decrease), after exposure to CCCP in four ciprofloxacin-adapted isolates and one ciprofloxacin-adapted isolate after exposure to PAβN (see Table 3.6). EPIs were able to reverse cross-resistance to Savlon™, Dettol®, TCP® in most cases, except for PAβN which had no effect on reversing resistance to Savlon™. Both EPIs had a modest impact on reversing cross-resistance to the other antibiotics tested, as well as to the biocide BKC (2-fold max, in 10-30% of cases).

3.5 Discussion

This chapter describes the phenotypic changes detected amongst ciprofloxacin-adapted and BKC-adapted variants of *C. jejuni* NCTC 11168 (*C. jejuni*). Variants were developed after continuously challenging the initially sensitive *C. jejuni* strain to progressively increasing concentrations of these antimicrobial agents in long term chemostat culture (Chapter 2). Adapted variants had MIC_{Agar} values significantly greater than the original strain, up to 2048-fold greater for ciprofloxacin and up to 8-fold greater for BKC. Changes in fitness and stability of strains as well as changes in antimicrobial susceptibility, the involvement of efflux, and the efficacy of commercial disinfectant Savlon™ and biocidal ingredient BKC against adapted strains were investigated.

Many antimicrobials target vital physiological processes that are essential for bacterial growth such as DNA, protein and cell wall synthesis (Andersson, 2003). Genetic changes as a result of resistance-conferring mutations or determinants may impose a biological cost resulting in a reduced growth rate and consequently affecting their competitiveness in antibiotic-free environments (Andersson, 2003; Fleming *et al.,* 1988; Luangtongkum *et al.,* 2009; Zhang *et al.,* 2006). In the absence of selection pressure, adaptation to antimicrobials in *Campylobacter* may or may not show a fitness burden (Luangtongkum *et al.,* 2009). The effect on the fitness of *C. jejuni* following adaptation to ciprofloxacin and BKC was assessed. The μmax was determined for ciprofloxacin-adapted (CR3; n=39) and BKC-adapted (CR5; n=20) variants taken from chemostats undergoing selection to ciprofloxacin and BKC.

The relative growth rates for ciprofloxacin-adapted variants differed greatly and the number of variants with a lower relative growth rate increased in the latter stages of chemostat culture (Figure 3.2). Once the selective pressure was removed from the media feed the relative growth rate decreased further (Figure 3.2). The removal of selection pressure from chemostats (via the media feed) would have been expected to allow adapted variants time to acquire mutations that would ameliorate the cost of resistance. Typically growth in chemostats in the absence of a specific selection pressure involves adaptation to culture conditions and results in the acquisition of mutations that give the organisms a selective advantage for growth thus increasing the

fitness of the organism (Gresham & Hong, 2015). However, these results (Figure 3.2) indicate that culture in the absence of ciprofloxacin had a negative effect on fitness. One possible explanation for decreased fitness could be that some ciprofloxacin-adapted variants present in the chemostat population undergoing selection for reduced susceptibility to ciprofloxacin had also acquired a deleterious mutation that had a negative effect on fitness. These ciprofloxacin-adapted strains could then have been selected for as a result of genetic hitchhiking i.e. the developed phenotype which had a reduced growth rate hitchhiked on the back of other mutations, which gave a advantage for survival in the presence of ciprofloxacin. The so-called hitchhiking effect was described by Dykhuizen and Hartl (1983), as "all genes present in the favored clone", including those encoding reduced susceptibility to ciprofloxacin (in this case) are selected for when selecting for strains with improved fitness when ciprofloxacin was present in the chemostat. These same mutations decreased fitness relative to the parent when ciprofloxacin was removed from the chemostat (via the media feed).

Ciprofloxacin resistance in *Campylobacter* has previously been associated with mutations in the gyrA gene of DNA gyrase, the molecular target of fluoroquinolone antibiotics (Luangtongkum *et al.,* 2009; Luo *et al.,* 2003; Payot *et al.,* 2006). Decreased fitness, in isogenetic variants of *C. jejuni* with resistant to ciprofloxacin, which carried the Thr-86-Ile mutation, was also found by Luo et al., (2005). The effect of Thr-86-Ile mutation in the gyrA gene on fitness appears to be dependant on the genetic background in which it occurs (Andersson & Hughes, 2010; Luo *et al.,* 2005). The same study showed that the Thr-86-Ile conferred a fitness advantage to other fluoroquinolone resistant isogenetic variants of *C. jejuni* (Luo *et al.,* 2005).

There is a lack of information in the literature on the effect of biocide resistance on bacterial fitness. In this study, adaption to BKC was shown to impose a biological cost. Adaptation to BKC has also been shown to result in decreased fitness in *Pseudomonas aeruginosa* (McCay *et al.,* 2010). Differences in growth rates were seen amongst isolates derived from the same culture sample from chemostat populations that had undergone selection to BKC or ciprofloxacin (Figure 3.2 - 3.3). Differences in growth rates are seen as an indicator of the genetic heterogeneity of variants present

in the bacterial populations undergoing adaptation to antimicrobials (Erickson *et al.,* 2015).

The dissemination of antimicrobial-resistant bacteria is a key contributor to the widespread emergence of problems in the treatment of infectious diseases (ESFA, 2009). The stability of the resistance mechanism in the absence of antimicrobials is an important factor to consider when discussing the emergence and dissemination of antimicrobial resistance in bacterial population (Andersson & Hughes, 2010). The stability of the developed phenotypes was determined after non-selective growth on CCDA base (Table 3.2-3.3).

Stability of resistance in the variants examined was found to depend at which stage variants were isolated from the chemostat i.e. during selection when the antimicrobial selective agent (ciprofloxacin) was present and when the antimicrobial selective agent was absent. It was observed that 40 % (8/20) of ciprofloxacin-adapted variants of *C. jejuni* were unstable after 5 serial transfers on CCDA base. Of these, 75 % (6/8) did not revert to the level of susceptibility of the original strain. A possible reason for this could be that ciprofloxacin-adapted variants evolved in the absence of antibiotic selective pressure resulted in mutational events favoring a reversion to a lower-level resistance phenotype associated with lesser fitness costs, rather than the acquisition of compensatory mutations that would maintain resistance while ameliorating the fitness burden (O'Regan *et al.,* 2010). A number of mechanisms other than mutation could have also played a role in decreased sensitivity to ciprofloxacin (Sánchez-Romero & Casadesús, 2014). Such mechanisms include efflux and reduced permeability of the cell membrane (Luo *et al.,* 2003; Han *et al.,* 2008; Wieczorek & Osek, 2013; Yan *et al.,* 2006). Alternatively, differences in stability from ciprofloxacin-adapted or BKC-adapted variants isolated from the same chemostat could be due to expressed phenotypic resistance mechanisms. Phenotypic resistance or adaptive resistance is a non-inherited form of resistance where strains become transiently resistant to antimicrobial agents and is not associated with genetic alteration (Corona & Martinez, 2013).

Adaptive resistance typically involves environmentally induced gene-expression changes that increase the ability of a bacterium to survive in the presence of an

antimicrobial (Sánchez-Romero & Casadesús, 2014). During selection in chemostat culture these variants may have expressed adaptive resistance mechanisms that selectively advantaged them to tolerate the high levels of ciprofloxacin present in the chemostat environment. However, when introduced into a novel environment i.e. subculture on agar plate and in the absence of selective agent, these advantages were unstable and were not maintained. The stability of the reduced susceptibility developed in chemostats may require the system for their maintenance (Andersson & Hughes, 2010). Another possible explanation is that variants showing decreased susceptibility to ciprofloxacin may have been derived from subpopulations in the chemostat showing niche specialization such as growth on the wall of the chemostat (Gresham & Hong, 2015). By contrast, when examining ciprofloxacin-adapted and BKC-adapted variants isolated after antimicrobial selective pressure was removed from the media feed, it was observed that reduced susceptibility to BKC or ciprofloxacin was stably maintained in these variants in the absence of selection pressure. This was observed in both chemostat culture (Chapter 2) and when individual variants were grown on agar (Table 3.2-3.3). Stable inheritance of reduced susceptibility to either BKC or ciprofloxacin in variants after prolonged culture in the absence of selective agent in the chemostat suggests that these strains had acquired mutations, which permanently affected their susceptibility to these agents (Sánchez-Romero & Casadesús, 2014).

The efficacy of biocides that are used for the decontamination of environmental surfaces can be evaluated by determining their bactericidal activity or minimum bactericidal concentration (MBC; SCENIHR, 2009; McDonnell & Russell, 1999). Standard efficacy tests guidelines set out by the European Standardization Body CEN recommend a 5-$\log_{10}$ fold reduction of bacterial counts within minimal exposure time of five minutes for the testing of disinfectants (Meyer *et al.,* 2010; Maillard, 2005). Studies have demonstrated the bactericidal activity (> 5 $\log_{10}$ fold reduction) of BKC against *C. jejuni* (Avrain *et al.,* 2003; Gutiérrez-Martín *et al.,* 2011). The bacteriostatic (Broth MIC) and bactericidal activity (> 5 $\log_{10}$ fold reduction) of BKC-based disinfectant Savlon™ and its active ingredient BKC was assessed against the chemostat- derived strains (BKC- and ciprofloxacin-adapted) and original strain to determine if adaptation to these agents altered the resistance to Savlon™. Survival to Savlon™ and BKC was carried out in the presence of broth to simulate natural

conditions under which cleaning of general equipment and surface disinfection occur (Gutiérrez-Martín *et al.,* 2011). Longer exposure times of fifteen and thirty minutes above the minimal recommended exposure time by CEN were used to quantify the time/minimum concentration of disinfectant required to inactivate ciprofloxacin-adapted and BKC-adapted variants of *C. jejuni*.

Adaptation of *C. jejuni* to BKC or ciprofloxacin increased MIC_{broth} values of adapted variants to Savlon^{TM} (Table 3.4) but did not alter the efficacy (bacteriocidal activity) of Savlon^{TM} at 'in use' concentrations (Figure 3.3-3.4). Exposure to concentrations of disinfectant lower than those recommended by the manufacturer may also in everyday situations and is one possible reason for disinfectant failure (Maillard, 2005). Dilution of the effective concentration of disinfectant may occur by contact with stagnant water can occur in selected niches on the farm (e.g., under objects or in cracks in wood and masonry; Cerf *et al.,* 2010; Karatzas *et al.,* 2007) or as a result of biodegradation (Tezel & Pavlostathis, 2015). The bactericidal activity of dilutions of use concentration of Savlon^{TM} was also assessed against BKC-adapted and ciprofloxacin-adapted variants of *C. jejuni*. Both ciprofloxacin- and BKC-adapted variants were able to survive longer (survivors detected after 30 minutes) and in greater numbers after exposure to 1:16 and 1:32 dilutions of disinfectant (Figure 3.3-3.4). When compared to the original strain, the bactericidal concentration of Savlon^{TM} after 15 minutes exposure against ciprofloxacin and BKC-adapted variants was 2-fold higher against ciprofloxacin-adapted (four strains) and was 4-fold higher against BKC- adapted variants (four strains) (Figure 3.3-3.4). BKC-adapted strains examined had MIC_{broth} values that were between 4-8 fold higher to BKC (Table 3.5) and the bactericidal concentration of BKC 4-fold higher for BKC adapted strains compared to the original strain which was in agreement with the results obtained for Savlon^{TM} (Figure 3.5). The risk of disinfectant failure may be augmented following adaptation to BKC or development of resistance to ciprofloxacin in *C. jejuni* in situations where disinfectants are diluted.

Cross-resistance between antibiotics and disinfectants may occur when both share a common resistance mechanism (Chapman, 2003). Cross-resistance between antibiotics and disinfectants has previously been reported to occur in *Campylobacter spp.* Randall *et al*., (2003) examined multidrug resistant *Campylobacter spp., C. jejuni*

and *C. coli* with reduced susceptibility to triclosan as well as ethidium bromide and acridine orange and found that strains were significantly more resistant (2-4 fold increases in MIC values) to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid and tetracycline. Multidrug resistance is defined as resistance to three or more different classes of antimicrobials (Piddock, 2006). In *Campylobacter*, ciprofloxacin-adapted variants (MIC_{Agar} values 32-256 mg l^{-1} ciprofloxacin) were examined for cross-resistance to other antimicrobials including antibiotics (erythromycin, cefotaxime and ampicillin), the biocide BKC, commercial disinfectants (Savlon™ and Dettol®), and a commercial antiseptic (TCP®). Cross-resistance between ciprofloxacin and erythromycin was examined, as both are important antibiotics for the treatment of campylobacterosis (Engberg *et al.,* 2001; Gibreel & Taylor, 2006; Luangtongkum *et al.,* 2009). β-lactam antibiotics act on the bacterial cell wall but mediate their effect by inhibiting penicillin-binding proteins preventing cell wall synthesis (Gilbert & Moore, 2005; Lovine, 2013; Luangtongkum *et al.,* 2009; McDonnell & Russell, 1999; Maillard, 2002). The mechanism of action of BKC has been described and involves the disruption of the bacterial cell membrane which results in leakage of intracellular constituents eventually leading to cell death (Gilbert & Moore, 2005). The commercial disinfectant Dettol® and commercial antiseptic TCP® both contain phenolic compounds (Chloroxylenol 4.8 % w/v, Dettol®; halogenated phenols 0.68% w/v, phenol 0.175 % w/v) which target the bacterial cell wall and membrane (Gnanadhas *et al.,* 2013; McDonnell & Russell, 1999).

Ciprofloxacin-adapted variants exhibited strain specific cross-resistance profiles to the range of antimicrobial agents with a two to four fold increase in MIC_{Agar} recorded to three or more of the antimicrobial agents (Table 3.6). Resistance to fluoroquinolones in *Campylobacter* is also shown to be associated with multidrug resistance (Griggs *et al.*, 2005; Hakanen *et al.,* 2003). Griggs et al., (2005) found that twenty-five percent (73 of 290) of ciprofloxacin-resistant isolates but only 13% (24 of 179) of susceptible isolates examined from commercial poultry flocks were resistant to three or more unrelated antimicrobial agents. Similarly, Hakanen et al., (2003) also found that multidrug resistance was occurred more frequently in clinical isolates with

ciprofloxacin resistance (33%; 57 of 174) than in 12% (24 of 202) of ciprofloxacin susceptible isolates tested.

Up-regulation of efflux may be one possible reason for multidrug resistance the observed in ciprofloxacin adapted variant. RND type and MFS efflux pumps has been shown to be involved in resistance to ciprofloxacin in *Campylobacter* and can also confer resistance to a wide variety of substrates including antibiotics, detergents, biocides and dyes (Jeon *et al.,* 2011; Lin *et al.*, 2002; Pumbwe *et al.,* 2005). Three have been functionally characterised, CmeABC and CmeDEF, cme for *Campylobacter* multidrug efflux, belonging to the resistance-nodulation-cell division (RND) family of transporters and the CmeGH, a major-facilitator super family (MFS) type efflux transporter (Jeon *et al.,* 2011; Lin *et al.*, 2002; Pumbwe *et al.,* 2005). Efflux pump inhibitors (EPIs) act to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (Lin & Martinez, 2006). The ability of the two efflux pump inhibitors, carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) and Phe-Arg β-naphthylamide dihydrochloride (PAβN), to reverse resistance to ciprofloxacin and cross-resistances and range of antibiotics and/or disinfectants was also examined. CCCP is a non-specific inhibitor of efflux and acts to dissipate the proton motive force, thus interrupting the main source of energy of PMF-dependant efflux including RND and MFS type efflux transporters (Ozaki *et al.,* 2008). PAβN on the other hand targets RND type transporters directly by reversibly binding an internal binding pocket inside the transporter thereby inhibiting its ability to catalyse the efflux of substrates (Lomovskaya *et al.,* 2006).

Both CCCP and PAβN failed to appreciatively reverse resistance to ciprofloxacin, erythromycin, cefotaxime and ampicillin in ciprofloxacin adapted variants (Table 3.6) despite the reported involvement of *Campylobacter* efflux systems in mediating resistance to antibiotics (Guo *et al.,* 2010; Jeon *et al.,* 2011; Lin *et al.*, 2002; Lovine, 2013; Pumbwe *et al.,* 2005; Quinn *et al.,* 2007). In *Campylobacter*, the involvement of efflux systems in decreased susceptibility to biocides including BKC, has also previously been inferred by use of EPIs CCCP and PAβN (Mavri & Možina, 2013). However, in this study cross-resistance to BKC was unaffected by both EPIs. This indicated that efflux did not contribute greatly to high-level ciprofloxacin resistance in

ciprofloxacin-adapted variants. For example CCCP but not PAβN was able to restore sensitivity to the commercial disinfectant Savlon™ (See table 3.6). This suggests that non-RND type efflux systems of *Campylobacter* are involved in mediating reduced susceptibility to Savlon™. Heterogeneous expression of different efflux pump systems can contribute to adaptive resistance to antimicrobials (Sánchez-Romero & Casadesús, 2014). In this study, heterogeneous expression of efflux pumps may also explain why efflux pump inhibitors had differing effects on reversing cross-resistance seen in ciprofloxacin-adapted variants.

Overall ciprofloxacin-adapted and BKC-adapted *C. jejuni* variants showed strain-specific differences in fitness, antimicrobial susceptibility profiles and efflux activity as a result of adaptation to these agents. These results also suggest that the risk of disinfectant failure may be augmented following adaptation to BKC or development of resistance to ciprofloxacin in *C. jejuni*.

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3.5 Bibliography

Andersson, D.I. (2003). Persistence of antibiotic resistant bacteria. *Curr. Opin. Microbiol., 6*(5), 452-456.

Andersson, D.I. & Hughes, D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol., 8*(4), 260-271.

Andersson, D.I. & Hughes, D. (2011). Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiol. Rev., 35*(5), 901-911.

Avrain, L., Allain, L., Vernozy-Rozand, C. & Kempf, I. (2003). Disinfectant susceptibility testing of avian and swine *Campylobacter* isolates by a filtration method. *Vet. Microbiol., 96*(1), 35-40.

Bloomfield, S.F. & Scott, E.A. (2013). A risk assessment approach to use of antimicrobials in the home to prevent spread of infection. *Am J Infect Control, 41*(Suppl 5), S87-93.

Bolton, D., Meredith, H., Walsh, D. & McDowell, D., 2014. Poultry food safety control interventions in the domestic kitchen. *J Food Saf, 34*(1), 34-41.

Cerf, O., Carpentier, B. & Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings., *Int. J. Food Microbiol. 136*(3), 247-254.

Chapman, J.S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation, 51*(4), 271–276.

Clinical and Laboratory Standards Institute (2006) *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guidelines. CLSI document M45-A2*, Wayne, PA: Clinical and Laboratory Standards Institute.

Corona, F. & Martinez, J.L. (2013). Phenotypic Resistance to Antibiotics. *Antibiotics, 2*(2), 237-255.

Dykhuizen, D.E. & Hartl, D.L. (1983). Selection in chemostats. *Microbiol. Rev., 47*(2), 150-168.

EFSA (2009). Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic infections. *EFSA Journal, 7*(11), 1372.

Engberg, J., Aarestrup, F.M., Taylor, D.E., Gerner-Smidt, P. & Nachamkin, I. (2001). Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. *Emerging Infect. Dis., 7*(1), 24-34.

Erickson, K.E., Otoupal, P.B. & Chatterjee, A., (2015). Gene expression variability underlies adaptive resistance in phenotypically heterogeneous bacterial populations. *ACS Infect Dis., 1*(11), pp.555-567.

Fallon, R., O'Sullivan, N., Maher, M., & Carroll, C. (2003). Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolates from broiler chickens isolated at an Irish poultry processing plant. *Lett. Appl. Microbiol., 36*(5), 277-281.

Fleming, G., Dawson, M.T. & Patching, J.W. (1988). The isolation of strains of *Bacillus subtilis* showing improved plasmid stability characteristics by means of selective chemostat culture. *J. Gen. Microbiol., 134*(8), 2095-2101.

Gibreel, A. & Taylor, D.E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 58*(2), 243-255.

Gilbert, P. & Moore, L.E. (2005). Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol., 99*(4), 703-715.

Gnanadhas, D.P., Marathe, S.A. & Chakravortty, D. (2013). Biocides-resistance, cross-resistance mechanisms and assessment. *Expert Opin Investig Drugs, 22*(2), 191-206.

Gresham, D. & Hong, J. (2015). The functional basis of adaptive evolution in chemostats. *FEMS Microbiol. Rev., 39*(1), 2-16.

Griggs, D.J., Johnson, M.M., Frost, J.A., Humphrey, T., Jørgensen, F. & Piddock, L.J., (2005). Incidence and mechanism of ciprofloxacin resistance in *Campylobacter spp.* isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. *J. Antimicrob. Chemother., 49*(2), 699-707.

Guo, B., Lin, J., Reynolds, D.L. & Zhang, Q. (2010). Contribution of the Multidrug Efflux Transporter CmeABC to Antibiotic Resistance in Different *Campylobacter* Species. *Foodborne Pathog. Dis.,* 7(1), 77-83.

Gutiérrez-Martín, C.B., Yubero, S., Martínez, S., Frandoloso, R. & Rodríguez-Ferri, E.F. (2011). Evaluation of efficacy of several disinfectants against *Campylobacter jejuni* strains by a suspension test. *Res. Vet. Sci., 91*(3), 44-47.

Hakanen, A.J., Lehtopolku, M., Siitonen, A., Huovinen, P. & Kotilainen, P., (2003). Multidrug resistance in *Campylobacter jejuni* strains collected from Finnish patients during 1995–2000. *J. Antimicrob. Chemother., 52*(6), 1035-1039.

Han, J., Sahin, O., Barton, Y.W. & Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog., 4*(6), e1000083.

Heres, L., Engel, B., Urlings, H.A., Wagenaar, J.A. & van Knapen, F. (2004). Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. *Vet. Microbiol., 99*(3-4), 259-267.

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., Rasschaert, G., Heyndrickx, M. & Pasmans, F. (2011). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol., 152*(3-4), 219-228.

Jeon, B., Wang, Y., Hao, H., Barton, Y.W. & Zhang, Q. (2011). Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni.* *J. Antimicrob. Chemother., 66*(1), 79-85.

Karatzas, K.A., Webber, M.A., Jorgensen, F., Woodward, M.J., Piddock, L.J. & Humphrey, T.J. (2007). Prolonged treatment of *Salmonella enterica serovar Typhimurium* with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J. Antimicrob. Chemother., 60*(5), 947-955.

Lin, J., Akiba, M., Sahin, O. & Zhang, Q. (2005). CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. *Antimicrob. Agents Chemother., 49*(3), 1067-1075.

Lin, J. & Martinez, A. (2006). Effect of efflux pump inhibitors on bile resistance and *in vivo* colonization of *Campylobacter jejuni*. *J. Antimicrob. Chemother., 58*(5), 966-972.

Lin, J., Michel, L.O. & Zhang, Q. (2002). CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob. Agents Chemother., 46*(7), 2124-2131.

Lomovskaya, O. & Bostian, K.A. (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic-a vision for applied use. *Biochem. Pharmacol., 71*(7), 910-918.

Lovine, N.M. (2013). Resistance mechanisms in *Campylobacter jejuni*. *Virulence., 4*(3), 230-240.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. & Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol, 4*(2), 189-200.

Luo, N., Sahin, O., Lin, J., Michel, L.O. & Zhang, Q. (2003). *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with

gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother., 47*(1), 390-394.

Luo, N., Pereira, S., Sahin, O., Lin, J., Huang, S., Michel, L. & Zhang, Q. (2005). Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *PNAS, 102*(3), 541-546.

Maillard, J.Y. (2002). Bacterial target sites for biocide action. *J. Appl. Microbiol., 92*(Suppl s1), 16S-27S.

Maillard, J.Y. (2005). Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Ther Clin Risk Manag, 1*(4), 307-320.

Mangen, M.J.J., Havelaar, A.H., Nauta, M.J., de Koeijer, A.A. & de Wit, G.A. (2005). Controlling *Campylobacter* in the chicken meat chain : cost-effectiveness and cost-utility analysis. RIVM report ; 250911007/2005.

Mavri, A. & Smole Možina, S. (2013). Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol., 160*(3), 304-312.

McCay, P.H., Ocampo-Sosa, A.A. & Fleming, G.T. (2010). Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. *Microbiology, 156*(Pt 1), 30-38.

McDonnell, G. and Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev., 12*(1), 147-179.

Meyer, B., Morin, V.N., Rödger, H.J., Holah, J. & Bird, C. (2010). Do European Standard Disinfectant tests truly simulate in-use microbial and organic soiling conditions on food preparation surfaces? *J. Appl. Microbiol., 108*(4), 1344-1351.

