Comparative Phenotypic and Transcriptional Differences of Campylobacter jejuni When Challenged with Low Molecular Weight Chitosan

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II Abstract

Campylobacter jejuni is a prominent food-borne pathogen and causative agent of Campylobacteriosis infection. This infection arises primarily from the consumption of foodstuffs/beverages previously contaminated with this microorganism. Despite current food control measures currently employed, this pathogen remains problematic despite its fastidious nature. To help control the spread of Campylobacter many hurdles are used in the food industry, including natural products such as modified atmosphere and salt. Other natural antimicrobials are shown to possess antibacterial activity.

Chitosan, a natural antimicrobial, has promising uses in food production. However, little is known about the transcriptional differences of C. jejuni upon chitosan exposure. Molecular responses may allow for generation of adaptive responses in an attempt to combat such a stressor, possibly altering phenotypic and virulence potential. The aims of this study were to establish the adaptive response of C. jejuni to low molecular weight chitosan by assessing the phenotypic and molecular differences arising from the chitosan challenge.

By exposing C. jejuni NTCT11168 to suboptimal levels of chitosan, based on MIC determination on a model system, an adaptive strain of C. jejuni has been developed. This newly developed isolate, referred to as the 'adapted' C. jejuni NCTC11168 was found to have enhanced antimicrobial tolerance to chitosan, with a 3.83 fold increase in MIC relative to the parental wild-type C. jejuni NCTC11168 cells (0.012% - 0.046% (w/v) respectively). Antimicrobial activity of chitosan was found to be pH dependant. Differences in motility were also apparent between the 'adapted' and wild-type strains. Consistent increases in motility were noted in the 'adapted' cells, especially in relation to wild-type after 24, 48 and 72 hours ($p<0.05$). Biofilm formation was also enhanced especially in the 'adapted' isolate relative to the wild-type NCTC11168 cells. This was indicated by significant increases, most notably after 3 days formation ($p<0.05$). Microarray analysis revealed significant alterations in transcriptional levels in response to chitosan exposure. Protein-encoding genes, believed to be associated with energy metabolism, solute/ion uptake/acquisition and efflux systems, were found to be differentially regulated when grown in the presence of low molecular weight chitosan ($p<0.05$). Overlapping between initial comparisons revealed several genes, which were differentially regulated. Most notably, PEB3 (major antigenic peptide), Cj0017c (disulphide bond formation) and Cj0294 (thiamine biosynthesis) genes were found to be up regulated in 'adapted' C. jejuni cells when compared to the wild-type ($p<0.05$). Whilst significant down regulation was found in the protein-coding genes, Cj0025c (putative sodium:dicarboxylate symporter) and Cj1608 (two-component regulator).

These results indicate that regulation of these genes may contribute to enhanced phenotypic responses observed in the 'adapted' isolate of C. jejuni NCTC11168. These findings are likely to account for increased growth and survival of 'adapted' C. jejuni NCTC11168 when challenged to this antimicrobial, when compared to the wild-type. The findings in this study provide useful information as to how C. jejuni NCTC11168 can develop an adaptive tolerance response to chitosan and that alterations in transcriptional aspects may facilitate this, contributing to an enhanced phenotypic response and virulence potential.

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VI - Abbreviations

Abbreviation	*Definition*
µl	*Microliter*
Ad	*Adapted*
AI	*Autoinducer*
ANOVA	*Analysis of Variance*
ATR	*Adaptive tolerance response*
Bp(s)	*Base pair(s)*
BSA	*Bovine Serum Albumin*
CD	*Chitosan dextran-based*
cDNA	*Complementary Deoxyribose Nucleic Acid*
CDT	*Cytolethal Distending Toxin*
CFU	*Colony Forming Unit(s)*
CFU/ml	*Colony forming units/millilitre*
Cm	*Centimetre*
CprRS	*Campylobacter planktonic growth regulation*
CSF	*Culture supernatant fluid*
Ct	*Cycle Threshold*
CV	*Chrystal Violet*
DASSs	*Divalent anion/sodium symporters*
DccRS	*Diminished ability to colonise*
DD	*Degree of Deacetylation*
DNA	*Deoxyribonucleic Acid*
EO	*Essential Oil*
EPSs	*Extracellular polymeric substances*
FTIR	*Fourier Transform Infrared Spectroscopy*
GBS	*Guillian Barré Syndrome*
gNDA	*Genomic DNA*
GRAS	*Generally Recognised As Safe*
IAM	*Invasion-associated marker*
IU	*International Unit*
kDa	*Kilodalton*
KOH	*Potassium Hydroxide*
log	*Logarithmic*
M	*Mole (unit)*
MBC	*Minimum Bactericidal Concentration*
mCCDA	*Modified charcoal-cefoperazone-deoxycholate agar*
MEM-a	*Modified Eagles Medium*
MFS	*Miller Fisher Syndrome*
mg	*Milligrams*
mg/ml	*Milligrams/millilitres*
MH	*Mueller Hinton*
MHA	*Mueller Hinton Agar*
MHB	*Mueller Hinton Broth*
MIC	*Minimum Inhibitory Concentration*

ml	*Millilitres*
Mm	*Millimetres*
mRNA	*Messenger Ribonucleic Acid*
Mw	*Molecular Weight*
NaOH	*Sodium Hydroxide*
NCTC	*National Collection of Type Cultures*
Nm	*Nanometre*
NO	*Nitric oxide*
PBS	*Phosphate Buffered Saline*
PCR	*Polymerase chain reaction*
pH	*Hydrogen ion concentration*
pKa	*Acid dissociation constant*
qRT-PCR	*Quantitative Real-time PCR*
RAPD	*Random Amplified Polymorphic DNA*
ReA	*Reactive Arthritis*
RNA	*Ribonucleic acid*
ROS	*Reactive oxygen species*
TCRs	*Two-component regulators*
UV	*Ultra Violet*
V	*Volts*
v/v	*Volume/volume*
VBNC	*Viable but non-culturable*
Wt	*Wild-type*
w/v	*Weight/volume*

Chapter 1 - Introduction

Campylobacter species, particularly C. jejuni account for the most commonly reported bacterial cause of food-borne gastroenteritis, worldwide (Moore et al., 2006). Infections resulting from this pathogen can cause symptoms such as abdominal pain, diarrhoea, haemorrhagic colitis and in rare cases Guillain-Barré syndrome (Papaconstantinou and Thomas, 2007). The majority of sporadic cases of human campylobacteriosis arise through the consumption of undercooked/raw poultry meat (Moore et al., 2006).

Owing to a chronic overuse of antibiotics in both human medicine and animal husbandry, an increase in the incidence of resistance to antibiotics has become evident of the years (Iovine, 2013). As a result of this issue, there has been a shift towards more alternative antimicrobials. In this respect, there is a preference for naturally occurring agents/derivatives which allow for a suitable decontamination (Burt, 2004; Chouliara et al., 2007). For food safety assurance, consumers frequently demand food products which contain natural alternatives to the traditional chemical additives, which still permit increases in food quality and shelf-life (Piskernik et al., 2011).

Chitosan, a naturally derived form of deacetylated chitin, has been seen to illustrate antimicrobial activity in a number of bacterial species (Wu et al., 2006). Due to the natural origin and Generally Recognised as Safe (GRAS) status of this biopolymer, it could be a desirable alternative to chemical decontaminants and food packaging or coatings (FDA, 2001). Much in the same

way that exposure to antibiotics and biocides can lead to enhanced resistance properties, having been previously documented in C. jejuni (Miflin et al., 2006; Mavri and Mozina, 2012), there may be a potential for C. jejuni to develop an increased tolerance towards chitosan and become stress hardened.

Limited gene expression analysis has been conducted following exposure of microorganisms to chitosan. For C. jejuni to illustrate an adaptive tolerance response, differences in molecular responses are likely to be present. Thus it seems appropriate to explore both the transcriptional response of C. jejuni to chitosan exposure and assess the impact of this in terms of altered phenotypic responses from an aspect of virulence.

Overall, the works undertaken in this study provide data highlighting the antimicrobial activity of low molecular weight chitosan against several C. jejuni strains/isolates. In addition the data allow for an assessment of C. jejuni NCTC11168 to adapt to sub-lethal low molecular weight chitosan and to subsequently explore the associated phenotypic and transcriptional differences resultant from challenge to this antimicrobial.

Chapter 2 - Literature Review

2.1 Campylobacter jejuni

2.1.1 Taxonomy, physiology and pathogenesis

Campylobacter jejuni is a Gram negative, non-spore forming, helical shaped, motile microorganism, belonging to the genus Campylobacter. Along with bacteria species from Arcobacter and Sulfurospirillum genera, Campylobacter spp. are designated under the family Campylobacteraceae (De Boer et al., 2013). C. jejuni is referred to as fastidious, requiring microaerobic conditions in order to grow optimally. An oxygen concentration of 3-15% and carbon dioxide concentration of 3-5% is accepted as being optimal for this growth (Ketley, 1997). C. jejuni has the ability to grow at temperatures ranging from 30-47^{o}C, with optimal growth at a temperature of 42^{o}C (Stintzi, 2003). With respect to pH, C. jejuni is said to grow optimally at a range of 6.5-7.5, with an inhibition in growth observed at pH 4.9 or less (Doyle and Roman, 1981).

*C. jejuni has been identified as the most common bacterial pathogen causing gastroenteritis and post infectious irritable bowel syndrome (PI-IBS) in humans (Zilbauer et al., 2008; Sung et al., 2013)***.** *These symptoms of gastroenteritis, sometimes labelled as Campylobacter enteritis or Campylobacteriosis include abdominal cramping/pain, nausea, fever, muscle pain and acute diarrhoea (CDC, 2011; Alazzam et al., 2011).*

Despite the well-documented pathogenesis of C. jejuni with respect to humans, birds and other animals; it is the microaerophilic and chemo-organotropic nature

which dictates the need for complex media when isolating and re-cultivating from environmental and clinical sources (Corry et al., 1995). Mechanisms involving the virulence and resistance to various environmental stresses remain either unknown or not fully understood. This is likely due to the relative fragility of this micro-organism (Garénaux et al., 2008; Alazzam et al., 2011; Corry et al., 1995). Therefore, gaining knowledge with respect to factors such as stress resistance, adaptation and virulence of C. jejuni are paramount for developing a greater understanding of this microorganism.

2.1.2 Epidemiology

Campylobacter infection with respect to humans is known to occur more often in summer months compared to winter in temperate climates. Approximately double the number of cases of Campylobacter infection occurs during summer when compared to winter (Butzler, 2004).

A similar trend is seen in the degree of flock colonisation of chickens. In many European countries, the amount of flock colonisation with Campylobacter appears to be seasonally dependant, with higher rates during in summer in contrast to autumn (EFSA, 2010).

The rate of notification of campylobacteriosis from archived census data from 1991 and 1996 for general population and aboriginal populations respectively in Western Australia revealed higher rates in spring months. Males were seen to have a higher rate of 89 cases of campylobacteriosis per 10,000 compared to that of females (78 per 10,000). The aboriginal population were typical of higher

incidence, as oppose to the general population, with young children and younger adults being particularly affected (Banmali et al., 2006).

True incidence of gastroenteritis resulting from Campylobacter spp. is not well known, with particular respect to low-income countries. In the case of high-income countries, the number of cases ranges from 44 and 93 per 10,000 individuals (WHO, 2013). Statistics suggest yearly increases in reported cases of around 30% in Scotland and 14% in England and Wales, according to data obtained in 2009. This number is likely to be markedly higher (450,000) with 10% of all cases being hospitalised (Strachan and Forbes, 2010). However, this number is likely to be greater due to under reporting (Thomas et al., 2006).

Zoonotic transfer via the faecal-oral route can lead to colonisation of the intestinal mucosa of host animals and humans alike (Jones, 2001; Newell et al., 2001). The environmental niche of choice is believed to be avian intestinal tracts (Newel and Fearnley, 2003). The main reservoir can involve either the domestic or wild animal populations. It has been recognised that poultry are seen to play a pivotal role when infection is concerned, with particular emphasis on contaminated poultry which are often a staple part of many diets worldwide (Butzler, 2004; Mullner et al., 2009; Stafford et al., 2008).

The main route of infection as identified in outbreak reports and case-control studies have uncovered other risk factors that can be involved. Examples includes the transmission of Campylobacter by means of flies as a vector which has led to the assumption that Campylobacter is readily transferred from organism to foodstuffs (Nichols et al., 2005; Ekdahl et al., 2005). However, it is often that contaminated foodstuffs are usually the culprit for human infection.

The handling of poultry is believed to be the focal route of infection with respect to developed countries (Gormely et al., 2008). When assessing Campylobacter infections in Ireland, the most pressing risk factor appeared to be consumption of chicken. Other factors to consider include other foodstuffs, such as lettuce and "fast food" as well as contact with sheep, bowel problems, hiatus hernia, peptic ulcers and consumption of bird pecked milk (Danis et al., 2009; Moore and Rooney, 2010). A case-control study in Sweden revealed an increase in campylobacteriosis infection concerning key risk behaviours including the consumption of unpasteurised milk, boned pork, barbecuing, hen/chicken breeding and farming (Studahl and Andersson, 2000). The above reiterate the potential risks associated with actions such as undercooking foodstuffs alongside other risk factors which may lead to an increased risk of the C. jejuni persistence in communities.

2.1.3 Historical aspects of Campylobacter

Although Campylobacter species were not acknowledged as being pathogenic to humans until the 1970s, they were likely to have been observed prior to this by the German paediatrician, Theodor Escherich. A spiral bacteria present in the colons of infants who had died from enteric disease, which was referred to as "cholera infatum". He then attempted to culture these microorganisms from the stool samples, and was unsuccessful. This was likely to be due to the fastidious nature of campylobacters (Escherich, 1886).

Additionally, campylobacters have been associated with veterinary disease. During 1909, two veterinary surgeons, McFadyean and Stockman repeatedly isolated unknown isolates originating from aborted ewe foetuses, resembling

vibrio characteristics with respect to morphology. They also presumed that the resultant infection was caused by entry into the mouth as opposed to other possible routes such as the genital tract (McFadyean and Stockman, 1913). Later in 1919, Smith isolated a bacterial species whilst investigating bovine abortions and was defined as Spirillum. Further work was undertaken, resulting in the species being termed "Vibrio fetus", owing to the morphological aspect and the isolation from a foetal origin (Smith, 1919; Smith and Taylor, 1919). Additionally, an organism termed Vibrio jejuni associated with winter dysentery in calves by Jones et al. (1931), was found to be very similar to an organism reported dysentery (Doyle (1944).

Following on from these microorganisms being established as veterinary pathogens, the first definitive human case was not identified until 1938, cases originating from a milk-borne originating enteritis outbreak. The species "V. jejuni" was identified in 10% of cases documented (Levy, 1946). Later in 1959, Florent categorised two distinct types of V. fetus according to their biochemical and pathogenic individualities, called V. fetus venerealis and V. fetus intestinalis (Florent, 1959).

Eventually, the genus Vibrio changed to Campylobacter, encompassing these species which were observed to be different than those in the Vibro genus. C. fetus and C. bubulus were two species which were immediately placed in the Campylobacter genus, based upon their microaerobic needs and also the low DNA base composition (Sebald and Véron, 1963). After this, there were many isolates confirmed as belonging to the Campylobacter genus, also aided by the development of a filtration technique facilitating the isolation of Campylobacter species from faecal samples (Dekeyser et al., 1972). This was also assisted with

development of selective isolation media particular to Campylobacter species as well as equipment and methodology advances tailored for the microaerobic requirements necessary for isolation (Skirrow, 1977).

2.1.4 Complications of Campylobacter infection

Manifestations of infection related complications arise if Campylobacter spp. disseminates from the gastrointestinal tract. Infections local to this including appendicitis, pancreatitis and cholecystitis can occur with haemorrhaging (Allos, 2001). Rarer still, other complications, namely meningitis, endocarditis, osteomyelitis and bacteraemia can occur as a result of Campylobacter infection, with the latter generally seen in around 1% of cases. These issues are naturally more prevalent in individuals who are at either end of the age spectrum or those of whom are immunocompromised (Allos, 2001).

Guillain-Barré syndrome (GBS), a leading case of acute flaccid paralysis can also occur as a result of post-infectious complications, and in the minority of cases can prove to be fatal (Moore et al., 2005). Compared to other complications associated with C. jejuni infection, GBS is understood to be the most abundant serious issue. There are between 0.6 and 4 cases per 100,000 population per annum worldwide (Hughes and Rees, 1997). More recent and vigilant European population-based studies revealed an incidence of 1.2-1.9 cases per 100,000 individuals (Hughes and Cornblath, 2005). A review conducted by Poropatich et al. (2010) of previously published findings of GBS rates in patients before and during Campylobacter infections indicated that approximately 31% of GBS cases arose from Campylobacter species.

Following the eradication of the poliovirus, GBS has become the most widespread cause of paralysis at a neuromuscular level (Nachamkin et al., 1998). This autoimmune disorder affects the peripheral nervous system and is characterised by atypical sensations involving numbness, tingling and weakening in muscles. The disease is self-limiting and generally individuals are observed as recovering completely (Nachamkin et al., 1998). However, approximately 15-20% of those affected appear to succumb to permanent neurological defects which are deemed severe, post recovery from the initial infection (Nachamkin et al., 1998).

Miller Fisher Syndrome (MFS) is a variant of GBS which is characterised by loss in coordination, tendon reflexes and paralysis of muscles in the eye. Similarly to GBS, MFS is perceived to be a molecular mimicry of C. jejuni glycoconjugates and host cell gangliosides (Ang et al., 2001; Ang et al., 2002). MFS is thought to account for approximately 5% of all GBS cases worldwide (McGrogan et al., 2009). As is often the case, within various regions/continents the proportion of cases attributable to MFS may be greater or smaller than predicted. A study assessing children admitted to two Hong Kong hospitals over a from 1976-2008, revealed 9% of GBS cases as MFS (Ma et al., 2010).In contrast, a paediatric sample from Argentina showed only 3 of the 179 (1.6%) of GBS cases were characteristic of MFS (Buompadre et al., 2006).

Reactive Arthritis (ReA) can also arise from C. jejuni and other microorganisms (e.g. Salmonella spp.) which are concerned with infections found primarily in the gut or urogenital tract (Hannu et al., 2002). This spondyloarthropathic disorder results in tissue and joint inflammation following infection (Townes, 2010). A mean incidence of ReA has been reported to be 9, 12 and 12 per 1000

worldwide for Campylobacter, Salmonella and Shigella, respectively (Ajene et al., 2013).

The acute cases of gastroenteritis which result from Campylobacter infection can occur from an infectious dose ranging from as little as 500-800 bacteria cells (Robinson, 1981; Black et al., 1988). The associated diarrhoea that Campylobacter causes is known to last for up to two weeks after initial infection, however in the vast majority of cases; the symptoms diminish after one week post infection (Young et al., 2007).

Prognosis is generally good with Campylobacter enteritis and in the absence of chemotherapy; faeces are still deemed Campylobacter positive from around 2-7 weeks after the illness. Patients with acute Campylobacteriosis, or have presented persistent fever, bloody diarrhoea, greater than 8 bowel movements per day or have over 7 days of persistent diarrhoea, will necessitate undergoing antibiotic therapy (Butzler, 2004).

Also, patients who are immunocompromised (I.e. HIV-positive individuals) and/or pregnant should receive antibiotic treatment following Campylobacter infection (Allos, 2001). In terms of antibiotics available, erythromycin is the drug of choice due to its effectiveness, low cost of manufacture and also the low toxicity when administered correctly (Butzler, 1992; Skirrow, 1977; Crushell et al., 2004; Salazar-Lindo et al., 1986).

The potential clinical manifestations of diseases which can arise from C. jejuni infections highlight the importance of reducing this problematic microorganism. Although the incidences of these complications are low, the developments of

novel alternative strategies are needed in order to decrease the likelihood of these resultant diseases.

2.2 Survival mechanisms

2.2.1 Biofilm Formation

The formation of bacterial biofilms is one survival mechanism employed in the presence of environmental stresses. Campylobacter spp. are able to form biofilms in three different forms whilst in liquid culture. There is an initial attachment to a given surface, an unattached aggregate and also pellicle formation. It is important to mention that all three types of biofilms seem to resemble one another when observed under scanning electron microscopy (Joshua et al., 2006). Past studies have reported that, when Campylobacter strains form biofilms, a decrease in antimicrobial susceptibility is present (Joshua et al., 2006; Kalmokoff et al., 2006). As biofilms often facilitate numerous microorganisms, such as Campylobacter species to survival in harsh environments which are not normally favourable and/or tolerable, this mechanism is invaluable towards persistence and subsequent colonisation of intestinal tract in both avian and animal hosts leading to further issues (Siringan et al., 2011).

Biofilm development of C. jejuni NCTC 11168 in microaerobic (5% oxygen, 10% carbon dioxide) and aerobic conditions (20% oxygen) after three days was assessed in wild-type and mutant variants of C. jejuni (Reuter et al., 2010). Biofilm formation by non-motile variants was found to be lower than that of the motile flagellated strain. Interestingly, under aerobic conditions a greater

development of biofilm was observed as opposed to strains under microaerobic conditions, customarily allowing for luxurious growth of C. jejuni (Reuter et al., 2010). Also, once the biofilms had formed, dissemination of viable planktonic C. jejuni cells was identified in the culture supernatant which was non-dependent upon the concentration of oxygen. This implies a passive release of cells into suspension instead of a mechanism regulated by changes from a desired microaerobic to an aerobic environment. Thus, it appears that C. jejuni biofilms facilitate the release of cells and can be thought of as reservoir when exposed to unfavourable conditions, and therefore is an important mechanism of survival (Reuter et al., 2010).

Moreover, protection of C. jejuni via formation of flocs has been documented in a study investigating the survival of flocs and planktonic cells in a range of strains. Essentially, cells incorporated in the biofilms were seen to survive for twice as long as free planktonic cells; of 24 and 12 days, respectively. This highlights the persistence of C. jejuni during a biofilm state may contribute to an enhanced degree of pathogenesis (Joshua et al., 2006).

It is thought that the ability of Campylobacter spp. to persist as a biofilm reservoir may lead to initial contamination in poultry. A study involving the use of C. jejuni cultured cells which were adherent to agar (biofilm model) was compared with that of C. jejuni cultured in broth (planktonic model) given to 1 day old broiler chicks, (after which cecal contents were analysed post 12 days.). 3-4 log cfu/ml^{-1} increases were observed from the initial starting inoculum (10^{5} cfu/ml^{-1}), as opposed to the chicks receiving the broth cultured C. jejuni. This implies that the phenotype may allow for differences in colonisation and therefore the virulence of C. jejuni (Hanning et al., 2009).

Another study by Hanning et al. (2008) investigated whether survival of C. jejuni outside the host was increased when pre-formed biofilms were formed. C. jejuni was inoculated into four biofilms and incubated at three temperatures prior to testing these encapsulated cells against planktonic cells cultured in a broth medium. Overall, the survival of C. jejuni after integration with biofilms appeared to have an increased survival capacity as oppose to those cells cultured in broths. This may be been due to the differences in temperatures when incubating the samples. Thus, to some extent it was found that pre-integration to biofilms may allow for C. jejuni to survive for a longer period of time, highlighting once more that biofilms may be the precursor to transmission and prevalence of this pathogen (Hanning et al., 2008).

2.2.2 Resistance to Bile Salts

There are several virulence factors associated with the induction of gastroenteritis and therefore the pathogenicity (Deun et al., 2007). It is thought that resistance to bile salts is an important factor which can enable C. jejuni to become more virulent (Malik-Kale et al., 2008). A study conducted by Lin et al. (2003) investigated the multidrug efflux pump in C. jejuni. This pump contributes to the resistance of C. jejuni against various antimicrobial challenges. Activation of the cmeABC operon which encodes the proteins involved in the efflux pump was seen to increase the resistance of C. jejuni to various bile salts. Additionally, with regards to the sections of chicken intestine, a correlation was observed between the overall bile concentration and Campylobacter. Conversely, cmeABC knock-out mutants failed to colonise the intestinal tract of the chickens inoculated with Campylobacter.

2.2.3 Cellular Invasion and Adherence

Invasion of C. jejuni into the epithelial cells within the intestine must also be considered when concerning pathogenesis (Deun et al., 2007). A study by Hu et al. (2008) aimed to determine the degree of interaction and also the translocation across differentiated Caco-2 cells. Both adherence and invasion was greater with in the case of INT407 cells with two thirds of these cells being subject to invasion over a two hour time period. However, in the case of Caco-2 only, 11-17% of host cells were found to have been invaded over the same period of time.

Nevertheless, when a supposed global posttranscriptional regulator; csrA was mutated in C. jejuni 81-176 strain and compared to the wild-type which possessed this gene, various differences were observed between the two strains. An example of this was that the csrA mutant less able to deal with and survive oxidative stress, whereas the wild-type strain appeared to have less susceptibility. To add to this, CsrA presence allowed for an increase in both adherence to and invasion of INT407 intestinal epithelial cells. This re-iterates the importance of such regulatory genes in the ability of C. jejuni to be pathogenic (Fields and Thompson, 2008).

Cell attachment and invasion have also been seen to be inhibited by various compounds. For example, a study investigating the invasion and adherence of C. jejuni upon Caco-2 cells at numerous concentrations of sugars and other chemicals was undertaken. At concentrations of 0.3 and 0.5 M of sugars including D-glucose, D-mannose and D-fucose, an inhibition in cell attachment and invasion was observed, conversely, when L-sugars were tested this

inhibition was not observed indicative of specificity. Additionally, an inhibition of cell attachment was observed at 4°C which was similar to a marginally decreased cell invasion and attachment in cells with a lack of potassium. This indicates that the degree of invasion and attachment are dependent upon resources which C. jejuni and other bacteria species alike utilise in order to increase pathogenicity (Russell et al., 1994).

2.2.4 Invasive and non-invasive Campylobacter

With adhesion and subsequent invasion of epithelial cells being the most imperative pathogenic mechanisms of Campylobacter diarrhea, isolates which do not occupy these virulence factors lack the ability to cause disease. A study assessment of the molecular characterization of invasive and non-invasive Campylobacter isolates from children with diarrhea and symptom free using random amplified polymorphic DNA techniques (RAPD) revelaed a distinct band of 1.6kb of DNA which was more prevalent in invasive isolates (63%) compared to that of non-invasive isolates (16%). This band was named the invasion-associated marker (IAM), and with specific primers, a 518bp fragment of the IAM locus was amplified. The degree of amplification was again higher in the invasive coupled with the non-invasive isolates, typical of 85% and 20%, respectively (Carvalho et al., 2001).

2.2.5 Survival at an intracellular level

After colonising the mucosal layer of the intestines, Campylobacter species can gain entry to the intestinal epithelial cells and use this as a survival mechanism. Inflammation and subsequent diarrhoea present upon infection (Park et al.,

2002). Once inside intracellular compartments, the degree of persistence is increased which thought to be attributable to the changing physiological processes Although C. jejuni can survive in the epithelial cells of the intestine, subsequent recovery was required involving specific oxygen-limited culturing conditions (Watson and Galán, 2008).

Generally it has been observed that C. jejuni loses viability after a 24 hour period after invasion into intestinal epithelial cells (Day et al., 2000). C. jejuni also enter macrophages through the action of upon being phagocytosed. In vitro invasion and survival of C. jejuni within mononuclear phagocytes can be observed under microscopy, with C. jejuni cells undergoing transformation into the coccoid form, as opposed to the more uniform spiral shape. Human monocytes were seen to demonstrate a greater efficiency of C. jejuni ingestion when compared to the murine equivalent monocytes. C. jejuni cells were seen to survive for up to seven days in mononuclear phagocytes (Kiehlbauch et al., 1985).

2.2.6 Responses to Environmental Stressors

With the limited ability for C. jejuni to grow in various environmental conditions, there is a necessity for the exploitation of specific nutritional requirements. Intermediates of the tricarboxylic acid cycle appear to be an alternate energy source, as opposed to carbohydrates which C. jejuni cannot metabolise (Stahl et al., 2012). This leads to the hypothesis that C. jejuni, unlike many Gram negative bacteria, are very sensitive to environmental stresses, as it seems to lack appropriate responses, notably the global regulator gene RpoS which oversees the adaptation to environmental challenges (Parkhill et al., 2000).

As a result of this, the utilisation of amino acids is paramount in order for C. jejuni to thrive within the host gastrointestinal tract. Campylobacter spp. also have the ability to exert adaptive responses to increases in acidity by the transformation to a viable but non-culturable (VBNC) cell morphology (Murphy et al., 2003; Chaveerach et al., 2003). With the vast majority of Campylobacter spp. being deemed microaerophilic, they are over sensitive to aerobic environments and as such have various regulators i.e. the transcription regulator Cj1556, in order to overcome such stresses (Gundogdu et al., 2011; Dasti et al., 2010).

Heat shock proteins are a co-regulated group of proteins which allow for numerous organisms to overcome exposure to the physiological stress of an increase in temperature, deviating from the optimum range of 37-42°C (Konkel et al., 1998). Genes which encode GroESL and DnaJ can be synthesised following exposure to extremes in temperature (amongst the 24 proteins which have been previously characterised) (Konkel et al., 1998; Thies et al., 1999). Mutants deficient in certain heat shock proteins are more susceptible to increases in temperature outside the optimal growth conditions. Overall growth of C. jejuni was seen to be diminished at 46°C and was not able to be isolated from infected chickens (Dasti et al., 2010). Expression of proteins driven by the RacRS regulon, a two-component heat shock regulatory system appeared to dictate the quantity of expression at 37^{o}C and 42^{o}C (Bras et al., 1999). Also, it is thought that the majority of bacteria also utilise osmo-regulatory mechanisms, although these systems have not yet been found in Campylobacter species (Parkhill et al., 2000).

2.3 Virulence factors

2.3.1 Motility and Chemotaxis

In order for an organism to be motile and present a chemotactic response to and from a stimulus, flagella are required. C. jejuni is no exception, possessing a polar flagellum composed of O-linked glycosylated flagellin (Thibault et al., 2001). This facilitates the attachment of C. jejuni cells onto epithelial cells within the intestine which is crucial to the colonisation and subsequent pathogenesis (Dasti et al., 2010; Wassenaar et al., 1993).

Basic flagella structure consists of a minimum of three parts, the rotary motor integrated into the cell wall matrix, a hook and helical propeller at outer most region (Minamino et al., 2008). The colonisation is dependent upon the presence of flagellin, comprising of both flaA and flaB subunits, which are subject to antigenic and phase variation alike (Wassenaar et al., 1993; Caldwell et al., 1985; Harris et al., 1987 Nachamkin et al., 1993).

Defects of O-linked glycosylation can lead to a total suppression in motility function, which thus decreases the likelihood of host cell adherence and subsequent invasion of the epithelial cells (Dasti et al., 2010). Non flagellated mutants have shown to be unable to colonise the intestinal cells, whilst the wild-type C. jejuni cells were seen to colonise this environment (Guerry et al., 1992; Yao et al., 1994).