Miles, A.A., Misra, S.S. & Irwin, J.O. (1938). The estimation of the bactericidal power of the blood. *J Hyg (Lond), 38*(6), 732-749.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G. & Allen, V.M. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter spp.* on poultry farms. *Appl. Environ. Microbiol., 77*(24), 8605-8614.

O'Regan, E., Quinn, T., Frye, J.G., Pagès, J.M., Porwollik, S., Fedorka-Cray, P.J., McClelland, M. & Fanning, S. (2010). Fitness costs and stability of a high-level ciprofloxacin resistance phenotype in *Salmonella enterica serotype enteritidis*: reduced infectivity associated with decreased expression of *Salmonella* pathogenicity island 1 genes. *Antimicrob. Agents Chemother., 54*(1), 367-374.

Ozaki, S., Kano, K. & Shirai, O. (2008). Electrochemical elucidation on the mechanism of uncoupling caused by hydrophobic weak acids. *Phys Chem Chem Phys, 10*(30), 4449-4455.

Payot, S., Bolla, J.M., Corcoran, D., Fanning, S., Mégraud, F. & Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in *Campylobacter spp*. *Microbes Infect., 8*(7), 1967-1971.

Peyrat, M.B., Soumet, C., Maris, P. & Sanders, P. (2008a). Phenotypes and genotypes of *campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses. *Vet. Microbiol., 128*(3-4), 313-126.

Peyrat, M.B., Soumet, C., Maris, P. & Sanders, P. (2008b). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol., 124*(2), 188-194.

Piddock, L. J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev., 19*(2), 382-402.

Pumbwe, L., Randall, L.P., Woodward, M.J. & Piddock, L.J. (2005). Evidence for multiple-antibiotic resistance in *Campylobacter jejuni* not mediated by CmeB or CmeF. *Antimicrob. Agents Chemother., 49*(4), 1289-1293.

Quinn, T., Bolla, J.M., Pagès, J.M. & Fanning, S. (2007). Antibiotic-resistant *Campylobacter*: could efflux pump inhibitors control infection? *J. Antimicrob. Chemother., 59*(6), 1230-1236.

Randall, L.P., Ridley, A.M., Cooles, S.W., Sharma, M., Sayers, A.R., Pumbwe, L., Newell, D.G., Piddock, L.J. & Woodward, M.J. (2003). Prevalence of multiple antibiotic resistance in 443 *Campylobacter spp.* isolated from humans and animals. *J. Antimicrob. Chemother., 52*(3), 507-510.

Saleha, A.A., Mead, G.C. & Ibrahim, A.L. (1998). *Campylobacter jejuni* in poultry production and processing in relation to public health. *Worlds Poult Sci J, 54*(1), 49-58.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). Assessment of the Antibiotic Resistance Effects of Biocides. 19-January 2009. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf (accessed 16-08-2013).

Sánchez-Romero, M.A. & Casadesús, J. (2014). Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proc. Natl. Acad. Sci. U.S.A., 111*(1), 355-360.

Tezel, U. & Pavlostathis, S.G., (2015). Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. *Curr Opin Biotechnol., 33*, 296-304.

Wieczorek, K. & Osek, J. (2013). Antimicrobial Resistance Mechanisms among *Campylobacter. Biomed Res Int*, 340605.

Yan, M., Sahin, O., Lin, J. & Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure., *J. Antimicrob. Chemother. 58*(6), 1154-1159.

Zhang, Q., Sahin, O., McDermott, P.F. and Payot, S. (2006). Fitness of antimicrobial-resistant *Campylobacter* and *Salmonella*. *Microbes Infect., 8*(7), 1972-1978.

An investigation into the role of proteomic changes in the adaptation of *C. jejuni* NCTC 11168 to ciprofloxacin and benzalkonium chloride

Olivia Duignan[1], Gerard Fleming[2] and Cyril Carroll[1].

[1]Enteric pathogen Research Laboratory, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland; [2]Microbial Oceanography Research Unit, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland.

4.1 Abstract

Campylobacter spp. are one of the leading bacterial etiologic agents of acute human gastroenteritis among industrialized countries. Increasing resistance to antibiotics well as for cleaning and disinfection compounds (biocides) is a public health concern. Sodium dodecyl gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2DGE) were used to determine changes in protein expression as a result of adaptation of *Campylobacter jejuni* NCTC 11168 (*C. jejuni)* to ciprofloxacin and biocide BKC (which is widely used in disinfectant formulations). No differences were observed in the banding pattern of adapted variants using SDS-PAGE. When examined by 2DGE, the the major changes observed were to proteins involved in metabolism and general and oxidative stress response in the ciprofloxacin-adapted *C. jejuni* variant while changes in the expression of oxidative stress proteins and metabolic proteins were found in the BKC-adapted variant. Differential expression of proteins involved in the oxidative stress response was observed in ciprofloxacin and BKC-adapted *C. jejuni*. Superoxide dismutase and thiol peroxidase were down-regulated in ciprofloxacin-adapted *C. jejuni*. Rubrerythrin was up-regulated in *C. jejuni* following a 25 % MIC challenge with ciprofloxacin. In addition, these same proteins together with alkyl hydroperoxide reductase also involved in the oxidative stress response were up-regulated in BKC-adapted *C. jejuni*. These results indicate that the oxidative stress response may play an important role in adaptation of *C. jejuni* to BKC but not to ciprofloxacin.

Keywords: *Campylobacter*, Ciprofloxacin, Benzalkonium chloride, oxidative stress response, SDS-PAGE, 2DGE.

4.2 Introduction

Campylobacter spp. remains the leading cause of food-borne illness worldwide with *C. jejuni* being responsible for the majority of illness. The major route of transmission of *Campylobacter* to humans is through the consumption of contaminated food produce, particularly undercooked poultry. Direct contact with animals/pets, recreational swimming or drinking water from streams or other natural water sources also play a role (Wagenaar *et al.,* 2006). Although *Campylobacters* are fastidious microaerophiles and exhibit poor survival outside their animal host, some *C. jejuni* strains appear to be more able to survive and persist in environmental niches than others (Bronowski *et al.,* 2014). The lack of effective control measures is a significant barrier in preventing human cases of infection caused by these pathogens (Bolton, 2015; FSAI, 2011). Coupled with this is the problem of fluoroquinolone -resistance in *Campylobacter spp*, which has increased in recent years (Ge *et al.,* 2013; EFSA/ECDC, 2014). This may have consequences on the successful treatment outcomes of infection in humans with these antibiotics (EFSA/ECDC, 2014). In 2006, the EU restricted the use of antibiotics in food production and antibiotics can now only be used in veterinary medicine of food production animals on a prescription only basis (ESVAC, 2013).

Antibiotics affect physiological processes that are vital for cell survival, for example protein or DNA synthesis, and exert their action against a single target site and have a high level of target specificity (Gilbert & McBain, 2003; Cerf *et al.,* 2010). The bacteriocidal antibiotic classes such as β-lactams, aminoglycosides and fluoroquinolones mediate their lethal effect, in part, by the production of hydroxyl radical production/oxidative stress (Dwyer *et al.,* 2014). Resistance mechanisms in bacteria to antibiotics have been described and include target protection, target substitution, antibiotic detoxification and blocking of intracellular accumulation by efflux (Bennett & Hughes, 2009; van Hoek *et al.,* 2011). Mutations leading to constitutive expression of the stress response regulator SoxR involved in oxidative stress has also been linked to antimicrobial resistance in *E. coli* and *Salmonella enterica* serovar Typhimurium (Poole, 2012).

In contrast to antibiotics, which have specific actions within the bacterial cell, biocides formulations contain antibacterial chemicals that are at sufficient

concentration so as to affect multiple rather then single target sites (Gilbert & McBain, 2003; McDonnell & Russell, 1999). The molecular targets for biocide action include outer and cytoplasmic membrane(s) of the bacteria as well as cellular components such as DNA, RNA and proteins (Cerf *et al.,* 2010; Gilbert & Mc Bain, 2003; Maillard *et al.,* 2002). Changes in the permeability of the cell wall as a result of changes in surface hydrophobicity, outer membrane ultra structure, outer membrane protein composition and changes in outer membrane fatty acid composition have been postulated to be responsible for acquired biocide tolerance in Gram- negative bacteria (Ortega Morente *et al.,* 2013). Furthermore, phenotype resistance or adaptive resistance, such as growth in biofilms, can result in transient resistant to antimicrobial agents including biocides and is a non-inherited form of resistance not associated with genetic alteration (Corona & Martinez, 2013; Meyer, 2006). Benzalkonium chloride is a member of the quaternary ammounium compounds (QACs) which are cationic surfactants with low- level disinfectant activity (McDonnell and Russell, 1999). It is widely used in the poultry industry as a disinfectant (Peyrat *et al.,* 2008). Survival of *C. jejuni* growing in mixed species -biofilms was shown to increase following treatment with a quaternary ammonium compounds-based disinfectant (Trachoo & Frank, 2002). Efflux activity has been also been shown to contribute to reduced susceptibility to BKC in *Campylobacter jejuni* (Mavri & Možina, 2013).

The aim of this study was to investigate the changes in protein expression following adaptive resistance of *C. jejuni* to the antibiotic ciprofloxacin and the biocide benzalkonium chloride using the proteomic techniques SDS-PAGE and two-dimensional gel electrophoresis. Proteins known to be involved in the oxidative stress response were examined to investigate changes in expression following adaptation of *Campylobacter* to ciprofloxacin.

4.3 Materials and methods

4.3.1 Strains and Antimicrobial Agents

Campylobacter jejuni NCTC 11168 (*C. jejuni*) was original strain used in this study and was obtained from Dr. Majella Mahar, the National Diagnositic Center, National University of Ireland, Galway. All other strains were derivatives of *C. jejuni* and were generated by means of selective/enrichment continuous cultures with either benzalkonium chloride or ciprofloxacin as described previously in section 2.3.5. (Chapter 2). Stocks were resusitated on Campylobacter blood-free agar (Oxoid Ltd., UK) under microaerobic conditions using an anerobic gas generating kit (BR0038B, Oxoid) at 42 ^{o}C for 48 h. Strains were cultured in Mueller Hinton broth (MHB; Lab114, LabM, UK) supplemented with 0.35 % (v/v) Isovitalex™ (211875, Becton Dickinson Sparks, MD, USA) for proteomic analysis. Antimicrobials used in this study included the antibiotics ciprofloxacin (Ciproxin ®, Bayer, UK) and chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and the biocide benzalkonium chloride (Sigma-Aldrich, Steinheim, Germany).

4.3.2 Sodium Dodecyl Gel Electrophoresis (SDS-PAGE)

4.3.2.1 Preparation of samples

Cultures growing exponentially for 18 h in MH broth supplemented with 0.35 % (v/v) Isovitalex™ were used to extract whole cell proteins from the original strain *C. jejuni* NCTC 11168 as well as BKC and ciprofloxacin adapted variants. OD_{600nm} of culture was determined prior to analysis by SDS-PAGE. Cultures were transferred into 50 ml tubes (Starsted, Germany) and centrifuged 3500 rpm for 20 min (Avanti TM J-20 XP centrifuge, Beckman Coulter). The supernatant was removed and pellets (pink, buff pellets) were resuspended 1 ml sterile PBS to wash cells. Resuspended pellets were then transferred into sterile 1.5 ml eppendorf tubes and washed pellets were collected by centrifuged at 15,600 g for 10 min (microcentrifuge, eppendorf). After removal of supernantant washed pellets were resuspended in lysis buffer (0.05 M Tris, 20 % glycerol, 4 % SDS, 3.5 % DTT, 0.1 % bromophenol blue) added in a ratio of 20 µl

lysis buffer for every 0.1 OD_{600nm} of culture. The tubes were vortexed and incubated at 37 ^{o}C for 30 min. This was followed by a 5 min incubation at 95 ^{o}C to lyse cells.

4.3.2.2 Electrophoretic analysis of the proteins by SDS-PAGE

SDS-PAGE used the method of Laemmli (1970) using 12 % (w/v) separating and 4 % (w/v) stacking gels. Ten µl of samples were electrophoresed at 120 V at 4 ^{o}C using a Mini - PROTEAN® III electrophoresis unit (Bio-Rad, California, USA) in tris glycine running buffer (0.15 M glycine; 0.025 M Tris-HCl; 0.1 % SDS, dissolved in MQ water) until the dye front had reached the bottom of the gel. Broad range protein molecular weight marker (5 µl, Pageruler™ Plus Pre-stained Protein Ladder, Thermo Scientific) was included on each gel. Following electrophoresis, gels were fixed by immersion in fixing solution (40 % Methanol; 5 % Acetic acid; 55 % dH_2O) for 15 min. After fixing gels were washed (three times) in Milipure water for 10 min and protein banding patterns were visualised by staining with Gelcode® blue reagent (Thermo Scientific). Where necessary gels were destained by washing with Milipure water for 1 hour to improve visualisation. Gel images were capured by scanning the gels with a Hewlett Packard Scanjet 5300C scanner at a resolution of 600dpi.

4.3.3 Two-Dimensional Gel Electrophoresis (2DGE)

4.3.3.1 Culture conditions

One litre MHB broths with or without a 25 % MIC challenge concentration of ciprofloxacin or BKC present (see table 4.1) and supplemented with 0.35 % Isovitalex™ (n=3) were inoculated with 25 ml of overnight culture of test strain (either original *C. jejuni* strain, or ciprofloxacin- or BKC-adapted *C. jejuni* variants). The chemostat adapted strains were challenged with 25 % of the minimum inhibitory concentrations of antimicrobial agent they were adapted to, to ensure the adaptation mechanisms of ciprofloxacin- and BKC-adapted *C. jejuni* were actively expressed. The original *C. jejuni* strain, was also challenged with a 25% MIC to BKC or ciprofloxacin and to see if protein expression differed from adapted strains. MIC_{broth} values were determined using the broth micro dilution method described previously in

section 2.3.3, chapter 2. Culture purity was assessed by Gram staining prior harvesting culture biomass.

Strain	MIC_{broth} (mg l^{-1} BKC) [25 % MIC_{broth} (mg l^{-1} BKC)][1]	MIC_{broth} (mg l^{-1} CIP) [25 % MIC_{broth} (mg l^{-1} CIP)][1]
C. jejuni original strain	0.2 [0.05]	0.06 [0.015]
BKC-adapted *C. jejuni* variant	0.8 [0.2]	-
Ciprofloxacin-adapted *C. jejuni* variant	-	25.6 [6.4]

Table 4.1 Strains (and corresponding MIC_{broth} values) used to elucidate the proteomic changes occuring in *C. jejuni* as a result of adaptation to ciprofloxacin or BKC. Note:-1. Proteins were isolated from strains were growth with or without antimicrobial agent concentration that was 25 % of their respective MIC_{broth} values shown in parenthesis. Abbreviations: CIP, ciprofloxacin; BKC, benzalkonium chloride

4.3.3.2 Protein extraction

Eighteen hour old broth cultures which had been grown with or without a 25 % MIC challenge to either ciprofloxacin or BKC in MHB, were transferred to 250 ml centrifuge tubes. Chlorampenicol (10 µg ml^{-1}) was added to inhibit further protein synthesis in the bacterial culture. Biomass was harvested by centrifugation at 13,000 g (4 °C) for 15 min (Avanti^{TM} J-20 XP centrifuge, Beckman Coulter). Supernatants were decanted and pellets were resuspended in 2 ml sonication buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM EDTA) containing 10 µg ml^{-1} chloramphenicol and 1 % (v/v) of protease cocktail inhibitor mix (Sigma, P2714). Samples were sonicated on ice using MSE Soniprep 150 at max amplitude (30 sec on; 30 sec rest) for 6 cycles. Samples were divided into two 1.5 ml Eppendorfs and soluble proteins were separated from any remaining intact cells and cell debris by centrifuging at 15,600 g for 20 min (micro centrifuge, Eppendorf). Supernatant containing cytosolic proteins were then transferred into fresh 1.5 ml Eppendorf tubes and Benzonase® endonuclease (Sigma, E1014; 44U ml^{-1}) added then samples incubated (37 ^{o}C, 30 min). This step was repeated a second time. Samples were centrifuged 15,600 g for 30 min. Protein concentration of the samples were determined using the D/C Lowry protein assay following manufacture's instructions with bovine serum albumin used as a standard. Protein extracts were stored at -20 ^{o}C until required.

4.3.3.3 First dimension- Isoelectric focusing (IEF)

The first dimension consisted of isoelectric focusing (IEF) using 7 cm IPG strips with linear pH gradients (pH 4 to 7; 17-6001-10, Amersham Biosciences). Prior to separation by IEF, proteins were precipitated using ice-cold acetone. A volume of protein sample containing 200 μg of protein was mixed with three volumes of ice-cold acetone and incubated at −20 °C for 1 h. Precipitated proteins were pelleted by centrifugation at 15,600 g for 15 min (Micro centrifuge, Eppendorf). Supernatants were discarded and protein was dried (42 ^{o}C, 15 min) with lids open. Dried pellets (200 μg protein) were resuspended in 135 μl IPG rehydration buffer supplemented with 2.5 mg ml^{-1} DTT and 0.4 % (v/v) IPG buffer pH 4 to 7 (17-6000-86, Amersham Biosciences). Samples were vortexed for two minutes every 10 min for 1 h to solubilise proteins. Samples were then centrifuged (15 min, 15,600 g) to remove insoluble material. The supernatant was pipetted onto the rehydration tray and IPG strips were placed face down on top of the sample avoiding trapping any air bubbles. The IPG strips were covered with non-conducting mineral oil (Ultra pure grade, Sigma). The rehydration tray was wrapped in tin foil and incubated overnight at room temperature to allow IPG strips to rehydrate before focusing.

Rehydrated IPG strips were removed from the rehydration tray and excess oil was allowed to drain before IEF focusing. Electrode wicks (Amersham Biosciences) cut to 0.5 cm and moistened with milipure water were placed onto the electrodes of the focusing tray (Bio-Rad, California, USA), ensuring no direct contact between the strips and the electrodes during IEF. IPG strips were placed gel side down on top of the wicks and the acidic side aligned with the positive electrode and the basic side aligned with the negative electrode and strips were covered with 2 ml of non-conducting mineral oil (Ultra pure grade, Sigma). IEF was carried out in three stages using PROTEAN IEF Cell (Bio-Rad, California, USA). A temperature of 20 °C was maintained in the IEF Cell throughout. An initial voltage of 50 V was applied and was increase to 250 V over 20 min. In the second stage, the voltage was increase to 4000 V over 2 h. The voltage was then maintained 4,000 V for 50,000 volt-hours. On completion, the IPG strips were dipped into milipure water to remove excess oil and IPG strips were equilibrated in 10 ml of 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) incubated with shaking on an orbital shaker (Heidolph unimax 1010) for 20 min 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).

4.3.3.4 Second dimension: SDS-PAGE

Once equilibrated a single IPG strip was positioned on top of a 1.5 mm gel (12 % acrylamide). A 0.5 cm electrode wick containing 5 µl of molecular weight marker (Pageruler™ Plus Pre-stained Protein Ladder, Thermo Scientific) was then placed next to the acidic side of the IPG strip. Both the IPG strip and electrode wick containing molecular weight marker were held in position by applying 0.8 % (w/v) agarose (Bio-Rad, California, USA) to the top of the gel and allowing it to set. Gels were electrophoresed, in pairs, at 120 V at 4 ^{o}C on a Mini - PROTEAN® III electrophoresis unit (Bio-Rad, California, USA) in tris glycine running buffer (0.15 M glycine; 0.025 M Tris-HCl; 0.1% SDS, dissolved in MQ water) until the dye front had reached the bottom of the gel.

4.3.3.5 Gel analysis

Following electrophoresis, gels were fixed by immersion in fixing solution (40 % methanol; 5 % acetic acid; 55 % dH_2O) for 15 min. After fixing gels were washed (three times) in milipure water for 10 min and protein banding patterns were visualised by staining with Gelcode® blue reagent (Thermo scientific) or Commasie blue stain (45 % methanol, 10 % glacial acetic acid; 0.1 g commassie brilliant blue R250). If stained with GelCode® Blue staining reagent, gels were washed in milipure water for several hours. Gels were often re-stained with the addition of more GelCode® Blue to help with visualisation of proteins. If the Commasie blue stain had been used gels were destained in destaining solution (10 % methanol; 10 % glacial acetic acid; 80 % milipure water) frequently changing the destaining solution until the background staining had decreased to the desired level. Gel images were captured by scanning the gels with a Hewlett Packard Scanjet 5300C scanner at a resolution of 600 dpi. Gels were kept at 4 °C in MQ water until further required. At least five gels were run for each strain investigated, representing samples extracted from at least three

independent cultures. Gels were systematically analysed by eye and with PDQuest-Advanced software version 8.0 (Bio-Rad, California, USA). Data were normalised using the Local Regression Model. This method, developed by Cleveland and Devlin (1988), is less susceptible to outliers than a simple linear regression. A curve was calculated in the scatter plot, which minimised the distance to all points in the plot. This curve was used to calculate the normalisation factor for each spot. Protein expression differences greater than two- fold that reproduced on the three pairs of replicate gels were considered significant.

4.3.3.6 Identification of proteins by MALDI-TOF-MS

Protein spots of interest were excised and a sequence determined using a combination of tryptic digestion and Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF) by the BSRC mass spectrometry and proteomics facility (St. Andrews University, Fife, 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 (Digilab) 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. The digest solution (0.5 µl) was applied to the MALDI target along with alpha-cyano-4- hydroxycinnamic acid matrix (0.5 µl, 10 mg/ml in 50:50 acetonitrile: 0.1 % TFA) and allowed to dry. MALDI MS was acquired using a 4800 MALDI TOF/TOF Analyser (ABSciex) equipped with a Nd:YAG 355 nm laser and calibrated using a mixture of peptides. The most intense peptides (up to 15) were selected for MSMS analysis and the MS data analysed using GPS Explorer (ABSciex) to interface with the Mascot 2.4 search engine (Matrix Science) and the MSMS data using Mascot 2.4 directly. Swiss-Prot (Dec 2012) or NCBInr (Aug 2013) databases were interrogated. No species restriction was applied. The data was searched with tolerances of 100 ppm for the precursor ions and 0.5 Da for the fragment ions, trypsin as the cleavage enzyme, assuming up to one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification

4.4 Results

4.4.1 Analysis of *C. jejuni* and chemostat derived ciprofloxacin- or BKC-adapted isolates of *C. jejuni* by SDS-PAGE

Whole cell proteomic extracts of *C. jejuni* (original strain) and representative chemostat-derived ciprofloxacin or BKC-adapted isolates of *C. jejuni* were analyzed by SDS-PAGE. No differences were observed in the banding pattern of adapted variants compared to the original parent strain was found using SDS-PAGE (data not shown).

4.4.2 Identification of proteins involved in BKC and ciprofloxacin adaptation in *C. jejuni*

Cytosolic protein from *C. jejuni* (original strain), BKC-adapted or ciprofloxacin adapted varients with or without a 25% MIC challenge to the adapted antimicrobial were compared. Proteins were extracted after 18 h growth at 42 °C in MHB. Cells were grown in the presence and absence of 25% concentration of their respective broth MIC to BKC or ciprofloxacin. All analyse were carried out on three independent biologial replicates for each culture condition used. Proteins were separated by two-dimensional gel electrophoresis (2-DGE) using a modified version of the O'Farrell method (O'Farrell, 1975). The first dimension consisted of isoelectric focusing (IEF) using 7 cm IPG strips with linear pH gradient of pH 4-7 (Amersham Biosciences, Uppsala, Sweden). For the second dimension, proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % acrylamide gels. The 2-D gel pattern of proteins extracted from original *C. jejuni* strain in exponential phase challenged with 25 % MIC to BKC in MH broth can be seen in Figure 4.1. This image was chosen as an example of a representative protein profile for the original *C. jejuni* strain to indicate the position of proteins showing altered expressed under the conditions tested over the course of this study. Actual changes in proteins are shown in Figure 4.2-4.3 and the results of the protein identification are summarised in Table 4.2-4.5.

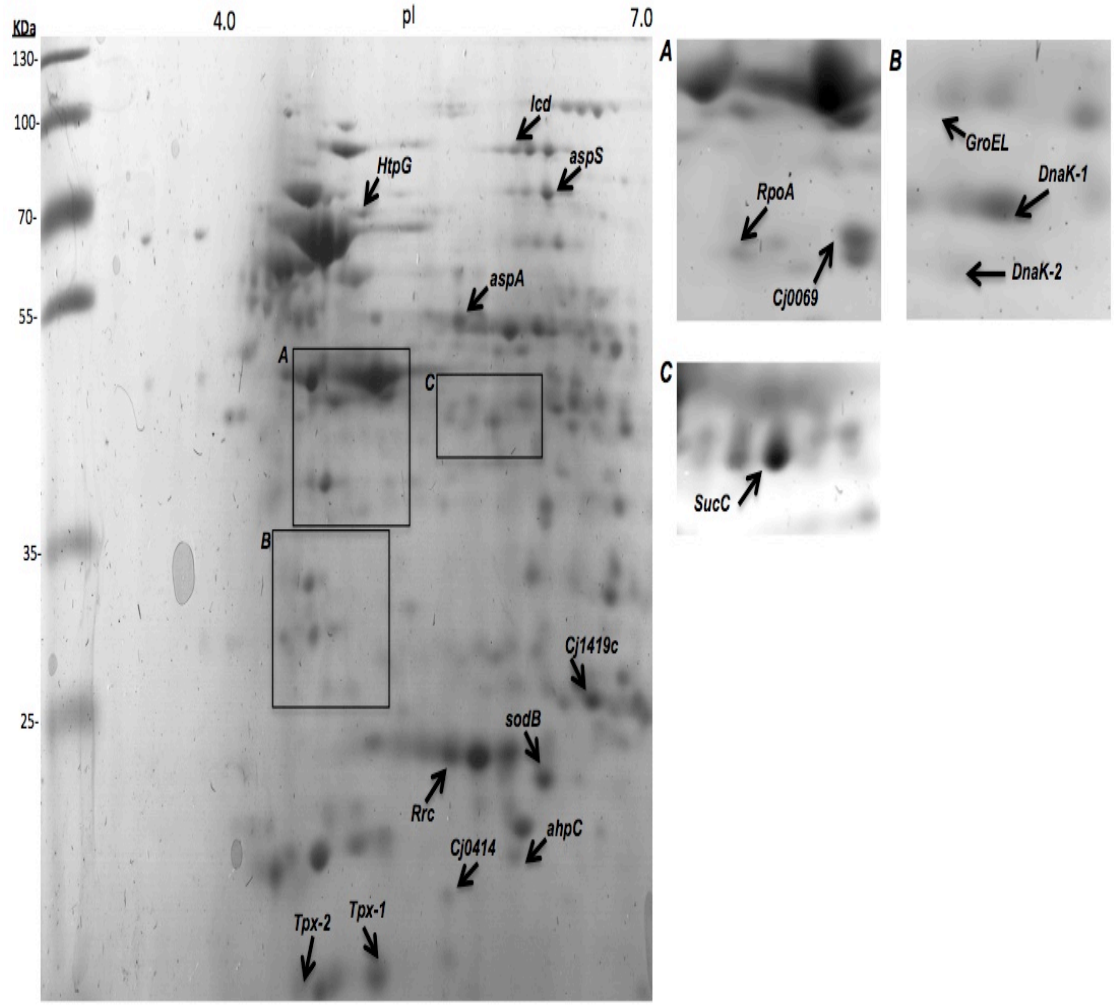

Figure 4.1 2-D gel pattern of proteins extracted from the original *C. jejuni* strain. Bacterial cells in exponential phase of growth were in MHB supplemented with 25 % concentration of its broth MIC to BKC. The proteins were visualised by Coomassie blue staining. Positions of proteins showing altered expression following adaptation to BKC or ciprofloxacin or following a 25 % MIC challenge to BKC/ciprofloxacin were identified by a combination of tryptic digestion and MALDI-TOF mass spectrometry are indicated by arrows. Highlighted region A was taken from 2D gel of proteins extracted from *C. jejuni* (original strain) and from region B & C from 2D gel of proteins extracted from ciprofloxacin-adapted *C. jejuni* variant (both in absence of challenge). Molecular weight marker values indicated on the left side of the gel image are in kDa and the 7 cm IPG strip used displayed a pH gradient ranged from 4 to 7 (indicated above gel picture).

The position of spots (not present on the gel) but expressed under other conditions are included and indicated in regions A (*C. jejuni* grown in the absence of a 25% MIC challenge), B and C (ciprofloxacin-adapted *C. jejuni* variant without a 25% MIC challenge). In addition, the representative 2-D gels taken from other conditions are presented in Supplemental materials in Section 4.5 of this chapter (Figure S4.1– Figure S4.2).

4.4.2.1 Identification of proteins that were altered upon adaptation of *C. jejuni* to BKC or following a 25 % MIC_{broth} challenge with BKC

Proteins spots identified that were differentially expressed following adaptation of *C. jejuni* to BKC and following a 25 % MIC_{broth} challenge with BKC are shown in Figure 4.2 and Table 4.2.-4.3.

	Expression ratio Original with 25 % MIC BKC [1]	Expression ratio BKC-adapted[2]	Expression ratio BKC-adapted with 25 % MIC BKC [3]
General stress (Heat shock) and oxidative stress			
SodB	1.03	**3.29**	**3.13** (0.80)
AhpC	1.17	**2.84**	**2.75** (0.82)
Tpx-1	1.4	**2.56**	**3.74** (1.08)
Tpx-2	NF	P	P(0.87)
Rrc	1.2	**3.03**	**2.42**(0.95)
HtpG	**-2.38**	-1.65	- 1.46 (1.15)
Energy Metabolism			
Icd	1.37	**2.22**	**2.54** (0.77)
AspA	**-2.05**	**-2.67**	**- 2.99** (1.14)
Cj0414	P	P	P(-1.64)
DNA and protein synthesis			
RpoA	**2.37**	**3.00**	**2.71**(1.01)
AspS	1.32	**2.53**	**2.37**(0.90)
Cj1419c	P	NF	NF
Unknown			
Cj0069	NF	NF	NF

Table 4.2 Analyses of spot intensities from proteins identified as differentially expressed in the BKC-adapted variant compared to the original *C. jejuni* strain and following a 25 % MIC challenge with BKC. The values were calculated from the average of the normalised spot intensity on three replicate 2-D gels provided by PDQuest software compared to the original strain without challenge and grouped by function. The ratios were calculated as follows: 1. Spot intensity of the original strain with 25 % MIC challenge to BKC/ Spot intensity of the original strain. 2 Spot intensity of the BKC-adapted variant/Spot intensity of original strain. 3. Spot intensity of the BKC-adapted variant with 25 % MIC challenge to BKC/Spot intensity of original strain. In brackets - Spot intensity of the BKC-adapted variant/BKC-adapted variant with 25 % MIC challenge to BKC. Abbreviations: P, present; NF, not found, up-regulation or down-regulation of protein spot intensities of greater than 2-fold are indicated in bold. Down-regulated proteins are expressed as a negative reciprocal of the expression ratio.

Gene	Name	Suggested Function	Molecular weight Predicted/observed	Isoelectric point Predicted/observed	%Coverage	Score
Proteins that were up-regulated following adaptation to BKC						
SodB	superoxide dismutase (sodB)	Oxidative stress response /detoxifiation	25.0/28	5.83/5.7	74	1087
Cj1419c	Methyltransferase (Cj1419c)	unknown	29.9/32	5.92/6.3	63	1167
AhpC	anti-oxidant AhpCTSA family protein (AhpC)	Tricarboxylic acid cycle/ aspartate metabolic processes	22.0/22	5.66/5.7	85	1070
Tpx-1	Thiol peroxidase (Tpx)	Cell redox homeostatis	18.8/18	5.13/4.7	96	1277
Tpx-2	Thiol peroxidase (Tpx)	Cell redox Homeostatis	18.8/17	5.13/4.9	96	1257
Rrc	Rubrerythrin (Rrc)	Iron ion binding/ oxidoreductase activity	25.0/25	5.49/5.6	81	1333
Cj0414	Gluconate 2-dehydrogenase gamma chain	unknown	27.0/20	5.50/5.7	53	1054
rpoA	DNA-directed RNA polymerase, alpha subunit	Transcription	37.7/40	4.97/5.1	66	1131
asps	aspartyl-tRNA synthetase	Aminoacyl tRNA synthetases and their modification	66.7/70	5.8/5.9	66	2468
Icd	isocitrate dehydrogenase, NADP-dependent (Icd)	Tricarboxylic acid cycle	82.6/85	5.84/5.7	35	1796
Proteins that were down-regulated following adaptation to BKC						
aspA	aspartate ammonia-lyase (aspA)	Tricarboxylic acid cycle/ aspartate metabolic process	52.1/52	5.5/5.6	36	1275
HtpG	Heat shock protein 90 (HtpG)	Heat Shock	69.7/62	5.09/5.3	65	2514
Cj0069	Hypothetical protein	Unknown	39/38	5.23/5.3	52	1016

Table 4.3 Differentially expressed proteins identified in original strain and BKC-adapted *C. jejuni* variant, using mass spectrometry. Protein spots were excised from 2-D gels and identified by MALDI-TOF mass spectrometry. All proteins were successfully identified as belonging to *C. jejuni.* Where protein function was not indicated, its probable function in other bacteria is supplied. Molecular weights (both observed and predicted) and isoelectric points are shown. The score is derived from ions scores that are equal to - 10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 48 were deemed significant ($P<0.05$). § Analysis of spot intensity data showing up-regulation or down-regulation in Table 4.2

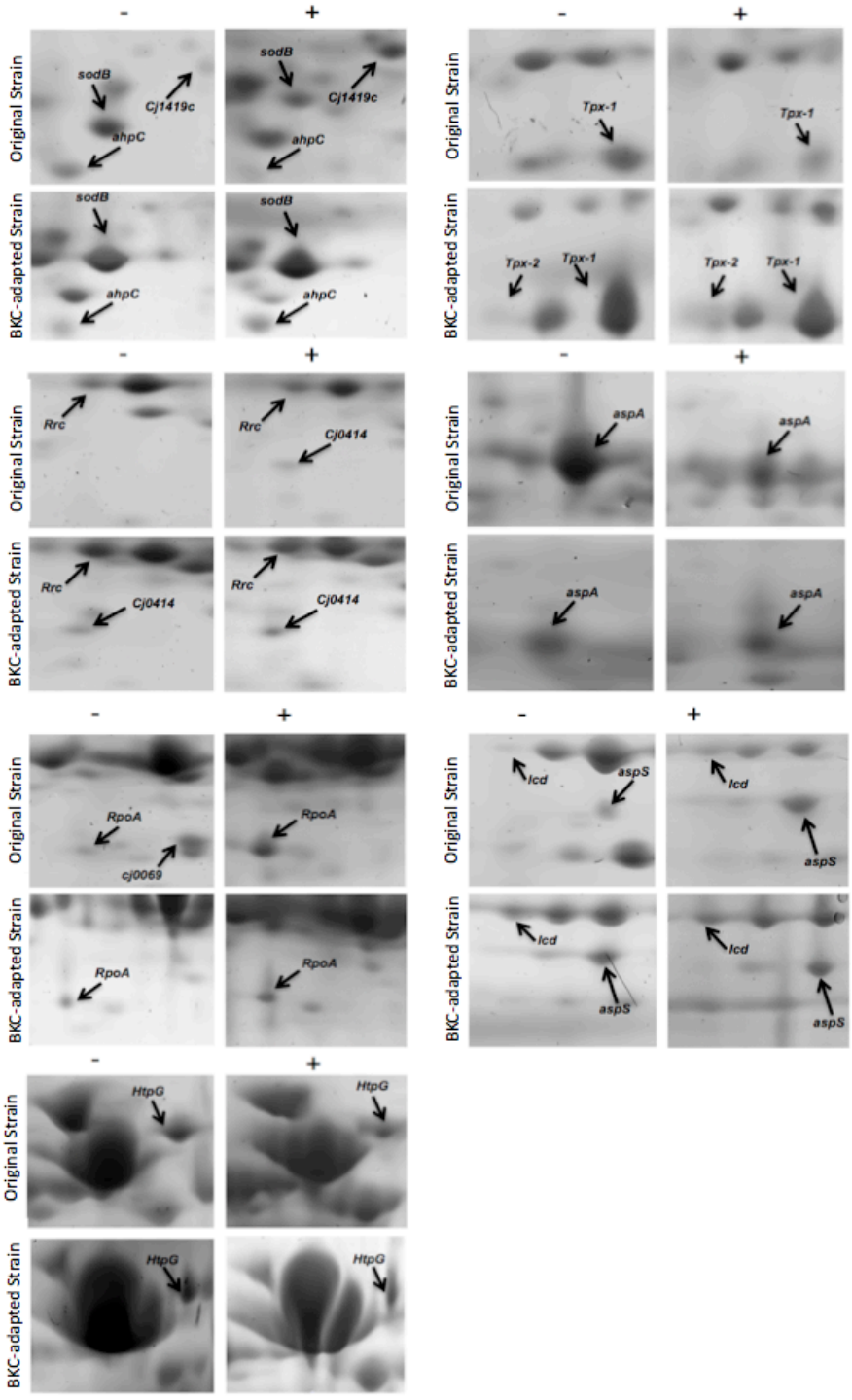

Figure 4.2 Differentially expressed proteins spots identified in original *C. jejuni* strain and its BKC-adapted counterpart, using 2D-gel electrophoresis. Note:- Protein spots of interest from original *C. jejuni* parent strain and BKC-adapted variants on 2-DGE gels with (+) and without (-) a 25% MIC concentration of BKC challenge of their respective MIC_{broth} values are depicted. Arrows indicate the locations of the proteins showing altered expression. Proteins were visualised by Coomassie blue staining. They were identified using a combination of tryptic digestion and mass spectrometry, the results of which are presented in Table 4.3 Analysis of spot intensity data showing up-regulation or down-regulation in Table 4.2.

Upon adaptation of *C. jejuni* to BKC, superoxide dismutase, thiol peroxidase, alkyl hydroperoxide reductase and rubrerythrin were upregulated (3.29-fold & 3.13-fold, SodB; 2.56-fold & 2.74-fold , Tpx-1; 2.84-fold & 2.75, AhpC; 3.03-fold 2.42-fold, Rrc; Table 4.4). A new protein spot identified as thiol peroxidase (Tpx-2) was found in the adapted strain. Expression of all of these proteins was unchanged in the original strain subjected to a BKC challenge at 25 % of its broth MIC concentration (1.03-fold, SodB; 1.40-fold, Tpx; 1.17-fold, AhpC; 1.2-fold, Rrc).

Isocitrate dehydrogenase was up-regulated 2.54-fold & 2.22-fold and aspartate ammonia-lyase was down-regulated 2.99-fold & 2.67-fold in the adapted variant with and without a 25% MIC challenge with BKC. When original strain was subjected to a 25 % MIC challenge with BKC, isocitrate dehydrogenase was upregulated 1.37-fold and aspartate ammonia-lyase was downregulated 2.05-fold.

A spot identified as the alpha subunit of DNA directed RNA polymerase and aspartyl tRNA synthase were upregulated (1.66-fold, RpoA; 2.53-fold, AspS) in the BKC-adapted variant and in the original strain (2.54-fold & 2.22-fold, RpoA; 2.37-fold & 2.53-fold, AspS). These same protein spots were unaltered after subjecting the adapted variant to a 25 % challenge of its broth MIC concentration to BKC (1.01-fold, RpoA; 0.90-fold, AspS).

Heat shock protein HtpG was down-regulated 1.56-fold in BKC-adapted variant and after a 25 % MIC challenge (-1.46-fold, HtpG). The same protein was down-regulated 2.38-fold following a 25 % MIC challenge in the original strain. Another protein identified as Cj0069 was absent in the BKC-adapted variant with or without a 25 % MIC challenge with BKC. The same protein was absent in the original strain subjected to a 25 % MIC challenge. Cj0414 was not present in the original strain but present in the BKC-adapted variant with or without a 25 % MIC challenge with BKC and when the original strain was subjected to a 25 % MIC challenge with BKC. It was down-regulated 1.64-fold following a 25 % MIC challenge with BKC in the adapted strain. A putative methyl transferase, cj1419c, was also identified in the original strain following a 25 % MIC challenge with BKC. When compared with other the level of expression of proteins identified as differentially expressed in BKC–adapted variant were similar under the two conditions tested (Table 4.2).

4.4.2.2 Identification of proteins that were altered upon adaptation of *C. jejuni* to ciprofloxacin or following a 25 % MIC_{broth} challenge with ciprofloxacin.

Proteins spots identified that were differentially expressed following adaptation of *C. jejuni* to ciprofloxacin and following a 25 % MIC_{broth} challenge with ciprofloxacin are shown in Table 4.4 - 4.5 and Figure 4.3.

Gene	Expression ratio original with ciprofloxacin challenge[1]	Expression ratio ciprofloxacin-adapted[2]	Expression ratio ciprofloxacin-adapted with ciprofloxacin challenge[3]
General stress (Heat shock)			
DnaK-1	1.70	**4.35**	1.32 (-1.83)
DnaK-2	P	P	P(-1.38)
GroEL	NF	P	NF
HtpG	**-2.43**	**-2.13**	-1.43 (0.94)
Oxidative stress[4]			
SodB	-1.98	**-2.97**	**- 2.36** (0.82)
Tpx-1	-1.79	**-2.28**	**- 2.91**(1.16)
AphC	1.08	1.20	0.97(1.30)
Rrc	**2.15**	1.08	0.93 (1.22)
Energy metabolism			
SucC	**-2.02**	**2.82**	**2.22** (0.88)
aspA	**-2.35**	**-6.82**	**-5.48** (1.50)
Protein synthesis			
Rpo A	0.97	**2.16**	1.65 (1.46)
Unknown			
Cj0069	NF	NF	NF

Table 4.4 Analyses of spot intensities from proteins identified as differentially expressed in the ciprofloxacin-adapted variant compared to the original *C. jejuni* strain and following a 25 % MIC challenge with ciprofloxacin. The values were calculated from the average of the normalised spot intensity on three replicate 2-D gels provided by PDQuest software compared to the original strain without challenge and grouped by function. The ratios were calculated as follows: 1. Spot intensity of the original strain with 25 % MIC challenge to ciprofloxacin/ Spot intensity of the original strain. 2 Spot intensity of the ciprofloxacin-adapted variant/Spot intensity of original strain. 3. Spot intensity of the ciprofloxacin-adapted variant with 25 % MIC challenge to ciprofloxacin/Spot intensity of original strain. In brackets - Spot intensity of the ciprofloxacin-adapted variant/ciprofloxacin-adapted variant with 25 % MIC challenge to ciprofloxacin. Abbreviations: P, present; NF, not found, up-regulation or down-regulation of protein spot intensities of greater than 2-fold are indicated in bold. Down-regulated proteins are expressed as a negative reciprocal of the expression ratio.

Gene	Name	Suggested Function	Molecular weight Predicted/observed	Isoelectric point Predicted/observed	%Coverage	Score
Proteins that were up-regulated following adaptation to ciprofloxacin						
RpoA	DNA-directed RNA polymerase, alpha subunit (rpoA)	Transcription	37.7/40	4.97/5.1	66	1131
DnaK	chaperone protein DnaK-fragment	Stress response	67.5/28	4.98/4.9	21	743
DnaK	chaperone protein DnaK-fragment	Stress response	67.5/28	4.98/4.9	14	313
Cpn 60	Chaperonin Cpn 60 (GroEL)	Protein refolding	44.1/36	5.17/5.1	28	698
Rrc	rubrerythrin (Rrc)	Iron ion binding/ oxidoreductase activity	25.0/25	5.49/5.6	81	1333
SucC	succinyl-CoA synthase, beta subunit (SucC)	Tricarboxylic acid cycle	41.9/44	5.61/5.4	58	1096
Proteins that were down-regulated following adaptation to ciprofloxacin						
AspA	aspartate ammonia-lyase (AspA)	Tricarboxylic acid cycle/ aspartate metabolic process	52.1/52	5.5/5.6	36	1275
HtpG	Heat shock protein 90 (HtpG)	Heat Shock	69.7/62	5.09/5.3	65	2514
SodB	superoxide dismutase (sodB)	Oxidative stress response /detoxifiation	25.0/28	5.83/5.7	74	1087
Tpx-1	Thiol peroxidase (Tpx)	Cell redox Homeostatis	18.8/17	5.13/4.9	96	1257
Cj0069	Hypothetical protein	Unknown	39/38	5.23/5.3	52	1016

Table 4.5 Differentially expressed proteins identified in original and ciprofloxacin-adapted strains ±25 % MIC challenge concentration of ciprofloxacin using mass spectrometry. Note:- Protein spots were excised from 2-D gels and identified by MALDI-TOF mass spectrometry. All proteins were successfully identified as belonging to *C. jejuni.* Where protein function was not indicated, its probable function in other bacteria is supplied. Molecular weights (both observed and predicted) and isoelectric points are shown. The score is derived from ions scores that are equal to - 10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 48 were deemed significant ($P<0.05$). Analysis of spot intensity data showing up-regulation or down-regulation in Table 4.4

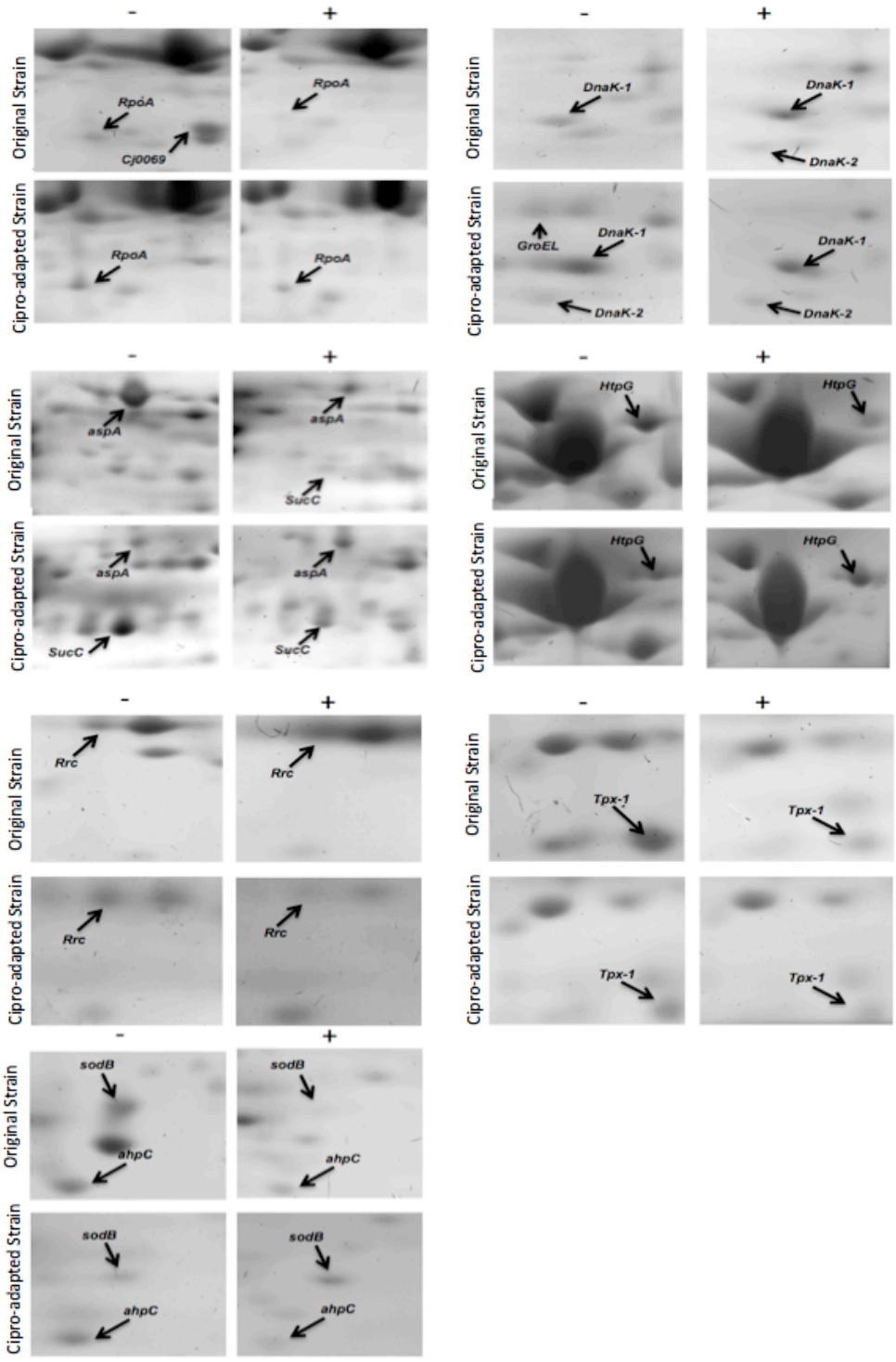

Figure 4.3 Differentially expressed proteins spots identified in original *C. jejuni* strain and its ciprofloxacin-adapted counterpart, using 2D-gel electrophoresis. Note:- Protein spots of interest from original *C. jejuni* strain and ciprofloxacin-adapted variants on 2-DGE gels with (+) and without (-) a 25% MIC concentration of ciprofloxacin challenge of their respective MIC_{broth} values are depicted. Arrows indicate the locations of the proteins showing altered expression. Proteins were visualised by Coomassie blue staining. They were identified using a combination of tryptic digestion and mass spectrometry, the results of which are presented in Table 4.5. Analysis of spot intensity data showing up-regulation or down-regulation in Table 4.4

Heat shock proteins involved in the general stress response were found to be altered upon adaptation of *C. jejuni* to ciprofloxacin and following a 25% MIC_{broth} challenge in both the original and ciprofloxacin- adapted variant (Table 4.5 and Figure 4.3). Heat shock protein DnaK was up-regulated upon adaptation of *C. jejuni* to ciprofloxacin (Dnak-1, 4.35-fold). The same protein was also up-regulated (1.70-fold & 1.32-fold; not significant, < 2-fold) in the original and ciprofloxacin-adapted strain following a 25% MIC challenge with ciprofloxacin. Heat shock protein HtpG was down-regulated both in the original strain following a 25% MIC_{broth} challenge to ciprofloxacin (-2.43-fold) and following adaptation of *C. jejuni* to ciprofloxacin in the ciprofloxacin-adapted strain (-2.13-fold and -1.43-fold).