A study using three chemotactic mutants which were previously assessed in terms of motility utilising semi-solid medium were not found to exert any

chemotactic responses to known chemo-repellents and chemo-attractants compared to the wild-type C. jejuni. All mutants generated were motile and thus had intact flagella. According to video tracking, the mutants did not appear to undergo the classic tumbling which the wild-type strain expressed. Suckling mice were orally given approximately 10^5 CFUs of the mutant strains and 10^2 CFUs of the wild-type strain separately. After 48 hours, the wild-type strains were seen to colonise the intestinal tract, whereas mutant strains lacked the ability to do so. Thus, the chemotactic movement was assumed to be partially responsible for the degree of colonisation, accounting for the virulence of the particular strain (Takata et al., 1992).

Whilst motility appears to a key aspect for colonisation, invasion and adherence, the degree of viability of Campylobacter jejuni can also vary. Black et al. (1988) fed 111 human volunteers a mixture of motile and non-motile variants of Campylobacter jejuni. Also in recovering isolates from the stool samples provided by the human subjects, only the motile forms were recovered. This implied that motility was an important factor which is required for colonisation of intestines. Interestingly, a study using C. jejuni 8116 and A3249 strains were shown to possess two-way transitions between aflagellated and flagellated phenotypes, with an increase in the ability of the C. jejuni to successfully passage through rabbit intestine in the latter case (Caldwell, et al. 1985).

2.3.2 Quorum sensing/cell signalling

Numerous bacteria are able to communicate via small signalling molecules, coined autoinducers (AI). Bacteria are able to regulate gene expression which

can ultimately affect their degree of virulence and constituting to adaptation as well as their colonisation potential (Gölz et al., 2012).

In order to produce a type of AI (AI-2), C. jejuni NCTC 11168 has been found to possess a gene which encodes an ortholog of luxS. When a luxS mutant was tested in comparison to C. jejuni expressing this gene, the latter strain was seen to have a higher degree of motility indicative of the diameter of haloes in semisolid agar medium. However the growth rate, Caco-2 invasion and oxidative stress susceptibility remained consistent between strains. This suggests that motility may be governed by quorum sensing, typical of the cellular communication (Elvers and Park, 2002).

Similar results were observed in a study investigating if C. jejuni biofilms are facilitated when quorum sensing is present. This experiment also used the luxS mutant, which was deficient AI-2 production. Here, a reduction in biofilm formation was observed at both 48 and 72 hours when comparing the luxS mutant strain to that of the wild-type. Interestingly, when confirming this finding, with an addition of culture supernatant fluid (CSF) to luxS mutant cultures, collected from wild-type cultures which had quorum sensing molecules present, an increase in biofilm formation of the luxS mutant was observed. Once more, growth was found to be the same as that of the wild-type and was not responsible for these data (Reeser et al., 2007).

Given that C. jejuni contains a homologue of the luxS gene, which has seen to be responsible for the AI-2 production, a study was investigated if AI-2 acted as a signal in quorum sensing and thus alters gene expression of C. jejuni. AI-2 was seen to be produced by the strain NCTC 11168 when cultured in Mueller

Hinton broth, opposed to none in to a more defined medium (Modified Eagles Medium, or MEM-α). Motility assays incubated at 37°C revealed a decrease in swarming with respect to the luxS mutant compared to the NCTC 11168 strain. In addition, analysis of microarray comparing wild-type and luxS mutants indicated that mRNA transcription was dependent upon the broth medium used to cultivate the initial samples. Also, exogenously produced AI-2 was not reported to cause differences in expression between the luxS and wild-type strain (Holmes et al., 2009). Although this decreased swarming on Mueller Hinton soft agar was also observed in another study, using C. jejuni 81-176 luxS null mutant (Delta luxS), in comparison to the wild-type strain, at 42°C indicated that there was no difference (He et al., 2008).

2.3.3 Toxin production

When discussing C. jejuni mediated enteritis, it is typically thought that toxins play a major role in this pathogenesis, and the various toxic activities have been described (Dasti et al., 2010).

The production and subsequent secretion of cytolethal distending toxin (CDT) is important for virulence of a pathogen and a number of species of Campylobacter (e.g. C. jejuni, C. coli, C. fetus) are known to produce this toxin (Mooney et al., 2001; Konkel et al., 2001) which was first observed in a study by Johnson and Loir, (1988). In this study, various cell-lines such as Vero, HeLA, Hep-2, Caco-2 were found to be susceptible as a result of swelling and elongation to approximately five times relative to the normal size was observed. Ultimately, the exposed cells underwent disintegration (Johnson and Loir, 1988; Whitehouse et al., 1998; Lin et al., 2003). Despite CDT being an important virulence factor

for C. jejuni, other cells for instance mouse Y-1 adrenal cells do not exhibit distension when exposed to this toxin from C. jejuni (Johnson and Lior, 1988; Dasti et al., 2010).

2.4 Antimicrobial resistance

Resistance to various components perceived be resultant from efflux mechanisms or plasmid transfer, have been previously documented (Padungton and Kaneene, 2003; Alfredson and Korolik, 2007).

Multidrug efflux pumps, CmeABC and CmeDEF are present in C. jejuni and are thought to be paramount in allowing resistance to be established to a variety of antimicrobial agents (Lin et al., 2002). CmeABC is known to be more widespread in both C. jejuni and C. coli alike, with the CmeDEF being less prolific due to reduced gene expression levels regarding the CmeDEF genes (Payot et al., 2006). Nevertheless, these efflux pumps are said to interact with CmeABC, conferring resistance to antimicrobials and toxic agents. The CmeDEF efflux pump is perceived to function as a secondary efflux mechanism (Akiba et al., 2006).

Insertional mutagenesis of the gene CmeB was seen to decrease the tolerance towards antibiotics including flouroquinolones, erythromycin, chloramphenicol, ampicillin and tetracycline (Lin et al., 2002; Pumbwe and Piddock, 2002). Inactivation of other presumed efflux pumps, for example RND and CmeDEF, were not seen to inhibit resistance to erythromycin, chloramphenicol, ampicillin, tetracycline and ciprofloxacin (Ge et al., 2005; Pumbwe et al., 2005). However, a study by Akiba et al. (2006) showed resistance to the antibiotics ciprofloxacin

and tetracycline with respect to the interaction between the CmeABC and CmeDEF protein operons in various strains. Thus, an inhibition in efflux pumps described in C. jejuni may have promising benefits in curbing antibiotic resistance (Quinn et al., 2007).

Often, fluoroquinolones such as ciprofloxacin are used in the treatment of a patient with symptoms of Campylobacter infection. Despite being effective, fluoroquinolone resistance has been observed in many regions of Europe (Piddock, 1995), South America (Fernandez, 2001), North America (McDermott et al., 2002) and Asia (Murphy et al., 1996).

Resistance to quinolones was investigated in Campylobacter isolates obtained from individuals during a six year time period. The 4953 isolates were tested for resistance to nalidixic acid along with the resistant and sensitive isolates also being tested for ciprofloxacin resistance. The number of isolates resistant to quinolone was found to be 10.2% in 1998 (in comparison to 1.3% in 1992). The increase in resistance of the isolates obtained over the period of 1996-1997 was partially attributable to foreign travel and also the individuals' use of quinolone before stool sample collection. Despite these factors thought to play a role in the increase in resistant population of Campylobacter isolates, the use of quinolone was found to account for a 15% of these cases from 1996-1998, in which the increase in quinolone resistance was also observed in domestic originating samples (Smith et al., 1999). With travel and prior quinolone use being important considerations in the resistance observed, the use of fluoroquinolones in the poultry processing was assumed to have a fundamental role in the increased likelihood of the resistant isolates from human origin.

2.5 C. jejuni Genetics

The concept of bioinformatics, first coined in 1978, involves the study of biological systems utilising computer-based processes (Hogeweg, 1978; Hogeweg and Searls, 2011). The primary use of bioinformatics has been for studying the genetic content within organisms of interest. Essentially, the development of in silico models which show degrees of complementation for in vitro and in vivo experimentation is the proposed outcome of bioinformatics as a whole (Cohen, 2004). With the rising number of completed microbial genome sequences, there is a necessity for the exploitation of the vast quantities of data generated through bioinformatics (Paine and Flower, 2002).

The primary use of bioinformatics lead to the genome sequencing of C. jejuni NCTC 11168 has exposed limited mechanisms by which enable the organism to become diverse from a genetic perspective. C. jejuni NCTC 11168, unlike the vast majority of pathogenic strains which have previously been sequenced contain a very small amount of repeat sequences, transposons or presence of phage associated sequences (Parkhill et al., 2000; Dorrel et al., 2001).

C. jejuni NCTC 11168 was the first Campylobacter species to be sequenced, encompassing a circular chromosome containing 1,641,481 base pairs (bp) encompassing a more recent estimation of 1,642 coding sequences in comparison to the previously value 1,654 originally stated in the first annotation of this organisms genome (Gundogdu et al., 2007; Parkhill et al., 2000). Other strains of Campylobacter have also been successfully sequenced including C. jejuni RM1221 (Fouts et al., 2005) and C. jejuni 81-176 (Hofreuter et al., 2006).

Campylobacter species are recognised as having AT-rich content within their genome along with a low GC content of approximately 32.5%, dependent on species (Taylor et al., 1992; Taylor et al., 1983). The genomic content of C. jejuni NCTC 11168 is very dense in relation to other species which have been sequenced. It has been deduced that 94.3% of this genome is believed to encode proteins. Additionally, the hypervariable sequences can render a high frequency of gene expression changes, thus allowing phase variation of genes responsible for biosynthesis and modification of structural surfaces (Parkhill et al., 2000).

2.6 Chitosan

2.6.1 Antimicrobial activity

With an ever increasing requirement for novel antimicrobials, there are a number of alternatives which may be used in order to meet desired antimicrobial strategies. In recent years, various biomolecules including chitosan have been undergoing integration within the food industry. As there have been numerous outbreaks of bacterial contamination in food products, there is a need for control of pathogens in order to address concerns from a public health perspective. The biodegradability and non-toxic properties which chitosan presents, allow for appropriate use with respect to the food, pharmaceutical, cosmetics and agricultural industries whether alone or in conjunction with other natural antimicrobials. (Dutta et al., 2012, Kim et al., 2005; Mohire and Yadav, 2010; Suman et al., 2011; Gaspar et al., 201; Kong et al., 2010).

Chitosan is a collective term for natural biopolymers derived from a deacetylated precursor; chitin. This biopolymer was first proposed by Allan and Hardwiger (1979) as having a broad antimicrobial activity against a variety of bacterial and fungal species alike, which has led to great scientific interest over recent years (Xu et al., 2007; Kong et al., 2010). Additionally, these biopolymers are integrated into various commercial settings and are of great potential mainly because they are considered GRAS, according to the Food and Drug Association (FDA, 2001).

The aminopolysaccharide chitin is a naturally abundant in nature, being a primary component of an arthropod skeletal structure. Chitin is a copolymer of β-(1-4)-linked N-acetyl-D-glucosamine and D-glucosamine and is difficult to dissolve. However, the deacetylated form, chitosan can be dissolved in various dilute organic acids (e.g. acetic, formic) forming a fluid of a non-Newtonian and shear-thinning nature (Thanh et al, 2007). Moreover, chitosan is the second-most ubiquitous polysaccharide on earth, and is often classed as a derivative of cellulose (the most ubiquitous) despite chitosan being absent from organisms which produce cellulose (Dutta et al., 2004). Structurally, is it identical to cellulose except the acetamide (-NHCOCH$_3$) on the C-2 position (Figure 2.1).

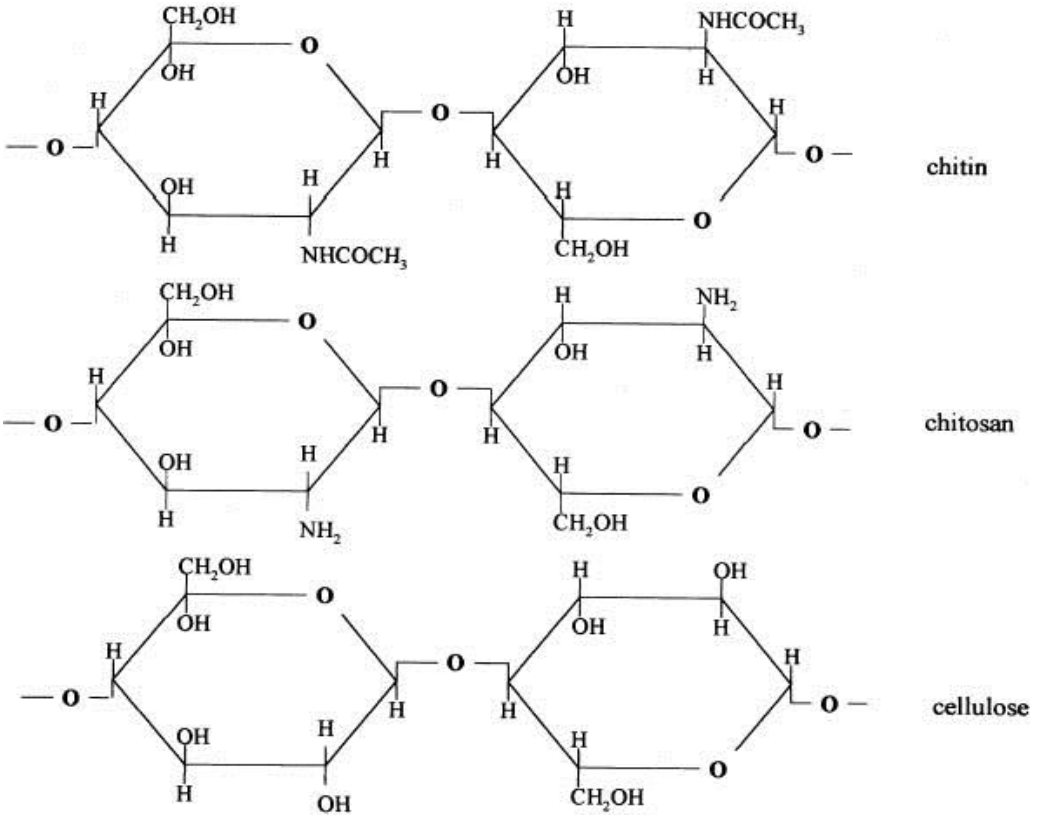

Figure 2.1: The structural similarities of cellulose and chitin, chitosan and cellulose (Allan et al., 1977).

Chitosan is mass produced commercially in many parts of the world including the United States, Poland, India, Norway, Russia and Japan (Singla and Chawla, 2001). Leading on from this, there is an estimated 10^9 - 10^{10} tons of chitosan produced per annum (Peter, 1997). Regardless of the encouraging applications of these biopolymers, there is a limited understanding as to the mode of action of chitosans relating to their antimicrobial nature. It has been argued that the mode of action is due to the cationic (possession of positively charged NH_3 groups) nature, interacting with the negatively charged bacterial cell surface, amounting to a disturbance of microbial activities (Raafat et al., 2008; Helander et al., 2001; Je and Kim, 2006; Zakrzewska et al., 2005). It had also been suggested that characteristics, for instance, water binding capacity, chelation of trace metals and the interaction with DNA may also be involved with the antimicrobial mode of action (Raafat et al., 2008; Rabea et al., 2003).

The antimicrobial efficiency of chitosan biopolymers and their constituent components may be subject to intrinsic and extrinsic factors, notably molecular weight (MW), degree of deacetylation (DD), presence or absence of cations, pK_a, pH and the microorganism exposed (Kong et al., 2010). Generally, it is assumed that chitosan exhibits a higher antibacterial activity towards Gram positive bacteria compared to Gram negative species (Jia et al., 2001).

The utilisation of chitosan in a solid state involves beads, fibres, films and hydrogels which are predominantly aimed towards biomedical perspective use (Kong et al., 2008; Kong et al., 2010). The effects of chitosan in a solid-state have received little attention, including determining the mode of action in this form (Kong et al., 2010).

2.6.2 Molecular weight

The molecular weight (Mw) of chitosan has been shown to influence its antimicrobial efficiency in some studies. For example, an increased antimicrobial efficacy of chitosan was observed with increasing Mw ranging from <5 kDa to 305 kDa against S. aureus. However, the opposite was seen in the case of E. coli, with an apparent decrease in activity with an increase in Mw (Zheng and Zhu, 2003).

Another study tested three chitosans with different Mw which were applied to cotton fabrics. When comparing the antibacterial activity those with a Mw of 100kDa and 210kDa were seen to inhibit S. aureus growth at 0.5% (w/v), whereas with the lower Mw chitosan (1.8kDa), the concentration needed was greater (1.0% (w/v)) for the same level of growth inhibition. A similar trend was

observed when applying these chitosans to E. coli, with a greater concentration needed for the inhibition (0.3% (w/v)) for the 100kDa and 210kDa chitosan, as opposed to a 0.5% (w/v) concentration required for the 1.8kDa chitosan. These findings indicated an increase in bacterial growth inhibition with an increase in Mw (Shin et al., 2001).

Another experiment involving six different types of chitosan including six oligomers, of different Mws were tested against a variety of Gram positive and Gram negative bacteria. All species illustrated growth inhibition, which appeared to be Mw and species dependant. According to the MIC values, in the case of the Gram negative bacteria, chitosan with a greater Mw (470 kDa) was seen to have a greater antimicrobial effect, in contrast to chitosan of 1106 kDa. In contrast, 1106kDa was seen to be inhibit Lactobacillus plantarum, Lactobacillus bulgaricus and Lactobacillus brevis to a greater extent (MIC 0.05-0.08% (w/v)), compared to a smaller Mw of 470 kDa, with an MIC value of 0.01% (w/v) or over required for inhibition. Interestingly, this trend was not observed with the extremes in chitosan Mws. Generally, when comparing the MIC values regarding chitosan of 1671 kDa and 28 kDa Mw, values were found to be identical, for instance the values the Gram negative species, Pseudomonas fluorescens having an MIC of 0.1% (w/v). This was also the case with the Gram positive species, Listeria monocytogenes (No et al., 2002).

In addition, an antibacterial study on E. coli was carried out with chitosans of different Mws (5.5×10^4 to 15.5×10^4 Da) with a similar degree of acetylation (80%±0.29). Antimicrobial action was observed at concentrations greater than 200ppm (0.02% (w/v)), and growth below this. The antimicrobial activity

appeared to be relative to the Mw, as previously described. Here, the lower the Mw, the higher the antimicrobial effect against E. coli. Additionally, the activity of chitosan was also found to be Mw dependant at lower concentrations ranging from 50 to 100 ppm. At the median Mw (9.0×10^4 Da) bacterial reduction was not apparent. Also, when introduced earlier with respect to growth stages, a greater activity of chitosan was observed. The mode of action of chitosan against this particular microorganism was deduced as flocculation (Liu et al., 2006).

In a study assessing the antimicrobial efficiency of three different chitosans, each with different Mws against three Gram positive and six Gram negative species of bacteria; the Gram negative species were found to be less susceptible and thus indicative of a lower antimicrobial activity of the chitosans. However, the most sensitive Gram negative species was found to be Campylobacter spp., independent of chitosan Mw. Additionally, the MIC of the chitosans against the Campylobacter spp. varied from 0.005-0.05% (w/v), indicating a high sensitivity towards these biopolymers. The physical effects to the bacterial cells upon exposure of chitosan were indicative of a loss in the membrane integrity. This study highlights the promising antimicrobial effects of the chitosan towards Campylobacter spp. as well as the other species (Ganan et al., 2009).

The findings presented above highlight ambiguous results when comparing the antimicrobial activity based on Mw. Also, these effects appear to be heavily dependent on species. No dissimilarities in antimicrobial activity when comparing a Gram positive and Gram negative species have also been observed, with E. coli and Bacillus subtilis having equal sensitivities regardless of the Mw of chitosan used (Tikhonov et al., 2006).

2.6.3 Degree of deacetylation (DD)

In an investigation using chitosan as a membrane purposely for separation engineering was undertaken, the antimicrobial activity was assessed via MIC values. When comparing the two organisms S. aureus and E. coli, the MIC values were approximately 200 and 40 (mg-chitosan/ml-bacterial suspension), respectively. Here, a greater antimicrobial activity of chitosan towards Gram positive was observed. Furthermore, this inhibition was recognised as bactericidal in both species, with this activity being reliant upon differences in membrane surface area and shape. Importantly, the DD seemed to alter the amount of growth, with the higher DD being indicative of greater inhibition. This highlights the dominant role of DD in the overall activity of chitosan biopolymers and useful applications which may arise when considering chitosan membranes (Takahashia et al., 2008).

2.6.4 pH

The antimicrobial activity of chitosan has been shown to vary with regards to the pH. The effect of lowering pH is considered to act synergistically with chitosan. This therefore challenges microorganisms with two hurdles, the antimicrobial effect of chitosan and acid stress (Aider, 2010; Rhoades and Roller, 2000).

Water-solubility of chitosan is important when considering antimicrobial activities. Due to its poor solubility above pH 6.5, antimicrobial effects are thought to only be observed with an acidic medium/environment (Li et al., 2002). Hence, the antimicrobial activity is inversely affected by an increase in pH, with an antimicrobial efficacy being greater at lower pH values (Qi et al., 2004).

According to some findings, the reduction or complete loss of antimicrobial activity at pH 7 can potentially be the result of decreased solubility of the chitosan during preparation and also a greater abundance of amino groups that are positively uncharged (Aiedeh and Taha, 2001; Papineau et al., 1991; Sudarshan et al., 1992).

2.6.5 Mode of action as an antimicrobial

Antimicrobial mechanisms of chitosan have been postulated to involve the interaction between the substance and bacterial cells outer cellular components, the cytoplasmic membrane and the cytoplasmic constituents (Raafat et al., 2009).

Interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes are thought to contribute to leaks with respect to various intracellular contents (Rabea et al., 2003). At lower concentrations (<0.2 mg/ml), chitosan is perceived to bind to the negatively charged surface of the cell membrane, causing agglutination. Contrary to this, higher concentrations may give rise to a larger number of positive charges which may cause an overall net charge which is positive, thus keeping them suspended into solution (Rabea et al., 2003).

Chitosan is also considered as a chelating agent whereby trace metals are bound and subsequently inhibit aflatoxin production in Apergillus spp. and also the microbial growth via the limitation of nutrient availability (Cuero et al., 1991). It is also postulated that chitosan can also interact with DNA. This is then thought

to interfere with successive mRNA synthesis and thus protein production (Sudarshan et al., 1992).

2.6.6 Applications of Chitosan

2.6.6.1 Chitosan films

Chitosan coatings are proven not only to be edible but also low cost, non-toxic, non-polluting, aesthetically acceptable, providing a barrier to gasses and illustrate biocompatibility (Vásconez et al., 2009). As a result of these desirable characteristics/properties, chitosan films have been produced with particular application in the food industry and their ability to be utilised as food coatings for instance can allow for extended shelf life of numerous food related products (Aider et al., 2010).

Antimicrobial packaging is one aspect of the process of active packaging. Active packaging serves as providing attributes aside from the more conventional remedial barrier properties used. Ingredients are added in the packaging system such as functional polymers (Flores et al., 1997; Han and Rooney, 2002).

The use of antimicrobial packaging provides this inhibition or killing of spoilage and/or pathogenic microorganisms, which present problems both from a public health and a financial standpoint. In comparison to the traditional food packaging outcomes including extending shelf life, maintenance of quality and safety assurance, antimicrobial packaging primarily focuses upon controlling microbial load on foodstuffs. Generally these microorganisms are detrimental to

the above goals, as foodstuffs which have a naturally prolonged shelf life do not often require an antimicrobial packaging system (Han, 2000).

Antimicrobial action of edible films composed of chitosan and incorporated garlic oil was investigated against a number of food pathogens (E. coli, S. aureus, Salmonella typhimurium, Listeria monocytogenes and Bacillus cereus). To provide a comparison to the above test conditions the conventional food preservatives potassium sorbate and nisin were also tested. Integration of garlic oil from as low as 100 µl/g, potassium sorbate at 100 mg/g or nisin at 51,000 IU/g of chitosan was seen to be active against all of the species tested except E. coli. At the concentrations the films were classed as being acceptable with respect to visual, mechanical and physical properties. Also, the garlic oil incorporation did not appear to alter either the physical or mechanical properties of the chitosan films, since it did not interact with chitosan in the film, as measured by Fourier Transform Infrared spectroscopy (FTIR) (Pranoto et al., 2005).

The use of chitosan alongside other antimicrobials has resulted in synergistic effects in some cases which can be more appropriate for use in some applications. For instance, the antimicrobial effectiveness of the combination of chitosan and essential oil (EO) was identified both in vitro and on processed meat products against L. monocytogenes and E. coli O157:H7 using the agar diffusion assay. The four EOs tested were seen to have the following activity from higher to lowest: oregano >> coriander > basil > anise. Consequentially, chitosan films containing oregano were added onto sausage bologna (an Italian delicacy) samples inoculated prior to application and stored for 5 days at 10^{o}C. Films which were exclusively composed of chitosan were effective producing a 2

log reduction of L. monocytogenes and films with 1% and 2% oregano accounted for 3.6 and 4 log reductions, respectively and a 3 log reduction E. coli. Interestingly, the addition of oregano resulted in the decreases in tensile strength, permeability of water and rigidity of the films. These types of films may be applicable in food preservation as they showed promising antimicrobial activities against pathogens and have further benefits due to their biodegradability (Zivanovic et al., 2006).

2.6.6.2 Chitosan Gels

Hydrogels are also a promising application of chitosan partly due to the additional high water content and the similarity to human tissues allowing them to be potentially useful for a number of biomedical applications since they are able to control bleeding, aid the conservation of tissue orientation and prevent adhesions, they lend themselves to use in postoperative healing processes (Aziz et al., 2012; Lim and Hudson, 2004).

Chitosan dextran-based (CD) hydrogels have been investigated for application such as endoscopic sinus surgery. The antimicrobial effect was tested against a range of microorganisms which can cause pathogenesis. CD hydrogels were found to be successful in terms of antimicrobial activity against S. aureus, E. coli, Streptococcus pyogenes and Clostridium perfringens when used at a surgical concentration of 50,000 mg/L. Scanning and transmission microscopy revealed cell wall damage, morphological disruption and loss of cytosolic contents which the hypothesis that chitosan binds to cells wall proteins and disrupt peptide bonds (Aziz et al., 2012).

Additionally, a study carried out by Ballal et al. (2009) analysed the antimicrobial capacity of 2% chlorhexidine (chemical antiseptic) and 2% chitosan (alone and together) gels against Candida albicans and Enterococcus faecalis. Initially, inoculae of the microorganisms was used in order to generate bacterial and fungal lawn on nutrient rich agar. Wells were made in each type of agar plate and the gels were incorporated and incubated for 37°C for 48 hours. Interestingly, chlorhexidine release was greater in absence of chitosan (determined by UV spectrophotometry). Also, the greatest antimicrobial effect was observed when the combination of chitosan and chlorhexidine was present in the gel (25.2mm zone of inhibition for C. albicans and 26mm for E. faecalis). Chlorhexidine alone was seen to have the next highest antimicrobial activity (20.6mm and 21.4mm, respectively), and chitosan the lowest (16.6mm and 11.0mm, respectively). Thus, chitosan gels both alone and in conjunction possess antibacterial and fungal effects may be of use in order to curb microbial persistence.

2.6.6.3 Bioremediation and Drug Delivery

Chitosan can also be exploited as a means of removing various substances which are not desirable in specific systems, for instance water remediation has a great An example involved the enzymatic removal of phenol compounds from model wastewater and the synergistic effect of mushroom tyrosinase with chitosan beads. Variables including pH, temperature, dosage of tyrosine and also the hydrogen peroxide-to-substrate ratio were all considered. A chitosan film incorporated in a p-crersol and tyrosinase mixture was found to adsorb quinone derivatives, typical of greater adsorption with the onset of immersion. Additionally, chitosan beads were also seen to adsorb the quinone derivatives

which were enzymatically generated. The percentage removal of the various phenol compounds increased to 93% when chitosan beads were used as opposed to the films (Yamada et al., 2005).

2.6.6.4 Nanotechnology

Nanomedicine as a whole consists of components at a nanometre-scale size used for a defined goal relating to diagnosis, treatments or prevention of disease (EMRC, 2004). Generally the nano-scale encompasses a size range from 1 to 100's of nanometres. Nanotechnology in a medical sense usually concerns drug carriers in the form of particles, liposomes and so forth, that play an important role in the delivery of active compounds (I.e. drugs), intended to target various sites within the body (Nagpal et al., 2010).

In order to treat a number of diseases, peptides and proteins have become the drugs of choice due to their advantages of high selectivity and subsequent effective action with fewer side effects (Frokjaer and Otzen, 2005). However, degradation of peptide and protein drugs occurs readily, as they are susceptible to enzymes found within the patient along with decreased permeability across the ileum (Zhang et al., 2010). In an attempt to combat this, the efficiency of oral uptake of drugs has been tested using various surfactants, protease inhibitors, enteric coatings, carrier systems and permeation enhancers (Nakashima et al., 2004; Carino et al., 2000).

Chitosan has been reported to be an appropriate candidate for use in nano and microparticles with respect to controlled drug release their advantages include

good stability, low toxicity, simple and mild preparation methods as well as providing versatile routes of administration (Tiyaboonchai, 2003).

Mucoadhesive nanoparticles have received much attention and have promising concepts (Takeuchi et al., 2001). In this case the degree of mucoadhesion relies on the particle size and also the superficial charge. Chitosan occupies this mucoadhesive property due to the cationic polyelectrolyte of this particular polysaccharide and has the ability to attach to the mucosal surface and consequently open the junction between epithelial cells (Qian et al., 2006).

With the somewhat limited application of chitosan due to restricted solubility in acidic conditions, the use of water-soluble chitosan has also been studied. This type of chitosan is easily soluble in neutral aqueous solution. With the advantage easily modified, it can be utilised in a variety of applications including as a gene or peptide drug carrier. Nanoparticles consisting of water-soluble chitosan with sodium tripolyphosphate were tested with respect to the efficiency of uptake of bovine serum albumin (BSA) as the model drug. In vitro results indicated that the nanoparticles enhanced and prolonged the intestinal absorption of BSA. These results also indicated that these nanoparticles could be used as a potential protein delivery system (Zhang et al., 2010).

2.6.6.6 Available chitosan-related products

A number of products are commercially available which use the characteristics of chitosan. Examples include Tegasorb™ (3M, USA) and Chitopack (Eisai, Japan) for wound-healing, Syvek NT Patch (Marine Polymer Technologies, USA) and Chito-Seal (Abott Vascular Devices, USA) for haemostatic dressing. Chitosan

hard capsules (Aicello Chemical, Japan) have also been produced for gastrointestinal drug delivery (Thanh et al., 2007).

2.7 Aim and Objectives

2.7.1 Aim

To identify any potential mechanism(s) of survival of Campylobacter jejuni against sub-lethal exposure to low molecular weight chitosan.