Two novel proteins identified as heat shock protein DnaK and GroEL (Cpn 60) were also found under the conditions tested. DnaK (DnaK-2, Figure 4.3, Table 4.5) was identified in the original strain following a 25 % MIC challenge with ciprofloxacin and upon adaptation of the original to ciprofloxacin. It was slightly down-regulated (< 2-fold) in the adapted strain following a 25 % MIC challenge with ciprofloxacin. A spot identified as GroEL (Cpn 60) was detected in the adapted strain but was absent following a 25 % MIC challenge with ciprofloxacin (Figure 4.5).

Proteins known to be involved in the oxidative stress response were also examined to investigate if their expression was altered in response to adaptation of *Campylobacter* to ciprofloxacin or as a result of a challenge with the antibiotic. These proteins were initially identified in BKC-adapted variant of *C. jejuni* (see Figure 4.2, Table 4.2). Proteins SodB and Tpx involved in the oxidative stress response were down-regulated following adaptation to ciprofloxacin (SodB, -2.36-fold & -2.97-fold; Tpx-1, -2.91 & -2.28-fold) in the ciprofloxacin-adapted variant with and without a 25% MIC challenge with ciprofloxacin. The same proteins were down-regulated (not significant; <2-fold) in the original strain following a 25 % MIC challenge with ciprofloxacin (SodB, 1.98; Tpx-1, 1.79). Rubrerythrin (Rrc) was up-regulated 2.15-fold in the original strain following a 25 % MIC challenge with ciprofloxacin (Table 4.5). Its expression relative to the original strain was unaltered in the ciprofloxacin-adapted variant (Rrc, 1.08-fold) and following a 25 % MIC challenge in the adapted strain (Rrc, 0.93-fold).

The expression of alkyl hydroperoxide reductase was unaltered both in the original strain following a 25 % MIC challenge with ciprofloxacin (AhpC, 1.08-fold) and following adaptation to ciprofloxacin (AhpC, 1.20-fold). Its expression was also unaltered following a 25 % MIC challenge in the ciprofloxacin-adapted strain (AhpC, 0.97-fold) when compared to the original un-challenged strain in the ciprofloxacin-adapted strain with a challenge with ciprofloxacin.

Proteins involved in energy metabolism were also altered following the adaptation of *C. jejuni* to ciprofloxacin. Succinyl-CoA synthase, beta subunit, (SucC) was up-regulated where as aspartate ammonia-lyase (AspA) was down-regulated in the ciprofloxacin-adapted variant (SucC, 2.22-fold & 2.82-fold; AspA, -5.48-fold & -6.82-fold). Both of these proteins were down-regulated in the original strain following a 25 % MIC challenge with ciprofloxacin (SucC, 2.02-fold; AspA, 2.35-fold). Expression of the DNA-directed RNA polymerase alpha subunit RpoA was up-regulated in the adapted strain (RpoA, 1.65-fold & 2.16-fold). The same protein was unaltered in the original strain (RpoA, 0.97-fold following a 25 % MIC challenge. When compared with other the level of expression of proteins identified as differentially expressed in ciprofloxacin–adapted variant were similar under the two conditions tested (Table 4.5).

4.5 Discussion

Proteomic analysis (SDS-PAGE and 2DGE) was carried out to determine changes induced by adaptation of *C. jejuni* NCTC 11168 to BKC and ciprofloxacin at the proteomic level following long-term chemostat culture (> 1400h). Expression of oxidative stress response proteins in the ciprofloxacin-adapted variant of *C. jejuni* NCTC 11168 was examined to determine if they played a role in mediating decreased sensitivity to ciprofloxacin.

Analysis of SDS-PAGE profiles of whole cell extracts of ciprofloxacin and BKC-adapted variants revealed similar banding pattern when compared to the original strain. To examine changes in protein expression two-dimensional gel electrophoresis (2DGE) was undertaken to investigate these differences. Proteins isolated from original *C. jejuni* strain, BKC-adapted and ciprofloxacin adapted variants (with/without a 25% MIC challenge of their respective MIC_{broth} values; Table 4.1.) were analyzed by 2DGE to detect differences in protein expression. Subjecting isolates to a 25% MIC challenge of their respective MIC ensured that resistance mechanisms of ciprofloxacin- and BKC-adapted *C. jejuni* variants were actively expressed. It also was used to compare whether resistance mechanisms expressed by the adapted strains were similar to those expressed by original strain when subjected to a 25% MIC challenge of its respective MIC_{broth} to BKC or ciprofloxacin.

Following analysis by 2DGE, fifteen proteins who's expression was altered more then 2-fold were identified during analysis (Figure 4.1-4.3 and table 4.2-4.5). Seven proteins, where expression was altered, were common to both BKC- and ciprofloxacin-adapted *C. jejuni* variants. Three proteins were unique to ciprofloxacin-adapted *C. jejuni* variant and four proteins were uniquely up-regulated following chemostat adaptation of *C. jejuni* to BKC. Finally one protein was identified as being novel to a 25% MIC challenge to BKC in original strain.

Using 2DGE, eleven proteins with altered expression in the BKC-adapted strain were identified (See Figure 4.2 and Tables 4.3-4.4). These included proteins associated with the general and oxidative stress response, metabolism, DNA and protein synthesis. The mechanism of action of BKC has been described and involves the disruption of

the bacterial cell membrane which results in leakage of intracellular constituents eventually leading to cell death (Gilbert & Moore, 2005). Disruption of the electron transport chain as a result of damage to the cell membrane following BKC exposure may result in an increased level of exposure of the cell to reactive oxygen species (ROS) (Bore *et al.,* 2007*)*.

Campylobacter possess unique regulatory mechanisms for response to oxidative stress (Kim *et al.,* 2015). The peroxide resistance regulator regulates the transcription of genes that confer protection against peroxide and oxidative stress. A second regulator, the campylobacter oxidative stress regulator (CosR) also controls the transcription of genes involved in oxidative stress (Hwang *et al.,* 2011; Kim *et al.,* 2015). Some of the proteins under the control of these regulators namely, superoxide dismutase, thiol peroxidase, alkyl hydroperoxide redutase and rubrerythrin were up-regulated following adaptation to BKC. Expression of the proteins superoxide dismutase, alkyl hydroperoxide redutase and rubrerythrin were unchanged in *C. jejuni* and BKC-adapted counterpart as a result of BKC challenge. Thiol peroxidase, however, was found to be up-regulated (1.4-fold, original; Table 4.3). These proteins are associated with oxidative stress resistance in *Campylobacter* and their role in oxidative stress has been described (Kim *et al.,* 2015).

C. jejuni encodes a single superoxide dismutase (SodB) which is a key enzyme involved in detoxification of O_2^- (Atack & Kelly, 2009; Garénaux *et al.,* 2007; Hwang *et al.,* 2011). SodB utilizes two protons (H^+) to convert two superoxide anion radicals to hydrogen peroxide (H_2O_2) (Atack & Kelly, 2009). In *Campylobacter*, SodB is negatively regulated by both the CosR and PerR regulons (Hwang *et al.,* 2011; Kim *et al.,* 2015). H_2O_2 itself is weakly reactive, however the single bond between the two oxygen molecules in H_2O_2 can fragment easily producing two hydroxyl radicals. Hydroxyl radicals are highly reactive and can cause damage to proteins, nucleic acids and membranes (Atack & Kelly, 2009). Both thiol peroxidase and alkyl hydroperoxide redutase are involved in the enzymatic removal of H_2O_2.

Thiol peroxidase has been identified as the major H_2O_2 scavanger in *Campylobacter* (Atack & Kelly, 2009). Thiol peroxidases are important in *Campylobacter* during conditions such as nutrient starvation. Additionally, thiol peroxidases have found to up-regulated in *Campylobacter* biofilms and have been shown to contribute to

aerotolerance in *C. jejuni* (Kim *et al.,* 2015; Murphy *et al.,* 2003). Alkyl hydroperoxide redutase (AhpC) is involved in H_2O_2 detoxification in *E. coli*, but AhpC does not appear to be essential for H_2O_2 removal in *Campylobacter* (Atack & Kelly, 2009). Expression of AhpC is positively regulated by the CosR regulon and negatively regulated by CosR in *Campylobacter* (Kim *et al.,* 2015).

Rubrerythrin is a non-heme iron protein which has shown to be extremely sensitive to reactive oxygen species. It has been also suggested to be involved in the oxidative stress response in *Campylobacter* and been shown to confer resistance to H_2O_2 and menadione (superoxide) (Kim *et al.,* 2015; Pinto *et al.,* 2011). In *Campylobacter*, rubrerythrin is negatively regulated by CosR. Ferric uptake regulator (fur), involved in iron homeostasis also regulates rubrerythrin expression. Its influence on expression of rubrerythrin is iron- dependant. Rubrerythrin is positively regulated by halo-fur (iron-bound fur) and negatively regulated by apo-fur (Kim *et al.,* 2015). Following adaptation to BKC, up-regulation of proteins involved in the oxidative stress response has also been shown to occur in *E. coli* and *B. cereus* (Bore *et al.,* 2007; Ceragioli *et al.,* 2010).

Acquired defense mechanisms normally relating to oxidative stress response also increase tolerance to antimicrobials including biocides and antibiotics in bacteria and may represent a possible cross-resistance mechanism (Bore *et al.,* 2007; Atack & Kelly, 2009). In BKC-adapted isolates it was noticed that strains developed low level cross-resistance (2-fold increase) to ciprofloxacin (Chapter 2). Up-regulation of the oxidative stress response in these strains could be mediating a protective effect against the action of ciprofloxacin resulting in the observed low-level cross-resistance. Up-regulation of oxidative stress response has been observed to confer low-level cross-resistance to antibiotics in BKC-adapted *E. coli* (Bore *et al.,* 2007).

The general stress protein, HtpG, involved in the heat shock response was down regulated in BKC-adapted *C. jejuni* (with/without a 25% MIC challenge to BKC) and original strain following a 25% MIC challenge with BKC. Little is known about the molecular function of heat shock protein HtpG, but under stress conditions it plays a supporting role in *de novo* protein folding in *E. coli* and suppresses the aggregation of the protein citrate synthase (Buchner, 2010). It has also shown to be required for the

assembly of large protein complexes called phycobilisomes (large protein complexes involved in light harvesting) in cyanobacteria (Buchner, 2010).

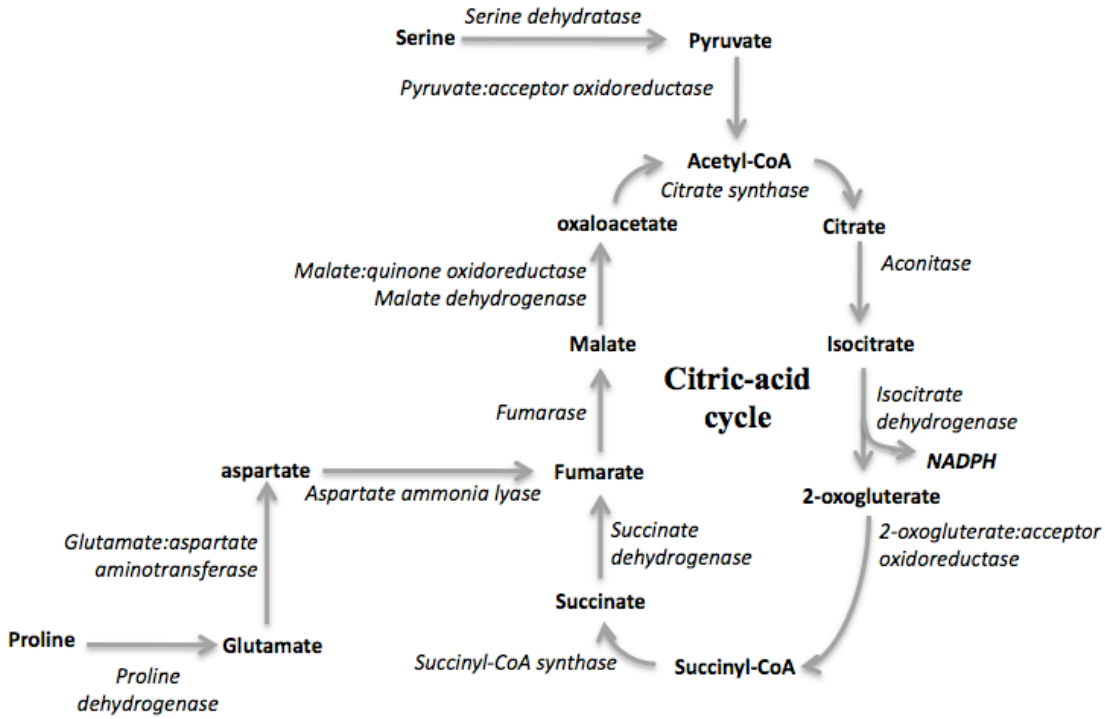

Figure 4.4. Amino acid metabolism and the citric acid cycle in *Campylobacter jejuni* adapted from Kendall *et al.*, 2014.

Mutations in genes that are associated with antimicrobial resistance may impose a biological cost to *Campylobacter* in the absence of antimicrobial selection pressure (Andersson, 2003; Zhang *et al.*, 2006). Reorganized metabolic networks may be one way to circumvent metabolic costs associated with development of antimicrobial resistance (Händel *et al.*, 2013). Levels of expression of the proteins isocitrate dehydrogenase, aspartate ammonia-lyase and gluconate dehydrogenase involved in energy metabolism were also altered upon adaptation to BKC. The TCA cycle enzyme isocitrate dehydrogenase was up-regulated in the BKC-adapted variant but not in the original following a BKC challenge. Its expression remained up-regulated following a BKC challenge in the adapted variant. *Campylobacter* is unable to utilize sugars as it lacks key glycolysis enzymes 6-phospho-gluconate dehydratase enzyme and 6-phosphofructokinase and, uses amino acids glutamate, aspartate, serine and proline as a main energy source (Guccione *et al.,* 2008; Kelly, 2005; Pajaniappan *et al.,* 2008; Parkhill *et al.,* 2000; Stahl *et al.,* 2014).

Amino acid utilization in the TCA cycle in *Campylobacter jejuni* is illustrated in Figure 4.4. In the TCA cycle, isocitrate dehydrogenase catalyses the conversion of isocitrate to 2-oxygluterate and CO_2 with the reduction of NADP to NADPH (Wang *et al.,* 2015). Up-regulation of isocitrate dehydrogenase may be protective under conditions of oxidative stress due to this production of NADPH as NADPH maintains a strong reducing environment in bacteria and is thus plays a role in the cellular defense against oxidative damage and the detoxification of ROS (Kashmiri & Mankar, 2014; Wang *et al.,* 2015). The protein aspartate ammonia-lyase (aspA) was down-regulated in the BKC-adapted variant and original strain following a 25 % MIC challenge with BKC. It remained down-regulated in the BKC-adapted variant after a 25 % MIC challenge with BKC. AspA is involved in the metabolism of the amino acids aspartate, glutamate and proline (Guccione *et al.,* 2008). This may indicate that in the BKC-adapted variant or after exposure to BKC the original strain both switched their metabolism to preferentially use serine as an energy source. In *Campylobacter*, serine is metabolized to pyruvate before it enters the TCA cycle. Pyruvate has been shown to protect cells from the damaging effects of super oxide anions and hydrogen peroxide and oxidative stress (Atack *et al.,* 2009).

A novel protein, gluconate-2-dehydrogenase gamma chain (Cj0414), part of the gluconate dehydrogenase enzyme, was identified in both the BKC-adapted *C. jejuni* variant and in the original strain following a 25 % MIC challenge with BKC. It was down-regulated (Cj0414, 1.64 –fold; Table 4.3) following a 25 % MIC challenge with the BKC in the adapted strain. It has been suggested that gluconate dehydrogenase is involved in energy metabolism during the oxidative phosphorylation stage of aerobic respiration in *Campylobacter* as an electron donor for the electron transport chain at the level of cytochrome c (Jeon *et al.,* 2010; Pajaniappan *et al.,* 2008). Gluconate is not utilized as a carbon energy source in *Campylobacter*. It lacks key glycolysis enzymes involved in both the Entner-Doudoroff pathway and Embden-Meyerhof-Parnas pathway namely 6-phospho-gluconate dehydratase enzyme and 6-phosphofructokinase (Kelly, 2005; Pajaniappan *et al.,* 2008). The gluconate-2-dehydrogenase enzyme was also found to be important for temperature-dependant colonization of chicken ceca and was up-regulated following acid stress in *Campylobacter* (Ried *et al.,* 2008; Pajaniappan *et al.,* 2008). Proteins involved DNA and protein synthesis were also up-regulated in the BKC adapted *C.jejuni* variant.

These proteins were identified as DNA-directed RNA polymerase and aspartyl-tRNA synthetase. Up-regulation of these proteins may be important for *de novo* protein synthesis in the adapted strain. In addition, a putative methyl transferase Cj1419c was also identified in original strain when challenged with 25% MIC to BKC. The gene encoding this protein is part of the capsule biosynthesis locus in *Campylobacter* and has previously been shown to be altered in the stringent response and acid stress (Gaynor *et al.,* 2005; Varsaki *et al.,* 2015).

Fluoroquinolones inhibit DNA synthesis in bacteria by targeting the topoisomerase enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV, which help control levels of DNA under- and over-winding in addition to removing knots and tangles from bacterial chromosomal DNA (Aldred *et al.,* 2014a; Hooper, 2001). DNA gyrase alters DNA topology by passing an intact double helix through a transient break (Aldred *et al.,* 2014b). There is growing evidence that DNA gyrase is the sole target for fluoroquinolone antibiotics (Luo *et al.,* 2003; Parkhill *et al.,* 2000; Payot *et al.,* 2006; Piddock *et al.,* 2003) whilst the secondary target of topoisomerase IV appears to be absent in *Campylobacter*. Ciprofloxacin and other fluoroquinolone antibioitics inhibit DNA replication by binding to DNA gyrase-DNA complex (after it has cleaved DNA) and inhibiting gyrase-mediated DNA re-ligation resulting in the formation of double stranded breaks in the bacterial chromosome. Treatment with fluoroquinolone antibiotics has been shown to result in increased production of reactive oxygen species (ROS; Hwang *et al.,* 2013). However, the exact molecular mechanism by which ROS is generated as a result of ciprofloxacin treatment in *Campylobacter* remains to be elucidated (Hwang *et al.,* 2013). Increased production of ROS by fluoroquinolone antibiotics can lead to destabilization of the iron-sulphur cultures in enzymes and the release of Fe^{2+} ions. Free Fe^{2+} ions can lead to the generation of hydroxyl radical species via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^-$) (Hwang *et al.,* 2013). Formation of ROS including the superoxide anion radical (0_2^-), hydrogen perioxide (H_2O_2) and highly reactive hydroxyl radicals ($OH\bullet$) can cause damage to proteins, nucleic acids and membranes (Atack & Kelly, 2009; Cabiscol *et al.,* 2000).

Proteins identified as differentially expressed during gel analysis between original strain and ciprofloxacin-adapted variant (with or without a 25% MIC challenge to ciprofloxacin) are listed in Table 4.4-4.5. Levels of proteins involved in the general and oxidative stress response, energy metabolism, protein synthesis and *Cj0069*, a protein with unknown function, were altered under conditions tested.

Heat shock proteins DnaK and GroEL and were found to be up-regulated in ciprofloxacin adapted strains. DnaK was also up-regulated in the parent strain following exposure to ciprofloxacin. Heat shock proteins serve vital roles in normal cell function by acting as chaperones to promote the folding of most cellular proteins and proteolysis of potentially deleterious, misfolded proteins (Konkel *et al.,* 1998). In *Campylobacter*, up-regulation of Dnak was also observed following exposure to high-pressure stress and acid stress (Bièche *et al.,* 2012; Murphy *et al.,* 2006). GroEL was also found to be up-regulated following adaptation to ciprofloxacin however, it was absent when the adapted strain was challenge with 25% MIC ciprofloxacin (see Figure 4.3). The reason why GroEL is absent in the adapted strain challenged with a 25% MIC of ciprofloxacin is unknown. On the gel, protein spots identified DnaK and GroEL migrated with a different mass to the predicted values (67.5 KDa and 28 kDa, DnaK; 44.1 Kda and 36 Kda, GroEL; respectively, Table 4.4) Exposure of the cell to ciprofloxacin could have resulted in a post translational modification of the heat shock proteins, inducing a shift in their position in the gels relative to their position in gels for cells grown in the absence of ciprofloxacin challenge. This has previously been suggested to explain the down-regulation of GroEL fragment following exposure of *Campylobacter* to 2.5 % bile salts (Fox *et al.,* 2006). Regula et al., (2000) also suggested that one possible reason for the occurance of DnaK fragments on 2DGE gels of *Mycoplasma pneumoniae* was post-translational modification. HtpG was found to be down-regulated in the ciprofloxacin-adapted variant was also down-regulated following adaptation to BKC in this study. Similarly down-regulation of the same protein spot was shown to occur in the original and adapted strain following a 25 % MIC challenge to both BKC and ciprofloxacin.

Bacteria may also decrease their metabolic energy production following ciprofloxacin treatment, which is thought to mitigate against the damaging effects of ROS production (Han *et al.,* 2008; Xia *et al.,* 2013). Two enzymes involved in energy

metabolism, succinyl Co-A synthase and aspartate ammonia-lyase (aspA), were altered in ciprofloxacin-adapted variant and following a 25 % MIC challenge with ciprofloxacin in the original. Succinyl Co-A synthase beta subunit (SucC) was down-regulated in the original following a 25 % MIC challenge. Han et al., (2008) and Hyytiäianen et al., (2013) using DNA microarrays similarly showed down-regulation of genes involved in citric acid cycle following a challenge with ciprofloxacin in *Campylobacter jejuni* in this study. SucC was up-regulated in the ciprofloxacin-adapted strain and remained up-regulated following a 25 % MIC challenge in the adapted strain with ciprofloxacin. Adaptation or a longer period of exposure to a lower concentration of ciprofloxacin may allow cells to naturally recover their ability to generate energy via the citric acid cycle. The protein aspartate ammonia-lyase (aspA) is involved in the metabolism of the amino acid aspartate, glutamate and proline (Guccione *et al.,* 2008). AspA was down-regulated in the ciprofloxacin-adapted *C. jejuni* variant (with or without a 25% MIC challenge with ciprofloxacin) and original strain following a 25% MIC challenge to ciprofloxacin. Down-regulation of AspA was also seen in BKC-adapted strains and in the original strain following a challenge with BKC (Figure 4.2). DNA-directed RNA polymerase involved in protein synthesis (rpoA; DNA-directed RNA polymerase), was up-regulated in ciprofloxacin-adapted *C. jejuni* variant (with or without a 25% MIC challenge with ciprofloxacin) but not in the parent strain exposed to a 25% MIC challenge to ciprofloxacin. Long-term adaptation to ciprofloxacin appears to require rpoA to perform protein synthesis.

One of the proposed mechanisms of action of ciprofloxacin involves ROS production as a result of DNA damage induced by these antibiotics (Dwyer *et al.,* 2014; Hyytiäinen *et al.,* 2013; Hwang *et al.,* 2013). The expression of proteins involved in the oxidative stress response which were examined to see if they also were altered either following a challenge with ciprofloxacin in the original strain or ciprofloxacin adapted variant. These proteins had previously been identified as altered in the BKC-adapted variant examined in this study. Four proteins were assessed namely thiol peroxidase (Tpx-1), superoxide dismutase (SodB), alkyl hydroperoxide reductase (AhpC) and rubrerythrin (rrc). Analysis of the 2D gels revealed that the expression of proteins involved in protection against oxidative stress, thiol peroxidase, superoxidase dismutase was reduced (Tpx-1, 1.79-fold; SodB 1.98-fold) following exposure of *C. jejuni* to ciprofloxacin or in the ciprofloxacin-adapted *C. jejuni* variant (with or without a 25% MIC challenge with ciprofloxacin) (Figure 4.3 and Table 4.5).

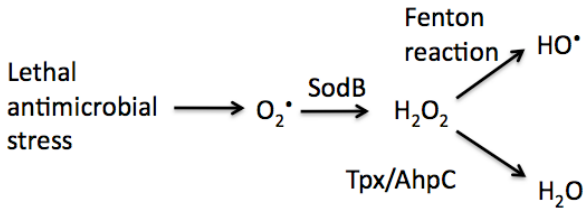

Figure 4.5 Proposed pathway by which bacterial antimicrobial stress modulates lethal oxidative damage. Adapted from Wang & Zhao (2009).

Superoxide dismutase generates peroxide as a result of the detoxification of superoxide anions generated as a result of antimicrobial stress (Wang & Zhao, 2009; Figure 4.7). This in turn could lead to increased exposure of the cell to hydroxyl radicals (HO•) generated by the Fenton reaction. Decreased expression of SodB may be protective mechanism to decrease exposure of *Campylobacter* to hydroxyl radicals. However in the same study down regulation of alkyl hydroperoxide reductase was shown to increase the lethal activity of antibiotics ampicillin and kanamycin in *E. coli* (Wang & Zhao, 2009). Although this protein was not down-regulated in *Campylobacter*, thiol peroxidase mediating the same function was down-regulated. However because SodB-mediated production of H_20_2 is reduced down-regulation of thiol peroxidase may not be as important to the accumulation of peroxide in the cell. Expression of AphC was unchanged in the original and ciprofloxacin-adapted variant upon adaptation to ciprofloxacin and following a 25% MIC challenge. It could also be playing a greater role in the detoxification of H_20_2 following the decreased expression of thiol peroxidaxe. Up-regulatation of the non-heme iron protein rubrerythrin in the original strain following a ciprofloxacin challenge may indicate that this protein is more sensitive to ROS generated by the antibiotic challenge (Pinto *et al.,* 2011; Kim *et al.,* 2015). The contribution of ROS to antibiotic lethality is most prominent at lower lethal antibiotic concentrations and at earlier time points after antibiotic exposure (Dwyer *et al.*, 2015). The fact that proteins involved in the oxidative stress were not up-regulated in the ciprofloxacin-adapted strain suggests that the oxidative stress response has a limited role in mediating resistance to ciprofloxacin in the long-term.

C. jejuni NCTC 11168 exhibited differences in expression of proteins following adaptation to BKC or ciprofloxacin. One of the most notable differences was to proteins involved in the oxidative stress resistance. These proteins were up-regulation following BKC adaptation and down-regulated in the ciprofloxacin-adapted variant of *C. jejuni* NCTC 11168.

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4.6 Bibliography

Andersson, D.I. (2003). Persistence of antibiotic resistant bacteria. *Curr. Opin. Microbiol., 6*(5), 452-456.

Atack, J.M. & Kelly, D.J. (2009). Oxidative stress in *Campylobacter jejuni*: responses, resistance and regulation. *Future Microbiol, 4*(6), 677-690.

Bennett, A.F. & Hughes, B.S. (2009). Microbial experimental evolution. *Am. J. Physiol. Regul. Integr. Comp. Physiol., 297*(1), R17-R25.

Bièche, C., de Lamballerie, M., Chevret, D., Federighi, M. & Tresse, O. (2012). Dynamic proteome changes in *Campylobacter jejuni* 81-176 after high pressure shock and subsequent recovery. *J Proteomics, 75*(4), 1144-1156.

Bore, E., Hébraud, M., Chafsey, I., Chambon, C., Skjaeret, C., Moen, B., Møretrø, T., Langsrud, Ø., Rudi, K. & Langsrud, S. (2007). Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. *Microbiology, 153*(Pt 4), 935-946.

Bronowski, C., James, C. & Winstanley, C. (2014). Role of environmental survival in transmission of *Campylobacter*. *FEMS Microbiol. Lett., 356*(1), 8-19.

Buchner, J. (2010). Bacterial Hsp90--desperately seeking clients. *Mol. Microbiol., 76*(3), 540-544.

Cabiscol, E., Tamarit, J. & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int. Microbiol., 3*(1), 3-8.

Ceragioli, M., Mols, M., Moezelaar, R., Ghelardi, E., Senesi, S. & Abee, T. (2010). Comparative transcriptomic and phenotypic analysis of the responses of *Bacillus cereus* to various disinfectant treatments. *Appl. Environ. Microbiol., 76*(10), 3352-3360.

Cerf, O., Carpentier, B. & Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int. J. Food Microbiol., 136*(3), 247-254.

Corona, F. & Martinez, J.L. (2013). Phenotypic Resistance to Antibiotics. *Antibiotics, 2*(2), 237-255.

Corso, J., Lugert, R., Groß, U. & Zautner, A.E. (2011). Is the *Campylobacter jejuni* secretory protein Cj0069 a suitable antigen for serodiagnostics? *Eur J Microbiol Immunol (Bp), 1*(1), 86-94.

Delcour, A.H. (2009). Outer membrane permeability and antibiotic resistance. *Biochim. Biophys. Acta, 1794*(5), 808-816.

Dwyer, D.J., Belenky, P.A., Yang, J.H., MacDonald, I.C., Martell, J.D., Takahashi, N., Chan, C.T., Lobritz, M.A., Braff, D., Schwarz, E.G., Ye, J.D., Pati, M., Vercruysse, M., Ralifo, P.S., Allison, K.R., Khalil, A.S., Ting, A.Y., Walker, G.C. & Collins, J.J. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U.S.A., 111*(20), E2100-E2109.

Dwyer, D. J., Collins, J. J. & Walker, G. C. (2015). Unraveling the physiological complexities of antibiotic lethality. *Annu. Rev. Pharmacol. Toxicol., 55*, 313-332.

EFSA/ECDC (2014). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal, 12*(3), 3590.

FSAI (2011). *Recommendations for a Practical Control Programme for Campylobacter in the Poultry Production and Slaughter Chain*. Food Safety Authority Ireland. Dublin, Ireland: Available at fsai.ie

Fox, E. M., Raftery, M., Goodchild, A. & Mendz, G. L. (2007). *Campylobacter jejuni* response to ox-bile stress. FEMS Immunology & Medical *Microbiology, 49*(1), 165-172.

Garénaux, A., Guillou, S., Ermel, G., Wren, B., Federighi, M. & Ritz, M. (2008). Role of the Cj1371 periplasmic protein and the Cj0355c two-component regulator in the *Campylobacter jejuni* NCTC 11168 response to oxidative stress caused by paraquat. *Res. Microbiol., 159*(9/10), 718-726.

Ge, B., McDermott, P.F., White, D.G. & Meng, J. (2005). Role of Efflux Pumps and Topoisomerase Mutations in Fluoroquinolone Resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob. Agents Chemother., 49*(8), 3347-3354.

Ge, B., Wang, F., Sjölund-Karlsson, M. & McDermott, P.F. (2013). Antimicrobial resistance in *Campylobacter*: susceptibility testing methods and resistance trends. *J. Microbiol. Methods, 95*(1), 57-67.

Gilbert, P. & McBain, A.J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin. Microbiol. Rev., 16*(2), 189-208.

Gilbert, P. & Moore, L.E. (2005). Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol., 99*(4), 703-715.

Guccione, E., Leon-Kempis Mdel, R., Pearson, B.M., Hitchin, E., Mulholland, F., van Diemen, P.M., Stevens, M.P. & Kelly, D.J. (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol.* Microbiol*., 69*(1), 77-93.

Han, J., Sahin, O., Barton, Y.W. & Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog., 4*(6), e1000083.

Han, J., Wang, Y., Sahin, O., Shen, Z., Guo, B., Shen, J. and Zhang, Q. (2012). A fluoroquinolone resistance associated mutation in gyrA Affects DNA supercoiling in *Campylobacter jejuni*. *Front Cell Infect Microbiol, 2*.

Händel, N., Schuurmans, J.M., Brul, S. and ter Kuile, B.H., (2013). Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in *Escherichia coli*. *Antimicrob. Agents Chemother.,* AAC-02096.

Hogan, D. & Kolter, R. (2002). Why are bacteria refractory to antimicrobials? *Curr. Opin. Microbiol., 5*(5), 472-477.

Hooper, D.C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infect. Dis., 7*(7), 337-341.

Hwang, S., Kim, M., Ryu, S. & Jeon, B. (2011). Regulation of oxidative stress response by CosR, an essential response regulator in *Campylobacter jejuni*. *PLoS ONE, 6*(7), e22300.

Hwang, S., Ryu, S. & Jeon, B. (2013). Roles of the superoxide dismutase SodB and the catalase KatA in the antibiotic resistance of *Campylobacter jejuni*. *J. Antibiot., 66*(6), 351-353.

Hyytiäinen, H., Juntunen, P., Scott, T., Kytömäki, L., Venho, R., Laiho, A., Junttila, S., Gyenesei, A., Revez, J. & Hänninen, M.L. (2013). Effect of ciprofloxacin exposure on DNA repair mechanisms in *Campylobacter jejuni*. *Microbiology., 159*(Pt 12), 2513-2523.

Jeon, B., Wang, Y., Hao, H., Barton, Y.W. & Zhang, Q. (2011). Contribution of CmeG to antibiotic and oxidative stress resistance in *Campylobacter jejuni*. *J. Antimicrob.* Chemother., 66(1), 79-85.

Kelly, D.J. (2005). Metabolism, electron transport and bioenergetics of *Campylobacter jejuni*: implications for understanding life in the gut and survival in the environment., in Ketley, J.M. and Konkel, M.E. (ed.) *Campylobacter: Molecular and Cellular Biology.* Norfolk, UK: Horizon Scientific Press., pp. 275-292.

Kendall, J.J., Barrero-Tobon, A.M., Hendrixson, D.R. & Kelly, D.J. (2014). Hemerythrins in the microaerophilic bacterium *Campylobacter jejuni* help protect key iron-sulphur cluster enzymes from oxidative damage. *Environ. Microbiol., 16*(4), 1105-1121.

Kim, J.C., Oh, E., Kim, J. & Jeon, B. (2015). Regulation of oxidative stress resistance in *Campylobacter jejuni*, a microaerophilic foodborne pathogen. *Front Microbiol., 6*.

Konkel, M. E., Kim, B. J., Klena, J. D., Young, C. R. & Ziprin, R. (1998). Characterization of the Thermal Stress Response of *Campylobacter jejuni*. *Infect. Immun., 66*(8), 3666-3672.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680-685.

Langsrud, S., Sundheim, G. & Holck, A.L. (2004). Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. *J. Appl. Microbiol., 96*(1), 201-208.

Lin, J., Akiba, M., Sahin, O. & Zhang, Q. (2005). CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. *Antimicrob. Agents Chemother., 49*(3), 1067-1075.

Luo, N., Sahin, O., Lin, J., Michel, L.O. & Zhang, Q. (2003). *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother., 47*(1), 390-394.

Maillard, J.Y. (2002). Bacterial target sites for biocide action. *J. Appl. Microbiol., 92*(Suppl.S1), 16S-27S.

Mavri, A. & Smole Možina, S. (2013). Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol., 160*(3), 304-312.

McDonnell, G. & Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev., 12*(1), 147-179.

Meyer, B. (2006). Does microbial resistance to biocides create a hazard to food hygiene? *Int. J. Food Microbiol., 112*(3), 275-279.

Murphy, C., Carroll, C. & Jordan, K.N. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol., 100*(4), 623-632.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem., 250*(10), 4007-4021.

Ortega Morente, E., Fernández-Fuentes, M.A., Grande Burgos, M.J., Abriouel, H., Pérez Pulido, R. & Gálvez, A. (2013). Biocide tolerance in bacteria. *Int. J. Food Microbiol., 162*(1), 13-25.

Pajaniappan, M., Hall, J.E., Cawthraw, S.A., Newell, D.G., Gaynor, E.C., Fields, J.A., Rathbun, K.M., Agee, W.A., Burns, C.M., Hall, S.J., Kelly, D.J. & Thompson, S.A. (2008). A temperature-regulated *Campylobacter jejuni* gluconate dehydrogenase is involved in respiration-dependent energy conservation and chicken colonization. *Mol. Microbiol., 68*(2), 474-491.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead, S. et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature, 403*(6770), 665-668.

Peyrat, M.B., Soumet, C., Maris, P. and Sanders, P. (2008). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol., 124*(2), 188-194.

Piddock, L.J., Ricci, V., Pumbwe, L., Everett, M.J. & Griggs, D.J. (2003). Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J. Antimicrob. Chemother., 51*(1), 19-26.

Pinto, A.F., Todorovic, S., Hildebrandt, P., Yamazaki, M., Amano, F., Igimi, S., Romão, C.V. & Teixeira, M. (2011). Desulforubrerythrin from *Campylobacter jejuni*, a novel multidomain protein. *J. Biol. Inorg. Chem., 16*(3), 501-510.

Poole, K. (2012). Bacterial stress responses as determinants of antimicrobial resistance. *J. Antimicrob.* Chemother., 67(9), 2069-2089.

Regula, J.T., Ueberle, B., Boguth, G., Görg, A., Schnölzer, M., Herrmann, R. & Frank, R., (2000). Towards a two-dimensional proteome map of *Mycoplasma pneumoniae. Electrophoresis*, *21*(17), 3765-3780.

Trachoo, N. & Frank, J.F. (2002). Effectiveness of chemical sanitizers against *Campylobacter jejuni*-containing biofilms. *J. Food Prot., 65*(7), 1117-1121.

Van Hoek, A.H., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P. & Aarts, H.J. (2011). Acquired antibiotic resistance genes: an overview. *Front. Microbiol., 28*(2), 203.

Varsaki, A., Murphy, C., Barczynska, A., Jordan, K. & Carroll, C. (2015). The acid adaptive tolerance response in *Campylobacter jejuni* induces a global response, as suggested by proteomics and microarrays. *Microb. Biotechnol.,* doi: 10.1111/1751-7915.12302

Wang, P., LV, C. Zhu, G. (2015). Novel Type II and Monomeric NAD+ Specific Isocitrate Dehydrogenases: Phylogenetic Affinity, Enzymatic Characterization, and Evolutionary Implication. *Sci. Rep.*, 5.

Wang, X. & Zhao, X. (2009). Contribution of oxidative damage to antimicrobial lethality. *Antimicrob. Agents Chemother., 53*(4), 1395-1402.

Wang, Y., Huang, W.M. & Taylor, D.E. (1993). Cloning and nucleotide sequence of the *Campylobacter jejuni* gyrA gene and characterization of quinolone resistance mutations. *Antimicrob. Agents Chemother., 37*(3), 457-463.

Xia, Q., Muraoka, W.T., Shen, Z., Sahin, O., Wang, H., Wu, Z., Liu, P. & Zhang, Q. (2013). Adaptive mechanisms of *Campylobacter jejuni* to erythromycin treatment. *BMC Microbiol., 13*(1), 133.

Zhang, Q., Sahin, O., McDermott, P.F. and Payot, S. (2006). Fitness of antimicrobial-resistant *Campylobacter* and *Salmonella*. *Microbes Infect., 8*(7), 1972-1978

S4.1 Representative 2D gels

These gels were taken for PDQuest analysis, where the protein expression in a chemostat ciprofloxacin and BKC challenged strains were compared with the protein expression to original parent strain *C. jejuni* NCTC 11168 (see Figure S4.1-S4.2). The position of proteins showing altered expressed under the conditions tested over the course of this study are indicated in Figure 4.1.

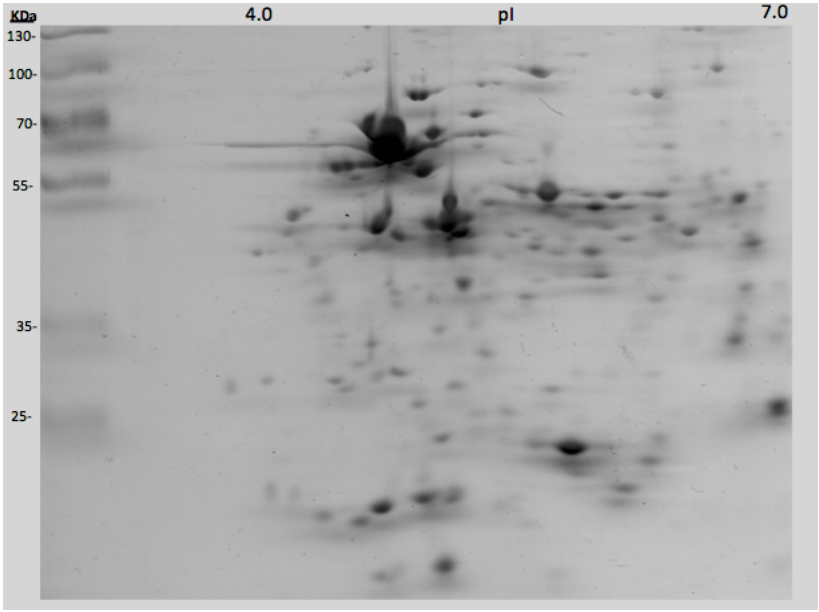

Figure S4.1. Two dimensional acrylamide gel representing protein expression in original *C. jejuni* strain

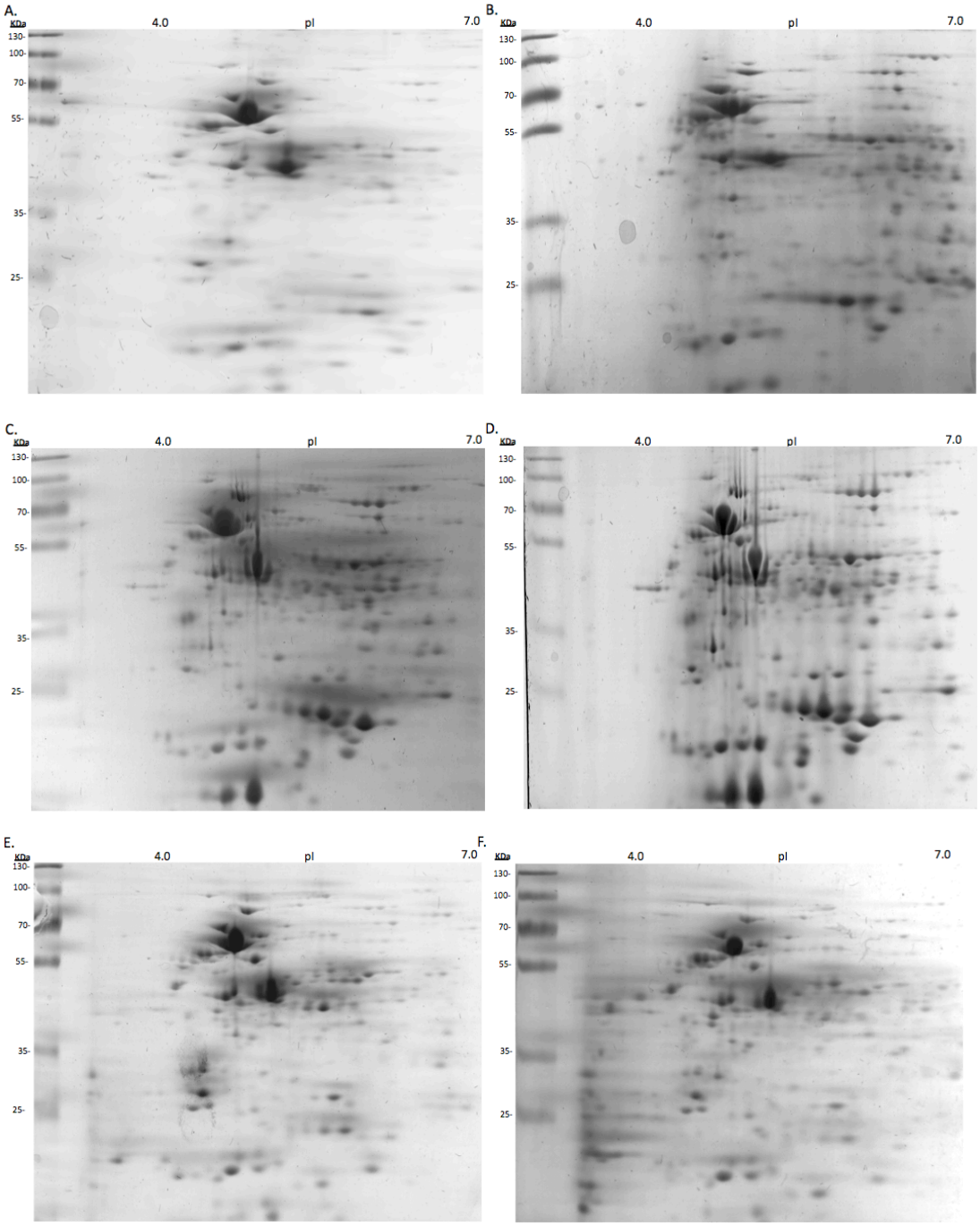

Figure S4.2 Two dimensional acrylamide gels representing protein expression under all conditions tested. A. Original with ciprofloxacin challenge B. Original with BKC challenge. C. BKC-adapted *C. jejuni* without challenge D. BKC-adapted *C. jejuni* variant with BKC challenge. E. Ciprofloxacin-adapted *C. jejuni* variant without challenge. F Ciprofloxacin-adapted *C. jejuni* variant with challenge.

An investigation into the role of target site mutations in the adaptation of *C. jejuni* NCTC 11168 to antibiotic ciprofloxacin.

Olivia Duignan[1], Gerard Fleming[2] and Cyril Carroll[1].

[1]Enteric pathogen Research Laboratory, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland; [2]Microbial Oceanography Research Unit, Microbiology Department, School of Natural Science, National University of Ireland, Galway, Ireland.

5.1 Abstract

The increasing resistance of *Campylobacter* to clinically important antibiotics, such as fluoroquinolones, is a serious public health problem. The quinolone resistance-determining region (QRDR) of gyrA gene encoding the alpha subunit of DNA gyrase in ciprofloxacin-adapted *C. jejuni* variants was sequenced to determine the role of target site mutations in the development of ciprofloxacin resistance in *C. jejuni.* Different levels of resistance to ciprofloxacin were found in strains with the same mutation. A single mutation in the GyrA subunit either Asp-90-Asn or Thr-86-Ile mutation was associated with moderate resistance to ciprofloxacin (between 4 mg l^{-1} and 32 mg l^{-1}). Double mutations (Thr-86-Ile and Asp-90-Asn) were associated with high level ciprofloxacin-resistant (MIC > 32 mg l^{-1}) in *Campylobacter.* No mutation was found in ciprofloxacin adapted variants with MIC > 4 mg l^{-1}. Mutations were not found in BKC-adapted *C. jejuni* variants exhibited low-level cross-resistance to ciprofloxacin suggesting that other mechanisms were involved in the low level cross-resistance to ciprofloxacin observed in these strains. Target site mutations are important in conferring high-level resistance to ciprofloxacin in *C. jejuni*. The acquisition of a single point mutation in gyrA (either Thr-86-Ile or Asp-90-Asn) was associated with a rapid increase in resistance to ciprofloxacin chemostat populations of *C. jejuni* NCTC 11168 undergoing adaptation to this antibiotic.

Keywords: *Campylobacter*, Ciprofloxacin, Benzalkonium chloride, cross-resistance target site mutations.

5.2 Introduction

Campylobacter jejuni is a major food-borne pathogen and is one of the leading causes of bacterial gastroenteritis worldwide. Eating undercooked chicken, cross-contamination of ready-to-eat foods from raw chicken, is the most common source of the infection (20-30%) (EFSA, 2010, Kennedy *et al.*, 2011; Luber, 2009; Nauta & Havelaar 2008; Silva & Teixeira, 2015). While the majority of cases are self-limiting and do not require therapeutic intervention, severe cases are normally treated with the antibiotics erythromycin or ciprofloxacin (Skirrow & Blaser, 2000; Wieczorek & Osek, 2013). The dramatic rise in the prevalence of fluoroquinolone resistance amongst *Campylobacter* isolates in recent years is of concern (EFSA/ECDC, 2014; Ge *et al.,* 2013; Silva & Teixeira, 2015). This may have consequences on the successful treatment outcomes of infection in humans with these antibiotics (EFSA/ECDC, 2014).

Fluoroquinolone antibiotics belong to a family of broad-spectrum antibiotics (Changkwanyeun *et al.*, 2016; Luangtongkum *et al.*, 2009). Fluoroquinolones inhibit DNA synthesis in bacteria by targeting the topoisomerase enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV, which help control levels of DNA under- and over-winding in addition to removing knots and tangles from bacterial chromosomal DNA (Aldred *et al.,* 2014a; Hooper, 2001). There is growing evidence that DNA gyrase is the sole target for fluoroquinolone antibiotics in *Campylobacters* (Luo *et al.,* 2003; Parkhill *et al.,* 2000; Payot *et al.,* 2006; Piddock *et al.,* 2003) whilst the secondary target of topoisomerase IV appears to be absent in *Campylobacter*. Chromosomally mediated changes caused by point mutations in the quinolone resistance-determining region (QRDR) of gyrA gene of DNA gyrase is one of the main mechanisms by which *Campylobacter* develops resistance to fluoroquinolones (Changkwanyeun *et al.*, 2016; Gibreel & Taylor, 2006).