2.7.2 Objectives

- *Develop an isolate of C. jejuni with an enhanced tolerance towards chitosan, relative to the parental wild-type strain.*

- *To establish the antimicrobial activity of chitosan against several C. jejuni strains/isolates under various environmental conditions.*

- *To establish any phenotypic differences of C. jejuni strains/isolates in order to gain knowledge of how the organism grows/functions and may be inhibited upon exposure to chitosan.*

- *To analyse the potential transcriptional differences of C. jejuni after exposure to low molecular weight chitosan using molecular biological techniques.*

- *To utilise bioinformatics tools to aid the analysis of the transcriptomics between the wild-type and 'adapted' isolates of C. jejuni NCTC11168.*

Chapter 3 - Materials and Methods

All chemicals were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) or Fisher Scientific UK Ltd (Loughborough, UK). Bacterial culture media was acquired from either Fisher Scientific UK Ltd (Loughborough, UK) or Oxoid Ltd (Basingstoke, UK). Identification kits were purchased from BioMérieux UK Ltd (Hampshire, UK) and Molecular biology kits from Qiagen, UK).

3.1 Bacterial strains and growth conditions

Campylobacter jejuni NCTC 11168 and NCTC 11828 (81116) were obtained from the National Collection of Type Cultures (NCTC), Health Protection Agency Culture Collection (Wiltshire, UK). Cells were incubated under microaerobic conditions at 42°C in both liquid and solid media for appropriate time periods unless stated otherwise.

Campylobacter jejuni RM1221 and 11168CH were kindly provided by Prof. Julian Ketley (University of Leicester) and were cultivated in the same way as described above.

3.2 Liquid media

3.2.1 Mueller-Hinton broth

Mueller-Hinton broth (CM0405: Oxoid Ltd, UK) was added at a concentration of 1.05g in 50ml of distilled water, mixed to dissolve completely prior to sterilising by autoclaving at 121°C for 15 minutes.

3.3 Solid media

3.3.1 Mueller-Hinton agar

Nineteen grams of Mueller-Hinton agar base (CM0337: Oxoid Ltd, UK) was added to 500ml of distilled water, dissolved entirely and sterilised by autoclaving at 121°C for 15 minutes. The agar was then allowed to cool to 50°C before the addition of 25ml of Defibrinated horse blood (SR0050: Oxoid Ltd, UK) giving a final concentration of 5% (v/v) of blood. After mixing, the agar was poured into sterile Petri dishes.

3.3.2 Campylobacter Blood-free Selective Medium (Modified CCDA-Preston)

Campylobacter Blood-Free Selective Agar Base (CM0739: Oxoid Ltd, UK) was added in a concentration of 22.75g in 500ml of distilled water and brought to the boil in order to dissolve. The media was then sterilised by autoclaving at 121°C for 15 minutes, cooled to 50°C prior to 1 vial of CCDA Selective Supplement

(SR0155: Oxoid Ltd, UK) being aseptically added, mixed thoroughly and then poured into sterile Petri dishes.

3.3.3 Agar Bacteriological

Bacteriological agar was prepared at concentrations at 0.8% (w/v) and 0.4% (w/v) with 0.8g and 0.4g per 100ml of distilled water which was then sterilised by autoclaving at 121°C for 15 minutes, cooled to 50°C and used accordingly.

3.4 CCDA Selective Supplement

CCDA Selective Supplement (SR0155: Oxoid Ltd, UK) was suspended by addition of 2ml of sterile distilled water using aseptic techniques according to manufacturer's instructions

3.5 Phosphate Buffered Saline (PBS)

Five PBS tablets (BR0014: Oxoid Ltd, UK) were dissolved in 500ml of distilled water, nine hundred microliter aliquots were transferred to micro-centrifuge tubes (Fisher Scientific, UK). These were then sterilised by autoclaving at 121°C for 15 minutes. PBS not distributed into mini-centrifuge tubes following sterilisation was stored at room temperature.

3.6 Chitosan

A 1% (w/v) stock solution of low molecular weight chitosan (448869: Sigma-Aldrich Company Ltd, UK) was prepared by dissolving 1g of 100ml diluent. Acetic acid (1% v/v) was used to dissolve the chitosan powder following titration to pH 5.5, 6.0 and 6.5 with 2% (w/v) NaOH. The mixture was then filter sterilised and refrigerated at 4°C until required for experimental purposes.

3.7 Culture techniques

3.7.1 Sub-culturing

Campylobacter jejuni NCTC 11168 was sent as a freeze-dried ampule and subsequently reconstituted as directed by the HPA (UK) guidelines. C. jejuni strains were aseptically streaked onto both Mueller-Hinton agar with 5% (v/v) defibrinated horse blood and Campylobacter selective agar following incubation at 42°C for 48-72 hours under microaerobic conditions utilising CampyGen gas generation sachets and Oxoid gas jars. Cultures were maintained by repeated re-streaking Mueller-Hinton agar for a maximum of three times, provided prior growth on the selective media was positive and incubated as above.

3.7.2 Long-term storage of cultures

Stock cultures of C. jejuni were stored at -80°C in vials containing Microbank beads (ProLab Diagnostics, Merseyside, UK) or in vials containing 20% glycerol and 80% PBS. Several colonies from Mueller-Hinton agar plates were aseptically picked using a sterile inoculating loop and added to respective vials. With regards to the Microbank beads vial, these were then closed and inverted a

number of times according to product guidelines after which the cryopreservation fluid was then aseptically removed by pipetting, closed and stored as above until needed. For glycerol stock vials, 800µl of suspension containing culture was aseptically added to 200µl of sterile glycerol, briefly vortexed and then stored as stated above.

3.7.3 Recovery of Campylobacter jejuni from Microbank beads

After three consecutive re-streaks on Mueller-Hinton agar plates enriched with 5% (v/v) defibrinated horse blood, Campylobacter jejuni cultures were regenerated from Microbank beads limiting phenotypic variation through over-passaging. Here, aseptic removal of a Microbank bead or a 100µl of suspension in the case of the glycerol stocks were re-streaked and spread onto Mueller-Hinton agar plate, respectively. These plates were then incubated under microaerobic conditions at 42°C for 48-72 hours after which plates were checked for correct morphology, Gram stained and re-streaked onto Campylobacter selective agar plates when necessary. Colonies were then treated as described in section 3.7.1 above.

3.7.4 Recovery of bacterial cultures from previous plates

On a weekly basis a single colony from a previously refrigerated stock Mueller-Hinton agar stock plate was aseptically streaked onto another agar plate (as before and all through) using a sterile inoculating loop and incubated in microaerobic conditions for 48-72 hours at 42°C.

3.7.5 Culture purity

Purity of cultures was routinely checked and C. jejuni was identified through Gram staining using light microscopy, KOH string test, oxidase test, catalase test and the use of API Campylobacter test strips (BioMérieux UK Ltd, Hampshire, UK). Other features including size and morphology were observed using microscopy. All of the stated tests were carried out following standard protocols and guidelines which in the case of the API identification kit, were provided. Alternatively, cells were streaked onto mCCDA agar, following incubation for 72 hours under microaerobic conditions (42°C).

3.8 Standard experimental procedures

3.8.1 Culture preparation

Cultures were prepared by aseptically transferring several colonies from a sub-cultured a Mueller-Hinton agar plate, to 50ml of Mueller-Hinton broth. The broth was incubated with shaking at 150rpm at 42°C under microaerobic conditions for 24 hours.

3.8.2 Cell suspension preparation

After 24 hours incubation cells were suspended in PBS to prevent organic matter interference. The culture-containing PBS suspension was centrifuged at 10,000 x g for 5 minutes at 25°C. Following centrifugation the supernatant was decanted and cell pellet re-suspended in 50ml of PBS by the use of a vortex.

3.8.3 Enumeration

Following serial dilutions in PBS cells were plated via the spread plating, Miles and Misra (Miles, Misra and Irwin 1938) or spiral plating method; after which plates were incubated at 42°C under microaerobic conditions for 72 hours and enumerated.

3.8.4 Spread plating

Unless stated elsewhere; 100µl of cell suspension was pipetted onto agar plates under aseptic conditions and evenly distributed across the plate surface and allowed to dry before being incubated at 42°C under microaerobic conditions and subsequently enumerated for 48-72 hours.

3.8.5 Drop plating

Twenty microliters of cell suspension from the relevant serial dilutions were aseptically pipetted onto pre-set agar plates in triplicate. Similarly to the above, drops were allowed to dry before being incubated at 42°C under microaerobic conditions and enumerated for 48-56 hours (Miles, Misra and Irwin, 1938).

3.9 Specific experimental procedures

3.9.1 Growth curves

Growth curves were carried out by inoculating 50ml Mueller-Hinton broth in centrifuge tubes with several colonies of C. jejuni. After inoculation, the capped

were loosened as to allow for gaseous exchange when placed in a gas jar. A CampyGen sachet was placed inside the gas jar and the lid closed immediately following incubation at 42°C for a total of 78 hours. One millilitre aliquots were then taken at various time intervals and diluted in PBS accordingly. These dilutions were then plated on Mueller-Hinton agar (Oxoid Ltd, UK) using the Miles, Misra and Irwin method (1938) and incubated at 42°C under microaerobic conditions for 72 hours.

3.9.2 Determination of Minimum Inhibitory Concentration (MIC)

3.9.2.1 Agar dilution method

The MIC of chitosan at pH 6.0 and 6.5 was determined using the agar dilution method, adapted from Wiegand et al. (2008). This involved a series of sterile Petri dishes having a homogenous mixture of Mueller-Hinton agar, 5% (v/v) defibrinated horse blood and sterile 1% (w/v) chitosan stock solution according the desired final test concentration.

One hundred microliter aliquots of an overnight culture of C. jejuni grown in Mueller-Hinton broth was added to each pre-set agar plates at a final concentration of 5 x 10^5 CFU/ml followed subsequent spreading, drying and incubation under microaerobic conditions at 42°C for 72 hours. The MIC value was determined as the concentration which no visible growth was observed.

3.9.2.2 Broth micro-dilution method

The broth micro-dilution method for determination of MIC was undertaken following an adapted method from Wiegand et al. (2008). Briefly, a doubling

concentration range of low molecular weight chitosan was incorporated in MH broth at a final pH of 5.5, 6.0 and 6.5.

Briefly, a $2x10^9$ overnight culture was diluted to a final starting count of $5x10^5$ CFU/ml and incubated at 42°C under microaerobic conditions for 48 hours and subsequently plated. The MIC value was determined as the lowest concentration required for no visible growth.

3.9.2.3 Minimum bactericidal concentration

In parallel to MIC values generated, the minimum bactericidal concentration (MBC) was carried out. Essentially, tubes which showed no visible growth were subject to MBC determination via three 100µl aliquots being spread onto separate MH broth plates supplemented with 5% (v/v) defibrinated horse blood, containing no chitosan. These plates were then incubated for 48-72 hours at 42°C under microaerobic conditions. After incubation, the MBC was determined as the lowest concentration in which no growth was present on the agar plate. Sterility controls, growth control and tubes containing the cells which were subjected to the concentrations tested were included for verification purposes.

3.10 Molecular techniques

3.10.1 Primer specificity

Forward and reverse primers were designed using the NCBI Primer-Blast software. Primer sequences were subsequently purchased from MWG Eurofins and were re-suspended in molecular grade Water to a stock concentration of

100pmol/µl. These stocks were further diluted to give a 10pmol/µl working stock concentration.

3.10.2 Polymerase Chain Reaction (PCR)

Endpoint PCR was performed using a Techne TC-512 thermal cycler (Bibby Scientific Ltd, Staffordshire, UK).

3.10.3 Gel electrophoresis

Samples were loaded in wells within a 1.2% (w/v) pre-set agarose gel. Samples were run for 30 minutes at 80v and carefully removed from the gel tank, following visualisation using a Gel Dock (SynGene).

3.11 Statistical analysis

All statistical analysis was undertaken using IBM SPSS Statistics version 20 (SPSS Science Inc., Chicago, Ill, USA) for Windows with a significance set at p = 0.05. Assumptions of normality were assessed using the Kolmorgorov-Smirnov test with variances of homogeneity checked by utilising Levine's test. Providing all assumptions of a normal Gaussian distribution were met, individual t-tests or one-way analysis of variance (ANOVA) was carried out. In the case of data analysed by ANOVA, additional post-hoc testing (Tukey correction) was used to determine significant differences between groups mean data.

Chapter 4 – Generation of adapted isolate to low molecular weight chitosan

4.1 Introduction

Campylobacter jejuni is often isolated from foodstuffs, despite the ongoing efforts currently employed to control this microorganism in the food industry. Foodstuffs which have been derived from animal origin, particularly poultry are thought to serve as the main vehicle of infection in humans (Blaser et al., 1983). The percentage of contaminated/infected broiler chickens at time of slaughter is currently high, allowing for the opportunity for C. jejuni to spread from the initial intestinal region (initial point of colonisation), to the carcasses. Multiple studies have revealed contamination in both frozen and fresh poultry alike, highlighting a major concern for both the public health and the poultry industry (Giessen et al., 1992).

Antimicrobial resistance is considered a significant problem with respect to food-borne pathogens such as C. jejuni which can amount to a serious problem (Mavri and Možina, 2012). Numerous biocides including triclosan, chlorhexidine and trisodium phosphate are commonly utilised in the prevention of bacterial contamination during food processing and also in a clinical setting (McDonnell and Russell, 1999; Møretrø et al., 2011).

However, despite the usage of multiple biocides and disinfectants, elimination of food-borne pathogens is somewhat challenging due to under-dosing of the chosen disinfectants and also the lack of inadequate cleaning of surfaces prior to

disinfection, reducing the overall antimicrobial efficiency (Mavri and Možina, 2012).

Exposure to sub-lethal concentrations of a given antimicrobial agent may increase the likelihood of adaptation of a more susceptible bacterium (Davidson and Harrison, 2002; Mavri and Možina, 2012).

The term resistance can be defined in several ways, Holah et al. (2002) describes this as the ability for microorganisms to become more suited to repetitive cleaning and disinfection programmes, leading to increased survival, which can be applied to antimicrobials/biocides use in general.

According to Fernández and Hancock (2012), there are three key types of resistance which can arise, intrinsic, acquired and adaptive. Intrinsic relates to the innate properties which limit a microorganism's susceptibility. Acquired resistance is achieved whereby incorporation of novel genetic material (e.g. plasmids and transposons) or mutational changes ensues. Lastly, adaptive resistance involves the temporary increase in survival due to changes in gene and/or protein expression through stressors namely, nutrient depletion and sub inhibitory levels of a given antimicrobial.

Rajkovic et al. (2009) explains that when disabling a proportion of the total microbial load, it is feasible that either individual cells or sub-populations of various pathogens can develop resistance, influencing the degree of persistence and subsequent virulence characteristics of these cells.

Moreover, injury of microorganisms can also be induced by various treatments involving sub lethal heating, freezing, drying, irradiation, aerosolisation, high hydrostatic pressure, antibiotics, sanitising compounds and other chemical and/or natural compounds which possess antimicrobial properties. With injury comes the potential issue of the microorganisms repairing themselves under appropriate conditions (Wu, 2008). The population of microorganisms which are able to survive the sub lethal onslaught from these treatments can give rise to either dead cells which are lethally injured or are subject to irreversible injury, seemingly uninjured cells and injured cells which are subsequently stress 'hardened', typical of reversible injury (Wu, 2008).

Although food-related antimicrobials have been used for many years there is limited data with respect to the development of microbial resistance for these compounds (Davidson and Harrison, 2002). The lack of data may suggest that the issue of microbial resistance is a rare occurrence and/or that this does not pose much of an issue. However, concerns remain because resistance is increasingly found against these antimicrobial compounds and sanitizers used to control pathogen growth in foods. Tolerance to antimicrobials, sanitizers and currently used in preservation processing may also be heightened through exposure to other stresses (Davidson and Harrison, 2002).

Therefore, it seems necessary to assess the ability for C. jejuni to develop an adaptive in response to chitosan, if this biopolymer is to be used as an effective control measure in such scenarios. By assessing a potential adaptive response which may result from chitosan challenge may provide additional information as to the role of stress induced adaptation on antimicrobial resistance (Kumar-Phillips, 2013).

4.2 Aims and Objectives

4.2.1 Aims

The aims of the study outlined in this chapter were to investigate the potential ability of C. jejuni to survive low molecular weight chitosan exposure and to highlight any adaptation to sub lethal exposure to this natural antimicrobial.

4.2.2 Objectives

- *To identify the differences in Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) for several C. jejuni isolates/strains.*

- *To assess the sub-lethal intensities needed for inhibition of C. jejuni cells.*

- *To assess the ability of C. jejuni cells to adapt to low molecular weight chitosan following stepwise training of cells.*

4.3 Methods

4.3.1 MIC Agar dilution method

Determination of the MIC of chitosan (pH 6.0) was determined as in section 3.9.2.1.

4.3.2 MIC Broth micro-dilution method

The broth micro-dilution method for determination as described in section 3.9.2.2.

4.3.3 Minimum bactericidal concentration (MBC)

In parallel to MIC values generated in MH broth, the MBC was carried according to section 3.9.2.3.

4.3.4 Determination of Treatment intensities

Firstly, the sub lethal treatment intensities were identified based on a 1-5 log cfu/ml reduction upon exposure to chitosan, centred on in-food reductions which were deemed realistic. These concentrations were then used in an attempt to generate resistant sub-populations of C. jejuni through exposure to low molecular weight chitosan. Methods adapted from Rajkovic et al. (2009) were carried undertaken.

Overnight cultures of C. jejuni NCTC 11168 were prepared as described in section 3.8.1 and aliquots transferred to tubes containing 10ml of MHB with chitosan present (pH 6.0) giving a final starting concentration of $2x10^7$cfu/ml.

These cultures were then incubated at 42°C for the time needed for a reduction in cell counts of 1-5 log cfu/ml.

After chitosan exposure, 100µl aliquots were then removed and were subjected to serial dilution in PBS and subsequent enumeration using the drop plating method (Miles et al., 1938). A second 100µl aliquot was also removed and added to fresh MHB containing no chitosan and incubated for 24 hours under standard conditions as described previously. Upon re-growth, the exposure of bactericidal concentrations of chitosan was repeated for a total of 20 cycles with plating and enumeration being undertaken after each point to determine potential deviation in the log reduction when compared to the first inactivation data for day 1 to assess potential resistance. This experiment was repeated several times on separate occasions.

4.3.5 Adaptation to sub lethal exposure via stepwise training

In a further effort to generate low molecular weight chitosan adapted strains against, a method similar to that described in Mavri and Možina (2012) was employed. Briefly the C. jejuni NCTC11168 strain underwent challenge to increasing concentrations of chitosan (pH 6.0) over a course of 15 days. This particular strain was chosen, as it occupied the lowest pre-determined MIC of all strains/isolates tested. Overnight cultures of C. jejuni NCTC11168 were prepared from the addition of several colonies into fresh MH broth following 24 hours incubation at 42°C under microaerobic conditions with shaking at 75rpm. After 24 hours, 100µl samples of the culture contents were added to tubes containing 10ml of MHB with sub-lethal concentrations of low molecular weight chitosan (pH 6.0), which were then incubated under the same conditions as previously

described. Cultures were then assessed for visual growth and 1ml samples were transferred to progressively higher sub-lethal concentrations, following the same incubation procedure. This was repeated for a total of 15 passages with subsequent increase as follows: 0.00004%, 0.00006%, 0.00008%, 0.00010%, 0.00020%, 0.00040%, 0.00060%, 0.00080% and 0.0010% (w/v) chitosan.

The potential adaptive resistance was determined after 5, 10 and 15 hours exposure to the gradually increasing concentrations of low molecular weight chitosan in MHB (pH 6.0). Here the MICs were carried out as previously described to highlight any changes present in relation to the control cells which had not been subjected to such stepwise exposure (control cell MICs determined in parallel). An alternative method of stepwise training was also used following the same basic principle as above, but using MH agar medium with supplementation of 5% (v/v) defibrinated horse blood.

In these instances, C. jejuni NCTC 11168 cells were subjected to sub-lethal concentrations of chitosan following a step-wise training. Cells were grown under the conditions mentioned previously for 72 hours after which the contents of the plates were partitioned by carefully swabbing plates with the increased concentration of chitosan, plates with the same concentration and also on plates containing chitosan-free plates in the potential event of no visible growth in the others two cases.

Cells generated through this method presenting an altered phenotype (visible growth above the wild-type MIC value) were stored temporarily on agar containing the maximum concentration of chitosan which permitted of growth. Multiple stocks were also prepared via transferring colonies from chitosan

containing plates and aseptically added to 20% (v/v) glycerol and 80% MH broth. Regular plating of wild-type C. jejuni NCTC 11168 on plates incorporating chitosan was performed in order to ensure the strain was not able to grow on the selective medium, which allowed for adapted cell growth.

4.3.6 Determination of adaptive resistance via comparison of Minimum inhibitory concentration.

Any adaptive resistance resulting from sub lethal exposure to low molecular weight chitosan was subject to MIC determination in parallel to the control strain, which was cultivated in chitosan-free medium.

4.3.7 Determination of stability of adapted resistance

In order to test the stability of any adaptive responses, the adapted isolate was repeatedly sub-cultured on MH agar supplemented with 5% (v/v) horse blood containing no chitosan (pH 6.0). Cultured cells from this agar were then suspended in PBS and subsequently plated onto MH agar with and also the addition of chitosan (pH 6.0) following incubation under microaerobic conditions (42°C). This was repeated until no growth was present on the chitosan containing agar. The MIC was determined for the control isolate during this passage and also plated on non-selective and chitosan containing agar in parallel to the adapted isolate. Moreover, the culture purity was analysed at each stage with plating onto mCCDA selective agar with the appropriate antibiotics needed.

4.4 Results

4.4.1 MIC/MBC data

In order to obtain a general indication of the antimicrobial nature of low molecular weight chitosan, it is useful to perform MIC/MBC assays in both agar and broth medium.

From the data presented in Table 4.1 the lowest MIC value was observed in the wild-type C. jejuni NCTC 11168 strain (0.0016% (w/v)) concerning pH 6.0 MH broth. This was followed by the RM1221 strain (0.0022% (w/v)), with the highest MIC found in both C. jejuni 81116 strain and the NCTC 11168CH isolate (both 0.0032% (w/v)). Interestingly, the MBCs for the wild-type NCTC11168, RM1221 and 81116 strains were consistent (0.0032% (w/v)) after 24 hours incubation at pH 6.0. The greatest MBC was seen for the isolate which had previously undergone passage through a chicken intestinal tract (NCTC11168CH). Due to the increase in low molecular weight chitosan concentration when comparing the MIC to the MBC for each isolate/strain (excluding the 81116 strain), it can be assumed that the antimicrobial action of chitosan is bacteriostatic.

Table 4.1: MIC and MBC values of C. jejuni strains/isolates when exposed low molecular weight chitosan (pH 6.0) in MH broth (n = 6, triplicate).

Isolate/strain type	*MIC (% (w/v))*	*MBC (% (w/v))*
C. jejuni wild-type NCTC11168	*0.0016*	*0.0032*
C. jejuni NCTC11168CH	*0.0032*	*0.0064*
C. jejuni RM1221	*0.0022*	*0.0032*
C. jejuni NCTC81116	*0.0032*	*0.0032*

Table 4.2: MIC values of C. jejuni strains/isolates when exposed low molecular weight chitosan (pH 6.0) on MH agar (n = 6, triplicate).

Isolate/strain type	*MIC (% (w/v))*
C. jejuni wild-type NCTC11168	*0.012*
C. jejuni NCTC11168CH	*0.028*
C. jejuni RM1221	*0.020*
C. jejuni NCTC81116	*0.020*

A similar trend was observed as in Table 4.1 with respect to the MICs generated using the agar dilution methods as described previously. The lowest MIC and therefore the most susceptible cells were that of the NCTC11168 strain of C. jejuni (MIC of 0.012% (w/v)). Equally values were observed in the RM1221 and NCTC81116 strains, with an MIC of 0.020% (w/v) low molecular weight chitosan. The greatest concentration of chitosan required to inhibit visible growth indicating the lowest susceptibility was that of the 11168CH isolate (Table 4.2).

4.4.2 Determination of sub-lethal intensities

In order to determine the extent of low molecular weight chitosan, time kill assays were also undertaken in order to deduce sub-lethal intensities (concentrations) sufficient to reduce cell recovery over a 1 hour period.

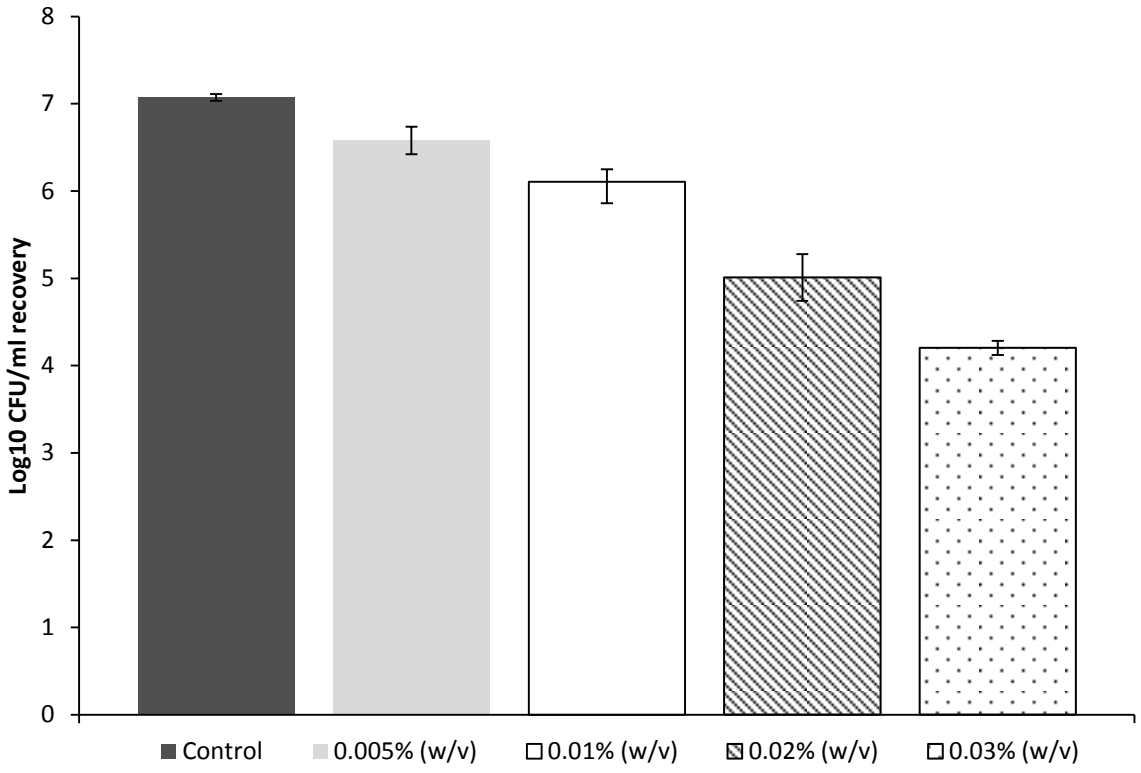

Figure 4.1: Mean recovery of C. jejuni NCTC 11168 upon 1 hour exposure to 0.005%, 0.01%, 0.02% and 0.03% (w/v) low molecular weight chitosan (pH 6.0). Error bars represent SEOM of 4 independent experiments, plated in triplicate.

Figure 4.1 represents the effect of low molecular weight chitosan exposure at various concentrations after one hour of contact. Upon exposure to 0.005% (w/v) chitosan, there was a significant difference of typically a 0.5 log CFU/ml difference was observed when compared to the control cells (p = <0.05). As expected, with an increase in concentration of chitosan, less recovery of cells

were found upon challenging to these sub-lethal concentrations of chitosan. Significant differences in log CFU/ml were found for 0.01% (w/v), 0.02% (w/v) and 0.03% (w/v) low molecular weight chitosan, with differences of 0.97, 2.06 and 2.87 differences compared to the control, respectively. It was decided that both 0.02% (w/v) and 0.03% (w/v) chitosan would be utilised for the stepwise exposure of sub-lethal concentrations to give a higher likelihood to generating resistant isolates of C. jejuni NCTC11168.

4.4.3 Effect of repeat sub-lethal exposure on adaptive resistance

The effect of repeated sub-lethal exposure to a given antimicrobial is also useful to investigate, as this may provide insight as to the microorganism's ability to undergo adaptive resistance when challenged with stressors such as chitosan.

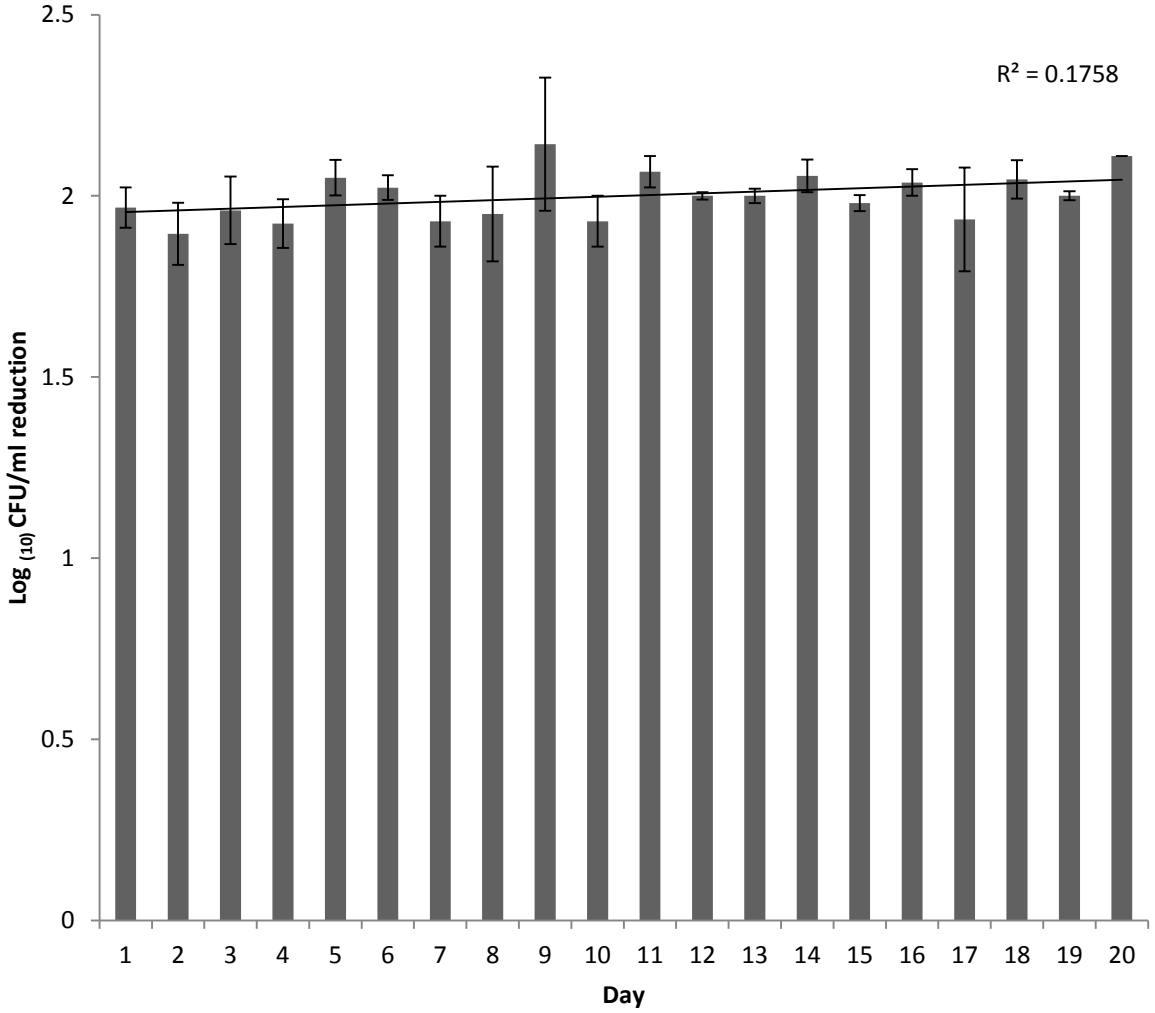

Figure 4.2: Mean log reductions of C. jejuni NCTC 11168 when subjected to 0.02% (w/v) low molecular weight chitosan. Errors bars relate to the SEOM of three independent experiments which were plated in triplicate. These log reductions were comparative to counts at time point '0'.

The failure of C. jejuni NCTC 11168 to demonstrate resistance characteristics based on repeated exposure and subsequent growth in chitosan-free media can be seen. When cells were grown for 24 hours followed by challenge of 0.02% (w/v) low molecular weight chitosan, after which an aliquot was taken for enumeration purposes, no change of the resistant sub-population was obvious over the 20 days tested, R^2 = 0.1758 (Figure 4.2).

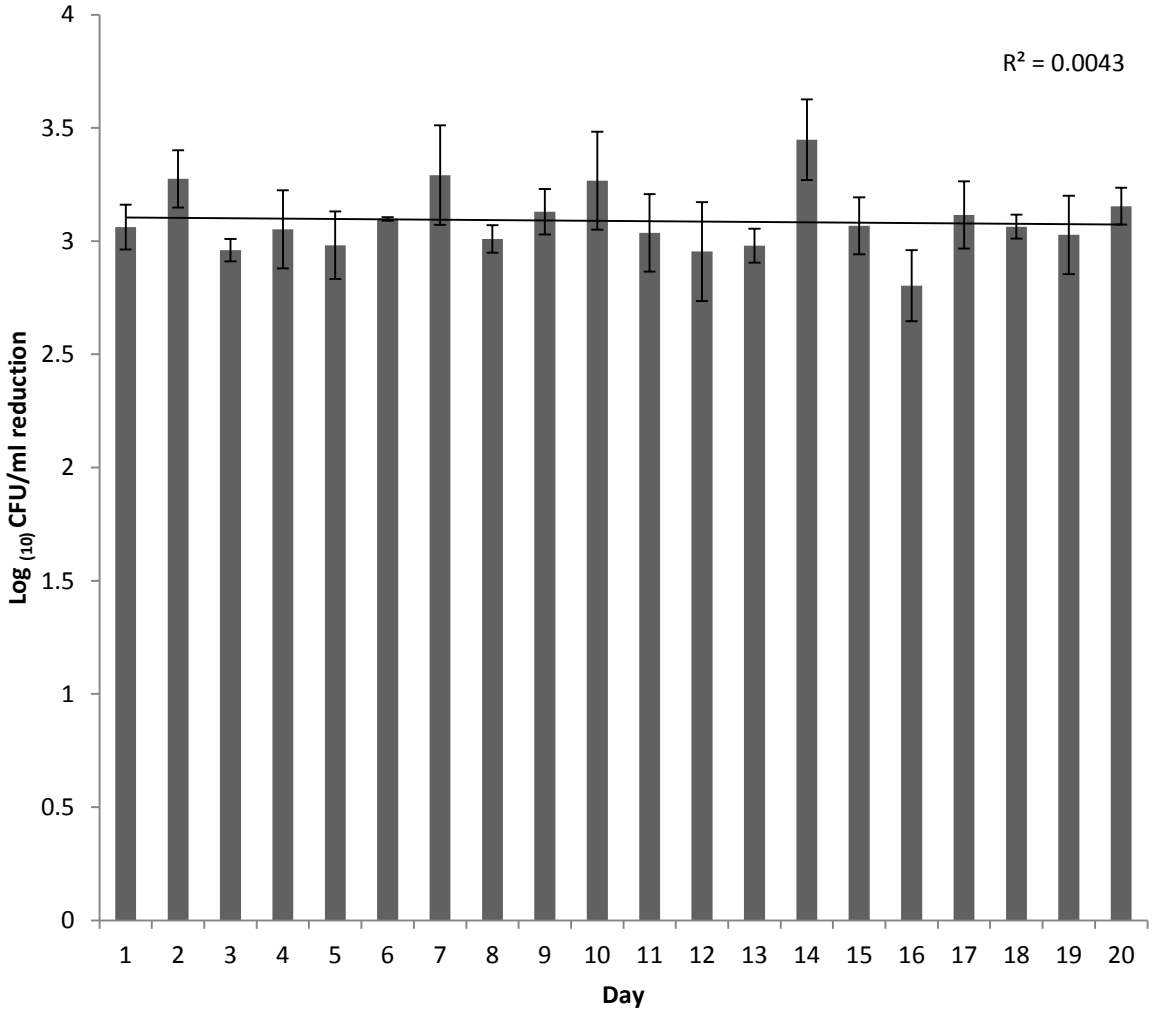

Figure 4.3: Mean log reductions of C. jejuni NCTC 11168 when subjected to 0.03% (w/v) low molecular weight chitosan. Errors bars relate to the SEOM of three independent experiments which were plated in triplicate. These log reductions were comparative to counts at time point '0'.

In addition, when applying the increased concentration of low molecular weight chitosan (0.03% (w/v)) in a separate set of experiments, upon repeated rounds of exposure over the course of 20 days, C. jejuni NCTC 11168 cells were unable to establish resistant sub-populations. With an R^2 value of 0.0043, this implies no substantial decrease in log reduction throughout repeated exposure to 0.03% (w/v) chitosan over the 20 days tested.

4.4.4 Effect of gradual sub-lethal increases in chitosan concentration upon MIC value

Table 4.3 Minimal Inhibitory Concentrations of C. jejuni NCTC 11168 when exposed to gradual increases in low molecular weight chitosan concentrations at 5, 10 and 15 days exposure. Experiments were performed on 4 separate occasions, MICs were conducted in triplicate.

	MIC (% (w/v)) after:		
Experiment set no.	*5 days exposure*	*10 days exposure*	*15 days exposure*
1	*0.0016*	*0.0014*	*0.0016*
2	*0.0016*	*0.0016*	*0.0016*
3	*0.0016*	*0.0016*	*0.0016*

After 5, 10 and 15 days exposure, no increase in MIC was found after upon gradually increased concentrations of low molecular weight chitosan in MH broth (pH 6.0). Interestingly, in the first set of experiments, there appeared to be a decrease in MIC after 10 days exposure, which was not expected. With an absence of MIC increases present when tested at 5, 10 and 15 days exposure to this stepwise training, it can be said that this method was unable to cells with decreased susceptibility with regards to low molecular weight chitosan (Table 4.3)

4.4.5 Effect of step-wise training in generating an adaptive response

Table 4.4: Effect of step-wise training of C. jejuni NCTC 11168 using gradually increasing sub-lethal concentrations of low molecular weight chitosan. Data represents 3 individual sets of experiments, plated in triplicate (n = 3).

Chitosan concentration (% (w/v))	*Control*	*Step-wise challenged*
0.001	+	+
0.002	+	+
0.004	+	+
0.008	+	+
0.012	-	-
0.016	-	-
0.018	-	-

(+ = visible growth present; - = no growth)

Upon undertaking the first attempt of increasing the concentration of low molecular weight chitosan in step-wise increments, C. jejuni NCTC11168 was not seen to adapt compared to the non-challenged cells. The absences of visible growth on the agar plates in the case of the 0.016% (w/v) chitosan were consistent with the respective MIC agar values above. In the likelihood that the chitosan concentration increase was too severe for an adaptive response to be generated, a second set of attempts were made typical of smaller increments of 0.002% (w/v) concentration (Table 4.4).

Table 4.5: Generation of step-wise trained C. jejuni NCTC 11168 isolate using gradually increasing sub-lethal concentrations of low molecular weight chitosan. Data represents 3 individual sets of experiments, plated in triplicate (n = 3).

Chitosan Concentration (% (w/v))	*Control*	*Step-wise challenged*
0.002	+/+/+	+/+/+
0.004	+/+/+	+/+/+
0.006	+/+/+	+/+/+
0.008	+/+/+	+/+/+
0.010	+/+/+	+/+/+
0.012	-/-/-	+/+/+
0.014	-/-/-	+/+/-
0.016	-/-/-	+/+/-
↓	-/-/-	+/+/-
0.040	-/-/-	+/-/-
0.042	-/-/-	+/-/-
0.044	-/-/-	+/-/-
0.046	-/-/-	-/-/-
0.048	-/-/-	-/-/-

(+ = visible growth present; - = no growth)

The second attempt at generating an adaptive isolate of C. jejuni NCTC 11168 was successful. A 3.83 fold increase in the MIC (0.012% (w/v) – 0.046% (w/v)) was observed when comparing this chitosan adapted isolate to those cells that did not undergo step-wise training.

4.4.6 Effect of repeated passage of 'adapted' isolate C. jejuni NCTC11168 on growth

In order to confirm whether the newly developed adapted isolate of C. jejuni NCTC11168 was stable and potentially indicating a mutational alteration, serial passaging was carried out as described previously (Table 4.6).

Table 4.6: Confirmation of growth regarding repeated passage on the chitosan "adapted" isolates of C. jejuni NCTC 11168. Each passage was performed on 3 plates.

Number of passages	*Growth on non-selective agar*	*"Adapted" isolate growth on selective agar*	*Wild-type growth on selective agar*
1	+/+/+	+/+/+	-/-/-
2	+/+/+	+/+/+	-/-/-
3	+/+/+	+/+/+	-/-/-
4	+/+/+	+/+/+	-/-/-
5	+/+/+	+/+/+	-/-/-
6	+/+/+	+/+/+	-/-/-
7	+/+/+	+/+/+	-/-/-
8	+/+/+	+/+/+	-/-/-
9	+/+/+	+/+/+	-/-/-
10	+/+/+	+/+/+	-/-/-
11	+/+/+	+/+/+	-/-/-
12	+/+/+	+/+/-	-/-/-
13	+/+/+	-/-/-	-/-/-

After a 12 successive passages from non-selective MH agar to selective plates incorporating a final concentration of 0.044% (w/v) chitosan, one of the three replicates showed no visible growth when compared to the isolate which did not undergo step-wise exposure, typical of growth on the respective non-selective agar (Table 4.6). When the resistant isolate of C. jejuni NCTC 11168 was passaged 13 times, no growth was observed for all replicates on 0.044% (w/v) chitosan containing agar. The non-stable resistance observed in the chitosan 'adapted' was referred to as an "adapted" isolate, as the cells did not succumb to permanent resistance, which may result from mutations.

4.4.7 Updated MIC/MBC values including newly generated 'adapted' isolate of C. jejuni NCTC11168.

To ensure that the MIC/MBC values remained consistent, these were tested a several occasions, alongside the newly developed 'adapted' isolate of C. jejuni NCTC11168, as presented is tables 4.7 and 4.8 (below).

Table 4.7: Updated MIC values of C. jejuni strains/isolates when exposed low molecular weight chitosan (pH 6.0) on MH agar (n = 6, triplicate).

Isolate/strain type	*MIC (% (w/v))*
C. jejuni wild-type NCTC11168	*0.012*
***C. jejuni* 'adapted' NCTC11168**	**0.046**
C. jejuni NCTC11168CH	*0.028*
C. jejuni RM1221	*0.020*
C. jejuni NCTC81116	*0.020*

Table 4.8: Updated MIC and MBC values of C. jejuni strains/isolates when exposed to low molecular weight chitosan (pH 6.0) in MH broth (n = 6, triplicate).

Isolate/strain type	*MIC (% (w/v))*	*MBC (% (w/v))*
C. jejuni wild-type NCTC11168	*0.0016%*	*0.0032%*
***C. jejuni* 'adapted' NCTC11168**	**0.0032%**	**0.0064%**
C. jejuni NCTC11168CH	*0.0032%*	*0.0064%*
C. jejuni RM1221	*0.0022%*	*0.0032%*
C. jejuni NCTC81116	*0.0032%*	*0.0032%*

4.5 Discussion

There has been evidence that foodborne pathogenic bacteria can adapt to sub-lethal stresses leading to an increased resistance to lethal levels of both the homologous and heterologous stresses. Many of these adapted microorganisms can also show an enhanced virulence (Rowan, 1999). In the past it has been documented that whilst under the appropriate sub-lethal conditions, cells can undergo 'stress-hardening'. Pathogens which can overcome various environmental stresses associated with foods and hosts based environments may lead to unforeseen shifts in physiological characteristics (Rowan, 1999).

In order to survive a number of environmental challenges, C. jejuni must possess the capability to tolerate increasing concentrations of various biocides/antimicrobials. It has been previously documented that several organisms have illustrated the ability to adapt to a given stress, including E.coli, L. monocytogenes following repetitive sub-lethal inactivation with lactic acid, chlorine dioxide and intense light pulses (Rajkovic et al., 2009). In this study, C. jejuni strains were unable to mount a tolerance response to repeated sub-lethal challenge.

Resistance in Campylobacter spp. remains relatively rare and despite the potential of chitosan for use in the food industry there is a surprising lack of reports on the effect of chitosan against this important foodborne pathogen (Ganan et al., 2009). It has been demonstrated that adaptive responses to biocides can account for an increased reduction in a microorganisms susceptibility both to the antimicrobial of choice and also dissimilar antimicrobials

and/or disinfectants, indicate of cross-protective resistance (Braoudaki and Hilton, 2005; Abdel Malek and Badran, 2010; Loughlin et al., 2002).

The results in this chapter successfully established the generation of an adaptive isolate of C. jejuni NCTC 11168 following step-wise increases in chitosan. No adaptive resistance to low molecular chitosan was observed when using repeated exposure following the methods adapted from Rajkovic et al. (2009) or to the step-wise training of cells following the methods described in Mavri and Možina (2012) in broth medium.

Upon exposing C. jejuni NCTC 11168 to repeated sub-lethal concentrations of 0.02% (w/v) and 0.03% (w/v) chitosan over the course of 20 days, indicative of 2 and 3 log reduction in log CFU/ml recovery, R^2 values of 0.1758 and 0.0043 were calculated, respectively. The C. jejuni strains used in Rajkovic et al. (2009) were typically producing non-culturable cells after subsequent exposure to repeated cycles of lactic acid and 2 cycles of liquid chlorine dioxide. The findings described in this chapter did not find complete reduction in cells numbers after the 20 cycles tested, suggesting that C. jejuni NCTC 1168 was able to survive the sub-lethal concentrations of chitosan used.

Moreover, the stepwise training of C. jejuni NCTC 11168 in MH broth with gradually increasing concentrations of low molecular weight chitosan, the lack of increased MIC values when testing after 5, 10 and 15 days exposure revealed that on these occasions C. jejuni NCTC 11168 was unable to develop increased resistance. Alternatively, it could be possible that the generation of an adapted isolate of C. jejuni NCTC 11168 may have been produced through prolonged exposure beyond the 15 day exposure used as well as increasing the number of cycles of inactivation when following a modified method from Rajkovic et al.

(2009). However, from the results observed, it was deemed unlikely that the successful generation of a resistant sub-population was to going to occur.

The findings described here suggest that the development of resistance populations are subject to potentially numerous intricate factors and may also be dependent upon chance. It has been suggested that the degree of increased resistance to antimicrobials and the time for which this resistance persists after the stressor is absent is largely dependent on the dosage, the time of which the microorganism is challenged and the bacterial species (Fernández et al., 2011; Rusell, 2004).

The generation of an adaptive isolate of C. jejuni NCTC 11168 was successful when applying a method to an agar based approach focused on gradual stepwise training typical of increasing the concentration of low molecular weight chitosan.

As was highlighted previously, this isolate was able to grow on 0.044% (w/v) chitosan containing MH plates (pH 6.0). In comparison to the wild-type C. jejuni NCTC 11168 strain, an increase of 3.83 fold (0.012% w/v – 0.046% w/v) was observed in the chitosan "adapted" isolate when comparing the MIC concentration of low molecular weight chitosan needed to inhibit visible growth when tested. This enhanced tolerance was seen to be stable for up to 12-13 passages onto chitosan-free agar medium after which no visible growth was seen when plated onto the agar medium containing 0.044% (w/v) chitosan in parallel. The unstable characteristics of the adaptive isolate generated have previously been reported, with a loss in enhanced resistance (Thomas et al., 2000; Mavri and Možina, 2012). Thus, this is consistent with previous findings, alongside the failure of adaptive response which have also been reported with respect to E. coli and C. jejuni when challenged to biocides, as in Thomas et al. (2000) and Mavri and Možina (2012). There have also been cases whereby adaptive resistance has

been sustained in Peudomonas spp. and Salmonella enterica (Tattawasart et al., 1999; Thomas et al., 2000 Braoudaki and Hilton, 2005).

The findings in this chapter are probably the first documented case of adaptive responses to chitosan through step-wise exposure and the second instance of adaptive resistance of C. jejuni. A major challenge for the food manufacturing industry is therefore to ensure the necessary quantity and spectrum of antimicrobials are used in order to inhibit microbial growth of food borne pathogens which minimise the likelihood of developing adaptive resistance (Ruston et al., 2013).

It is important to mention that the inability of C. jejuni to develop resistance in the majority of cases may be due to the possible existence of viable but non-culturable (VBNC) populations of cells after treatment to stressors, in this instance, low molecular weight chitosan. If this phenomenon was to occur during stepwise training of cells under the various methods used there may be certain cells which may be 'hidden' to an extent, whereby any adaptive responses which may have arisen may not be quantified by a noticeable increase in MIC values for example. This arises from the inability of cells to form colonies on both agar and broth media (Rajkovic et al., 2009). In the particular case for C. jejuni, the rapid loss of culturability may not necessarily be directly correlated to death of exposed cells (Guillou et al., 2008).

The newly developed chitosan 'adapted' isolate of C. jejuni NCTC11168 was seen to have the greatest MIC agar value when compared to the other strain/isolates tested (Table 4.6). When compared to the MIC values obtained using broth medium, the 'adapted' isolate was equally as high as the isolate which

underwent passage in a chicken (Table 4.7). This would suggest the previous exposure to low molecular weight chitosan can induce an increased survival potential, which may account for an increased degree of persistence. Nevertheless, the corresponding MBC for the 'adapted' isolate of C. jejuni NCTC 11168 was also found to be equally the greatest in comparison to the other strains tested.

The identification of Campylobacter spp. which have a degree of antimicrobial resistance, such as that seen in the generated 'adapted' isolate of C. jejuni NCTC11168, highlight concerns in terms of treatment of campylobacteriosis. Due to the decreased susceptibility, those strains which happen to develop resistance to a given antimicrobial, even temporarily, may give rise to prolonged illness associated with subsequent infection (Travers and Barza, 2002).

Chapter 5 – Comparative antimicrobial activity of low molecular weight chitosan against *C. jejuni* strains/isolates

5.1 Introduction

Despite the fastidious nature of Campylobacter jejuni, requiring microaerobic conditions and alongside specific nutrients allowing for ample growth in vitro, it poses an enormous burden to the public health, which raises the question as to how they are able to cause such an issue (Crushell et al., 2004). As with other pathogenic bacteria, there is the pressing issue of resistance which is perceived to only escalate in terms of severity with the chronic overuse of various antimicrobials/antibiotics. C. jejuni is no exception, with antimicrobial resistance having previously been observed (Wieczorek and Osek, 2013; Luangtongkum et al., 2009; Mavri and Možina, 2012).

Low molecular weight chitosan has attracted an increasing amount of attention, owing to the potential for a variety of biological activities, including fat-binding, antithrombotic activity, antitumor and antimicrobial activity (Kim and Rajapakse, 2005). Alongside other useful properties it the potential for use as a food preservative which has proven to be particularly appealing, mainly due to its natural origins and known antimicrobial activity against a variety of microorganisms (Devlieghere et al., 2004).

The emergence and persistence of pathogenic microorganisms such as C. jejuni are often thought to found to have originated from a food origin (Altekruse et al.,

1999). With the ever increasing need for more natural and sustainable antimicrobials in such environments as the food industry, chitosan may be an important biopolymer to consider with regards to reducing the bacterial load of C. jejuni, which could have a positive impact in reducing the incidence of human infections arising from this pathogen.

The antimicrobial activity of chitosan is known to be dependent on numerous factors. This includes microbial factors, relating to the type of microorganism and respective cell age, intrinsic factors (I.e. positive charge density, Mw, concentration, chelating capacity and hydrophilic/hydrophobic nature), physical state and environmental factors, namely ionic strength, pH and temperature (Kong et al., 2010).

Moreover, the antimicrobial activity of chitosan is fairly complex, with differences in reported efficacy with respect to Gram positive and Gram negative bacteria species. A number of studies have observed a greater antibacterial activity towards Gram negative bacteria, as oppose to Gram positive species (Chung et al., 2004; No et al., 2002). Whilst in other studies, the opposite was found, with Gram positive bacteria being more susceptible (Zhong et al., 2008). Despite this, a study conducted by Wang et al. (2004) found no significant differences in antimicrobial activity, suggesting a generic degree of variance between bacteria species.

In more recent times, there has been an increasing interest in the development of film forming materials which harbour antimicrobial activity (Tripathi et al., 2009). Chitosan films have a promising potential application with respect to antimicrobial packaging, one of the most encouraging active packaging systems

(Dutta et al., 2012). These packaging systems have illustrated a high degree of inhibition or direct killing of pathogenic microorganisms associated with contaminating foodstuffs (Salleh et al., 2007).

The overall goals of any antimicrobial are to limit or prevent microbial growth either by extending the lag phase with a reduction in growth rate or a decrease in viable counts (Han, 2000). It therefore seemed appropriate to assess the antimicrobial activity of chitosan at concentrations which would be likely both permit and the inhibition of growth in C. jejuni strain/isolates and also at concentrations which are lethal, resulting in loss a total loss of cell viability over a shorter time period.

5.2 Aims and Objectives

5.2.1 Aim

The aim of this study was to investigate the general antimicrobial efficacy of low molecular weight chitosan against different strains and isolates of C. jejuni, highlighting any trends/discrepancies in susceptibility.

5.2.2 Objectives

- *To determine the MICs and MBCs of chitosan against C. jejuni strains/isolates in broth and agar medium under different pHs.*

- *To assess the effect of chitosan films regarding several C. jejuni strains/isolates.*

- *To establish the effect of lethal chitosan exposure upon cell viability/recovery in nutrient rich (MH broth) and limited (PBS) environments.*

- *To compare the growth of C. jejuni strains/isolates when exposed to sub-lethal concentrations of chitosan.*

5.3 Methods

5.3.1 Microorganisms

C. jejuni strains and isolates are summarised in section 3.1. The culture conditions and media utilised are described as in section 3.8

5.3.2 Chitosan

Low molecular weight chitosan was prepared as described in the materials and methods chapter and pH adjusted to pH 5.5, 6.0 or 6.5 consistent with the pH of MH broth/PBS diluent utilised, dependent upon the experiment required.

5.3.3 Determination of Minimum Inhibitory Concentration (MIC)

5.3.3.1 Agar dilution method

The agar dilution methods were carried out as described in section 3.9.2.

5.3.3.2 Broth micro-dilution method

Broth micro-dilution methods were carried as in section 3.9.2.

5.3.4 Time Kill assay

Time kill experiments were conducted utilising pre-grown early stationary phase C. jejuni cultures grown in MH broth (incubated at 42°C under microaerobic conditions). In the case of the PBS assay, the contents of the broth were then placed into a sterile 50ml centrifuge tube and centrifuged for 8 minutes at 10,000 x g. The supernatant was discarded and the same volume of PBS was added into the tube as compared to the initial volume of the culture media. The pellet was then vortexed and re-suspended in diluent. Several 10ml aliquots were transferred from the initial culture into sterile 15ml tubes along with a specific volume of chitosan from a 1% (w/v), giving a final starting cell concentration of approximately $2x10^7$ CFU/ml at a final concentration of 0.01% (w/v) and 0.02% (w/v) chitosan (final of pH 6.0 or 6.5), depending on the experiment. PBS with no chitosan added was used for control samples. Aliquots were taken from these respective tubes at various time intervals during incubation (150rpm shaking) under conditions mentioned previously, serial diluted and plated utilising the Miles and Misra technique (Miles, Misra and Irwin, 1938). The same procedure was undertaken with in the case of studies concerning the incorporation of chitosan in MH broth (nutrient rich conditions).

5.3.5 Preparation of chitosan films

Chitosan was dissolved in 1% (w/v) acetic acid in order to prepare a 1% (w/v) low molecular weight chitosan solution. Ten millilitres of the solution was poured into a Petri dish at and dried in a lamina flow hood at room temperature for 48 hours. The resulting film was then washed with 2% (w/v) NaOH for neutralisation purposes and then with distilled water and added into a 2% (w/v) H_2SO_4 cross-linking solution for 1 hour. After fully cross-linking, the film was then washed with distilled water and left to dry for a further 24 hours (adapted from Tripathi et al., 2009).

5.3.6 Chitosan Film Assay

One centimetre chitosan films were aseptically cut using a sterile borer. The plugs were subsequently transferred to centrifuge tubes containing MH broth (pH 6.0) with a final concentration of approximately 2 x 10^5 CFU/ml from an overnight culture and shaken at 150rpm. Aliquots were taken at 0, 4, 8, 24, 48 and 52 hours, plated and enumerated described previously.

5.3.7 Growth Inhibition Study

Several colonies of respective C. jejuni cultures were inoculated into 50 ml of Mueller Hinton broth and were incubated for 24 hours (early stationary phase) under microaerobic conditions at 42°C.

After these overnight cultures of C. jejuni had been grown, aliquots were taken and diluted to a final concentration of approximately 2x10^6 CFU/ml (final volume

of 10ml consisting of MH broth (adjusted to pH 6.0) and a volume low molecular weight chitosan added (also pH 6.0).

At various time intervals, aliquots were taken from samples, serially diluted and subsequently plated at 42°C for a maximum of 56 hours under microaerobic conditions. Counts were then enumerated as described previously.

5.4 Results

To reinforce the notion that chitosan antimicrobial activity is dependent aspects including pH, a comparison of susceptibility of C. jejuni strains/isolates with in relation to this was investigated (below).

5.4.1 MIC broth micro-dilution

Table 5.1: MIC and MBC values of C. jejuni when exposed low molecular weight chitosan (pH 5.5) in MH broth (n = 6, triplicate).

Isolate/strain type	*MIC (w/v)*	*MBC (w/v)*
C. jejuni wild-type NCTC11168	*	*0.0001%*
C. jejuni 'adapted' NCTC11168	*	*0.0001%*
C. jejuni NCTC11168CH	*	*0.0001%*
C. jejuni RM1221	*	*0.0001%*
C. jejuni NCTC81116	*	*0.0001%*

** = not quantifiable (lowest concentration tested = 0.0001% (w/v))*

The MIC values for all of the strains/isolates of C. jejuni upon exposure to chitosan at pH 5.5 were unable to be deduced, as no visual turbid growth was observed in the treated samples (as represented by the asterisk in Table 5.1). After the aliquots from the plates were spotted onto MH agar with 5% (v/v) defibrinated horse blood, the same MBC values regarding chitosan were concluded for all C. jejuni isolates/strains tested (0.0001% w/v). Only the control samples showed recovery upon spot plating onto the non-chitosan containing media.

Table 5.2: MIC and MBC values of C. jejuni when exposed low molecular weight chitosan (pH 6.0) MH broth (n = 6, triplicate).

Isolate/strain type	*MIC (w/v)*	*MBC (w/v)*
C. jejuni wild-type NCTC11168	*0.0016%*	*0.0032%*
C. jejuni 'adapted' NCTC11168	*0.0042%*	*0.0064%*
C. jejuni NCTC11168CH	*0.0032%*	*0.0064%*
C. jejuni RM1221	*0.0022%*	*0.0032%*
C. jejuni NCTC81116	*0.0032%*	*0.0032%*

From the MIC data presented in Table 5.2 (above), the lowest MIC value was observed in the wild-type C. jejuni NCTC11168 strain (0.0016% (w/v)) concerning pH 6.0 MH broth. This was followed by the RM1221 strain (0.0022% (w/v)). Equally greater MIC values were typical of both the C. jejuni NCTC81116 strain and the NCTC11168CH isolate (both 0.0032% (w/v)). The highest MIC concerned the 'adapted' NCTC11168 isolate of C. jejuni (0.0042% (w/v)).

Interestingly, the MBCs for the wild-type NCTC 11168, RM1221 and 81116 strains were consistent (0.0032% (w/v)) after 24 hours incubation at pH 6.0. The greatest MBC was found in both the 'adapted' isolate NCTC11168 isolate and NCTC11168CH, which had previously undergone passage through a chicken. With an increase in low molecular weight chitosan concentration when comparing the MIC to the MBC for each isolate/strain (excluding the 81116 strain), it can be assumed that the antimicrobial activity of chitosan is bacteriostatic.

Table 5.3: MIC and MBC values of C. jejuni when exposed to low molecular weight chitosan (pH 6.5) in MH broth (n = 6, triplicate).

Isolate/strain type	*MIC (w/v)*	*MBC (w/v)*
C. jejuni wild-type NCTC11168	*0.0260%*	*0.0700%*
C. jejuni 'adapted' NCTC11168	*0.0480%*	*0.0960%*
C. jejuni NCTC11168CH	*0.0400%*	*0.0940%*
C. jejuni RM1221	*0.0300%*	*0.0920%*
C. jejuni NCTC81116	*0.0300%*	*0.0840%*

When subjected to concentrations of chitosan with an increase in overall pH (6.5), increases in MICs were identified in all strain/isolates of C. jejuni tested. After being incubated at the pH 6.5 with exposure to a range of concentrations of low molecular weight chitosan, the greatest MIC and subsequent MBC value was observed in the 'adapted' isolate of C. jejuni NCTC11168 (0.0480% (w/v)), which was followed by the NCTC11168CH isolate with a concentration of 0.0400% (w/v) needed. The RM1221 and 81116 C. jejuni strains were seen to possess equal MICs (0.0300% (w/v)) and the wild-type NCTC 11168 occupying to lowest MIC of all the above tested. Similar to the MIC generated under pH 6.5, the MBC

value was also seen to be the lowest with regard to the wild-type NCTC 11168 strain, which was then followed by the NCTC81116 strain, RM1221 strain, NCTC11168CH and the 'adapted' NCTC11168 cells occupying the highest MBC of all the tested strains/isolates (0.0940% (w/v)) (Table 5.3).

5.4.2 MIC agar dilution results

The antimicrobial effects of low molecular chitosan were also undertaken at pH 6.0 and 6.5 in agar medium to establish if alterations in final pH affected chitosan activity.