Biocides are frequently employed to control transmission of this food borne pathogen and when used properly, biocide use in domestic environments could be helpful in reducing the public health and food poisoning risks associated with kitchen cross-contamination with *Campylobacter spp*. (Humphrey et al., 2001; Azevedo et al., 2014). Quaternary ammonium compound (QAC)-based disinfectants are widely used

in the food industry due to their excellent non-tainting, non-toxic and non-corrosive (to human skin as well as hard surfaces) nature (Holah et al., 2002; Langsrud *et al.*, 2003). Reduced susceptibility to biocides is associated with cross-resistance to antibiotics particularly the membrane active QAC benzalkonium chloride (BKC) (Braoudaki & Hilton, 2004; Christensen *et al.*, 2011; Randall *et al.,* 2003). The aim of this study was to investigate the role of target site mutations in the development of *C. jejuni* to the fluoroquinolone ciprofloxacin and determine their role in mediating cross-resistance to ciprofloxacin in BKC-adapted *C. jejuni* variants.

5.3 Materials and methods

5.3.1 Strains and Antimicrobial Agents

Campylobacter jejuni NCTC 11168 (*C. jejuni*) was original strain used in this study and was obtained from Dr. Majella Mahar, the National Diagnositic Center, National University of Ireland, Galway. All other strains were derivatives of *C. jejuni* and were generated by means of enrichment cultures with ciprofloxacin and BKC described previously in section 2.3.5. (Chapter 2). A 100 μl aliquot of stock from a chemostat culture sample, taken at various time intervals during and after removal of selection pressure from chemostat culture, was spread-plated on campylobacter blood-free agar base (CCDA base; CM0739, Oxoid Ltd., UK) and incubated at 42 ^{o}C for 48 h under microaerobic conditions using an anerobic gas generating kit (BR0038B, Oxoid Ltd., UK). Individual colonies, chosen at random, were isolated for further analysis. Strains were cultured on CCDA base from which stocks of isolates (50 % (v/v) glycerol and trypic soya broth; TSB, 214889, Becton Dickinson, Sparks, MD) were made. Stocks were then maintained in cryovials at -20 ^{o}C for short-term storage up to 1 month and at -70 ^{o}C for long-term storage. Prior to analysis, stocks of adapted variants were resuscitated on CCDA base at 42 ^{o}C for 48 h under microaerobic conditions. Antimicrobials used in this study included Ciprofloxacin (Ciproxin®, supplied as a 2 mg ml^{-1} solution, Bayer, UK), and BKC (Sigma-Aldrich, Steinheim, Germany). BKC was diluted to appropriate working concentrations in sterile deionized water and filter-sterilized through a 0.2 μm Millipore^{TM} membrane filter before use.

5.3.2 Antimicrobial Susceptibility Testing

MIC determinations were carried out in duplicate on Muller Hinton agar (MHA; Lab039, LabM, UK) using 2-fold serial dilutions of antimicrobial agent on MHA plates (supplemented with 5 % lysed horse blood, (Charles River Laboratories, Ireland) as described previously (section 2.3.3, chapter 2).

5.3.3.1 Isolation of chromosomal DNA from *C. jejuni*

Ciprofloxacin-adapted and BKC-adapted variants of *C. jejuni* NCTC 11168 were used to innoculate 28 ml of MH broth and cultures were incubated at 42 ^{o}C for 18 h. Cells

were harvested by centrifugation at 3,500 g for 15 min (Megafuge 1.0, Heraeus, Sepatech) and pellets were resuspended in 1 ml PBS. The cell suspension was transferred into 1.5 ml sterile eppendorfs and centrifuged at 15,600 g for 5 min (Sigma, Harz, Germany). The supernatant was decanted and pellets were washed in 1 ml SET buffer (100 mM NaCl, 15 mM EDTA, 10 mM Tris-HCl; pH 8) and centrifuged again at 13,000 rpm for 5 min. The supernatant was removed and pellets were resuspended in 570 µl SET buffer with subsequent addition of 30 µl SDS (10 % stock in SET buffer) and 3 µl proteinase K (20 mg ml^{-1} stock in milipure water) to give final concentrations of 0.5 % SDS and 100 µg ml^{-1} proteinase K respectively. The tubes were vortexed briefly and then incubated overnight at 50 ^{o}C to lyse cells. Following incubation, protein and other cellular debris was removed by addition of 600 µl of a solvent mixture of phenol: chloroform: isoamylalcohol (25:24:1). Eppendorf tubes were vortexed (yellowline TTS 2, USA) for 5-10 seconds. The aqueous and organic phases were then separated by centrifugation at 15,600 g for 8 min. Aqueous phase (500-500 µl) was transferred in a fresh 1.5 ml eppendorf tube. An aliquot of 550 µl chloroform (at 4^{o}C) was added to the supernatant and the eppendorf was vortexed prior to centrifugation at 15,600 g for 8 min. Approximately 500 µl of the supernatant (upper aqueous phase containing nucleic acid) was transferred into a 1.5 ml fresh sterile eppendorf tube. DNA was precipitated by adding 50 µl of 3 M sodium acetate (pH 5.3; at 4 ^{o}C) and 1 ml absolute ethanol (at 4^{o}C). The tubes were placed at -20^{o}C overnight to allow the DNA to precipitate. The DNA was pelleted by centrifugation at 15,600 g for 10 min. The supernatant was discarded and the pellet was washed once in 70 % ethanol and the centrifugation step was repeated. The supernatant was removed and the tubes were left facedown on tissue paper for 1 hour to allow excess ethanol to drain and to dry the DNA pellets. The pellet was resupended in 100 µl milipure water. RNA was removed by incubating the samples with 3 µl of RNase (10 mg ml^{-1}) for 1 h at 37 ^{o}C. The DNA was allowed to solvate by placing the tubes at 4^{o}C overnight. The quantity of DNA present in the samples was determined by running 10 µl of DNA and 10 µl of λ DNA-Hind III molecular weight standard II (Roche) on 0.8 % agarose gel electrophoresis containing 0.5 µg ml^{-1} ethidium bromide using a tris-borate-EDTA buffer (0.44 M Tris-HCl, 0.44 M broic acid, 0.01 M EDTA) at 80 V constant for 1 h. Gels were imaged using ultraviolet light (G:box, Syngene).

5.3.3.2 Sequencing the quinolone resistance-determining region of gyrA

PCR reaction was setup to amplify the quinolone resistance-determining region of gyrA subunit of DNA gyrase spanning all published mutations in this region from codon 11 to codon 157 and was a modified version of the one used by Zirnstein et al. (1999). Reactions were carried out in 0.5 ml sterile eppendorf tubes with a final reaction volume of 100 µl. PCR reactions contained 2 µl of chromosomal DNA (~ 75 ng DNA template), 10 mM Tris-HCl (pH 8.3), 1X reaction buffer, a 200 µM concentration of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 30 pmol primers (see table 5.2) and nuclease-free water (Sigma, U.K). A PCR negative control containing PCR reaction mixture without template DNA was included in each PCR reaction. The PCR reactions were carried out in a Mastercycler gradient (Eppendorf, Germany).

Primer	Sequence 5' to 3'	Source
QRDRgyrAF	TAG AAG GTA AAA CAT CAG GT	This study[1.]
QRDRgyrAR	TTT TTA GCA AAG ATT CTG AT	Zirnstein *et al.,* 1999

Table 5.1 Primers used in this study for amplification of the quinolone resistance-determining region of gyrA. Note:- 1. Primers designed with specificity toward the genome of *Campylobacter jejuni subsp. jejuni* NCTC 11168 strain using Primer-BLAST tool available at www.ncbi.nlm.gov.

Figure 5.1 Primer annealing sites on gyrA gene of *C. jejuni* NCTC 11168 visualized using the SnapGene Viewer software available at www.snapgene.com. Note:- Primers were designed with specificity toward the genome of *Campylobacter jejuni subsp. jejuni* NCTC 11168 strain using Primer-BLAST tool available at www.ncbi.nlm.gov.

The PCR cycling conditions used with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Ten- microliter aliquots of each of the PCR products were run on 0.8 % agarose gel electrophoresis containing 0.5 µg ml^{-1} ethidium bromide using a tris-borate-EDTA buffer (0.44 M Tris-HCl, 0.44 M broic acid, 0.01 M EDTA) at 80 V constant for 1 h. A DNA molecular weight standard XIV (Roche) was included on each gel. Gels were imaged using ultraviolet light (G:box, Syngene) and their images were captured using Genesnap software (Syngene). PCR products to be subjected to sequencing were purified with a QIAquick PCR Purification Kit (QIAGEN Ltd, West Sussex, UK), according to the manufacturer's instructions. The PCR products were sequenced by GATC Biotech (Konstanz, Germany). All sequenced PCR products were analysed using SnapGene Viewer software (available at www.snapgene.com).

5.4 Results

5.4.1 Mutations in gyrA conferring resistance to ciprofloxacin

The QRDR of gyrA was sequenced, Table 5.2. Isolates were derived from chemostat samples taken during selection to ciprofloxacin (CR3; n=17) and after selection pressure was removed (CR2, n=10; CR3, n=11). The QRDR of gyrA was also sequenced from isolates (n=5) derived from samples taken from CR5, which underwent adaptation to BKC.

Chemostat[1]	gyrA sequence mutations	MIC_{Agar} values	No. and Percentage of isolates[2]
CR2[a]	Thr-86-Ile	32 mg l^{-1} ciprofloxacin	n=10; 23.3%
CR3[b]	None	< 2 mg l^{-1} ciprofloxacin	n=5; 11.6%
	Thr-86-Ile	4-32 mg l^{-1} ciprofloxacin	n=4; 9.3%
	Asp-90-Asn	4 mg l^{-1} ciprofloxacin	n=1; 2.3%
	Thr-86-Ile, Asp-90-Asn	> 32 mg l^{-1} ciprofloxacin	n=18; 41.9%
CR5[c]	None	0.25 mg l^{-1} ciprofloxacin	n=5; 11.6%

Table 5.2 Mutations detected in the QRDR of gyrA gene and corresponding ciprofloxacin MIC_{Agar} values of ciprofloxacin-adapted and BKC-adapted variants. 1. Ciprofloxacin-adapted variants were isolated from two independent chemostats operated using ciprofloxacin as a selective agent (denoted as CR2 and CR3; chapter 2). a. Ciprofloxacin-adapted variants sequenced from CR2 (n=10) were derived from samples taken 400 h and 500 h after removal of selective pressure. b. Ciprofloxacin-adapted variants sequenced from CR3 were taken from samples while the chemostat was undergoing selection (n=17) and 700 h and 850 h after selection pressure was removed (n=11). 1, c. BKC-adapted variants were isolated from one chemostat population using BKC as a selective agent (CR5; chapter 2) strains sequenced were taken 350 h and 650 h after selection pressure was removed. 2. Expressed as a percentage of the total number of isolates sequenced.

Chemostat derived isolates from CR3 that had MIC_{Agar} values less then 4 mg l^{-1} ciprofloxacin did not contain any mutation in the QRDR region of gyrA gene. In CR3 isolates with MIC_{Agar} values between 4 mg l^{-1} and 32 mg l^{-1} ciprofloxacin a single mutation (Thr-86-Ile or Asp-90-Asn) was observed. Isolates from CR3 with MIC_{Agar} values greater than 32 mg l^{-1} ciprofloxacin, carried a double mutation (Thr-86-Ile and Asp-90-Asn) regardless of whether they were derived from samples taken during

selection or after selection pressure was removed. Isolates derived from samples taken from CR2 at 400h and 500h after the ciprofloxacin selection pressure was removed carried a single (Thr-86-Ile) mutation in the QRDR of gyrA and all had MIC_{Agar} values of 32 mg l^{-1} ciprofloxacin. Isolates derived from samples taken from CR5 which was not adapted to ciprofloxacin but to the biocide BKC taken at 350h and 650h after selection pressure was removed did not have a mutation in the QRDR of gyrA and all had MIC_{Agar} values of 0.25 mg l^{-1} ciprofloxacin.

4.5 Discussion

Chromosomal mutations in the molecular target of fluoroquinolone, DNA gyrase, are the main mechanism by which *Campylobacter* develops resistance to fluoroquinolones (Gibreel & Taylor, 2006). These point mutations are clustered in the quinolone resistance-determining region (QRDR) of gyrA subunit of DNA gyrase (Yan *et al.,* 2006). DNA sequencing of the QRDR of gyrA subunit of DNA gyrase gene is sufficient for detecting such mutations (Gibreel & Taylor, 2006). The quinolone resistance-determining region (QRDR) of the gene encoding the alpha subunit of gyrA was sequenced to elucidate the role mutation in conferring ciprofloxacin resistance in ciprofloxacin-adapted *C. jejuni* variants.

A number of different mutations in the QRDR have been associated with resistant strains and different gyrA mutations confer different levels of resistance to fluoroquinolone antibiotics (Yan *et al.,* 2006). In this study, PCR products of the QRDR of gyrA gene of isolates spanning from codon 11 to codon 157 and containing all published mutations in this region reported by Payot *et al.,* (2006) and Piddock *et al.,* (2003) was sequenced (Table 5.2). The mutations found here leading to ciprofloxacin adaptation in *C. jejuni* are the same as described previously (Alfredson & Korolik, 2007; Luo *et al.,* 2003; Payot *et al.,* 2006; Piddock *et al.,* 2003). The Thr-86-Ile mutation was the most common point mutation present in ciprofloxacin-adapted *C. jejuni* variants. The threonine residue, at position 86 in the QRDR of gyrA subunit of DNA gyrase (equivalent to the serine residue at position 83 in *E. coli*), together with acidic residues located four amino acids downstream, are important in mediating fluoroquinolone-enzyme interactions (Aldred *et al.,* 2014a; 2014b; Piddock *et al.,* 2003). Partial disruption of the interactions between antibiotic and enzyme, resulting from mutation of the serine or acidic residue, significantly decreased the affinity of the DNA gyrase to clinically relevant quinolones (Aldred *et al.,* 2014b). This mutation was found in adapted variants with MIC_{Agar} values ranging between 4 mg l^{-1} and 32 mg l^{-1} ciprofloxacin. A single isolate was found to carry the Asp-90-Asn mutation and was also associated with moderate resistance (4 mg l^{-1} ciprofloxacin). MIC_{Agar} values were slightly lower then those reported Piddock *et al.,* (2003) in isolates with the same mutation. In addition, the acquisition of a single point mutation in gyrA in the chemostat population (either Thr-86-Ile or Asp-90-Asn) was associated

with a rapid increase in chemostat population MIC_{Agar} (Chapter 2). Furthermore a double mutation (Thr-86-Ile and Asp-90-Asn) was observed in ciprofloxacin-adapted variants with MIC_{Agar} values greater than 32 mg l^{-1} ciprofloxacin. Double mutations of gyrA combining Thr-86-Ile and Asp-90-Asn have been reported to lead to high levels of resistance to floroquinolones (Alfredson & Korolik, 2007; Luo *et al.,* 2003; Payot *et al.,* 2006). The Thr-86-Ile mutation has previously been associated with high-level resistance (MIC_{Agar} values ≥16 mg l^{-1} ciprofloxacin; Payot *et al.*, 2006; Piddock *et al.*, 2003). However differences level of ciprofloxacin resistance in isolates with the same mutation in the gyrA gene may be due to the presence of other resistance mechanisms (Corcoran *et al.*, 2005; Payot *et al.*, 2002).

Resistance-associated gyrA mutations occur spontaneously at a relatively high frequency in *Campylobacter* and fluoroquinolone treatment rapidly selects for pre existing FQ-resistant mutants in bacterial populations containing greater then 10^6 to 10^9 colony-forming units (CFU) (Luangtongkum *et al.,* 2009; Wassenaar *et al.,* 2011). Overexpression of the *Campylobacter* multidrug efflux pump CmeABC has been shown to increase the frequency of emergence of fluoroquinolone resistant *C. jejuni* by 17-fold (Yan *et al.,* 2006). Emergence of mutations conferring fluoroquinolone resistance in *C. jejuni* has also been associated with Mfd (Mutant Frequency Decline), a transcription-repair coupling factor involved in strand-specific DNA repair. Mfd has also been shown to be up-regulated in *C. jejuni* following treatment with ciprofloxacin (Han *et al.,* 2008).

The selective pressure exerted by biocides has been shown to activate the expression of genes in antibiotic multidrug resistance leading to low-level cross-resistance to antibiotics in bacteria (Braoudaki & Hilton, 2004; Karatzas *et al.*, 2007; Moken *et al.*, 1997, SCENIHR, 2009). In this study, BKC-adapted *C. jejuni* variants exhibited low-level cross-resistance to ciprofloxacin. The role of gyrA mutations in mediating this resistance was assessed. No mutation was found in the QRDR of gyrA of BKC-adapted variants from chemostats population adapted to BKC in this study suggesting that other mechanisms were involved in the low level cross-resistance to ciprofloxacin observed in these strains. The acquisition of a single point mutation in gyrA (either Thr-86-Ile or Asp-90-Asn) was associated with a rapid increase in resistance to ciprofloxacin chemostat population of *C. jejuni* NCTC 11168.

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5.6 Bibliography

Aldred, K.J., Kerns, R.J. & Osheroff, N. (2014a). Mechanism of quinolone action and resistance. *Biochemistry, 53*(10), 1565-1574.

Aldred, K.J., Breland, E.J., Vlčková, V., Strub, M.P., Neuman, K.C., Kerns, R.J. & Osheroff, N. (2014b). Role of the water-metal ion bridge in mediating interactions between quinolones and *Escherichia coli* topoisomerase IV. *Biochemistry, 53*(2), 5558-5567.

Alfredson, D.A. & Korolik, V. (2007). Antibiotic resistance and resistance mechanisms in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol. Lett., 277*(2),123-132.

Azevedo, I., Albano, H., Silva, J. & Teixeira, P., (2014). Food safety in the domestic environment. *Food Control*, *37*, 272-276.

Braoudaki, M. & Hilton, A.C., (2004). Adaptive resistance to biocides in *Salmonella* enterica and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. *J. Clin. Microbiol., 42*(1), 73-78.

Christensen, E. G., Gram, L., & Kastbjerg, V. G. (2011). Sublethal Triclosan Exposure Decreases Susceptibility to Gentamicin and Other Aminoglycosides in *Listeria monocytogenes*. *Antimicrob. Agents Chemother., 55*(9), 4064-4071.

Changkwanyeun, R., Yamaguchi, T., Kongsoi, S., Changkaew, K., Yokoyama, K., Kim, H., Suthienkul, O., Usui, M., Tamura, Y., Nakajima, C. & Suzuki, Y., (2016). Impact of mutations in DNA gyrase genes on quinolone resistance in *Campylobacter jejuni*. *Drug Test Anal.* 2016.

Corcoran, D., Quinn, T., Cotter, L. & Fanning, S., (2005). Relative contribution of target gene mutation and efflux to varying quinolone resistance in Irish *Campylobacter* isolates. *FEMS Microbiol. Lett., 253*(1), 39-46.

EFSA. (2010). Scientific Opinion on Quantification of the risk posed by broiler meat to human Campylobacteriosis in the EU. *EFSA Journal, 8*(1):1437.

EFSA/ECDC (2014). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal, 12*(3), 3590.

Ge, B., McDermott, P.F., White, D.G. & Meng, J. (2005). Role of Efflux Pumps and Topoisomerase Mutations in Fluoroquinolone Resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Antimicrob. Agents Chemother., 49*(8), 3347-3354.

Ge, B., Wang, F., Sjölund-Karlsson, M. & McDermott, P.F. (2013). Antimicrobial resistance in *Campylobacter*: susceptibility testing methods and resistance trends. *J. Microbiol. Methods, 95*(1), 57-67.

Gibreel, A. & Taylor, D.E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 58*(2), 243-255.

Han, J., Sahin, O., Barton, Y.W. & Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog., 4*(6), e1000083.

Holah, J., Taylor, J., Dawson, D., & Hall, K. (2002). Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *J. Appl. Microbiol., 92*(s1), 111S-120S.

Hooper, D.C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infect. Dis., 7*(7), 337-341.

Humphrey, T.J., Martin, K.W., Slader, J. & Durham, K., (2001). *Campylobacter spp.* in the kitchen: spread and persistence. *J. Appl. Microbiol.*,90(S6).

Karatzas, K. A., Webber, M. A., Jorgensen, F., Woodward, M. J., Piddock, L. J., & Humphrey, T. J. (2007). Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance,

increased efflux and reduced invasiveness. *J. Antimicrob. Chemother., 60*(5), 947-955.

Kennedy, J., Nolan, A., Gibney, S., O'Brien, S., McMahon, M.A.S., McKenzie, K., Healy, B., McDowell, D., Fanning, S. & Wall, P.G., (2011). Determinants of cross-contamination during home food preparation. *British Food Journal, 113*(2), 280-297.

Langsrud, S., Sundheim, G., & Holck, A. (2004). Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. *J. Appl. Microbiol., 96*(1), 201-208.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. & Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol, 4*(2), 189-200.

Luber, P. (2009). Cross-contamination versus undercooking of poultry meat or eggs— which risks need to be managed first? *Int. J. Food Microbiol., 134*(1), 21-28.

Luo, N., Sahin, O., Lin, J., Michel, L.O. & Zhang, Q. (2003). *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with gyrA mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother., 47*(1), 390-394.

Moken, M.C., McMurry, L.M. & Levy, S.B., (1997). Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and acrAB loci. *Antimicrob. Agents Chemother., 41*(12), 2770-2772.

Nauta, M. J., & Havelaar, A. H. (2008). Risk-based standards for *Campylobacter* in the broiler meat chain. *Food Control, 19*(4), 372-381.

Payot, S., Bolla, J.-M., Corcoran, D., Fanning, S., Mégraud, F., & Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in *Campylobacter spp*. *Microbes Infect., 8*(7), 1967-1971.

Payot, S., Cloeckaert, A. & Chaslus-Dancla, E., (2002). Selection and characterization of fluoroquinolone-resistant mutants of *Campylobacter jejuni* using enrofloxacin. *Microb. Drug Resist., 8*(4), pp.335-343.

Piddock, L.J., Ricci, V., Pumbwe, L., Everett, M.J. & Griggs, D.J. (2003). Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J. Antimicrob. Chemother.*, 51(1), 19-26.

Randall, L., Ridley, A., Cooles, S., Sharma, M., Sayers, A., Pumbwe, L., & Woodward, M. J. (2003). Prevalence of multiple antibiotic resistance in 443 *Campylobacter spp.* isolated from humans and animals. *J. Antimicrob. Chemother., 52*(3), 507-510.

SCENIHR. (2009). *Assessment of the Antibiotic Resistance Effects of Biocides*. Scientific Committee on Emerging and Newly Identified Health Risks. Brussels, Belgium: Retrieved from http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf

Skirrow, M., & Blaser, M. (2000). Clinical aspects of *Campylobacter* infection. In I. Nachamkin & M. J. Blaser (Eds.), *Campylobacter* (pp. 68-88): ASM Press.

Silva, J. & Teixeira, P., (2015). Tackling *Campylobacter*: a Review. *Am. J. Adv. Food Sci. Technol., 3*(2), 107-124.

Wassenaar, T.M. (2011). Following an imaginary *Campylobacter* population from farm to fork and beyond: a bacterial perspective. *Lett. Appl. Microbiol.*, 53(3), 253-263.

Wieczorek, K., & Osek, J. (2013). Antimicrobial resistance mechanisms among *Campylobacter*. *Biomed Res. Int.,* 2013.

Yan, M., Sahin, O., Lin, J. & Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J. Antimicrob. Chemother.*, 58(6), 1154-1159.

Zirnstein, G., Li, Y., Swaminathan, B., & Angulo, F., (1999). Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of gyrA resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. *J. Clin. Microbiol. 37*, 3276–3280.