Table 5.4: MIC values of C. jejuni when exposed low molecular weight chitosan (pH 6.0) on MH agar (n = 3, triplicate).

Isolate/strain type	*Concentration (w/v)*
C. jejuni wild-type NCTC11168	*0.012%*
C. jejuni 'adapted' NCTC11168	*0.046%*
C. jejuni NCTC11168CH	*0.028%*
C. jejuni RM1221	*0.020%*
C. jejuni NCTC81116	*0.020%*

As expected, the lowest MIC value in using the MIC agar dilution method at pH 6.0 was also found in the wild-type NCTC11168 strain (0.012% (w/v)), whilst the greatest MIC was typical of the 'adapted' NCTC11168 isolate (0.046% (w/v)). Identical values were found with respect to the RM1221 and NCTC81116 strains (0.020% (w/v)). Likewise, the second highest MIC value was observed in the NCTC11168CH isolate of C. jejuni upon exposure to chitosan concerning pH 6.5 (Table 5.4).

Table 5.5: MIC values of C. jejuni when exposed low molecular weight chitosan (pH 6.5) on MH agar (n = 3, triplicate).

Isolate/strain type	*Concentration (w/v)*
C. jejuni wild-type NCTC 11168	*0.090%*
C. jejuni 'adapted' NCTC 11168	*0.160%*
C. jejuni 11168CH	*0.120%*
C. jejuni RM1221	*0.120%*
C. jejuni NCTC 11828 (81116)	*0.120%*

An increase in pH from 6.0 to 6.5 also accounted for greater MIC values in all strains/isolates of C. jejuni when employing the MIC agar dilution method, as expected (Table 5.5). Once again, the lowest MIC of chitosan was found in the wild-type NCTC11168 strain (0.090% (w/v)). This was followed by equal values of 0.120% (w/v) characteristic of the NCTC81116, RM1221 and NCTC11168CH isolate. Moreover, the highest MIC was typical of the 'adapted' isolate of NCTC11168 (0.160% (w/v)).

5.4.3 Chitosan film activity

Addition of 1cm chitosan films into MH broth medium (pH 6.0) accounted for an inhibition of growth relative to the respective control cells which were not subject to film exposure. The degree of growth inhibition in the presence of the chitosan film was found to be similar in all C. jejuni strains/isolates tested. Individual t-tests comparing growth regarding the various strains/isolates revealed no significant differences from 0-52 hours as tested ($p>0.05$). Significant reductions were apparent during 4-48 hours for all strain/isolates of C. jejuni, relative to the respective control cells ($p<0.05$). Interestingly, after 52 hours incubation, all

strains/isolates exposed to chitosan films illustrated a substantial recovery, indicated by no significant differences in relation to their respective controls (Figure 5.1).

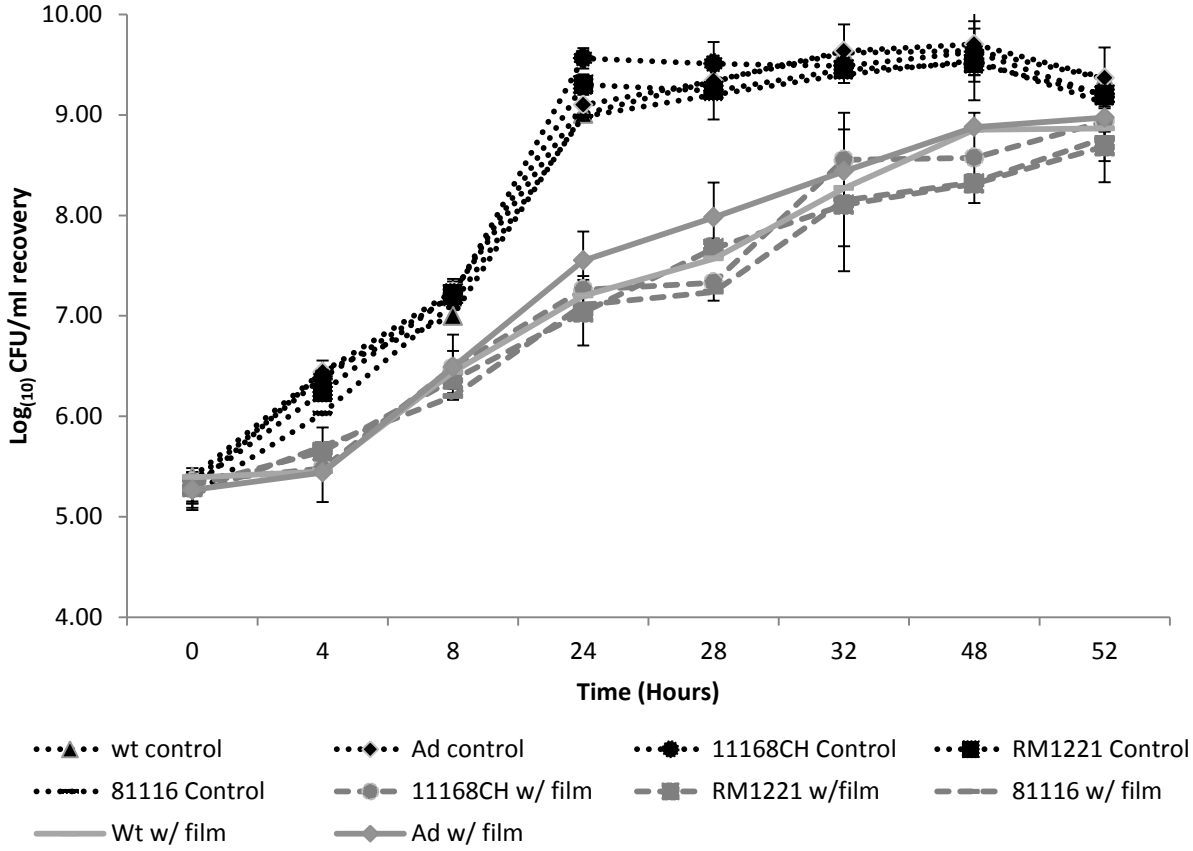

Figure 5.1: Effect of chitosan film exposure with regards to C. jejuni strains/isolates relative to the untreated controls over the course of 52 hours at 42°C under microaerobic conditions (pH 6.0). Experiments were conducted n = 3 and plated in triplicate. Data represents mean values; error bars indicate SEOM.

5.4.4 Time kill inactivation results (MH broth)

Time kill experiments were undertaken in both nutrient rich (MH broth) and nutrient limited (PBS) conditions to establish the extent to which nutrient availability may have on antimicrobial efficacy of chitosan.

The figure overleaf (Figure 5.2) illustrates the difference in cell recovery following exposure to 0.01% (w/v) and 0.02% (w/v) chitosan with respect to C. jejuni strains/isolates under nutrient rich conditions, as provided by MH broth.

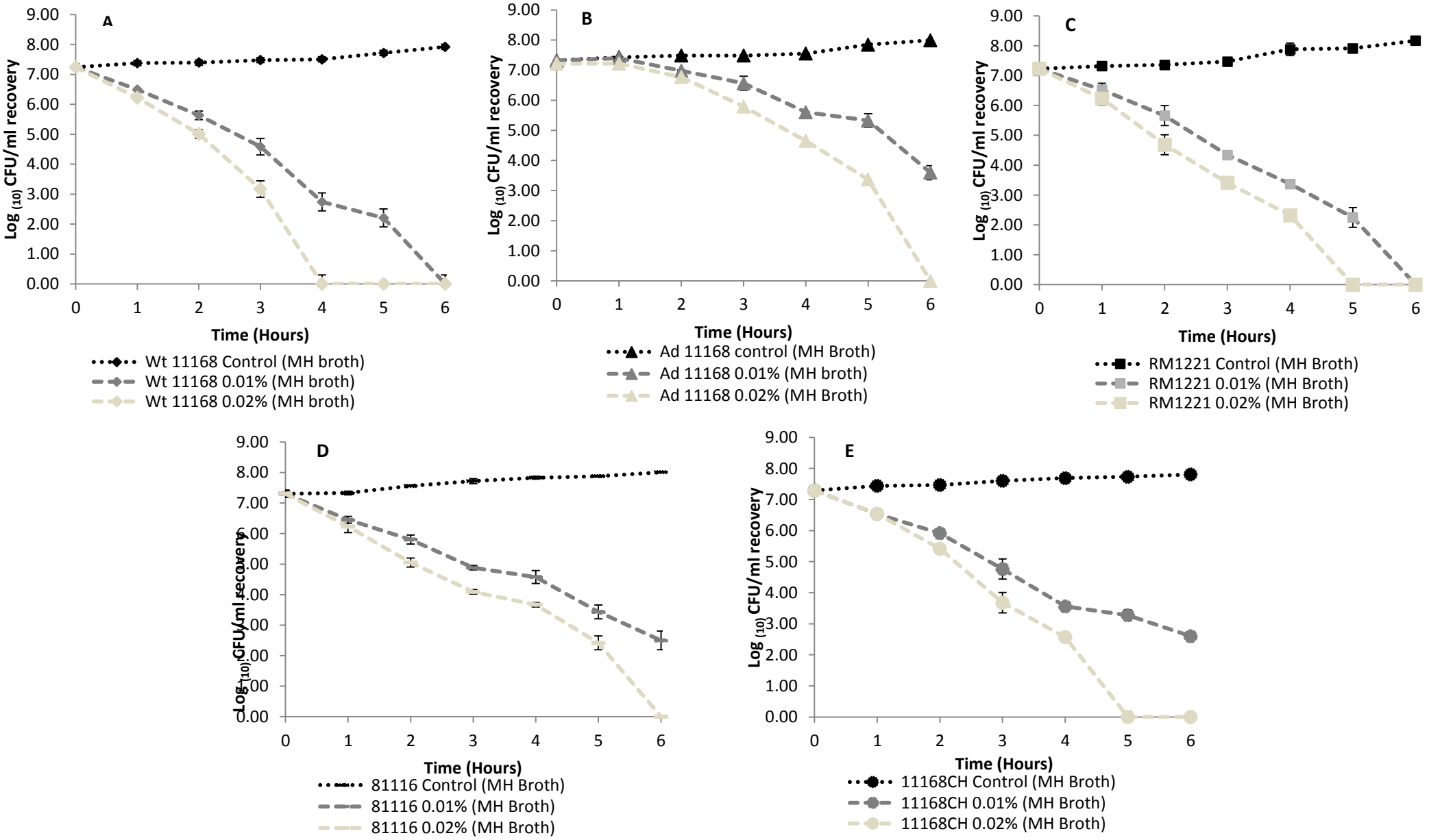

Figure 5.2: Effects of 0.01% (w/v) and 0.02% (w/v) chitosan exposure against wild-type NCTC11168 (A), "adapted" NCTC11168 (B), RM1221 (C), 81116 (D) and 11168CH (E) C. jejuni strains/isolates over a time period of 6 hours, relative to untreated control samples (pH 6.0). Experiments were conducted n = 4, plated in triplicate. Data represents mean values; error bars indicate SEOM.

Upon exposure to 0.01% (w/v) and 0.02% (w/v) chitosan in MH broth (pH 6.0) with respect to the wild-type NCTC11168 strain of C. jejuni, no detectable cells were found to be viable after 6 hours and 4 hours, respectively (detection limit of 1.50E+02 CFU/ml). Significant differences were observed when comparing the untreated control cells relative to those which were subject to 0.01% (w/v) chitosan ($F(13, 154) = 5370$, $p<0.0001$) and 0.02% (w/v) chitosan treatment ($F(13, 154) = 8251$, $p<0.0001$), revealed by ANOVA analysis. Post hoc analysis revealed significant reductions in both 0.01% and 0.02% (w/v) chitosan treated cells, as oppose to the respective controls from 1-6 hour time intervals ($p<0.05$). However, no significant differences were found in relation to the control and respective treated cells at time point 0, as expected ($p>0.05$).

Upon exposure of the chitosan 'adapted' isolate of C. jejuni NCTC11168 to 0.01% and 0.02% (w/v) chitosan incorporated in MH broth resulted in noticeable reductions from 2 – 6 hours, relative to the respective untreated cells (Figure 5.2). When compared to the control cells, the respective chitosan treated cells illustrated a significant reductions in cell viability with respect to 0.01% (w/v) chitosan ($F(13, 154) = 3824$, $p<0.0001$) and 0.02% (w/v) chitosan ($F(13, 154) = 10596$, $p<0.0001$). Additional post hoc analysis revealed statistically significant decreases in cells recovery in all chitosan treated cells in relation to the respective untreated cells from 2-6 hours ($p<0.05$). A greater degree of antimicrobial activity was also found with an increase in chitosan concentration utilised (0.02% (w/v)), as expected.

Interestingly, the 'adapted' treated cells were seen to be viable after 6 hours exposure to chitosan, whereas no wild-type NCTC11168 cells treated with chitosan were recovered after 6 hours when treated with 0.01% (w/v) chitosan

as shown in Figure 5.2. Moreover, substantial decreases were apparent with respect to 0.02% (w/v) chitosan exposure, with no viable cells recovered after 4 hours exposure in the wild-type NCTC11168 cells, whilst for the 'adapted' NCTC11168 cells, viability cells were absent after an increased exposure time of 6 hours.

Similar to the wild-type NCTC11168 cells exposed to 0.01% (w/v) chitosan (Figure 5.2), cells regarding the RM1221 strain of C. jejuni were not found to be viable after 6 hours of exposure, suggesting that the RM1221 was also more susceptible to chitosan when compared to the 'adapted' NCTC11168 cells (Figure 5.2). Significance decreases in cell viability were observed when comparing the treated control cells of RM1221 C. jejuni, relative to those exposed to 0.01% ($F(13, 154) = 6616$, $p<0.0001$) and 0.02% (w/v) chitosan ($F(13, 154) = 19018$, $p<0.0001$). Significant decreases in recovery were found as a result of post hoc analysis from 1-6 hours in respective chitosan treated cells of RM1221 ($p<0.05$). As expected, greater reductions were found in a time dependant manner with regards to those cells exposed to 0.02% (w/v) chitosan. Significant differences were found when comparing 0.02% (w/v) exposed cells to those treated with 0.01% (w/v), from 3-6 hours ($p<0.05$).

Additionally, significant differences in cell recovery was identified concerning the 81116 C. jejuni strain when exposed to 0.01% (w/v) ($F(13, 154) = 2918$, $p<0.0001$) and 0.02% (w/v) chitosan ($F(13, 154) = 9239$, $p<0.0001$), relative to the untreated control cells. These decreases in recovery were seen to increase in a time dependant manner, with post hoc testing highlighting significantly lower recovery in respective chitosan treated cells versus the control from 1-6 hours ($p<0.05$). Interestingly, an absolute loss in cell recovery was not observed in the

RM1221 strain during the 6 hour time period (Figure 5.2), unlike the wild-type NCTC11168 and RM1221 strains which underwent treatment with chitosan at 0.01% (w/v). However, when exposed to 0.02% (w/v) chitosan, no recoverable cells were detected after 6 hours. This absence in viable cells was similar with respect to the 'adapted' NCTC11168 cells upon treatment with 0.02% (w/v), accounting for no cell recovery after the same contact time.

A similar inability of total cell viability loss after 6 hours exposure was typical of those treated with 0.01% (w/v) chitosan with regards to the 11168CH isolate (Figure 5.2), as in the 'adapted' NCTC11168 and 81116 cells. Once again, significant decreases in cell viability were indicated by ANOVA testing when comparing 0.01% (w/v) and 0.02% (w/v) chitosan treated cells relative to the control cells ($F(13, 154) = 3974$, $p<0.0001$; $F(13, 154) = 4110$, $p<0.0001$). Upon exposure to 0.02% (w/v) chitosan, no cells were recovered above the detection limit after 5 hours exposure, which was also the case for 0.02% (w/v) chitosan treated RM1221 cells.

5.4.5 Time kill inactivation results (PBS)

Results in Figure 5.3 represent the effect of low molecular weight chitosan upon the same C. jejuni strains/isolates tested previously, but in nutrient limited conditions:

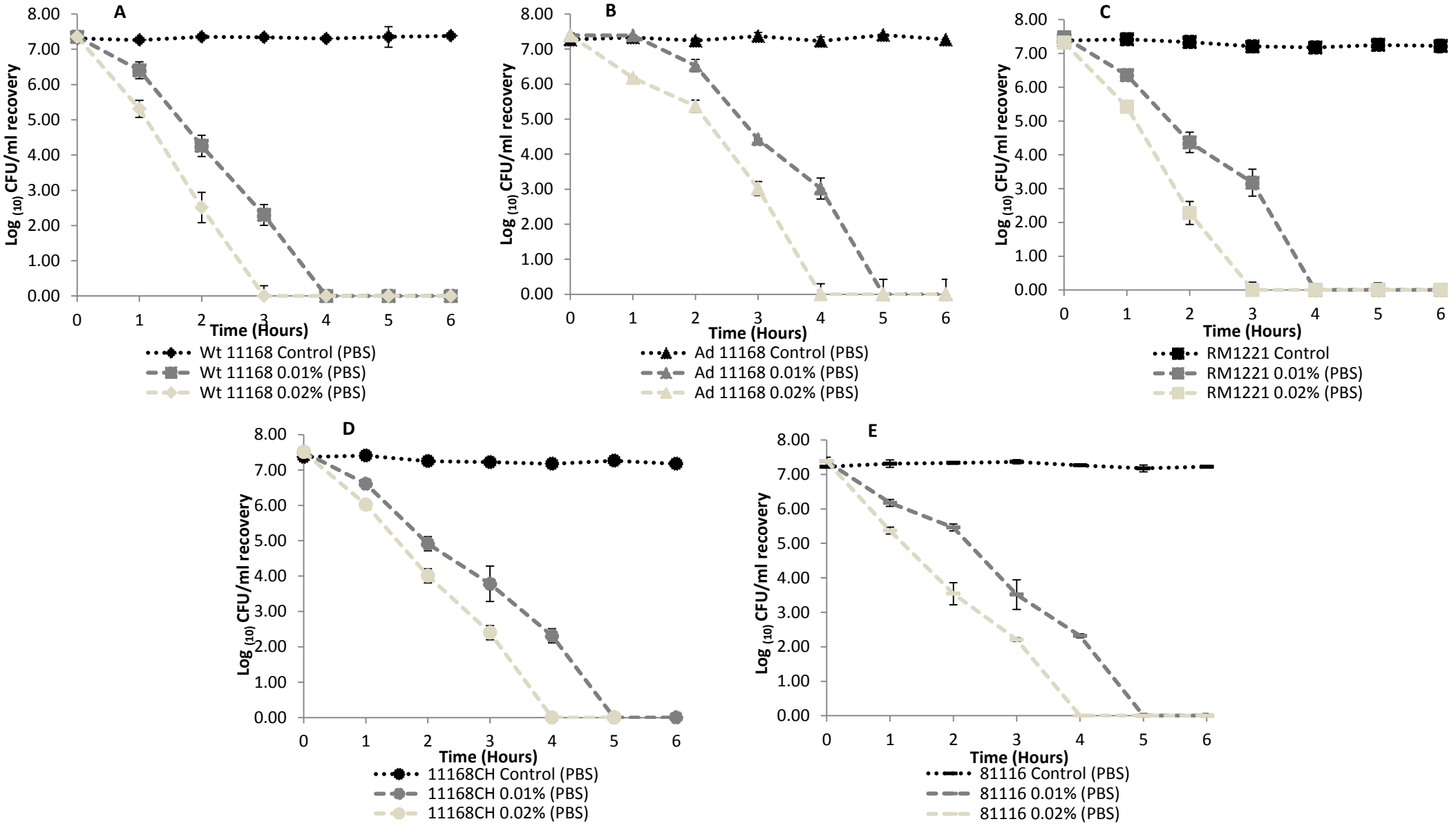

Figure 5.3: Effects of 0.01% (w/v) and 0.02% (w/v) chitosan exposure against wild-type NCTC11168 (A), "adapted" NCTC11168 (B), RM1221 (C), 81116 (D) and 11168CH (E) C. jejuni strains/isolates under nutrient limiting conditions (from 1-6 hours), relative to untreated control samples (pH 6.0). Experiments were conducted n = 4, plated in triplicate. Data represents mean values; error bars indicate SEOM.

No detectable cells were found after 4 and 3 hours when exposed to 0.01% (w/v) and 0.02% (w/v) chitosan in nutrient limited conditions (PBS), respectively (Figure 5.2). Significant differences were observed when comparing the untreated control cells in relation to those exposed to 0.01% (w/v) chitosan ($F (13, 154) = 5264$, $p<0.0001$) and also 0.02% (w/v) chitosan ($F (13, 154) = 9264$, $p<0.0001$). As with illustrated in Figure 5.2 previously, the antimicrobial efficacy was also found to be dose and time dependant, with post hoc testing highlighting significant decreases in cells treated with both 0.01% and 0.02% (w/v) chitosan after 1 hour exposure onwards ($p<0.05$).

When compared to the wild-type NCTC11168 treated cells under nutrient depleted conditions (Figure 5.3), a decreased susceptibility was generally observed in the 'adapted' NCTC11168 cells (Figure 5.3). Significant decreases were found in relation to the chitosan treated cells of the 'adapted' NCTC11168 cells when treated to 0.01% (w/v) and 0.02% (w/v) chitosan in relation to the untreated control cells from 2-6 hour exposure ($F (13, 154) = 24826$, $p<0.0001$) and from 1-6 hours ($F (13, 154) = 15224$, $p<0.0001$), respectively. No viable cells were viable, above the detection limit after 5 hours and 4 hours exposure to 0.01% (w/v) and 0.02% (w/v) chitosan, when compared to the wild-type NCTC11168 treated cells in which no viable cells were found after 4 hours and 3 hours under the same concentrations, respectively.

Exposed C. jejuni RM1221 cells to concentrations of 0.01% (w/v) and 0.02% (w/v) chitosan caused significant decreases in cell recovery with respect to untreated cells from 1-6 hours in both cases ($F (13, 154) = 26151$, $p<0.0001$) and ($F (13, 154) = 19492$, $p<0.0001$), respectively ($p<0.05$). This was similar to treated wild-type NCTC11168 cells (Figure 5.3), indicative by a lack of viability

after 4 hours and 3 hours exposure to 0.01% (w/v) and 0.02% (w/v) chitosan, respectively.

An increased chitosan tolerance, comparable to the 'adapted' NCTC11168 cells appeared was apparent with regards to the 81116 C. jejuni cells upon exposure to 0.01% (w/v) and 0.02% (w/v) chitosan (Figure 5.3). Significant differences were found from 1-6 hours exposure to chitosan at both 0.01% (w/v) ($F(13, 154) = 24819$, $p<0.0001$) and 0.02% (w/v) of chitosan concentrations used ($F(13, 154) = 2611$, $p<0.0001$). Similarly, a total loss of cell recovery was also observed after 4 and 5 hours chitosan exposure to 0.02% (w/v) and 0.01% (w/v) chitosan in nutrient limiting conditions for NCTC11168CH cells, respectively (Figure 5.3). Nevertheless, significant decreases were also observed from 1-6 hours upon exposure to 0.01% (w/v) ($F(13, 154) = 14330$, $p<0.0001$) and 0.02% (w/v) chitosan ($F(13, 154) = 17212$, $p<0.0001$), relative to untreated control, confirmed by post hoc testing ($p<0.05$).

5.4.6 Effects of chitosan on growth inhibition

The effects of sub-lethal concentrations of low molecular weight chitosan equal to 0.5X and 1X wild-type NCTC11168 MIC (0.0008% and 0.0016% (w/v), respectively) were also investigated over a period of 26 hours (Figure 5.4).

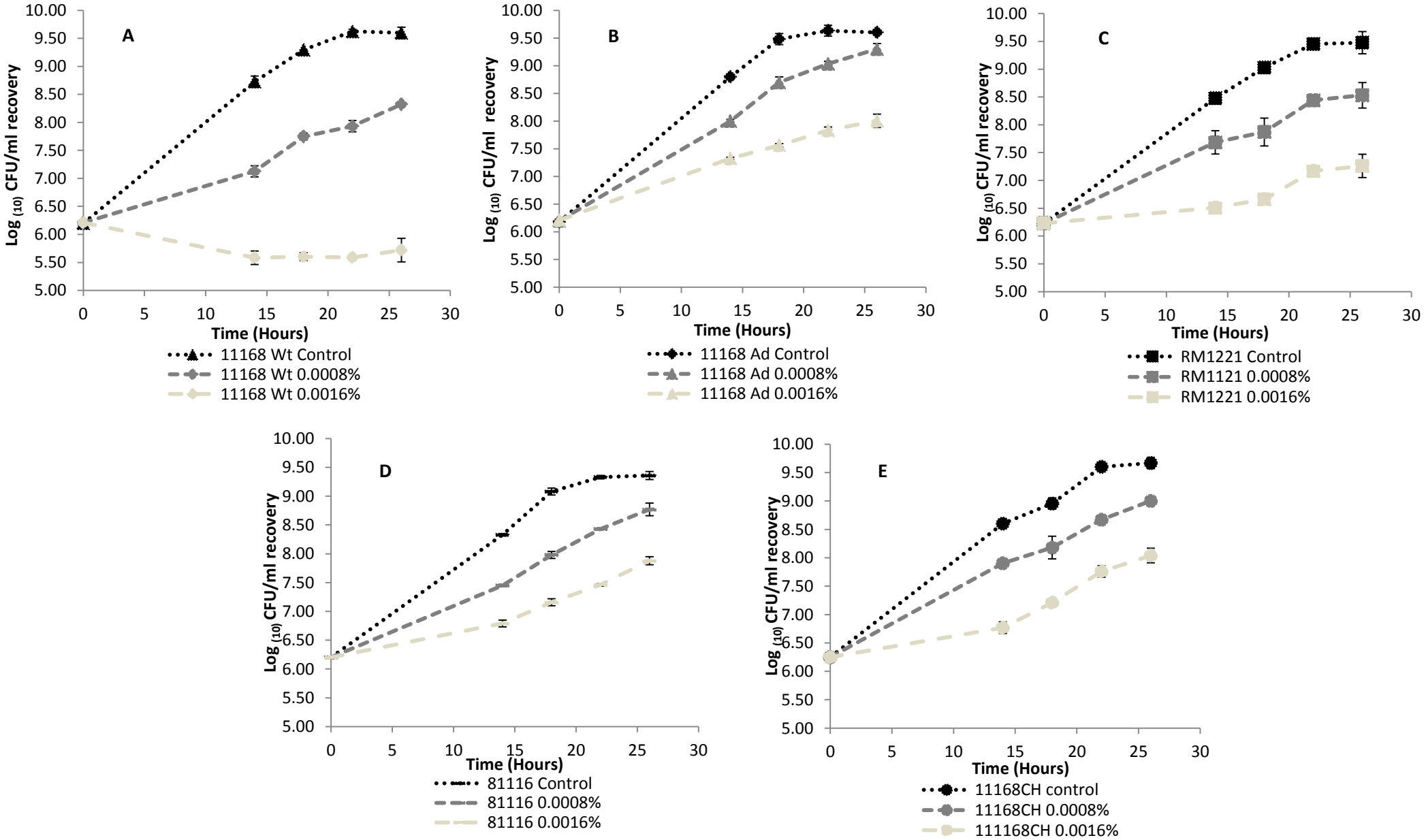

Figure 5.4: Inhibition of growth following exposure to 0.0008% (w/v) and 0.0016% (w/v) chitosan with regards to wild-type NCTC11168 (A), 'adapted' NCTC11168 (B), RM1221 (C), 81116 (D) and 11168CH (E) C. jejuni cells grown under control conditions (pH 6.0). Experiments were performed n = 4 and plated in triplicate. Data represents mean values; error bars indicate SEOM.

Growth in MH broth (pH 6.0) with final concentrations of 0.0008% (w/v) and 0.0016% (w/v) chitosan were found to significantly inhibit wild-type C. jejuni NCTC11168 recovery in comparison to the untreated control cells as expected. Log reductions of 1.6, 1.55, 1.7 and 1.28 were typical upon exposure to 0.0008% (w/v) chitosan after 14, 18, 22 and 26 hours exposure, respectively. As expected, when a greater inhibition was observed in relation to cells subjected to 0.0016% (w/v) chitosan, with log reductions typical of 3.15, 3.70, 4.04 and 3.88 after 14, 18, 22 and 26 hours exposure, respectively (Figure 5.4). Significant reductions in viable cells were also found from 14-26 hours sampled for both chitosan concentrations used relative to the respective controls at the given time point $F (8, 80) = 7043$, $p<0.0001$), confirmed by post hoc analysis.

Lesser significant decreases in cell recovery were also found in relation to the 'adapted' NCTC11168 cells when grown in the presence of chitosan at 0.0008% (w/v) and 0.0016% (w/v) chitosan ($F (8, 80) = 936.5$, $p<0.0001$). This was seen to be dose dependant, with log reductions typical of 0.8, 0.78, 0.65 and 0.30 when exposed to 0.0008% (w/v) chitosan and 1.47, 1.92, 1.8 and 1.59 with regards to 0.0016% (w/v) chitosan exposure at 14, 18, 22, and 26 hours, respectively (Figure 5.4).

Growth was also seen to be significantly inhibited with respect to RM1221 cells exposed to 0.0008% (w/v) and 0.0016% (w/v) chitosan over the course of 26 hours tested ($F (8, 80) = 1431$, $p<0.0001$). Upon exposure to 0.0008% (w/v) chitosan, log reductions typical of 0.8, 1.16, 1.01 and 0.95 were seen after 14, 18, 22 and 26 hours exposure relative to the respective control counts ($p<0.05$). Greater reductions were found observed with respect to the cells grown in the presence of 0.0016% (w/v) chitosan, typical of log CFU/ml decreases totalling 1.97, 2.37, 2.28 and 2.22 after 14, 18, 22 and 26 hours growth (Figure 5.4).

Growth was also found to be inhibited when the 81116 cells were exposed to chitosan, with a greater inhibition when grown in 0.0016% (w/v) chitosan (Figure 5.4). Significant differences were observed from 14-26 hours incubation, with log reductions of 0.88, 1.11, 0.9 and 0.59 at 14, 18, 22 and 26 hours growth relative to the untreated control samples ($p<0.05$). Log reduction in relation to the control cells of the 81116 strain were found to be 1.54, 1.92, 1.87 and 1.48 when grown in the presence of 0.0016% (w/v) chitosan ($F (8, 80) = 1246$, $p<0.0001$).

Upon exposure of the NCTC11168CH isolate to 0.0008% (w/v) and 0.0016% (w/v) chitosan, significant decreases in growth were observed as indicated by a reduction in viable cells during 14, 18, 22 and 26 hours ($p<0.05$). After 14, 18, 22 and 26 hours of growth, log reductions of 0.7, 0.77, 0.93 and 0.67 were found when cells were grown in the presence of 0.0008% (w/v) chitosan ($p<0.05$). Whereas, when exposed to 0.0016% (w/v) chitosan, greater significant log decreases in growth were observed after 1.83, 1.74, 1.84 and 1.63 during 14, 18, 22 and 26 hours growth ($F (8, 80) = 5030$, $p<0.0001$).

When comparing the viable cells after 26 hours of incubation when grown in 0.0008% (w/v) chitosan, the greatest growth inhibition was established in the wild-type NCTC11168 cells (8.32 log CFU/ml), followed by the RM1221 cells (8.53 log CFU/ml), 81116 cells (8.77 log CFU/ml), NCTC11168CH cells (9.00 log CFU/ml) and lastly the 'adapted' NCTC11168 cells (9.30 log CFU/ml), found to represent the lowest amount of total growth inhibition. These differences were identified to be significantly different ($F (4, 40) = 259.2$, $p<0.0001$), as confirmed by post hoc testing ($p<0.05$).

A similar trend in the overall growth inhibition typical of cells grown for 26 hours under an increased concentration of 0.0016% (w/v) chitosan was evident. Once again, the wild-type NCTC11168 was seen to illustrate the greatest susceptibility in viable cells recovered (5.72 log CFU/ml), followed by RM1221 cells (7.26 log CFU/ml), 81116 (7.88 log CFU/ml), 'adapted' NCTC11168 (8.01 log CFU/ml) and the lowest in the NCTC11168CH cells (8.04 log CFU/ml). One way ANOVA revealed that a significant differences between mean log CFU/ml values between strains/isolates tested ($F(4, 40) = 6415$, $p<0.0001$). Post hoc analysis indicated that cell recovery in relation to the 'adapted' and NCTC11168CH isolates was not significantly different ($p>0.05$).

5.5 Discussion

The antimicrobial activities of chitosan are widespread, having been identified in a range of microorganisms including fungi, yeasts and bacteria species alike (Raafat and Sahl, 2009). This chapter demonstrated there are indeed differences in the antimicrobial susceptibility of low molecular weight chitosan against the C. jejuni strains/isolates tested.

When comparing MIC values of the C. jejuni strains/isolates tested, the antimicrobial activity of chitosan was found to be dependent upon the pH. MIC Fold increases of 16.25, 10.43, 13.6, 12.5 and 9.4 were found with regards to the wild-type NCTC11168, 'adapted' NCTC11168, RM1221, 11168CH and 81116 upon increasing to pH 6.5, as oppose to the MIC values obtained at pH 6.0.

This coincides with previous studies have previously indicated that antimicrobial activity for chitosan is pH dependent (Kong, 2008). This is thought to be the case, as chitosan is only soluble in an acidic environment and becomes polycationic at pH values below the pKa (6.3-6.5) (Lim and Hudson, 2004). It has also been reported that chitosan only exerts antimicrobial activity under acidic conditions exclusively (Helander et al., 2001).

The rationale for performing the time kill and growth inhibition studies exclusively at pH 6.0 was based on recurrent issues with solubility when adjusting low molecular weight chitosan to a final pH of 6.5. As a result of this, experiments utilising pH6.5 media/chitosan were avoided. At pH greater than 6.5, chitosan solutions is said to exhibit phase separation, whereas when at a pH

below 6.5, chitosan is readily soluble, carrying an positive charge via protonation of amino groups (Kumirska et al., 2011; Elsabee et al., 2009). Chitosan is considered as a strong base due to the possession of amino groups which have a pKa value of around 6.3 (Saied and Aider, 2014). The presence of these amino groups suggest that pH may change the state of both charge and properties of the chitosan, when the pH is below the pKA of chitosan, it is polycationic whilst at pH of 4 and below, chitosan is completely protonated (Jayakumar et al., 2010).

To summarise, the time kill assays concerning chitosan exposure over a period of 6 hours revealed clear differences in susceptibility between the C. jejuni strains/isolates tested. Exposure to both 0.01% (w/v) and 0.02% (w/v) chitosan revealed varying degrees of total cell viability/recovery over this period of 6 hours exposure. In general, the most susceptible C. jejuni strains were found to be the wild-type NCTC11168 and RM1221 strains, both indicating an absence viable cells detected after 6 hours exposure to 0.01% (w/v) chitosan when incorporated in MH broth. Similar observations were found upon challenging to the same concentrations of chitosan in the time kill experiments involving nutrient limited conditions, as simulated by a PBS environment. Both the wild-type NCTC11168 and RM1221 cells were not recovered after 4 hour and 3 hours when challenged to 0.01% (w/v) and 0.02% (w/v) chitosan, respectively.

The greatest tolerance to chitosan indicated by the time kill experiments was observed in the 'adapted' NCTC11168 cells of C. jejuni. Viable cells were found even after 6 hours exposure to 0.01% (w/v) chitosan (3.59 log CFU/ml), which was found to be significantly higher than the recovery found after 6 hours exposure with respect to the 81116 cells (2.50 log CFU/ml detected) and

NCTC11168CH cells (2.60 log CFU/ml detected). Exposure to 0.02% (w/v) chitosan indicated a similar susceptibility pattern between the 'adapted' NCTC11168 and the 81116 cells challenged, indicative of a total loss of viable cells after 6 hours exposure in all cases.

Moreover, comparable losses in cell viability were observed when concerning the 'adapted' NCTC11168, 81116 and NCTC11168CH cells to 0.01% (w/v) and 0.02% (w/v) chitosan when suspended in PBS (nutrient limitation conditions), with no detectable cells following 5 and 4 hours, respectively for all strains/isolates. In contrast, an increased susceptibility was apparent with regards to the wild-type NCTC11168 and RM1221 exposed cells, with a complete loss in cell recovery after 4 and 3 hours exposure to 0.01% (w/v) and 0.02% (w/v) chitosan, respectively for both strains. These findings suggest that under nutrient depleted conditions (PBS), all strains/isolates of C. jejuni were found to have a greater degree of susceptibly when challenged to chitosan in MH broth, indicated by prolonged cell viability with respect to the latter condition.

Time kill experiments were also seen to be consistent with findings with respect to chitosan susceptibility in growth inhibition studies, at sub-lethal concentrations of both 0.0008% (w/v) and 0.0016% (w/v) chitosan. For example, after 26 hours of incubation when growth in 0.0008% (w/v) chitosan, the greatest growth inhibition was identified for the wild-type NCTC11168 cells with the 'adapted' NCTC11168 cells occupying the lowest degree of inhibition under the same conditions. The resulting inhibition following growth in 0.0016% (w/v) chitosan accounted for a substantial decrease in cell recovery with respect to the wild-type cells, whereas both the 'adapted' and NCTC11168CH isolates were seen to have an enhancement in growth following 26 hours exposure.

Generally, these above findings support the MIC values obtained at pH 6.0, with the lowest MIC values, with the particular respect to the wild-type NCTC11168 strain when compared to the other strains/isolates tested. Results also coincide with greater tolerance seen in the 'adapted' NCTC11168 isolate in particular comparison to the wild-type NCTC11168 cells upon challenge to chitosan.

Previous studies on the antimicrobial activity of chitosan with respect to Campylobacter are limited. One study conducted by Ganan et al. (2009), illustrated differences in the antimicrobial activity of chitosan against strains of Campylobacter even when utilising chitosan previously adjusted to pH 7.0. In this study, all strains of C. jejuni were found to be inhibited at a chitosan concentration of 0.05% (w/v), although at a lower concentration of 0.01% (w/v), C. jejuni LP1 strain did not show any significant decrease in cell number when challenged at pH 7.0 relative to the untreated control. In contrast, the C. jejuni NCTC11168 and NCTC11351 strains illustrated varying degrees of inhibition upon exposure to the same concentration of chitosan. Results in this study concluded the Campylobacter species in a general sense illustrated greater sensitivity to chitosan, reinforced by a lower susceptibility found in P. aeruginosa, S. aureus and Lactobacillus spp. under the same conditions (Ganan et al., 2009).

Discrepancies between findings in this chapter with respect to results found in the above study are more than likely due to the differences in the pH of chitosan and diluent used. Chitosan has been shown to illustrate a stronger inhibition towards bacterial species at lower pHs, with a weakening at pH values above the pKa (Kong et al., 2008). Although it seems chitosan is not completely inactive in terms of antimicrobial activity at pH 7, as indicated by Ganan et al. (2009), an

increased susceptibility in C. jejuni strains/isolates established in this chapter may reflect the reduced ability of chitosan to remain bactericidal at pH 7. This may be due to the presence of a larger quantity of uncharged amino groups and well as poor solubility at this pH (Sudarshan et al., 1992). Thus, it seems relatively small shifts in pH can account for a drastic alteration in the antimicrobial activity of chitosan (Jumaa et al., 2002; Devlieghere et al., 2004). The differences in sensitivity of C. jejuni strains/isolates tested may explain how such fragile microorganisms can potentially become much more of an issue through decreases in susceptibility to antimicrobials such as chitosan. With particular attention to the generated 'adapted' isolate of C. jejuni NCTC11168, it is clear that an adaptive tolerance response towards low molecular weight chitosan can allow for significant increases in the ability to withstand such sub-inhibitory and lethal concentrations of chitosan.

With respect to the antimicrobial activity of chitosan films when added to MH broth (pH 6.0), although effective in significantly reducing growth of all C. jejuni strains/isolates tested, no significant differences between the strains/isolates tested were established. It can therefore be concluded that chitosan film activity is broadly effective towards C. jejuni in a global sense. Additionally, chitosan films could be of use in decreasing the bacterial load of Campylobacter species with respect to foodstuffs, for instance during poultry processing. This suggests the need for further study using food models to determine if these films exert similar effects in a real world scenario. Surprisingly, there appears to be no literature with regards to the antimicrobial efficacy of chitosan film activity against Campylobacter species.

C. jejuni strains have previously been found to have differing degrees of sensitivity to a range of antibiotics, which has been shown to be isolate dependant (Kumar-Phillips et al., 2013). Exposure to sub-lethal stresses may give rise to increasing amounts of resistance of C. jejuni, assisting in an increased survival potential. This can potentially prove to play a major role differences in aspects such as cross protection towards different stressors (Kumar-Phillips et al., 2013). Bacteria exposed to non-lethal (sub-inhibitory) concentrations of antibiotics have been suggested to play an important role upon generation of resistance; such changes may induce variability in genotype and phenotype (Andersson and Hughes, 2014).

With the above in mind, the exploration of the phenotypic and genotypic aspects resulting from C. jejuni challenge to chitosan was subsequently tested in the following chapters (chapter 6 and 7, respectively).

Chapter 6 – Phenotypic differences of *C. jejuni* in relation to biofilm, production, motility and chemotaxis

6.1 Introduction

In order for C. jejuni to become established in a given host and food-related environment, it must also be able to undergo phenotypic alterations which can ultimately lead to an increased survival potential. Several factors are believed to have a bearing upon survival, virulence and subsequent pathogenesis of C. jejuni. These include, but are not limited to biofilm formation, motility and chemotaxis.

Many microorganisms are thought to be able to form biofilms. The primary feature of this phenotype involves the production of extracellular materials which function as a protective mechanism of cell embedment (Flemming and Wingener, 2010). Food contact surfaces are ideal environments which can allow for the formation of biofilms. Despite strict cleaning and disinfection regimes, planktonic cells can often settle in hard to access regions due to surface irregularities for instance (Orgaz et al., 2011). In the event of improper cleaning, microbial cell transfer can occur from the biofilm onto foodstuffs, which is hazardous from a food safety and quality perspective (Shi and Zhu, 2009).

Usually, in natural, industrial or clinical environments, bacteria species are more likely to be found as a biofilm, as oppose to a planktonic state (O'Toole et al., 2000; Reeser et al., 2007). Increased protection from various stresses such as sanitizers and antibiotics/antimicrobials can occur as a result of biofilm

formation. This is thought to be due partly to an increased nutrient access as well as genetic exchange between the cellular community within the biofilm (Hanning and Slavik, 2009). Formation of biofilms has been found to increase following stresses related to nutrient depletion (Ryu et al., 2004). Also it has been previously documented that Campylobacter species can form biofilm in a range of environments including aquatic environments, on stainless steel and also on glass (Somers et al., 1994: Buswell et al., 1998; Joshua et al., 2006).

Motility observed in C. jejuni is often associated with the process of chemotaxis, another factor thought to contribute to the overall virulence potential. Essentially, chemotaxis has been shown to facilitate the migration of C. jejuni towards favourable conditions and also away from unfavourable environmental conditions (Hermans et al., 2011). A number of genes are believed to be associated with the chemotactic response in C. jejuni, these include energy taxis response genes, cetA and cetB (Golden and Archeson, 2002). In terms of overall regulation of the chemotactic response to stimuli, the cheY gene, coding for a response regulator, has been found also to govern flagellar rotation (Stephens et al., 2006). In one study, mutants deficient in cheY have been reported to affect the degree of colonisation of the intestinal tract of chicks (Hendrixson and DiRita, 2004).

Additionally, flagella are often associated with bacterial biofilm formation, in many microorganisms including Pseudomonas (Barken et al., 2008), Yersinia (Kim et al., 2008) and Vibrio species (Watnick et al., 2001). There has also been evidence to suggest that biofilm formation is dependent upon flagella in C. jejuni (Reuter et al., 2010). C. jejuni mutant deficient in FlaAB have been shown to occupy a reduced ability to form biofilms when compared to motile wild-type C.

jejuni NCTC11168. Additionally, when comparing a non-motile variant of C. jejuni against a motile NCTC11168 variant, after 48 hours of biofilm growth, a reduction of over 50% was observed with respect to the non-motile variant (Reuter et al., 2010). Interestingly, a study has also suggested the potential role of flagella as an adhesion factor during biofilm formation (Kirov et al., 2004). Nevertheless, flagellar components have also been recognised as important initiators of biofilm formation both in terms of solid surfaces (attached) and also pellicle formation within liquid cultures. This was indicated by an enhanced expression of proteins thought to be involved in the flagellar motility complex (Kalmokoff et al., 2006).

Therefore, it can be assumed that these phenotypes present in C. jejuni are linked. This chapter attempted to assess the phenotypes regarding motility, biofilm and chemotaxis phenotypes was undertaken to illustrate any trends/discrepancies which may occur amongst C. jejuni strains/isolates previously tested.

6.2 Aims and objectives

6.2.1 Aim

This chapter aimed to assess any differences in phenotypes related to motility, chemotaxis and biofilm formation.

6.2.2 Objectives

- *To determine any differences in motility between C. jejuni strains/isolates.*

- *To establish the chemotactic response of C. jejuni to the presence of low molecular weight chitosan in relation to control chemicals.*

- *To assess potential differences in biofilm formation/eradication in relation to several C. jejuni strains/isolates in the presence and the absence of chitosan.*

6. 3 Materials and Methods

6.3.1 Motility assay

In order to establish the degree of motility between strains/isolates of C. jejuni, methods adapted from Tareen et al., (2010) were employed. Briefly, 2µl of an overnight culture of approximately 1x10^{8} was stabbed into the centre of an agar plate containing 0.4% MH agar (pH 7.0) with a sterile pipette tip. Plates were then incubated at 42°C under microaerobic conditions for 24, 48 and 72 hours. The low concentration of Mueller-Hinton agar allowed the bacteria to swarm within the agar. Each experiment was performed 3 times in triplicate.

6.3.2 Chemotaxis assay

A chemotaxis assay was undertaken as described by Hugdahl et al. (1988) and Vegge et al. (2009). Overnight cultures of C. jejuni Mueller-Hinton broth were washed in phosphate-buffered saline (PBS) and the bacterial concentration adjusted to approximately 2x10^{9} cfu/ml with temperate soft Bacteriological agar (0.4% v/v). Ten millilitres of bacterial suspension was poured into a sterile petri dish and 2cm filter discs (Whatman, UK) saturated with test chemicals (0.1 M

and pH 6) were placed on the solidified soft agar comprising of the bacterial suspension. Following 4 hours of incubation at 42°C under microaerobic conditions, the chemotactic activity was examined over a light source provided by the GeneSys Gel Dock (SynGene). Bacterial accumulations or clearing zones were interpreted as zones of chemo-attraction and migration away from a test chemical was considered as chemo-repulsion.

6.3.3 Biofilm formation assay

Biofilm formation experiments were performed adapted from Reeser et al. (2007). The wells of 96-well polystyrene plates contained either 200µl Mueller-Hinton broth (pH 6.0) of a mixture of MH broth and low molecular chitosan (pH 6.0) to a final concentration ranging from 0.002% (w/v) – 0.032% (w/v) following inoculation from prepared overnight cultures of C. jejuni to a final concentration of $1x10^7$ cfu/ml. Plates were then incubated under microaerobic conditions at 42°C for 48 and 72 hours.

The well contents were then removed via pipetting and residues were dried for 30 minutes at 55°C, after which 250µl 0.1% (w/v) crystal violet (CV) was added for 5 min at room temperature. Unbound CV was then removed, and the wells were washed twice with sterile distilled water. The wells were dried once again at 55°C for 15 minutes prior to bound CV undergoing de-colourisation with 250µl of an 80% ethanol-20% acetone mix. One hundred microliters of the solution from each well was removed and placed in a 96-well plate, and the absorbance at 595nm was determined using a Bio-Rad 680XR Microplate reader.

6.3.4 Biofilm eradication assay

Biofilm eradication was investigated using a similar methodology as above, with the exception 48 hours prior incubation in the absence of chitosan. This was then followed by media replacement with a subsequent 24 hour incubation to follow. With respect to control pre-formed biofilms, chitosan-free MH media replenishment was administered. The remaining biofilms were exposure to the same range of chitosan concentrations as above and exposed for 24 hours. This was then followed by crystal violet staining and absorbance quantification, as before.

6.4 Results

6.4.1 Motility comparison in C. jejuni strains/isolates

Data in Figure 6.1 demonstrates an increase in motility over the course of incubation time period, ranging from 24 hours to 72 hours. This was indicated by highly significant differences which was apparent upon conducting one way ANOVA regarding all strains/isolates tested ($F (14, 255) = 159.6$, $p<0.0001$). Post hoc testing confirmed this, with all respective C. jejuni strains/isolates illustrating significant increases in motility with an increase in incubation time, as expected ($p<0.05$).

The greatest overall motility was generally observed in the RM1221 strain, the "adapted" C. jejuni NCTC11168 isolate was the next most motile, followed by the NCTC11168 isolate which had previously undergone passage through a

chicken (11168CH). The 81116 and the wild-type NCTC11168 cells were found to be least motile. This trend was representative at all time points.

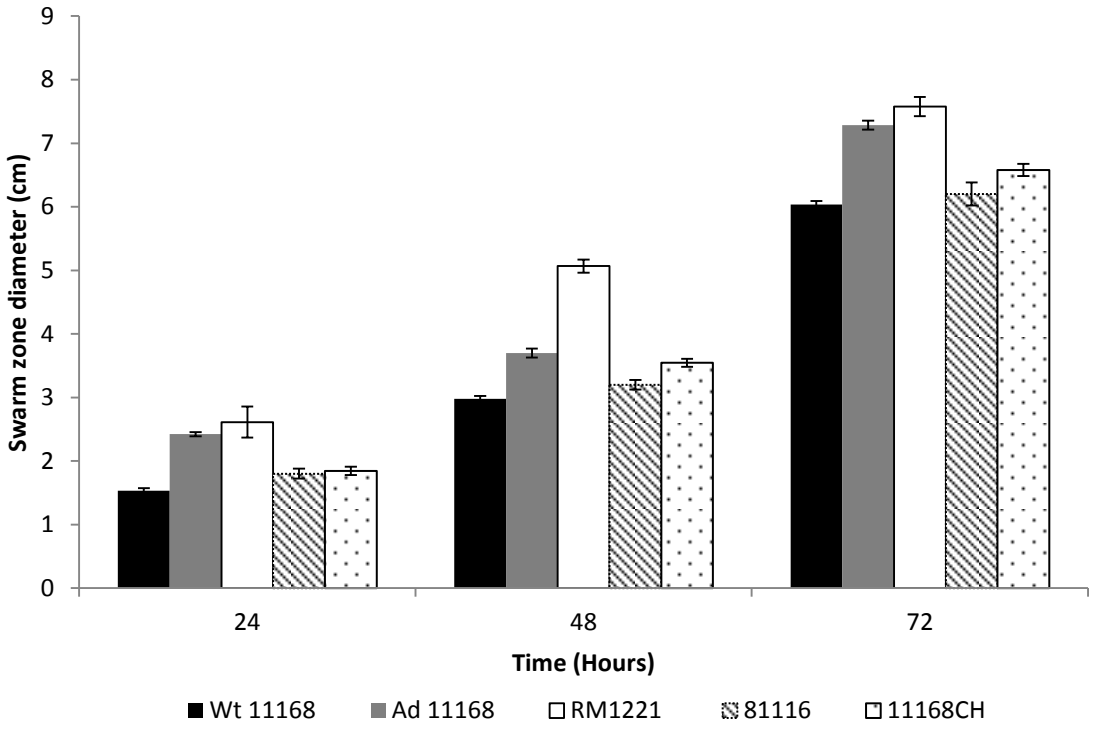

Figure 6.1: Motility comparison between several strains/isolates of C. jejuni. Experiments were performed n = 6, triplicate. Data represents mean values; error bars indicate SEOM.

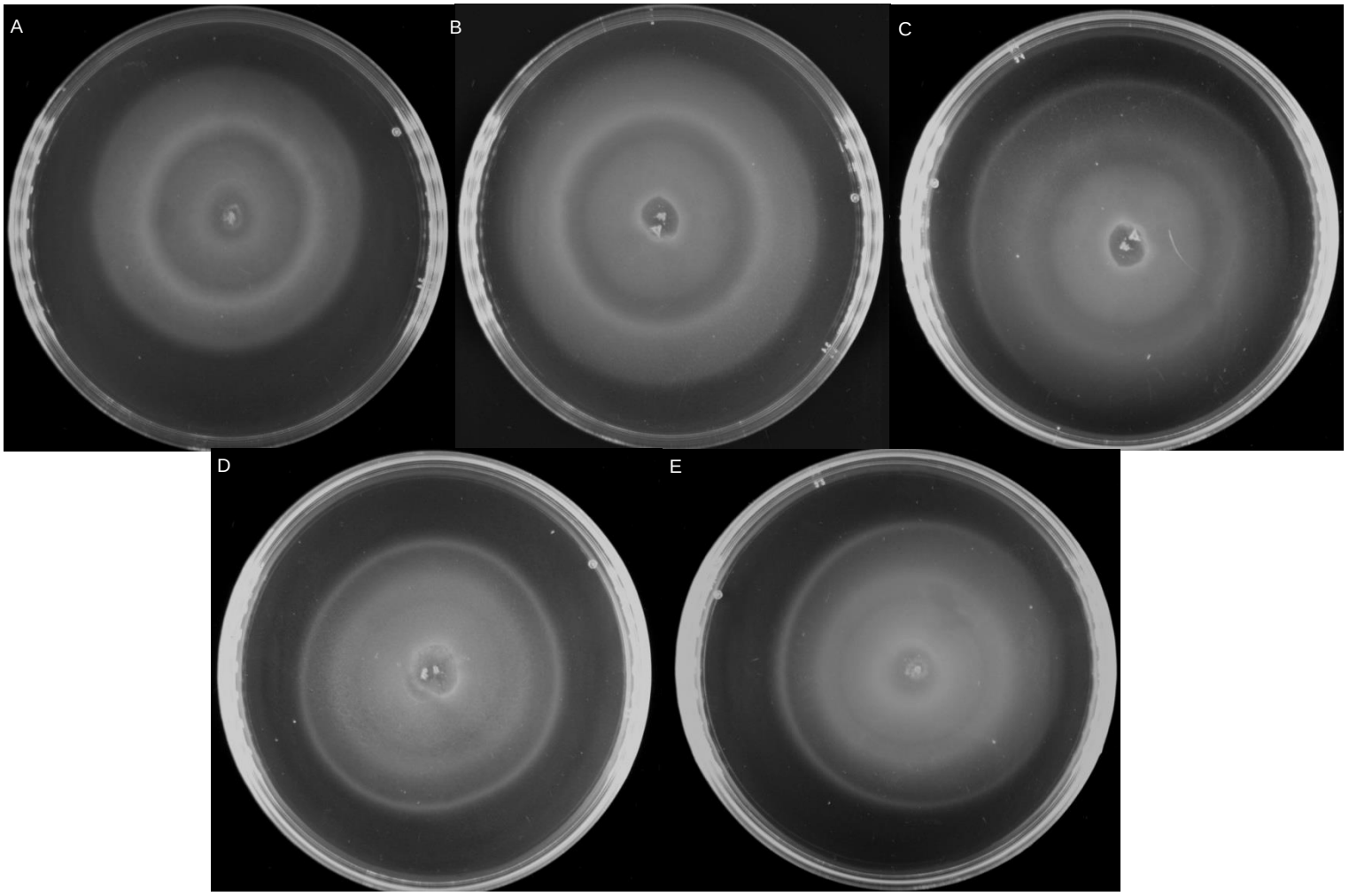

Figure 6.2: Representative motility comparison between (A) wild-type NCTC11168, (B) 'adapted' NCTC11168, (C) RM1221, (D) 81116 and (E) 11168CH strains/isolates of C. jejuni representative of 72 hours incubation under microaerobic conditions at 42°C.

One way ANOVA testing identified significant differences between motility in the C. jejuni strains/isolates tested were typical after 24 hours ($F(4, 85) = 30.07$, $p<0.0001$), 48 ($F(4,85) = 260.2$, $p<0.0001$) and 72 hours incubation ($F(4,85) = 14.78$, $p<0.0001$).

Further post hoc analysis revealed that the amount of motility observed after 24 hours of incubation was seen to be significantly different when comparing all C. jejuni strains/isolates, with the exception of the "adapted" NCTC11168 isolate versus the RM1221 strain ($p<0.05$). Overall, the greatest difference in motility was seen in the RM1221 strain, with an increase of 1.08cm relative to the wild-type isolate of C. jejuni NCTC11168.

With respect to motility post 48 hours incubation, post hoc analysis indicated significance when comparing C. jejuni strains/isolates tested, excluding the negligible difference between the chitosan "adapted" and 11168CH isolates, with a slight variance 0.16cm in diameter ($p<0.05$). Similar to the degree of motility after 24 hours incubation, the greatest decrease in motility was found in the wild-type C. jejuni NCTC 11168 isolate relative to the RM1221 strain (-2.09cm). Additionally, after 72 hours of incubation, post hoc analysis suggested significant differences in C. jejuni strains/isolates compared bar the wild-type NCTC11168 versus the 81116 strain, "adapted" NCTC11168 isolate in relation to the RM1221 strain and also the 81116 strain when compared to the 11168CH isolate ($p<0.05$). Similar to the above, the greatest significant decrease in motility between strains/isolates was typical of the wild-type NCTC11168 cells in relation to the RM1221 strain (-1.54cm).

6.4.2 Chemotaxis of C. jejuni strains/isolates

The following figure represents chemotactic activity of C. jejuni strains/isolates after 4 hours incubation at 42°C under microaerobic conditions (Figure 6.3)

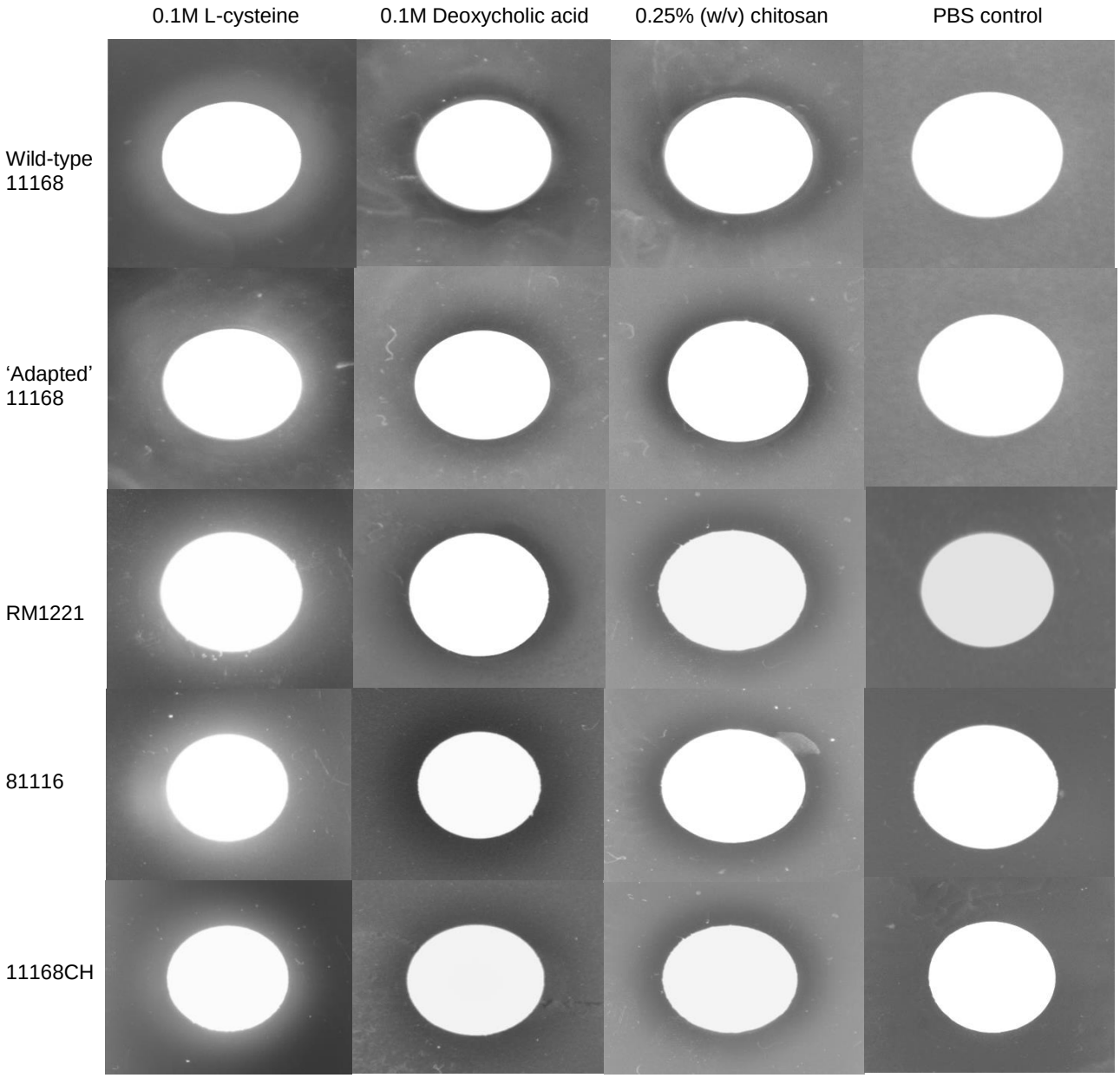

Figure 6.3: Representative Chemotaxis activity of C. jejuni strains/isolates in response to 0.1M L-cysteine, 0.1M deoxycholic acid, 0.25% (w/v) chitosan and PBS (pH 6.0) for 4 hours. Experiments were performed at 42°C under microaerobic conditions and repeated on three independent times in triplicate.

The chemotactic response was found to be similar in all C. jejuni strains/isolates tested (Figure 6.3). Chemo-attraction was observed when C. jejuni were exposed to positive control, 0.1M L-cysteine, as indicated by the accumulation of cells around the saturated disk. Chemo-repulsion was typical in response to 0.1M deoxycholic acid (negative control), typical of a clearing of cells from this stimulus. 0.25% (w/v) of chitosan produced a chemo-repulsive phenotype for all strains of C. jejuni, whilst no response was found with regards to PBS.