6.1 General discussion

Campylobacter jejuni (*C. jejuni*) is a zoonotic pathogen of major public health importance and is the leading cause of acute bacterial gastroenteritis worldwide. Most cases of *Campylobacter* enteritis in humans are caused by *C. jejuni* (c. 90%) and by *C. coli* (10%) (Bolton, 2015; Bronowski *et al.,* 2014; Wagenaar *et al.,* 2013). Typically symptoms of infection include bloody diarrhoea, cramping, abdominal pain and fever and may be accompanied by nausea and vomiting (Moore *et al.,* 2005). While most cases are self-limiting and do not require therapeutic intervention, antimicrobial therapy is indicated in persistent or complicated cases and in those affecting immunocompromised patients (Engberg *et al.,* 2001; Ge *et al.,* 2013; Gibreel & Taylor, 2006). Macrolides (primarily erythromycin, or alternatively one of the newer macrolides, such as clarithromycin or azithromycin) remain the frontline agents for treating laboratory confirmed *Campylobacter* infections. Fluoroquinolones (FQ, e.g., ciprofloxacin) are also commonly used because they are the drugs of choice for empirical treatment of diarrhoeal illness, such as travelers' diarrhoea when a microbiological diagnosis is absent (Engberg *et al.,* 2001; Ge *et al.,* 2013; Gibreel & Taylor, 2006). Therefore, antimicrobial resistance in *Campylobacter* is a challenge for food safety and public health. Owing to the high prevalence of FQ resistance in *Campylobacter*, FQ antimicrobials are losing effectiveness in the clinical treatment of *Campylobacter* enteritis (Bolton, 2015; Mavri *et al.,* 2012). This is a worrisome development as FQ antibiotics are among the few antibiotic classes used to treat *Campylobacter* (Luangtongkum *et al.,* 2009).

Campylobacter are fastidious microaerophiles and exhibit poor survival outside their animal host, however some *C. jejuni* strains appear to be more able to survive and persist in environmental niches than others (Bronowski *et al.,* 2014; Murphy *et al.,* 2006; Park, 2002). The main routes of transmission of *Campylobacter* to humans are by (i) consumption of contaminated food, particularly undercooked chicken, (ii) direct contact with animals/pets, (iii) recreational swimming or (iv) drinking water from streams or other natural water sources (Jacobs-Reitsma *et al.,* 2008; Wagenaar *et al.,* 2006; Wassenaar, 2011). Research has shown that between 20% and 30% of human cases of *Campylobacter* enteritis may be attributed to the handling, preparation or consumption of broiler meat and 50% to 80% may be attributed to the chicken

(broiler) reservoir as a whole (EFSA, 2010). Infected birds can carry extremely high loads of *Campylobacter* in their gastrointestinal tract, particularly in their caeca (up to 10^{10} colony-forming units per gram of infected intestine) (Hermans *et al.,* 2011; Young *et al.,* 2007). Reducing the carriage rates of *Campylobacter* has been correlated with a reduction in the incidence of *Campylobacter* infections in humans (Rosenquist *et al.,* 2003; Messens *et al.,* 2007). Eliminating *Campylobacter* entry on the farm would have the double benefit of preventing the transmission of *Campylobacter* to humans not only via meat but also via other (environmental) pathways (O'Mahony *et al.,* 2011; Newell *et al.,* 2011; Wagenaar *et al.,* 2013).

To date, prevention measures including biosecurity and good hygiene practices (including the use of disinfectant) are currently considered the most economically viable intervention strategies available in controlling the transmission of *Campylobacter* to poultry (Hermans *et al.,* 2011; Heres *et al.,* 2004; Mangen *et al.,* 2005; Newell *et al.,* 2011). These measures however, are not completely effective and the majority of broiler flocks are infected within the third and fourth week of rearing (Bolton, 2015). This is despite the fact that *Campylobacter* are considered sensitive to the action of disinfectants (Peyrat *et al.,* 2008). Plausible reasons that may explain the persistence and survival of *Campylobacter* in the food chain include the ability of *Campylobacter* to form biofilm on different surfaces and/or entry into a viable but nonculturable state (VBNC) making them more resistant to the action of disinfectants (Bolton, 2015; Humphrey *et al.,* 2007; Murphy *et al.,* 2006; Park, 2002). The localization of *Campylobacter* in selected niches on the farm (e.g., under objects or in cracks in wood and masonry) may also prevent direct contact with disinfectant (Cerf, 2010; Karatzas *et al.,* 2007). The development of biocide tolerance in *Campylobacter* may also hinder efforts to control its transmission and promote its persistence in the food-chain (Larsen *et al.,* 2014). A limited amount of information is available regarding biocide tolerance in *Campylobacter* (Peyrat *et al.,* 2008a,b; Mavri *et al.,* 2012; 2013a,b). It has been postulated that the increasing use of chemical disinfection, particularly of quaternary ammonium compounds, might impose a selective pressure and contribute to the emergence of disinfectant-resistant microorganisms (Langsrud *et al.,* 2003) since biocides might also select for strains expressing antibiotic resistance mechanisms. There is an urgent need for new research on this topic (Russell, 2000).

5.2 Aims and objectives

The aim of this undertaking was to study the adaptation of a single strain *C. jejuni* NCTC 11168, here after referred to as *C. jejuni*, to the antibiotic ciprofloxacin and the biocide benzalkonium chloride (BKC). A chemostat was used to culture an initially BKC- and ciprofloxacin-sensitive strain and allow it to evolve and adapt to these agents following prolonged exposure (> 1000 h). Chemostat populations were monitored before, during and after adaptation (OD_{625nm}, total viablity counts, antimicrobial susceptibility) to observe changes in the evolving chemostat population as a result of adaptation (Chapter 2). This thesis set out to investigate adaptation of *Campylobacter* and its proteome and genome response to stress caused by exposure to the antibiotic ciprofloxacin and disinfectant BKC. The objectives of this project were:

(i) To characterize of the development of resistance of *C. jejuni* to ciprofloxacin and BKC in chemostat culture (Chapter 2)
(ii) To characterize phenotypic changes (changes in fitness and stability) following adaptation to ciprofloxacin and BKC (Chapter 3).
(iii) To determine if development of resistance to antibiotics leads to cross-resistance to biocides and *vice versa*, and/or alters the efficacy of a disinfectant (Savlon^{TM} and its active biocidal ingredient BKC) against adapted variants (Chapter 3).
(iv) To investigate if efflux mechanisms were involved in conferred resistance to ciprofloxacin and/or cross-resistance to other antimicrobials in ciprofloxacin resistant variants (Chapter 3)
(v) Finally, to evaluate the mechanisms of resistance to both these antimicrobials at the proteomic and genetic level (Chapter 4 and Chapter 5)

6.3 Ciprofloxacin

Research into the underlying mechanisms of resistance in *C. jejuni* generated in this study confirmed previous findings about the role of DNA mutations in mediating ciprofloxacin resistance (Griggs *et al.,* 2005; Humphrey *et al.,* 2005; Martinez & Lin, 2006). FQ resistance is most commonly associated with mutations in its molecular target of DNA gyrase (Gibreel & Taylor, 2006). Target site mutations conferring resistance to FQs in the gene encoding the GyrA subunit of DNA gyrase are clustered

in the region now referred to as the quinolone resistance determining region (QRDR) of the gene (Yan *et al.,* 2006). Sequencing of the QRDR of the gryA gene in this study revealed that ciprofloxacin-adapted variants derived from this study carried two mutations namely Thr-86-ile and Asp-90 –Asn (Chapter 5). These gyrA mutations were reported previously (Griggs *et al.,* 2005; Piddock *et al.,* 2003; Yan *et al.,* 2006). The acquisition of a single point mutation in gyrA (either Thr-86-Ile or Asp-90-Asn) was associated with a rapid increase in MIC_{Agar} to ciprofloxacin in the chemostat population (16-32-fold increase; Chapter 2). MIC_{Agar} to ciprofloxacin also correlated with the occurrence of particular combinations of mutations in the chemostat population. A single mutation in the gyrA subunit either Asp-90-Asn or Thr-86-Ile mutation was associated with moderate resistance to ciprofloxacin (between 4 mg l^{-1} and 32 mg l^{-1}). Double mutations (Thr-86-Ile and Asp-90-Asn) were observed in ciprofloxacin-adapted isolates in the chemostat population with MIC_{Agar} values greater than 32 mg l^{-1} (Chapter 5). The Thr-86-Ile mutation had previously been reported as being associated with high-level resistance to ciprofloxacin (MIC_{Agar} values ≥16 mg l^{-1} ciprofloxacin; Payot *et al.,* 2006; Piddock *et al.,* 2003), however, in this study the Thr-86-Ile mutation occurred in ciprofloxacin adapted variants with MIC_{Agar} values as low as 4 mg l^{-1} ciprofloxacin. Sub-MIC concentrations of ciprofloxacin were not found to alter the population MIC to ciprofloxacin in chemostat cultures. Differences in the level of ciprofloxacin resistance in isolates with the same mutation in the gyrA gene may be due to the presence of other resistance mechanisms (Corcoran et al., 2005; Payot et al., 2002).

C. jejuni rapidly developed resistance to the FQ antibiotic ciprofloxacin in chemostat culture. Ciprofloxacin-adapted strains had MIC_{Agar} values of 256 mg l^{-1} i.e. up to 2048-fold higher than the original parent strain (Chapter 2). This is in agreement with *in vivo* data, which also confirmed rapid emergence of FQ resistance in *Campylobacter* from commercial poultry flocks following treatment with difloxacin or enrofloxacin (Humphrey *et al.,* 2005). Mutations in the gyrA gene encoding the alpha subunit of DNA gyrase occur spontaneously at a relatively high frequency in *Campylobacter*. Spontaneous mutation frequency (average fraction of mutant bacteria in a few replicate cultures) among quinolone-sensitive *C. jejuni* and *C. coli* strains has previously has been found to range between ~4 x 10^{-9} and ~7 x 10^{-3} (Hänninen & Hanula, 2007; Wang *et al.,* 2001; Yan *et al.,* 2006). In *C. jejuni* NCTC 11168, the

frequency of emergence of gyrA mutations occurs at a rate of ~1 x 10^{-6} (Yan *et al.,* 2006). FQ treatment rapidly selects for pre-existing FQ -resistant mutants in bacterial populations in containing greater then 10^6 to 10^9 colony-forming units (Luangtongkum *et al.,* 2009; Yan *et al.,* 2006). In chemostats operated in this study with ciprofloxacin selection (CR1, CR2 & CR3), population sizes typically ranged between 10^8 and 10^{10} individuals (based on the total viable counts ranging between 10^6 to 10^8 CFU ml^{-1} and culture volumes of 520 ml; Chapter 2).

Resistance to ciprofloxacin was stably maintained both in the chemostat population and ciprofloxacin-adapted variants isolated from culture samples after removal of selective pressure from the media feed (Chapter 2 & Chapter 3). Some instability of resistance was observed in ciprofloxacin-adapted variants derived from culture samples taken from the chemostat undergoing selection. However, it was noteworthy that the susceptibility of all ciprofloxacin-adapted variants did not revert to the level of the original strain after the allotted period of non-selective growth (5 serial subcultures on CCDA base). Ciprofloxacin-adapted variants evolved in the absence of antibiotic selective pressure may have resulted in mutational events favoring a reversion to a lower-level resistance phenotype associated with lesser fitness costs, rather than the acquisition of compensatory mutations that would maintain resistance while ameliorating the fitness burden (O'Regan *et al.,* 2010).

Interestingly, in the present study it was found that the presence of sub-MIC concentrations in chemostat culture did not alter the chemostat population MIC_{Agar} to ciprofloxacin. It was only after the concentration of ciprofloxacin in the chemostat exceeded the ciprofloxacin MIC_{broth} for the strain that the population MIC_{Agar} to ciprofloxacin began to rise. One of the factors affecting the outcome of carrying a mutation on the fitness of strains is the presence of antibiotic in the growth media.

In this study strain mutations were shown to confer a fitness cost (as seen by a reduced growth rate in the absence of antibiotic; Chapter 3), which may also have disadvantaged them initially at sub-MIC concentrations of ciprofloxacin. At a certain of ciprofloxacin, however, termed the minimal selection concentration, *C. jejuni* strains carrying mutations would become growth rate advantaged compared to their sensitive counterparts. The minimal selection concentration (MSC) has been defined

previously as the lowest concentration of the drug that gave the resistant strain a selective advantage when competed against the sensitive phenotype (McCay *et al.,* 2010; O'Reilly & Smith, 1999). In chemostat competition experiments operated by Fleming et al., (2002), exposure to 0.03 mg l^{-1} levofloxacin (close to 50 % of the MIC_{broth} of the sensitive strain) resulted in a levofloxacin resistant *E. coli* 35218 outcompeting its sensitive counterpart. It may be that the MSC for ciprofloxacin-adapted mutants was also close to the MIC_{broth} of the original strain to ciprofloxacin and resulted in a population exhibited a decreased susceptibility to ciprofloxacin.

Mechanisms widely associated with high-level FQ resistance, such as multiple topoisomerase mutations and overexpression of MDR efflux pumps, have been associated with fitness costs (O'Regan *et al*., 2010). Fitness cost is associated with high-level resistance was also observed in this study and might limit the emergence and spread of highly resistant variants in the absence of antibiotic selection. However FQ-resistant *Campylobacters* have been shown to persist on poultry farms up to 4 years after FQ antibiotics usage had ceased (Zhang *et al.,* 2006). Luo *et al*., (2005) examined the effect of gyrA mutation, Thr-86-Ile on *Campylobacter* on persistence and found a positive effect biological fitness *in vivo.* The positive effect the Thr-86-Ile on fitness was not present in all strains examined and the effect may be strain dependant (Luo *et al*., 2005) suggesting multiple mechanisms conferring resistance to ciprofloxacin and affected fitness of these strains were present.

Control in the dissemination of ciprofloxacin resistance strains through the food-chain and preventing the transmission of *Campylobacter* to humans is best achieved by the effective implementation of good hygiene practices. Residual concentrations of disinfectants may occur on food contact surfaces after cleaning and disinfection. Exposure of food pathogens to residual concentrations of biocide has been shown to increase the expression of antimicrobial resistance mechanisms (SCENIHR, 2009). Conversely, antimicrobial resistance mechanisms expressed by ciprofloxacin-adapted variants could also aid their survival when exposed to disinfectants and render disinfectants less efficacious. This was investigated by examining the efficacy (defined as greater than 5 Log_{10} fold reduction in bacterial counts after 15 minutes exposure) of the commercial disinfectant Savlon™ against ciprofloxacin -adapted strains. "In use" (relating to the concentrations prepared according to the

manufactures instructions, 2% v/v was the in use concentration, the concentration recommended by the manufacturer, and half-strength is half that concentration) and 1:2-1:32 strength dilutions of Savlon™ disinfectant liquid containing BKC (1.2-1.4% w/w) were tested. It was found that some (4/8) ciprofloxacin-adapted variants showed enhanced survival at more dilute concentrations (1:16, 1:32) of Savlon™ when compared to the original strain. However, "in use" dilutions of Savlon™, half strength, quarter strength and one eight-strength dilutions were found to be efficacious with no survivors detected after 15 minutes exposure. These results suggest that that development of ciprofloxacin resistance in *Campylobacter* might augment the risk for disinfectant failure if disinfectants are not used properly.

Cross-resistance was also evaluated in ciprofloxacin-adapted variants of *C. jejuni* to a range of antimicrobial agents including erythromycin, cefotaxime, ampicillin, BKC, TCP, Savlon™ and Dettol® (Chapter 3). Ciprofloxacin-adapted variants exhibited strain-specific cross-resistance profiles to the range of antimicrobial agents tested with a two to four fold increase in MIC_{Agar} recorded to three or more of the antimicrobial agents. The role of efflux pumps in ciprofloxacin resistance and cross-resistance to other antimicrobials was examined (Chapter 3). Phe-Arg β-naphthylamide dihydrochloride (PAβN) is a competitive inhibitor of the resistance nodulation division (RND) family efflux pumps. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is known to act on efflux systems that use the proton motive force (pmf), such as the RND type efflux pump. PAβN and CCCP are considered efflux pump inhibitors (EPIs) that block different types of efflux pump systems. The same ciprofloxacin adapted variants used to evaluate cross-resistance were used in evaluating the role of efflux in ciprofloxacin resistance and cross-resistance to other antimicrobials. CCCP and PAβN, were unable to completely reverse cross-resistance to antibiotics and to the biocides BKC and TCP in the same variants. EPIs had differed in their ability to restore susceptibility to the commercial disinfectants Savlon™ and Dettol®. CCCP but not PAβN was able to restore sensitivity in 67 % (6/9) of ciprofloxacin-adapted variants showing cross-resistance to the commercial disinfectant Savlon™. CCCP was able to restore sensitivity in all (5/5) and PAβN was able to restore sensitivity in most (4/5) of the ciprofloxacin-adapted variants showing 2- fold increases in resistance to Dettol®. In two adapted

variants Dettol® sensitivity was increased a further 2-fold in the presence of an EPI. With regard to developed ciprofloxacin resistance, EPI CCCP was found to reduce resistance (2-fold increase in susceptibility) in four (4/10) ciprofloxacin-adapted variants while the presence of PAβN partially reversed resistance (2-fold increase in susceptibility) in one (1/10) ciprofloxacin- adapted variant. The ability of EPIs to reverse resistance in adapted strains may have been as a result of heterogeneous expression of efflux pumps in adapted strains.

Proteomic changes, induced during adaptation of *C. jejuni* to ciprofloxacin, was examined using two-dimensional gel electrophoresis. Changes in expression of levels of proteins involved in protection against oxidative stress and general stress response, metabolism, and protein synthesis were found (Chapter 4). Antimicrobials are growth-inhibiting stressors that often elicit protective responses in bacteria (Poole, 2012). One of the proposed mechanisms of action of ciprofloxacin, besides its inhibitory effect on DNA gyrase, involves the generation of reactive oxygen species (ROS) (Dwyer *et al.,* 2014; Hyytiäinen *et al.,* 2013; Hwang *et al.,* 2013). However it was found that some proteins involved in the oxidative stress response namely thiol peroxidase and superoxidase were downregulated in ciprofloxacin-adapted variant. Up-regulation of the non-heme iron protein rubrerythrin in the original strain following a ciprofloxacin challenge may indicate that this protein is more sensitive to ROS generated by the antibiotic challenge (Pinto *et al.,* 2011; Kim *et al.,* 2015). The contribution of ROS to antibiotic lethality is most prominent at lower lethal antibiotic concentrations and at earlier time points after antibiotic exposure (Dwyer *et al.,* 2015). The fact that proteins involved in the oxidative stress were not up-regulated in the ciprofloxacin-adapted strain suggests that the oxidative stress response has a limited role in mediating resistance to ciprofloxacin. Up-regulation of the oxidative stress response could be protective against the bactericidal action of ciprofloxacin and may be responsible for low-level cross-resistance to ciprofloxacin in BKC-adapted *C. jejuni* variant (see below). Adaptation to ciprofloxacin, as well as exposure of the original strain, resulted in the up-regulation of heat shock proteins DnaK and GroEL. Both DnaK and GroEL may play a protective role in reversing damage to proteins as a result of ROS production following ciprofloxacin treatment in adapted variants (Dwyer *et al.,* 2009, 2014). DnaK binds and protects exposed regions on unfolded or

partially folded protein chains protecting them from proteolysis and aggregation and GroEL provides a protective microenvironment for protein folding (Cardoso *et al.,* 2010). HtpG, another heat shock protein, was down-regulated in adapted variants. HtpG has previously been shown to be important for protein synthesis in *E. coli* (Buchner, 2010).

Mutations in genes that are associated with antimicrobial resistance may impose a biological cost to Campylobacter in the absence of antimicrobial selection pressure (Andersson, 2003; Zhang *et* al., 2006). Reorganized metabolic networks may be one way to circumvent metabolic costs associated with development of antimicrobial resistance (Händel *et al.*, 2013). Changes in the levels of expression of proteins involved in metabolism were also observed in the ciprofloxacin adapted variant. *Campylobacter* is unable to utilize sugars as they lacks key glycolysis enzymes 6-phospho-gluconate dehydratase enzyme and 6-phosphofructokinase and uses amino acids glutamate, aspartate, serine and proline as a main energy source via the citric acid cycle (Guccione *et al.,* 2008; Kelly, 2005; Pajaniappan *et al.,* 2008; Parkhill *et al.,* 2000). The citric acid cycle enzyme, succinyl Co-A synthase (SucC), and aspartate ammonia-lyase were both downregulated in the wild type strain following antibiotic challenge with ciprofloxacin. Decreased metabolic energy production following ciprofloxacin treatment, is thought to mitigate against the damaging effects of ROS production (Han *et al.,* 2008; Xia *et al.,* 2013). SucC was shown to be upregulated in the adapted strain. Adaptation or a longer period of exposure to a lower concentration of ciprofloxacin may allow cells to naturally recover their ability to generate energy via the citric acid cycle. AspA was downregulated in the ciprofloxacin-adapted *C. jejuni* variant This may indicate that ciprofloxacin-adapted variants (or exposure to ciprofloxacin) switch their metabolism to preferentially use serine as an energy source. In *Campylobacter*, serine is metabolized to pyruvate which enters the TCA cycle. Pyruvate has been shown to protect cells from the damaging effects of super oxide anions and hydrogen peroxide and oxidative stress (Atack *et al.,* 2009). It may also be protective against ROS generated as a result of antibiotic challenge with ciprofloxacin. Proteins involved in protein synthesis (DNA-directed RNA polymerase and aspartyl-tRNA synthase) were also up-regulated and may indicate protein synthesis is important in adaptation to ciprofloxacin.

5.4 BKC

To date little work has been carried out to understand the development of increased resistance to biocides in *Campylobacter.* Studies have shown that *Campylobacter* can survive and persist on surfaces after cleaning and disinfection (Kudirkiene *et al.,* 2011; Peyrat *et al.,* 2008). Many commercial disinfectants used in the poultry industry contain quaternary ammonium compounds as one of the active biocidal ingredients (Gutiérrez-Martín *et al.,* 2011; Peyrat *et al.,* 2008a) Adaptation of *C. jejuni* NCTC 11168 to BKC (a quaternary ammounium compound) in chemostat culture was carried out to gain a better understanding of potential of the development of biocide tolerance in this pathogen. Exposure to BKC led to a gradual step-wise increase in tolerance to the biocide (8-fold increase; MIC_{Agar} from 8 to 64 mg l^{-1} BKC; MIC_{broth} 0.2 mg l^{-1} to 1.6 mg l^{-1}; over 20 generations growth in chemostat culture). Mc Cay *et al.,* (2010) similarly reported a slow stepwise adaption of *Pseudomonas aeruginosa* to BKC during chemostat enrichment studies (12-fold increase: 25 mg l^{-1} to > 350 mg l^{-1} over 33 generations growth in chemostat culture). The level of adaptation to BKC achieved in this study (64 mg l^{-1} BKC) was similar to that reported in a BKC-adapted variant of *Salmonella* (50 mg l^{-1}; Condell *et al.,* 2012).

Similar to ciprofloxacin adaptation, development of increased tolerance to BKC was stably maintained in the absence of selection but associated with a fitness burden (reduced growth rate). This was observed both in the chemostat population and in individual BKC-adapted variants after selective pressure was removed from the media feed (chapter 2 & chapter 3). BKC-adapted chemostat populations also showed concomitant development of low-level cross-resistance (2-fold increase) to both the ciprofloxacin and cefotaxime. Cross-resistance to cefotaxime but not ciprofloxacin was found to be unstable in the absence of BKC selection pressure. Development of increased tolerance to biocides (stable or otherwise) has been shown to lead to reductions in the susceptibilities to dissimilar disinfectants or antimicrobials in other bacteria (Marvi *et al.,* 2013a).

Survival of foodborne pathogens on food contact surfaces after cleaning and disinfection can increase the risk of cross-contamination of products and represents a potential food safety hazard (Langsrud *et al.,* 2003; Lavilla Lerma *et al.,* 2013;

Meyer, 2006; Peyrat *et al.,* 2008). The efficacy of the BKC-containing commercial disinfectant Savlon™ against BKC-adapted variants was examined to ascertain if adaptation enhanced their survival and be a possible reason for disinfectant failure. However, because situations can occur where disinfectants may become diluted (e.g. in food-baths where the organic content increases over time (due to use) and rain or by stagnant water present under objects or in cracks in wood and masonry; Cerf *et al.,* 2010; Karatzas *et al.,* 2007) concentrations below the recommended use concentration of disinfectant for Savlon™ were also examined in the same experiment. Antimicrobial susceptibility testing was also carried out to determine the lowest dilution of Salvon™ tolerated. Dilutions below this value were not included as they would result in 100% survival of the test strain. Recommended use concentrations (relating to the concentrations prepared according to the manufactures instructions, where in use concentration is the concentration recommended by the manufacturer, 2% v/v solution, and half-strength is half that concentration) of Savlon™ were still effective against BKC-adapted strain. Dilute solutions of Savlon™ (1:2, 1:4 and 1:8 strength dilutions of Savlon™ were similarly efficacious after 15 minutes exposure). However, when compared to the original strain, BKC-adapted variants were able to survive longer and in greater numbers after exposure to 1:16 and 1:32 dilutions of Savlon™. These results suggest that the risk of disinfectant failure may be augmented following adaptation to BKC if Savlon™ is not applied as directed.