6.4.3 Biofilm formation after 48 hours incubation

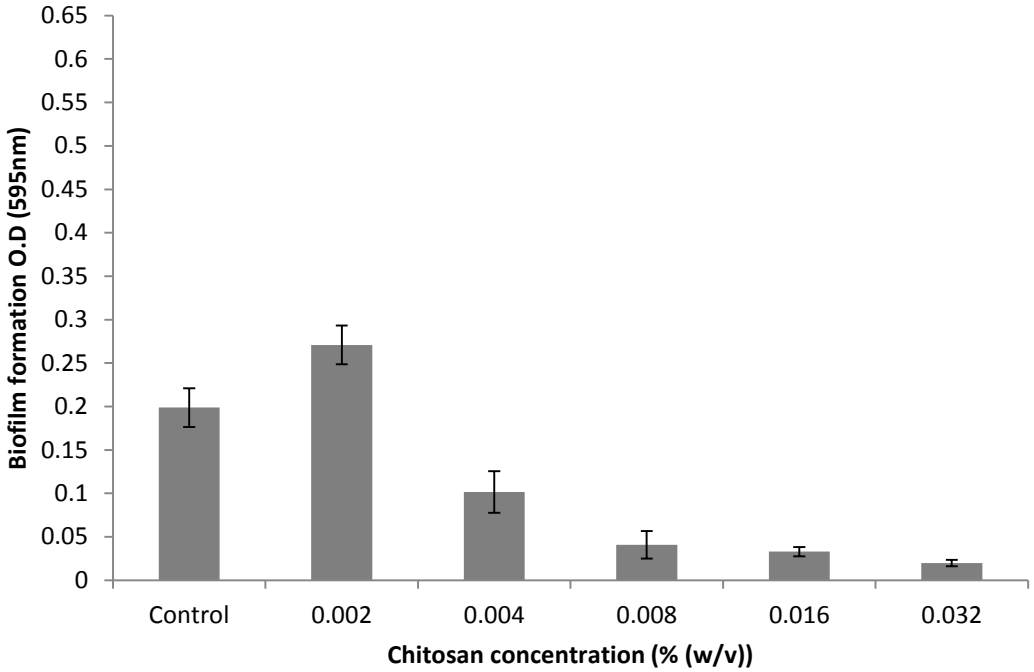

Figure 6.4: Comparison of wild-type C. jejuni NCTC 11168 biofilm formation after 48 hours incubation in the absence or presence of various concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

The degree of biofilm formation with respect to wild-type C. jejuni NCTC 11168 after 48 hour incubation at pH 6.0, as represented by the amount of absorbance was found to be significantly different when comparing those grown in the absence of chitosan (control) against those which were formed in the presence of low molecular weight chitosan ($F (5, 48) = 65.05$, $p<0.0001$). Post hoc analysis revealed significant differences in biofilm formation with respect to all those formed in all concentrations of chitosan when compared to the control ($p<0.05$). Interestingly, an increase in biofilm formation (0.08nm) was found with regards to the 0.002% (w/v) chitosan, relative to the cells which were not exposed to chitosan. A decrease in biofilm formation was typical from 0.004% (w/v) to 0.032% (w/v) relative to those formed under control conditions. These decreases were found to be 0.09nm, 0.15nm, 0.15 and 0.18, respectively (Figure 6.4).

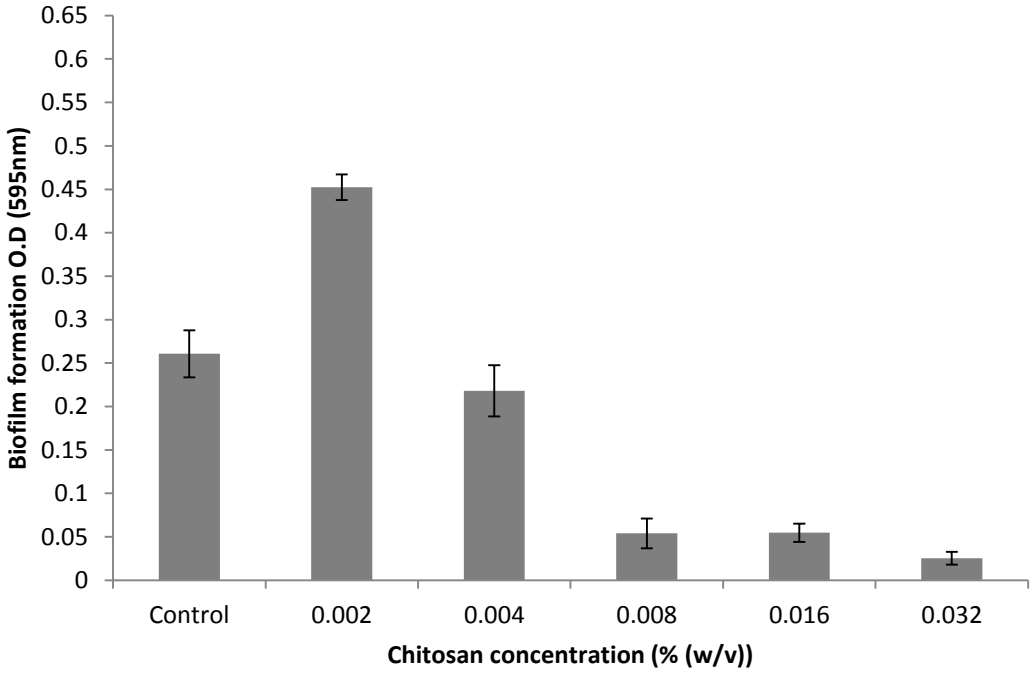

Figure 6.5: Comparison of 'adapted' C. jejuni NCTC 11168 biofilm formation after 48 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

Similar to the biofilm formation seen in the wild-type C. jejuni NCTC 11168, the chitosan 'adapted' isolate of C. jejuni NCTC 11168 was seen to differ in terms formation when exposed to different concentrations of chitosan over the course of 48 hours. Biofilm formation was found to be substantially increased in the presence of 0.002% (w/v) chitosan in comparison to the control cells grown in the absence of chitosan. An apparent decrease in formation was typical for those biofilms which were grown in final concentrations of 0.004% (w/v) – 0.032% (w/v) chitosan, as oppose to those in the control conditions. These differences were found to be statistically significant ($F(5, 48) = 107.6$, $p<0.0001$) and typical of 0.05, 0.21, 0.21 and 0.24nm, respectively. Post hoc analysis reinforced these observations with the exception of those biofilms formed in 0.004% (w/v) chitosan, which was not deemed significantly different from the control samples ($p>0.05$) (Figure 6.5).

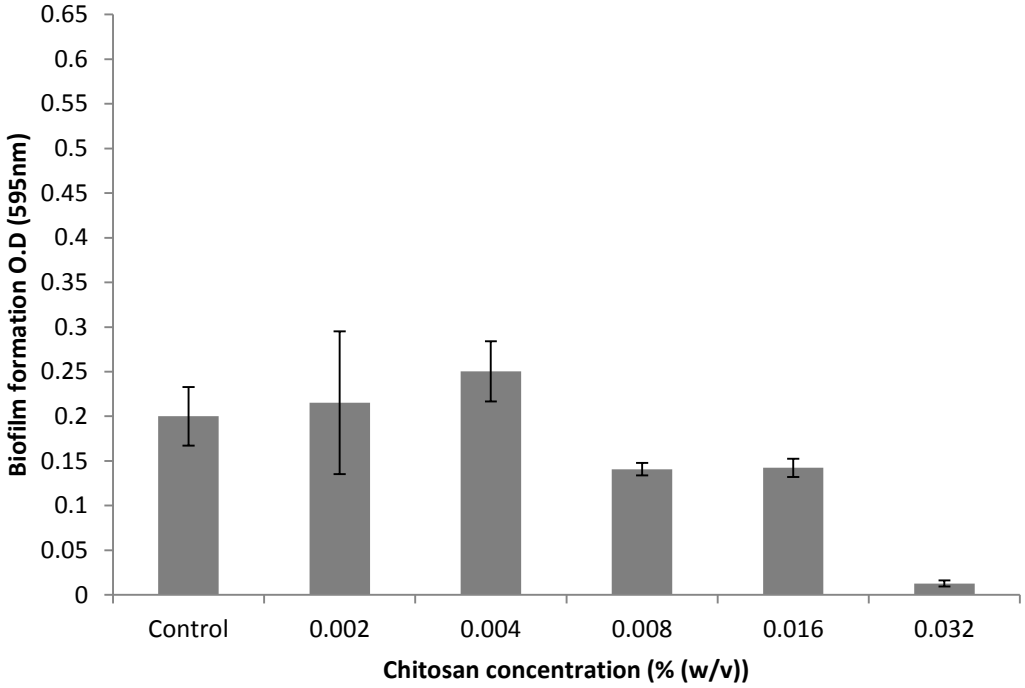

Figure 6.6: Comparison of C. jejuni RM1221 biofilm formation after 48 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

After 48 hours of biofilm formation with respect to the RM1221 strain of C. jejuni, statistically significant differences in the absorbance from biofilm formation was observed when comparing those cells grown in the presence of chitosan against the cells characteristic of the control ($F(5, 48) = 11.63$, $p<0.0001$). However, post hoc analysis identified no significant difference between the biofilm formed under control conditions in relation to 0.002% (w/v), 0.004% (w/v), 0.008% (w/v) or 0.016% (w/v) chitosan ($p>0.05$). The only significant difference was found in relation to the biofilms formed under control conditions versus those exposed to 0.032% (w/v) chitosan ($p<0.05$). Interestingly, the amount biofilm formation was found to be fairly consistent when comparing the control against those formed in concentrations of chitosan ranging from 0.002% (w/v) – 0.016% (w/v) (Figure 6.6).

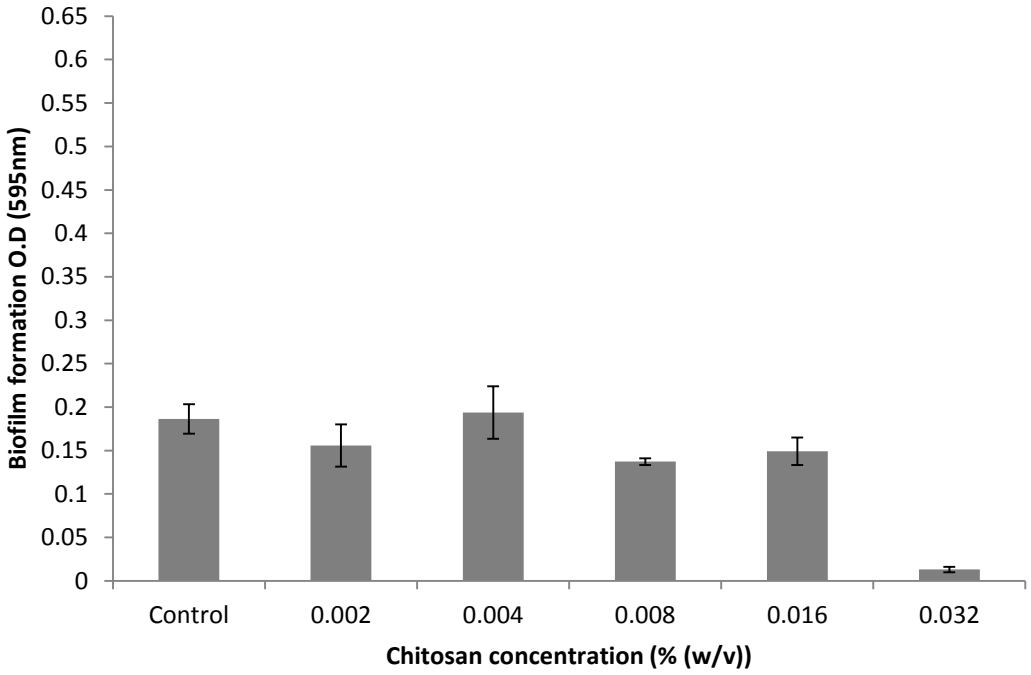

Figure 6.7: Comparison of C. jejuni 81116 biofilm formation after 48 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

A similar trend was observed in Figure 6.7, concerning the 81116 strain of C. jejuni with regards to consistent biofilm formation under control conditions and chitosan concentrations of 0.002% (w/v) – 0.016% (w/v). Although one-way ANOVA analysis found a significant difference between the control biofilm formation against those formed under chitosan conditions ($F(5, 48) = 33.32$, $p<0.0001$), post hoc testing suggested no significant difference in relation to the biofilm formation under control conditions against those concerned with 0.002% (w/v), 0.004% (w/v) and 0.016% (w/v) chitosan exposure ($p>0.05$). However, a significant difference in absorbance was reported between the control and both the 0.008% (w/v) and 0.032% (w/v) conditions ($p<0.05$) and were typical of 0.05nm and 0.17nm, respectively.

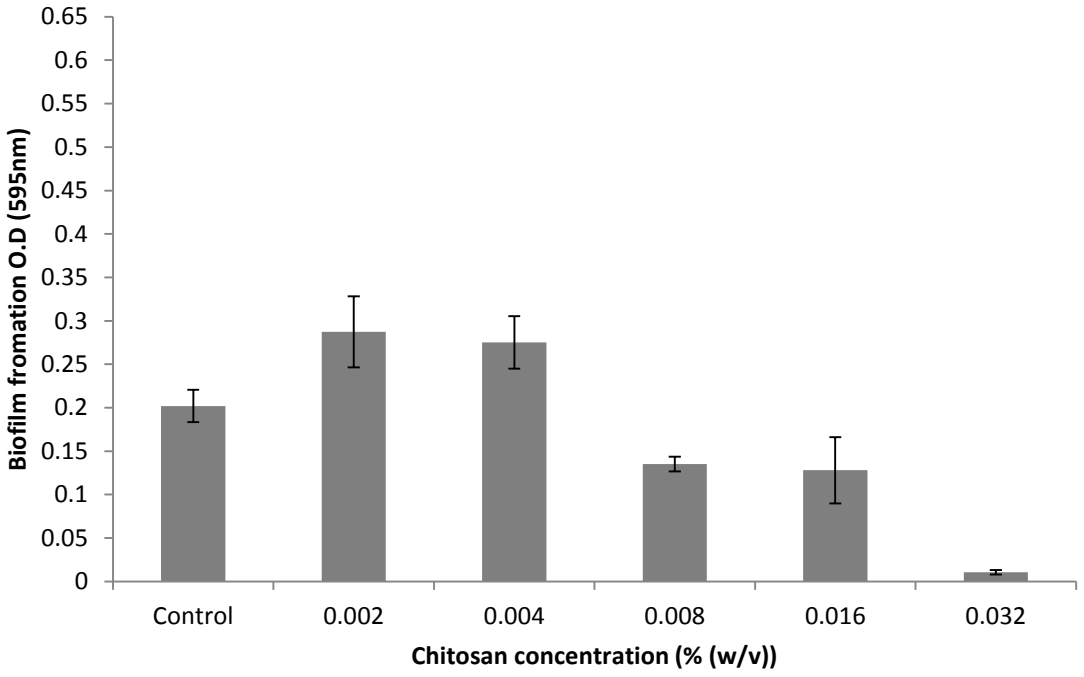

Figure 6.8: Comparison of C. jejuni 11168CH biofilm formation after 48 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

The degree of biofilm formation regarding the 11168CH isolate was found to follow a similar trend as in the 'adapted' isolate of C. jejuni NCTC 11168. This was typical of an increase significant increase in formation of biofilms between control and 0.002% - 0.004% (w/v) chitosan samples, followed by a decrease from 0.008% (w/v) – 0.032% (w/v) chitosan conditions ($F(5, 48) = 40.65$, $p<0.0001$). However, post hoc analysis found that no significant difference was typical when comparing the control biofilm formation against both that of 0.004% (w/v) and 0.016% (w/v) chitosan exposure ($p>0.05$) (Figure 6.8).

6.4.4 Biofilm formation after 72 hours incubation

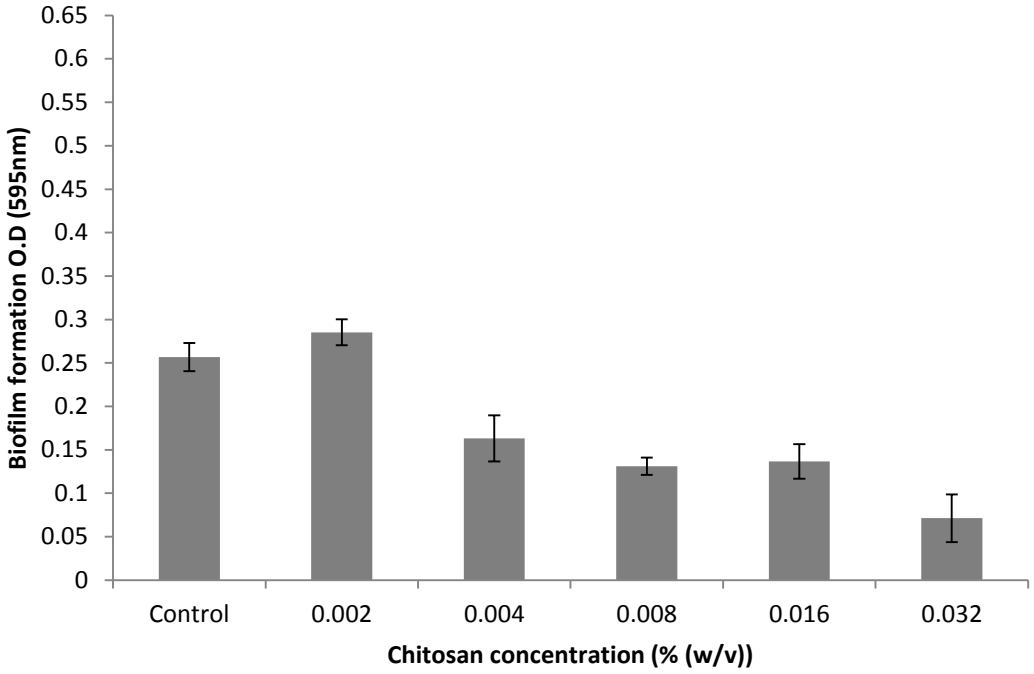

Figure 6.9: Comparison of C. jejuni wild-type NCTC11168 biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

After 72 hours of incubation, the degree of biofilm formation was seen to be somewhat similar to wild-type C. jejuni NCTC 11168 cells after 48 hours (Figure 6.9). A small initial increase in the degree of biofilm formation as indicated by the relative increase in absorbance was observed with respect to growth under 0.002% (w/v) chitosan. Biofilms grown in 0.004% (w/v) – 0.032% (w/v) chitosan were seen to be diminished, when compared to that of the control. One-way ANOVA accounted for a significant difference between control and chitosan exposed biofilms ($F(5, 48) = 42.15$, $p<0.0001$). Although post hoc analysis highlighted significant differences when comparing the biofilm formation under control conditions versus those grown under 0.004%, 0.008%, 0.016%, and 0.032% (w/v) chitosan ($p<0.05$), the increase in biofilm formation seen in the 0.002% (w/v) chitosan relative to the control was not deemed significant ($p>0.05$). Although one-way ANOVA indicated significant differences between the biofilm formation between 48 and 72 hours ($F(11, 96) = 55.27$, $p<0.0001$), significant differences were typical when comparing biofilms formed in the presence of 0.008% (w/v) and 0.016% (w/v) chitosan ($p<0.05$).

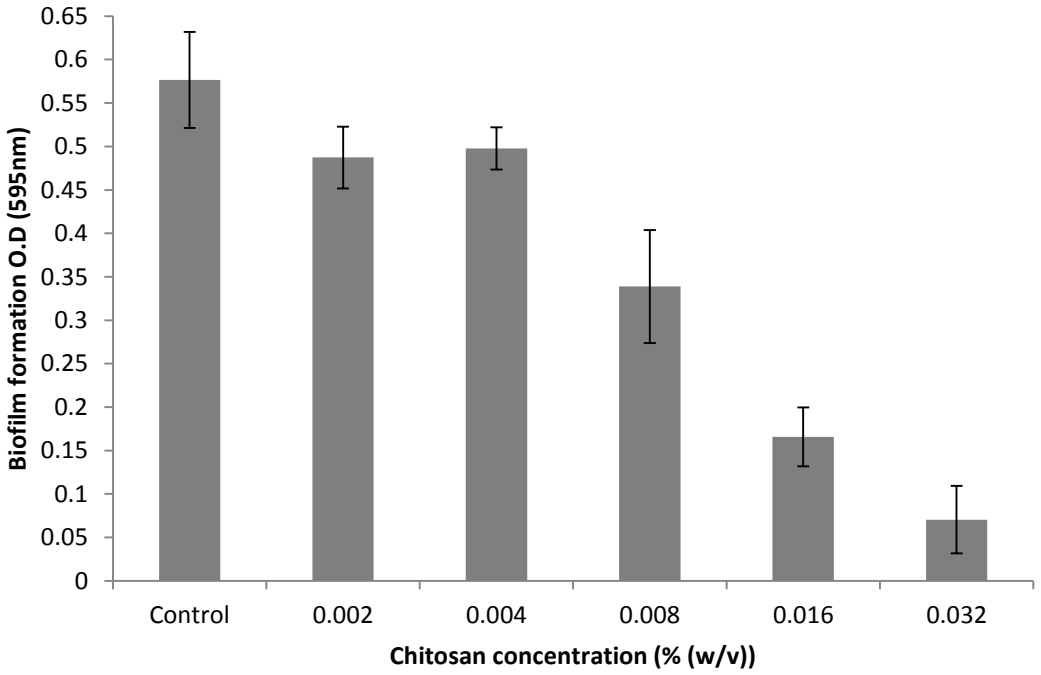

Figure 6.10: Comparison of C. jejuni 'adapted' NCTC11168 biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

When comparing the biofilm formation after 72 hours incubation compared to 48 hours with regards to the 'adapted' isolate of C. jejuni NCTC 11168, one-way ANOVA analysis revealed significant increases in formation ($F(11, 96) = 45.75$, $p<0.0001$). Post hoc analysis confirmed this significance when comparing the respective control, 0.004%, 0.008%, 0.016% and 0.032% (w/v) chitosan grown biofilms ($p<0.05$). However, no significant difference was found when comparing the 0.002% (w/v) chitosan grown biofilms ($p>0.05$).

Significant changes in biofilm formation were observed with respect to those grown under control conditions and those which can formed under 0.002% (w/v) – 0.032% (w/v) chitosan ($F(5, 48) = 42.15$, $p<0.0001$). Significant decreases

in biofilm formation were apparent when applying post hoc analysis with regards to the control biofilms formed versus those grown in 0.008% (w/v), 0.016% and 0.032% (w/v) chitosan ($p<0.05$). However, no significant differences in biofilm formation were typical of those grown in the presence of chitosan at a final concentration of 0.002% (w/v) and 0.004% (w/v) ($p>0.05$) (Figure 6.10).

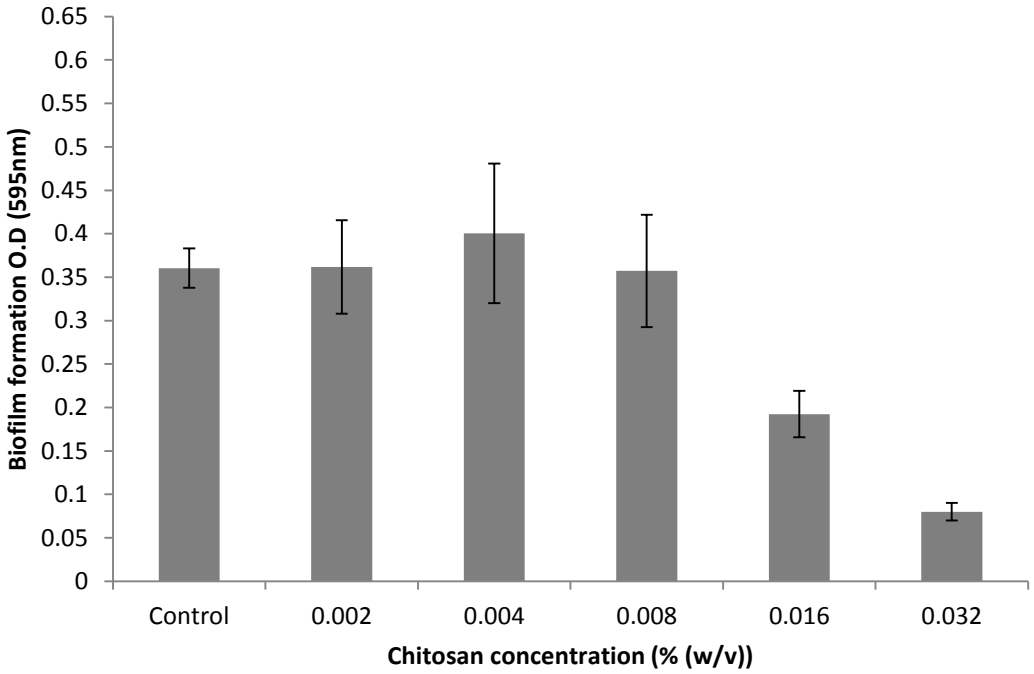

Figure 6.11: Comparison of C. jejuni RM1221 biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

The degree of biofilm formation was also found to be significantly different when comparing all conditions in the RM1221 strain of C. jejuni when concerning 48 and 72 hours incubation ($F (11, 96) = 18.64$, $p<0.0001$). Post hoc analysis revealed that significant increases in biofilm formation were typical of under control, 0.008% (w/v) and 0.032% (w/v) chitosan conditions ($p<0.05$).

No significant difference in the amount of biofilm formed was characteristic of those concerning control when compared to 0.002% (w/v), 0.004% (w/v) and 0.008% (w/v) chitosan conditions after 72 hours incubation ($p>0.05$). A significant decrease in biofilm formation was only apparent when formed in wells containing 0.016% (w/v) and 0.032% (w/v) chitosan, relative to these control conditions ($p<0.05$) (Figure 6.11).

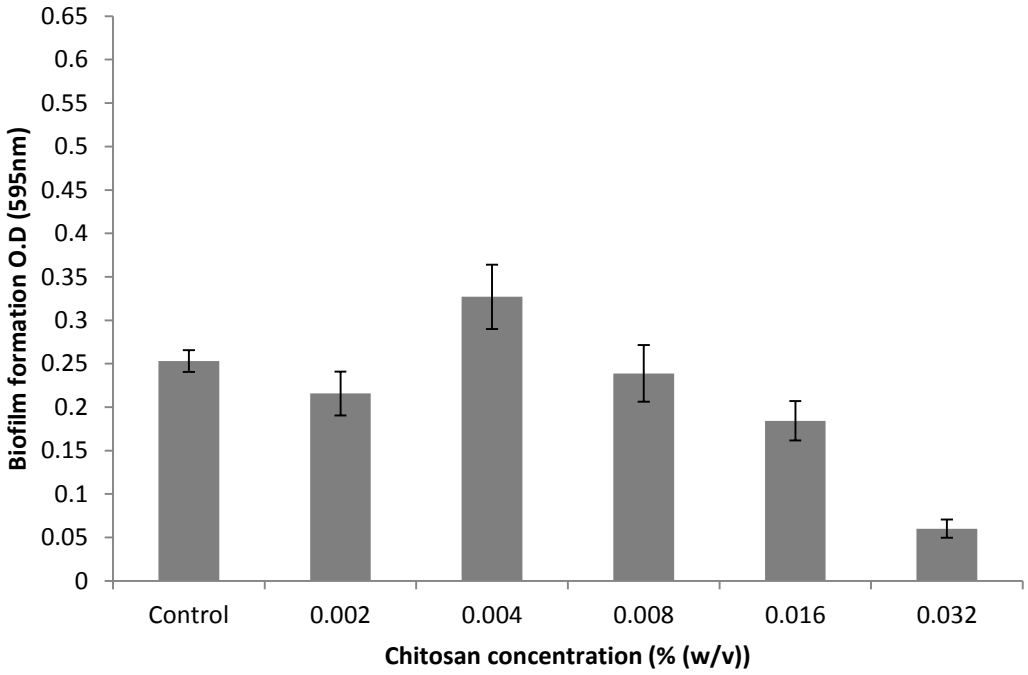

Figure 6.12: Comparison of C. jejuni 81116 biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

After incubation for 72 hours, the degree of biofilm formation was seen to be statistically different when comparing control conditions and those which were formed in the presence of chitosan at concentrations from 0.002% (w/v) –

0.032% (w/v) (F (5, 48) = 28.29, p<0.0001). Further analysis implied that the formation of biofilms significantly decreased with respect those grown in 0.016% (w/v) and 0.032% (w/v) chitosan when compared to under control conditions (p<0.05) (Figure 6.12).

Additionally, when comparing formation after 72 hours incubation to that concerning 48 hours, a significant difference was apparent (F (11, 96) = 33.51, p<0.0001). Although the post hoc testing identified a significant increase in biofilm formation when comparing those grown under 0.008% (w/v) chitosan after 72 hours, as oppose to 48 hours incubation (p<0.05).

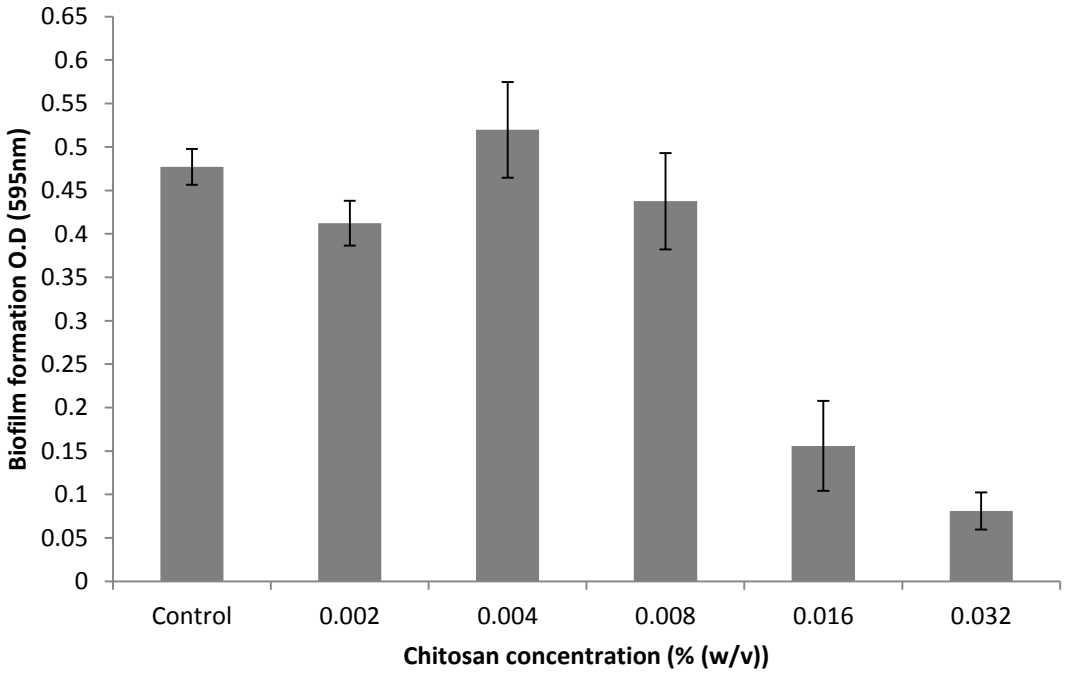

Figure 6.13: Comparison of C. jejuni 11168CH biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

When concerning biofilm formation after 72 hours incubation in the 11168CH isolate of C. jejuni (Figure 6.13), one-way ANOVA revealed that significant differences were observed under control and 0.002% (w/v) – 0.032% (w/v) growth conditions ($F (5, 48) = 48.01$, $p<0.0001$). However, when compared to the biofilms formed in under control conditions, only those grown in 0.016% (w/v) and 0.032% (w/v) chitosan occupied a significant decrease biofilm production, as highlighted by post hoc analysis ($p<0.05$). Additionally, when comparing formation of biofilms in after 72 hours versus to those formed post 48 hours incubation, significant differences were indicated ($F (11, 96) = 64.17$, $p<0.0001$). Further analysis via post hoc testing indicated significant increases in biofilm formation when comparing all conditions, excluding the comparison between 0.032% (w/v) exposed cells ($p>0.05$). A summary of biofilm formation regarding all C. jejuni strains/isolates after 48 and 72 hours incubation can be seen overleaf (Figure 6.14 and 6.15, respectively).