Analysis of proteomic changes revealed that proteins involved in the oxidative stress response were up-regulated following adaptation of *C. jejuni* to the biocide BKC as has also been shown to occur in *E. coli* and *B. cereus,* other known food-borne and food spoilage bacteria (Bore *et al.,* 2007; Ceragioli *et al.,* 2010). The proteins thiol peroxidase, superoxidase dismutase, alkyl hydroperoxide reductase and rubrerythrin are involved in the oxidative stress response and were up-regulated following adaptation to BKC (Chapter 4). This was in contrast to the effect that ciprofloxacin exposure/adaptation had on the expression of proteins in the ciprofloxacin-adapted *C. jejuni* variant involved in the oxidative stress response. In BKC-adapted populations, development of increased resistance to BKC was associated with low-level (2-fold) cross-resistance to ciprofloxacin and cefotaxime (Chapter 2). Up-regulation of the oxidative stress response in these adapted variants could be mediating a protective effect against the action of ciprofloxacin/cefotaxime resulting

in the observed low-level cross-resistance (Dwyer *et al.,* 2014). This concept needs to be examined further. Other proteins identified include those associated with DNA and protein synthesis, metabolism and heat-shock response In addition, a putative methyl transferase was also identified in original strain when challenged with 25% MIC to BKC. Protein spots that were down-regulated, involved in the heat shock response and metabolism (namely, HtpG and aspartate ammonia-lyase) were also found to be decreased under stress conditions as a result of exposure to or adaptation to ciprofloxacin in *C. jejuni*.

6.5 Future work

Expanding our current understanding of how *C. jejuni* NCTC 11168 adapts to ciprofloxacin and BKC is important not only for the prevention of transmission of this food-borne pathogen in the food chain but also for the development of effective treatment regimes. While the results here provide some insight into the processes involved in adaptation to both these antimicrobial agents in *Campylobacter* this study was limited. A single strain*, C. jejuni* NCTC 11168 was used to investigate the adaptation. The nature of evolution in the chemostat introduced a degree of random chance that may have affected the outcome of evolution experiments. In this study adapted variants with decreased fitness were found following adaptation to ciprofloxacin and BKC. However, this may represent one possible outcome of evolution. Luo et al., (2005) showed that adaptation to FQ antibiotics by acquisition of a single resistance mutation Thr-86-Ile conferred resistance to FQs. The Thr-86-Ile mutation had a positive effect on fitness in certain strains of *Campylobacter* and the observed effect on fitness was strain-dependant. Evidence of intra-phenotypic diversity within chemostat populations was found when examining efflux activity and antimicrobial susceptibility in Chapter 3. High genetic and phenotypic diversity exists among *Campylobacter spp.* and may explain the strain specific responses observed in this study and by other authors (Dorrell *et al.,* 2001; Joen *et al.,* 2010; Wassenaar & Newell, 2000). Future work should focus on addressing this issue.

Future work relating to this project

The survival of other isogenic or clonally related BKC- and ciprofloxacin-adapted stains to other BKC-containing disinfectants commonly used in the food industry and

in particular those used on poultry farms should be determined. This is particularly relevant to determining the likelihood of survival of biocide-adapted and antibiotic – adapted strains in the food chain. One of the major proteomic changes in BKC-adapted strains was up-regulation of proteins involved the oxidative stress response. Future work could focus on identifying a regulator(s) responsible for this up-regulation and to examine its role in mediating reduced susceptibility to BCK in other *C. jejuni* and *C. coli* strains. *Campylobacter* has previously been shown to possess an adaptive tolerance response to acid and aerobic stresses (Murphy *et al.,* 2003). It would be worth investigating if the observed up-regulation of the oxidative stress response in BKC-adapted variants conveyed cross-protection (enhanced survival) to other commonly encountered stresses in the food production environment (Garénaux *et al.,* 2008; Murphy *et al.,* 2003). Examination of the stability and fitness of BKC-adapted variants taken from samples taken while the chemostat undergoing selection was not carried out due to time limitations in this study. Carrying out this will provide important information relating to the likelihood of dissemination of biocide resistance among BKC-adapted variants following short-term exposure to BKC.

Examining factors affecting the survival of Campylobacter in the environment.

Measures to adequately control the entry of *Campylobacter* in to the food chain remain elusive, despite 30 years of research (Bolton, 2015). In general, *Campylobacter* are considered sensitive to the action of disinfectants yet despite this strains have been isolated from processing plants and transport crates after cleaning and disinfection (Peyrat *et al.,* 2008; Ellerbroek *et al.,* 2010). Adaptation to biocides is only part of the story and may not be the only risk factor for disinfectant failure in reducing or eliminating *Campylobacter* contamination of surfaces. Other risk factors for disinfectant failure such as application at an incorrect temperature, insufficient contact time and ineffective cleaning and removal organic material all are critical for effective disinfection action (Buffet-Bataillion, 2012; Miallard, 2005). The contribution of *Campylobacter* biofilms on survival and persistence of *Campylobacter* in the food-chain should also be evaluated further (Bolton, 2015; Humphrey *et al.,* 2007; Murphy *et al.,* 2006; Park, 2002)

Transcriptomic and genomic profiling of adapted strains

The mechanisms underlying the variety of antimicrobial resistance profiles in ciprofloxacin-adapted variants were not fully elucidated in this study (chapter 3). Transcriptomic profiling of adapted variants using DNA microarrays is one method of achieving a wider view of differential gene expression occurring as a result of adaptation. One of the major advantages of using *C. jejuni* NCTC 11168 in this study is that its genome has already been sequenced. Coupled with this is the fact that *C. jejuni* NCTC 11168 specific DNA microarrays (MYcroarray, 5692 Plymouth Road, Ann Arbor, MI 48105, USA) which up to 97% coverage of the genes present in this strain are already commercially available. Analysis of transcriptomic changes will facilitate identification of changes in gene expression profiles in adapted strains exhibiting stable resistance and simultaneously identify any novel resistance mechanisms which may explain the variety of resistance phenotypes seen in this study. It will also complement to proteomic analysis already carried out. Progress in DNA sequencing technologies have increased the capacity to sequence whole bacterial genomes and may allow for detailed analyses of large collections of clonally related bacteria generated in this study (Palmer & Kishnoy, 2013). Whole genome sequening (WGS) provide a top down approach that allow the comparision of inter- and intra-individual genome variation within a species on a routine basis (Palmer & Kishnoy, 2013). WGS has previously been used to identify SNPs responsible for resistance to teicoplanin (a glycopeptide antibiotic) in *Staphylococcus aureu*s and to chloramphenicol, doxycyline and trimethoprim in *E. coli* (Renzoni *et al.,* 2011; Toprak *et al.,* 2011). Comparing the genomes of ancestral and evolved strains and would allow for the precise genetic changes that underlie adaptive evolution to BKC and ciprofloxacin to be defined (Palmer & Kishnoy, 2013).

6. 6 Bibliography

Andersson, D.I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance?, *Nat. Rev. Microbiol., 8*(4), 260-271.

Andersson, D.I. (2003). Persistence of antibiotic resistant bacteria. *Curr. Opin. Microbiol., 6*(5), 452-456.

Atack, J.M. & Kelly, D.J. (2009). Oxidative stress in *Campylobacter jejuni*: responses, resistance and regulation. *Future Microbiol, 4*(6), 677-690.

Bolton, D.J. (2015). *Campylobacter* virulence and survival factors. *Food Microbiol., 48*, 99-108.

Bore, E., Hébraud, M., Chafsey, I., Chambon, C., Skjaeret, C., Moen, B., Møretrø, T., Langsrud, Ø., Rudi, K. & Langsrud, S. (2007). Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. *Microbiology, 153*(Pt 4), 935-946.

Bronowski, C., James, C. & Winstanley, C. (2014). Role of environmental survival in transmission of *Campylobacter. FEMS Microbiol. Lett., 356*(1), 8-19.

Buchner, J. (2010). Bacterial Hsp90--desperately seeking clients. *Mol. Microbiol., 76*(3), 540-544.

Buffet-Bataillon, S., Tattevin, P., Bonnaure-Mallet, M. and Jolivet-Gougeon, A. (2012). Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds--a critical review. *Int. J. Antimicrob. Agents, 39*(5), 381-389.

Cardoso, K., Gandra, R.F., Wisniewski, E.S., Osaku, C.A., Kadowaki, M.K., Felipach-Neto, V., Haus, L.F. & Simão Rde, C. (2010). DnaK and GroEL are induced in response to antibiotic and heat shock in *Acinetobacter baumannii*. *J. Med. Microbiol., 59*(Pt 9), 1061-1068.

Ceragioli, M., Mols, M., Moezelaar, R., Ghelardi, E., Senesi, S. & Abee, T. (2010) Comparative transcriptomic and phenotypic analysis of the responses of *Bacillus*

cereus to various disinfectant treatments. *Appl. Environ. Microbiol., 76*(10), 3352-3360.

Cerf, O., Carpentier, B. and Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int. J. Food Microbiol., 136*(3), 247-254.

Condell, O., Iversen, C., Cooney, S., Power, K.A., Walsh, C., Burgess, C. and Fanning, S. (2012). Efficacy of biocides used in the modern food industry to control *Salmonella enterica*, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds., *Appl. Environ. Microbiol. 78*(9), 3087-3097

Corcoran, D., Quinn, T., Cotter, L. & Fanning, S., (2005). Relative contribution of target gene mutation and efflux to varying quinolone resistance in Irish *Campylobacter* isolates. *FEMS Microbiol. Lett., 253*(1), 39-46.

Dorrell, N., Mangan, J.A., Laing, K.G., Hinds, J., Linton, D., Al-Ghusein, H., Barrell, B.G., Parkhill, J., Stoker, N.G., Karlyshev, A.V., Butcher, P.D. & Wren, B.W. (2001). Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res., 11*(10), 1706-1715.

Dwyer, D.J., Belenky, P.A., Yang, J.H., MacDonald, I.C., Martell, J.D., Takahashi, N., Chan, C.T., Lobritz, M.A., Braff, D., Schwarz, E.G., Ye, J.D., Pati, M., Vercruysse, M., Ralifo, P.S., Allison, K.R., Khalil, A.S., Ting, A.Y., Walker, G.C. & Collins, J.J. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U.S.A., 111*(20), E2100-E2109.

Dwyer, D.J., Kohanski, M.A. and Collins, J.J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr. Opin. Microbiol., 12*(5), 482-489.

EFSA (2010). Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. Part A: *Campylobacter* and *Salmonella* prevalence estimates. *EFSA Journal 8*(3), 1503.

Ellerbroek, L.I., Lienau, J.A. & Klein, G. (2010). *Campylobacter spp*. in broiler flocks at farm level and the potential for cross-contamination during slaughter. *Zoonoses Public Health, 57*(7-8), e81-88.

Engberg, J., Aarestrup, F.M., Taylor, D.E., Gerner-Smidt, P. & Nachamkin, I. (2001). Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates., *Emerging Infect. Dis. 7*(1), 24-34.

Garénaux, A., Jugiau, F., Rama, F., de Jonge, R., Denis, M., Federighi, M. & Ritz, M. (2008). Survival of *Campylobacter jejuni* strains from different origins under oxidative stress conditions: effect of temperature., *Curr. Microbiol. 56*(4), 293-297.

Ge, B., Wang, F., Sjölund-Karlsson, M. & McDermott, P.F. (2013). Antimicrobial resistance in *Campylobacter*: susceptibility testing methods and resistance trends. *J. Microbiol. Methods, 95*(1), 57-67.

Gibreel, A. & Taylor, D.E. (2006). Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*., *J. Antimicrob. Chemother. 58*(2), 243-255.

Gilbert, P. & Moore, L.E. (2005). Cationic antiseptics: diversity of action under a common epithet., *J. Appl. Microbiol. 99*(4), 703-715.

Griggs, D.J., Johnson, M.M., Frost, J.A., Humphrey, T., Jørgensen, F. & Piddock, J.L. (2005). Incidence and mechanism of ciprofloxacin resistance in *Campylobacter spp*. isolated from commercial poultry flocks in the United Kingdom before, during, and after fluoroquinolone treatment. *Antimicrob. Agents Chemother., 49*(2), 99-707.

Guccione, E., Leon-Kempis Mdel, R., Pearson, B.M., Hitchin, E., Mulholland, F., van Diemen, P.M., Stevens, M.P. & Kelly, D.J. (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate., *Mol. Microbiol. 69*(1), 77-93.

Han, J., Sahin, O., Barton, Y.W. & Zhang, Q. (2008). Key role of Mfd in the development of fluoroquinolone resistance in *Campylobacter jejuni*. *PLoS Pathog., 4*(6), e1000083.

Han, J., Wang, Y., Sahin, O., Shen, Z., Guo, B., Shen, J. & Zhang, Q. (2012). A fluoroquinolone resistance associated mutation in gyrA Affects DNA supercoiling in *Campylobacter jejuni*. *Front Cell Infect Microbiol, 2*.

Händel, N., Schuurmans, J.M., Brul, S. and ter Kuile, B.H., (2013). Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in *Escherichia coli. Antimicrob. Agents Chemother.*, AAC-02096.

Hänninen, M.L. & Hannula, M. (2007). Spontaneous mutation frequency and emergence of ciprofloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother., 60*(6), 1251-1257.

Heres, L., Engel, B., Urlings, H.A., Wagenaar, J.A. & van Knapen, F. (2004). Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. *Vet. Microbiol., 99*(3-4), 259-267.

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., Rasschaert, G., Heyndrickx, M. & Pasmans, F. (2011). *Campylobacter* control in poultry by current intervention measures ineffective: urgent need for intensified fundamental research. *Vet. Microbiol., 152*(3-4), 219-228.

Humphrey, T.J., Jørgensen, F., Frost, J.A., Wadda, H., Domingue, G., Elviss, N.C., Griggs, D.J. & Piddock, L.J. (2005). Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter spp.* in commercial poultry flocks before, during, and after treatment with fluoroquinolones. *Antimicrob. Agents Chemother., 49*(2), 690-698.

Humphrey, T., O'Brien, S. and Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol., 117*(3), 237-257.

Hwang, S., Ryu, S. & Jeon, B. (2013). Roles of the superoxide dismutase SodB and the catalase KatA in the antibiotic resistance of *Campylobacter jejuni*. *J. Antibiot.*, 66(6), 351-353.

Hyytiäinen, H., Juntunen, P., Scott, T., Kytömäki, L., Venho, R., Laiho, A., Junttila, S., Gyenesei, A., Revez, J. & Hänninen, M.L. (2013). Effect of ciprofloxacin

exposure on DNA repair mechanisms in *Campylobacter jejuni*., *Microbiology., 159*(Pt 12), 2513-2523.

Jacobs-Reitsma, W., Lyhs, U. & Wagenaar, J. (2008). *Campylobacter* in the food supply. in Nachamkin, I., Szymanski, C.M. and Blaser, M.J. *Campylobacter*, ASM Press, Washington, DC.

Jeon, B., Muraoka, W.T. & Zhang, Q. (2010). Advances in *Campylobacter* biology and implications for biotechnological applications. *Microb Biotechnol, 3*(3), 242-258.

Karatzas, K.A., Webber, M.A., Jorgensen, F., Woodward, M.J., Piddock, L.J. & Humphrey, T.J. (2007). Prolonged treatment of *Salmonella enterica serovar Typhimurium* with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J. Antimicrob. Chemother., 60*(5), 947-955.

Kelly, D.J. (2005). Metabolism, electron transport and bioenergetics of *Campylobacter jejuni*: implications for understanding life in the gut and survival in the environment. in Ketley, J.M. and Konkel, M.E. (ed.) *Campylobacter: Molecular and Cellular Biology*. Norfolk, UK: Horizon Scientific Press, 275-292.

Kudirkienė, E., Bunevičienė, J., Brøndsted, L., Ingmer, H., Olsen, J.E. & Malakauskas, M. (2011). Evidence of broiler meat contamination with post-disinfection strains of *Campylobacter jejuni* from slaughterhouse. *Int. J. Food Microbiol., 145* (Suppl 1), S116-S120.

Langsrud, S., Sidhu, M.S., Heir, E. & Holck, A.L. (2003). Bacterial disinfectant resistance—a challenge for the food industry. *Int. Biodeterior. Biodegradation, 51*(4), 283-290.

Larsen, M.H., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., Møretrø, T., Možina, S.S., Rychli, K., Wagner, M., Wallace, J.R., Zentek, J. & Jordan, K. (2014). Persistence of foodborne pathogens and their control in primary and secondary food production chains. *Food Control, 44*, 92-109.

Lavilla Lerma, L., Benomar, N., Gálvez, A. & Abriouel, H. (2013). Prevalence of bacteria resistant to antibiotics and/or biocides on meat processing plant surfaces throughout meat chain production. *Int. J. Food Microbiol., 161*(2), 97-106.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. & Zhang, Q. (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol, 4*(2), 189-200.

Maillard, J.Y. (2005). Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Ther Clin Risk Manag, 1*(4), 307-320.

Mangen, M. J. J., Havelaar A. H., Nauta M. J., de Koeijer A. A., & de Wit G .A. (2008). Controlling *Campylobacter* in the chicken meat chain: cost-effectivity and cost-utility analysis. *RIVM report 250911007*, 1-58.

Martinez, A. & Lin, J. (2006). Effect of an efflux pump inhibitor on the function of the multidrug efflux pump CmeABC and antimicrobial resistance in *Campylobacter*. *Foodborne Pathog. Dis., 3*(4), 393-402.

Mavri, A. & Smole Možina, S. (2012). Involvement of efflux mechanisms in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. *J. Med. Microbiol., 61*(Pt 6), 800-808.

Mavri, A. & Smole Možina, S. (2013a). Development of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. *Int. J. Food Microbiol., 160*(3), 304-312.

Mavri, A. & Smole Možina, S. (2013b). Effects of efflux-pump inducers and genetic variation of the multidrug transporter cmeB in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. *J. Med. Microbiol., 62*(Pt 3), 400-411.

Messens, W., Hartnett, E., Gellynck, X., Viaene, J., Halet, D., Herman, L. & Grijspeerdt, K. (2007). Quantitative risk assessment of human campylobacteriosis through the consumption of chicken meat in Belgium., in *XVIII European Symposium on the Quality of Poultry Meat and XII European Symposium on the Quality of Eggs and Egg Products*, Prague, Czech Republic.

Meyer, B. (2006). Does microbial resistance to biocides create a hazard to food hygiene? *Int. J. Food Microbiol., 112*(3), 275-279.

Moore, J.E., Corcoran, D., Dooley, J.S., Fanning, S., Lucey, B., Matsuda, M., McDowell, D.A., Mégraud, F., Millar, B.C., O'Mahony, R., O'Riordan, L., O'Rourke, M., Rao, J.R., Rooney, P.J., Sails, A. & Whyte, P. (2005). *Campylobacter*. *Vet. Res., 36*(3), 351-382.

Murphy, C., Carroll, C. & Jordan, K.N. (2003). Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. *FEMS Microbiol. Lett., 223*(1), 89-93.

Murphy, C., Carroll, C. & Jordan, K.N. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni. J. Appl. Microbiol., 100*(4), 623-632.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G. & Allen, V.M. (2011). Biosecurity-based interventions and strategies to reduce *Campylobacter spp.* on poultry farms. *Appl. Environ. Microbiol., 77*(24), 8605-8614.

O'Mahony, E., Buckley, J.F., Bolton, D., Whyte, P. & Fanning, S. (2011). Molecular epidemiology of *Campylobacter* isolates from poultry production units in southern Ireland. *PLoS ONE, 6*(12), e28490.

O'Regan, E., Quinn, T., Frye, J.G., Pagès, J.M., Porwollik, S., Fedorka-Cray, P.J., McClelland, M. & Fanning, S. (2010). Fitness costs and stability of a high-level ciprofloxacin resistance phenotype in *Salmonella enterica serotype enteritidis*: reduced infectivity associated with decreased expression of *Salmonella* pathogenicity island 1 genes. *Antimicrob. Agents Chemother., 54*(1), 367-374.

O'Reilly, A. & Smith, P. (1999). Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. *Aquaculture, 180*(1-2), 1-11.

Pajaniappan, M., Hall, J.E., Cawthraw, S.A., Newell, D.G., Gaynor, E.C., Fields, J.A., Rathbun, K.M., Agee, W.A., Burns, C.M., Hall, S.J., Kelly, D.J. & Thompson, S.A. (2008). A temperature-regulated *Campylobacter jejuni* gluconate dehydrogenase is involved in respiration-dependent energy conservation and chicken colonization. *Mol. Microbiol., 68*(2), 474-491.

Palmer, A.C. & Kishony, R. (2013). Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat. Rev. Genet.*, 14(4), 243-248.

Park, S.F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int. J. Food Microbiol., 74*(3), 177-188.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead, S. et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature, 403*(6770), 665-668.

Payot, S., Bolla, J.M., Corcoran, D., Fanning, S., Mégraud, F. & Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in *Campylobacter spp*. *Microbes Infect., 8*(7), 1967-1971.

Payot, S., Cloeckaert, A. & Chaslus-Dancla, E., (2002). Selection and characterization of fluoroquinolone-resistant mutants of *Campylobacter jejuni* using enrofloxacin. *Microb. Drug Resist., 8*(4), pp.335-343.

Peyrat, M.B., Soumet, C., Maris, P. & Sanders, P. (2008a). Phenotypes and genotypes of *Campylobacter* strains isolated after cleaning and disinfection in poultry slaughterhouses. *Vet. Microbiol., 128*(3-4), 313-326.

Peyrat, M.B., Soumet, C., Maris, P. & Sanders, P. (2008b). Recovery of *Campylobacter jejuni* from surfaces of poultry slaughterhouses after cleaning and disinfection procedures: analysis of a potential source of carcass contamination. *Int. J. Food Microbiol., 124*(2), 188-194.

Piddock, L.J., Ricci, V., Pumbwe, L., Everett, M.J. & Griggs, D.J. (2003). Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J. Antimicrob. Chemother., 51*(1), 19-26.

Poole, K. (2012). Bacterial stress responses as determinants of antimicrobial resistance., *J. Antimicrob. Chemother. 67*(9), 2069-2098.

Renzoni, A., Andrey, D.O., Jousselin, A., Barras, C., Monod, A., Vaudaux, P., Lew, D. and Kelley, W.L. (2011). Whole genome sequencing and complete genetic analysis reveals novel pathways to glycopeptide resistance in *Staphylococcus aureu*s. *PLoS ONE, 6*(6), e21577.

Rosenquist, H., Nielsen, N.L., Sommer, H.M., Nørrung, B. and Christensen, B.B. (2003). Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Microbiol., 83*(1), 87-103.

Russell, A.D. (2000). Do biocides select for antibiotic resistance? *J. Pharm. Pharmacol., 52*(2), 227-233.

SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks. (2009). *Assessment of the antibiotic resistance effects of biocides.* European Commission, Brussels, Belgium: http://ec.europa.eu/health/ph risk/committees/04 scenihr/docs/scenihr o 021.pdf [accessed 16.08.13]

Toprak, E., Veres, A., Michel, J.B., Chait, R., Hartl, D.L. & Kishony, R. (2011). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet., 44*(1), 101-105.

Wagenaar, J.A., French, N.P. & Havelaar, A.H. (2013). Preventing *Campylobacter* at the Source: Why Is It So Difficult? *Clin. Infect. Dis., 57*(11), 1600-1606.

Wagenaar, J.A., Mevius, D.J. & Havelaar, A.H. (2006). *Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis. *Rev Sci Tech, 25*(2), 581-594.

Wassenaar, T.M. (2011). Following an imaginary *Campylobacter* population from farm to fork and beyond: a bacterial perspective. *Lett. Appl. Microbiol., 53*(3), 253-263.

Wassenaar, T.M. & Newell, D.G. (2000). Genotyping of *Campylobacter spp.*, *Appl. Environ. Microbiol. 66*(1), 1-9.

Wang, G., Wilson, T.J., Jiang, Q. & Taylor, D.E. (2001). Spontaneous mutations that confer antibiotic resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother., 45*(3), 727-733.

Xia, Q., Muraoka, W.T., Shen, Z., Sahin, O., Wang, H., Wu, Z., Liu, P. & Zhang, Q. (2013). Adaptive mechanisms of *Campylobacter jejuni* to erythromycin treatment. *BMC Microbiol., 13*(1), 133.

Yan, M., Sahin, O., Lin, J. & Zhang, Q. (2006). Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J. Antimicrob. Chemother., 58*(6), 1154-1159.

Young, K.T., Davis, L.M. & Dirita, V.J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol., 5*(9), 665-679.

Zhang, Q., Sahin, O., McDermott, P.F. & Payot, S. (2006). Fitness of antimicrobial-resistant *Campylobacter* and *Salmonella*. *Microbes Infect., 8*(7), 1972-1978.