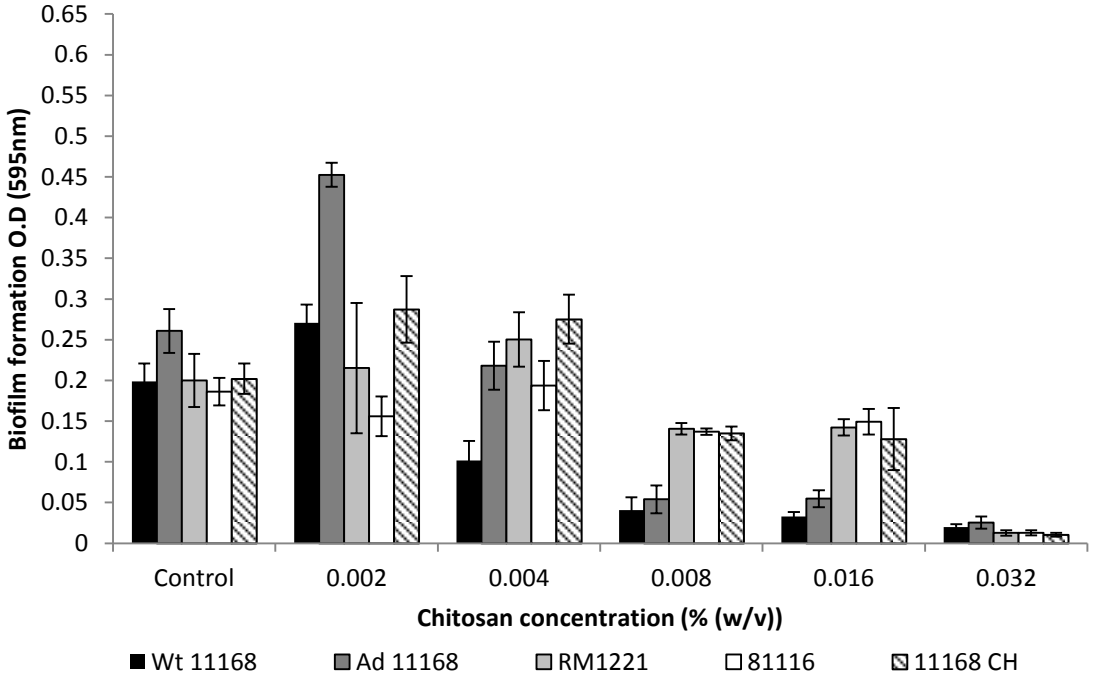

Figure 6.14: Summary of biofilm formation after 48 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

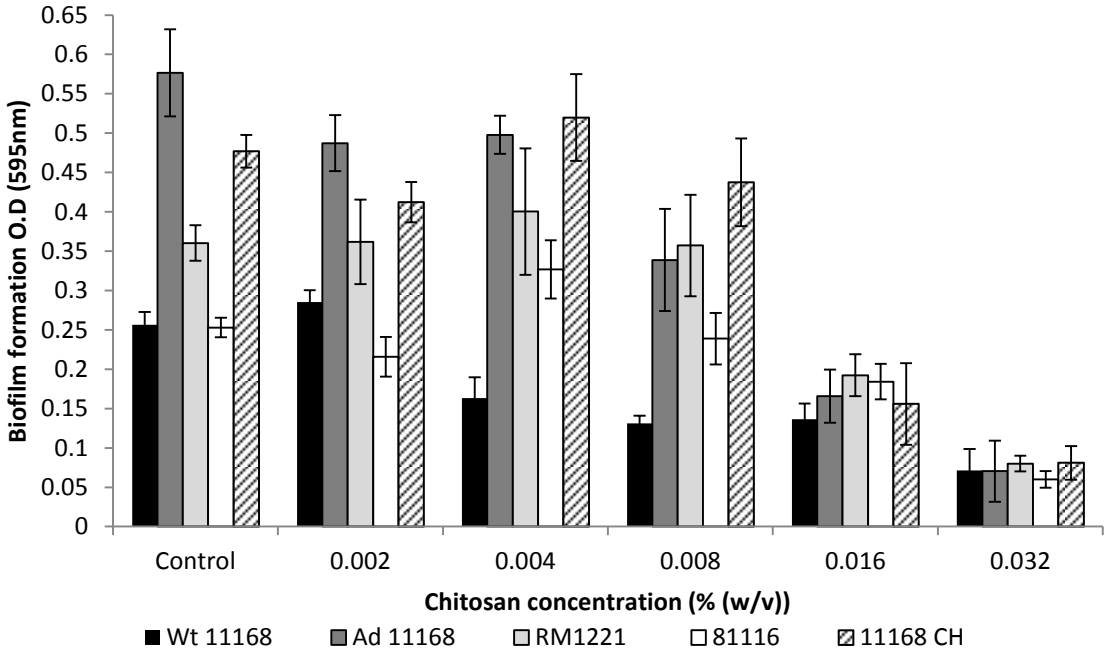

Figure 6.15: Summary of biofilm formation after 72 hours incubation in the presence or absence of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

With respect to Figure 6.15, a significant increase in biofilm formation was consistently observed with respect to the 'adapted' isolate of C. jejuni NCTC11168 in relation to all strains/isolates under control conditions and when formed under 0.02% (w/v) chitosan ($p<0.05$). Conversely, the wild-type NCTC11168 cells were generally found to form the least amount of biofilm with respect to control (no chitosan added) and 0.002% (w/v) – 0.008% (w/v) whereby significant decreases were apparent with in contrast to all strains/isolates tested ($p>0.05$).

6.4.5 Eradication of pre-formed biofilms following 24 hours exposure to chitosan

In order to assess the effect of low molecular weight chitosan upon pre-formed biofilms of C. jejuni, exposure to 0.002% - 0.032% (w/v) chitosan for a period of 24 hours was tested.

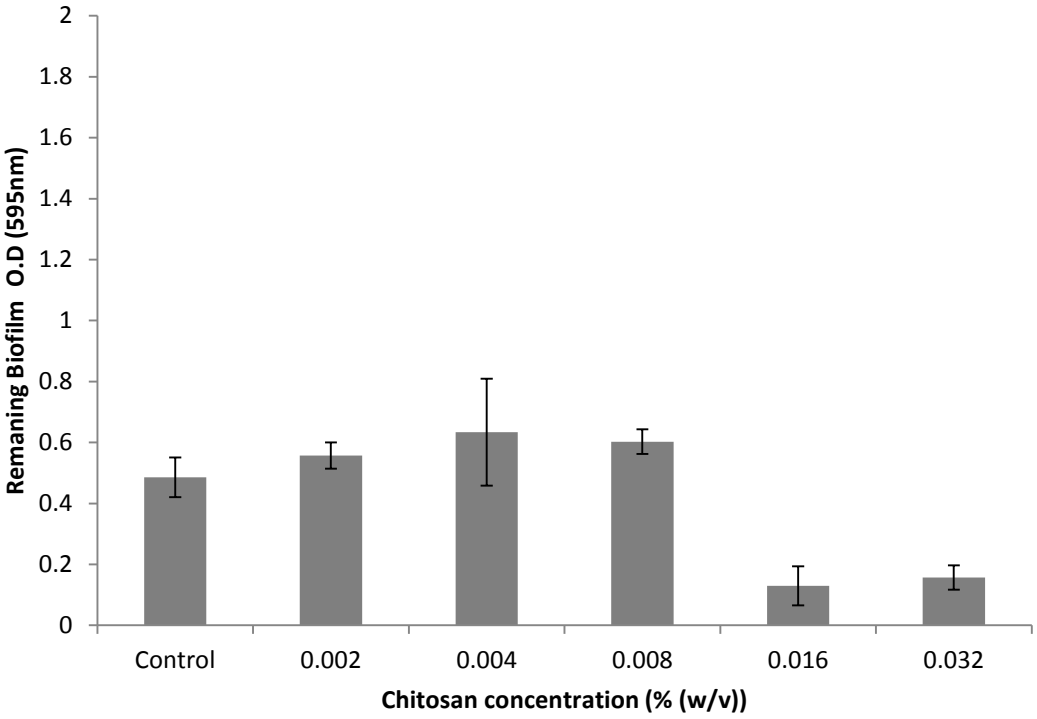

Figure 6.14: Eradication of C. jejuni wild-type NCTC11168 pre-formed biofilms after 24 hours exposure to concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

Following 24 hours exposure of varying concentrations of chitosan ranging from 0.002% (w/v) – 0.032% (w/v) of pre-formed biofilms previously grown in the absence of chitosan, statistically significant differences in biofilm production was apparent ($F (5, 48) = 17.60$, $p<0.0001$). Post hoc testing indicated that although no significant difference was found when comparing the control versus the 0.002% (w/v), 0.004% (w/v) and 0.008% (w/v) chitosan exposed biofilm, significant differences resulted from the addition of 0.016% (w/v) and 0.032% (w/v) addition for 24 hours after an initial 48 hours of growth in the absence of chitosan ($p<0.05$). Thus biofilm eradication was found in biofilms exposure to 0.016% (w/v) and 0.032% (w/v) chitosan (Figure 6.14).

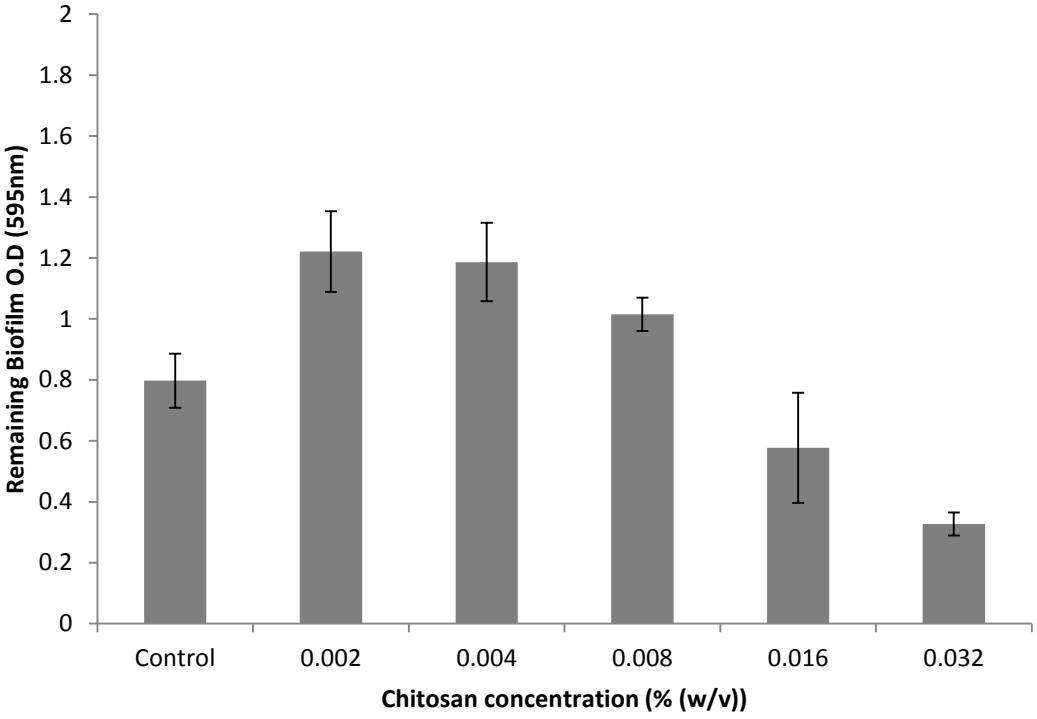

Figure 6.15: Eradication of C. jejuni 'adapted' NCTC11168 pre-formed biofilms after 24 hours exposure to concentrations of chitosan whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

Significant differences were observed in relation to the 'adapted' C. jejuni NCTC 11168 biofilms when comparing those challenged for 24 hours exposure to varying concentrations of chitosan to the control biofilms, which had non chitosan-containing media replenishment ($F (5, 48) = 30.44$, $p<0.0001$). Supplementary testing revealed a significant increase in the amount of biofilm remaining with respect to those treated with 0.002% (w/v) and 0.004% (w/v) chitosan for 24 hours ($p<0.05$). Whereas, biofilm eradication was found occur when comparing the 0.032% (w/v) challenged biofilms relative to control samples ($p<0.05$). Despite an apparent increase in biofilm growth following exposure to 0.008% (w/v) and reduction in remaining biofilm when exposed to 0.016% (w/v) challenged biofilms, their differences in relation to the control were not deemed significant ($p>0.05$) (Figure 6.15).

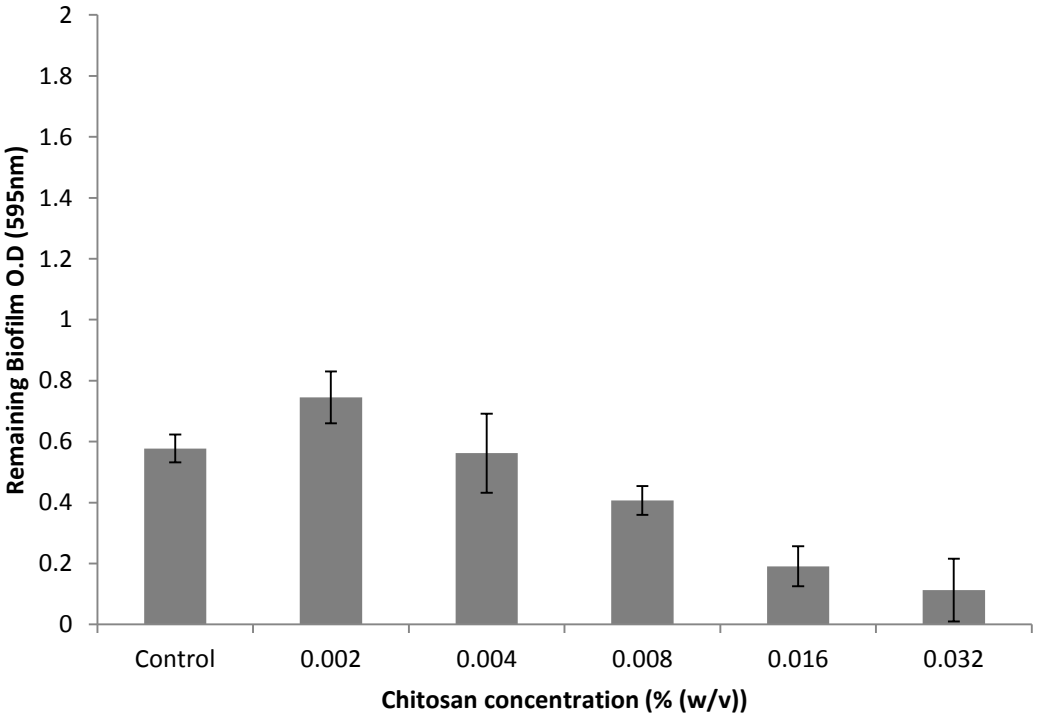

Figure 6.16: Eradication of C. jejuni RM1221 pre-formed biofilms after 24 hours exposure to concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

After previously formed biofilms were subject to 24 hours exposure to chitosan concentrations typical of 0.002% (w/v) – 0.032% (w/v), differences in the amount of biofilm production were found ($F (5, 48) = 21.12$, $p<0.0001$). Post hoc analysis revealed significant decrease in the amount of biofilm present following 24 hour exposure to 0.016% (w/v) and 0.032% (w/v) chitosan, relative to the control samples ($p<0.05$). No significant differences were accountable between 0.002% (w/v) – 0.008% (w/v) exposure for chitosan for the same period of time ($p>0.05$) (Figure 6.16)

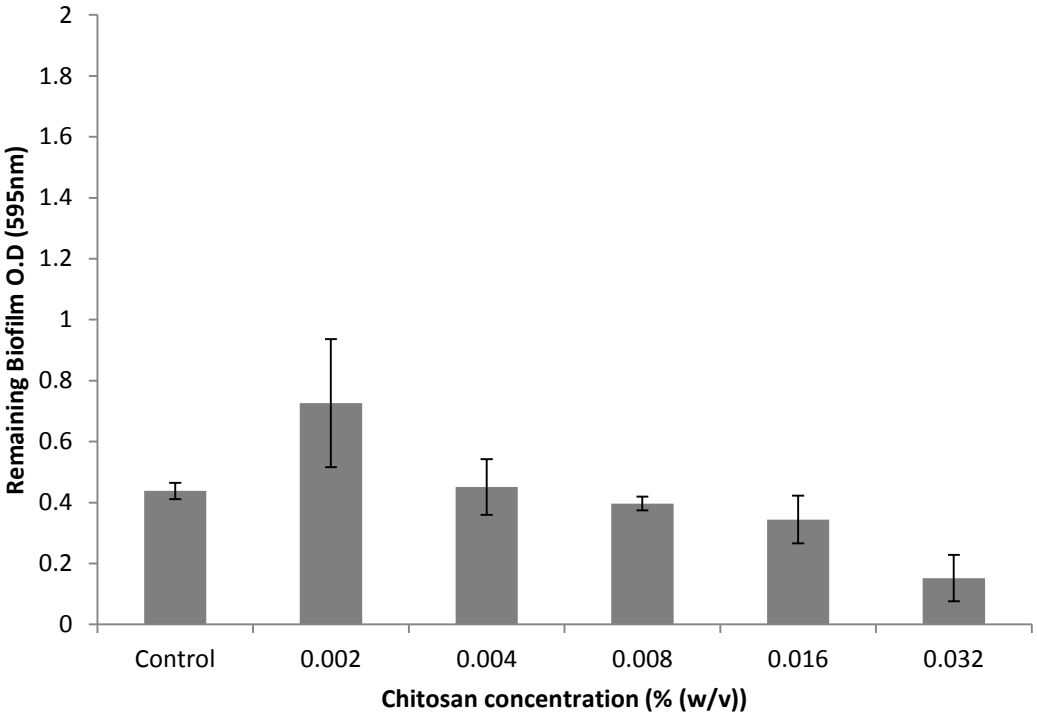

Figure 6.17: Eradication of C. jejuni 81116 pre-formed biofilms after 24 hours exposure to concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

Pre-formed biofilms which were subject to chitosan exposure for 24 hours were deemed significantly different following ANOVA analysis ($F(5, 48) = 10.80$, $p<0.0001$), as seen in Figure 6.17. However, subsequent post hoc analysis indicated that the only significant decrease in the amount of biofilm in relation to the control was found when biofilm were subject to 0.032% (w/v) chitosan for 24 hours ($p<0.05$). Interestingly, the opposite was found in the case of 0.002% (w/v) exposure, characteristic of an increase in the quantity of biofilm relative to the control ($p<0.05$). No significant difference was found when comparing biofilms treated with concentrations ranging from 0.004% (w/v) – 0.016% (w/v)

chitosan when compared with biofilm which received chitosan-free media replenishment (p>0.05).

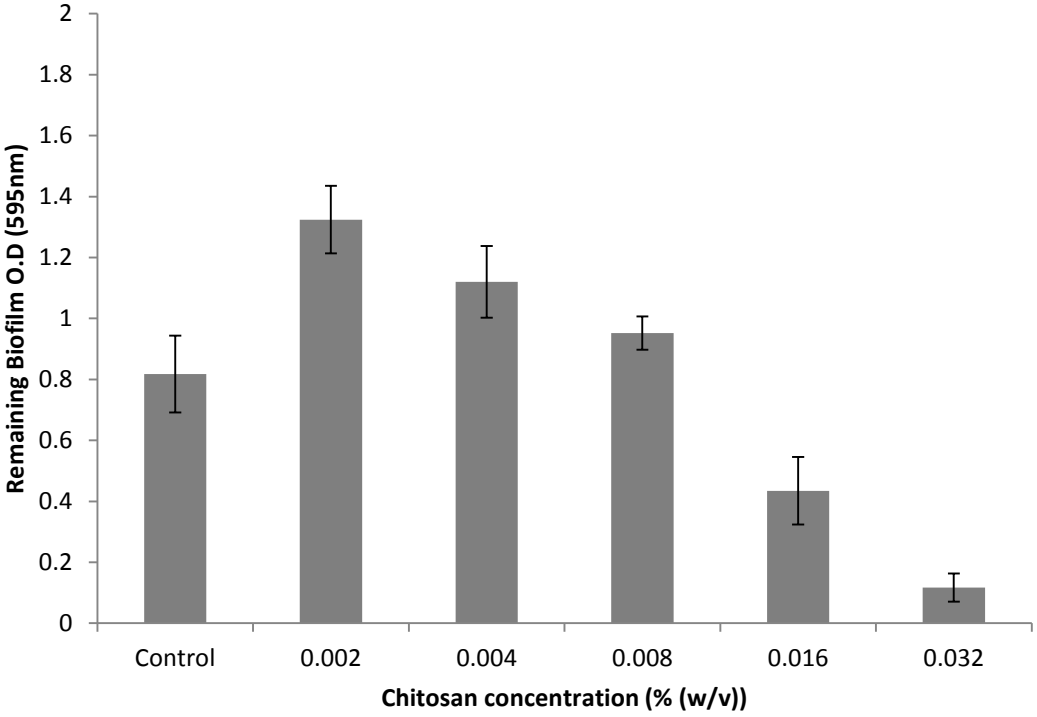

Figure 6.18: Eradication of C. jejuni 11168CH pre-formed biofilms after 24 hours exposure to concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

Twenty four hours exposure of pre-formed biofilms to chitosan at concentrations ranging from 0.002% (w/v) – 0.032% (w/v) with respect to the 11168CH isolate of C. jejuni NCTC 11168 were found to be significantly different ($F(5, 48) = 67.73$, $p<0.0001$). Further post hoc analysis indicated that biofilms exposed to 0.002% (w/v) and 0.004% (w/v) allowed for a significant increase in the amount of biofilm present in comparison to the control biofilm typical of chitosan absent media replenishment ($p<0.05$). Significant decreases in biofilm production were typical of 0.016% (w/v) and 0.032% (w/v) chitosan exposed

pre-formed biofilms in relation to those concerning the control ($p<0.05$). In contrast, no significant difference was observed when comparing the control versus the biofilms challenged with 0.008% (w/v) chitosan ($p<0.05$) (Figure 6.18).

A summary of findings with regards to biofilm eradication can be seen in Figure 6.19:

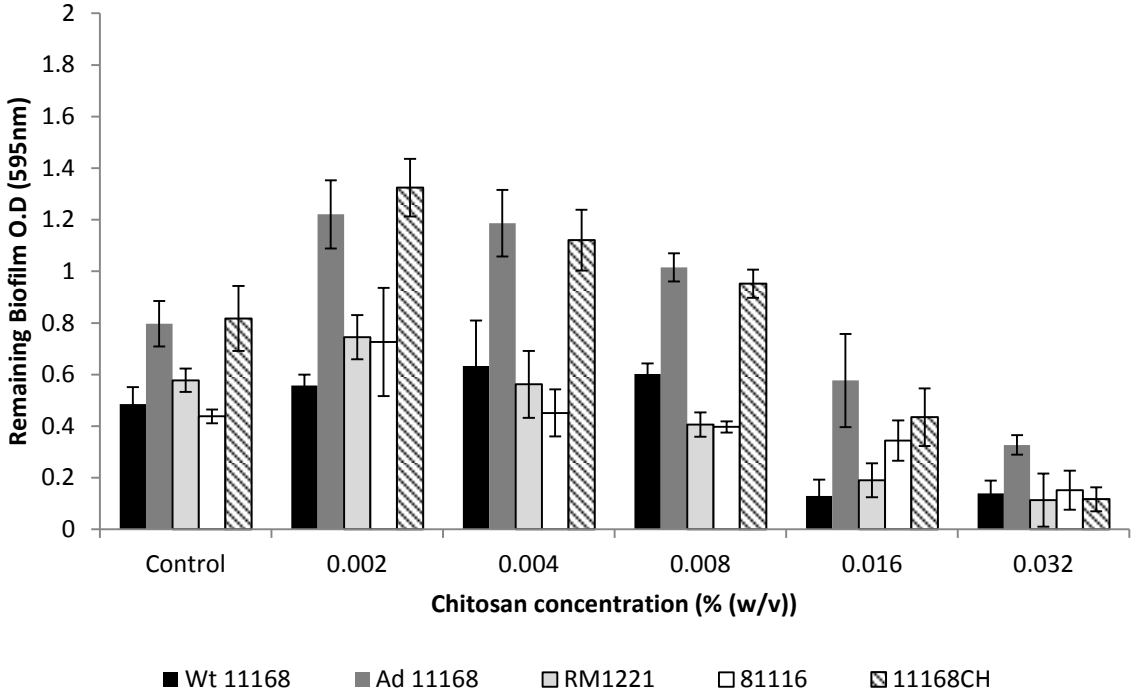

Figure 6.19: Summary of pre-formed biofilm eradication in several C. jejuni strains/isolates after 24 hours exposure to concentrations of chitosan, whilst under microaerobic conditions at 42°C (pH 6.0). Data shown concerns mean absorbance values. Experiments were performed three times in triplicate, error bars indicate the SEOM. (Note: negative control wells containing non-inoculated MH broth accounted for an average mean absorbance of 0.043nm at 595nm).

The general trend with regards to the remaining pre-formed biofilm after 24 hours exposure to chitosan revealed that the both the 'adapted' isolate and 11168CH isolate were seen to occupy the greatest amount of remaining biofilm

the other strains/isolates. Significant increases in remaining biofilm were observed in both isolates 0.002% (w/v) – 0.008% (w/v) chitosan, including controls which were subject to media replacement with no chitosan present ($p<0.05$). No significant difference was observed between these two isolates with respect to the subject to control conditions (no chitosan addition) and exposure to 0.002% (w/v) – 0.016% (w/v) chitosan for a period of 24 hours ($p<0.05$) (Figure 6.19).

6.5 Discussion

In summary, the chemotactic responses were found to be consistent amongst all strain/isolates of C. jejuni tested, with chemo-attraction in response to L-cysteine, chemo-repulsion following deoxycholic acid and chitosan exposure and no attraction/repulsion observed in relation to PBS. Chemotactic responses in the positive, negative and neutral controls were found to be consistent with taxis in NCTC11168 as in Vegge et al. (2012). It was established that no isolate/strain tested illustrated a deficiency in chemotaxis.

As mentioned previously, motility is said to play an important part in Campylobacter colonisation, as this organism must penetrate through mucus before subsequently adhering and invading host epithelial cells. Reduction in motility has previously been seen to account for a diminished ability of C. jejuni 81-176 to infect human epithelial cells, which in turn has a negative impact on pathogenesis (Stef et al., 2013).

With respect the overall findings in this chapter, the greatest degree of motility after 72 hours incubation was observed in the RM1221 strain of C. jejuni (figure

6.1, 6.2). This was followed by the previously generated chitosan 'adapted' isolate of C. jejuni NCTC 11168, despite this no significant difference was found between the two groups ($p>0.05$). In contrast, the lowest motility as indicated by the zone diameter was found in the wild-type C. jejuni NCTC 11168 cells after 24, 48 and 72 hours incubation at 42^{o}C under microaerobic conditions.

It is possible that lower degree of motility in particular relation to 24 hours and 48 incubation may link to the lower MIC values as seen in the wild-type C. jejuni NCTC 11168 strain, as oppose to the other strains/isolates tested, especially with regards to the 'adapted' isolate of C. jejuni NCTC 11168.

The increase motility in the chitosan 'adapted' C. jejuni isolate particularly when compared to the wild-type equivalent cells could also potentially be associated with an increase the significant increase in biofilm formation after 72 hours incubation with respect to control conditions and those formed under 0.002% - 0.008% (w/v) chitosan. This general trend was also accounted for with respect to 48 hours incubation excluding significant increases in biofilm formed under 0.008% (w/v) chitosan ($p<0.05$). Such findings may be resultant from alteration in specific genes for example.

Although many genes relating to the pathogenicity of C. jejuni have been previously reported, the relationships between these genes are not obvious (González-Hein et al., 2013). C. jejuni is known to possess regulatory systems which function in the adaptation to external stress (Gundogdu et al., 2011). It has been suggested that regulatory proteins such as CsrA (carbon starvation regulator), may be particularly vital in both the regulation of virulence and also stress responses. For instance, this regulator has been show enhance biofilm

formation, intestinal cell adherence and survival to oxidative stress (Fields and Thompson, 2008). Therefore, in order to allow for an increased understanding of various alterations in aspects such as biofilm formation, analysis of transcriptome may prove to be advantageous.

As expected, biofilm formation was seen to generally increase after 72 hour incubation, as oppose to 48 hours. In addition, biofilm formation appeared to be absent with respect to that of the wild-type C. jejuni NCTC11168 strain from as low as 0.008% (w/v) after 48 hours incubation, whereas the other strains/isolates show varying amount of biofilm. This implied that the wild-type C. jejuni NCTC11168 strain occupied the greatest sensitivity when grown in chitosan, which is in accordance with previous MIC data, found to be most susceptible in broth and agar-based susceptibility testing (chapter 4).

In the case of the chitosan 'adapted' isolate, the enhanced phenotypes in both biofilm formation and motility may be a potentially resultant of step-wise exposure to low molecular weight chitosan. Additionally, the 'adapted' isolate may through an enhanced phenotype, be more able to not only from chitosan induced stress but perhaps cross tolerance to other antimicrobials. However, in order to confirm this, additional susceptibility testing would need to be carried out to establish potential differences.

The greatest amount of remaining pre-formed biofilms exposed a range of concentrations of chitosan for a 24 hour time period was generally observed with regards to the 'adapted' isolate and also the 11168CH isolate of C. jejuni NCTC 11168. Surprisingly, the resultant amount of remaining biofilm was found to be increased after exposure to 0.002% (w/v) – 0.008% (w/v) chitosan for 24 hours

in relation to control samples which were subject to media replacement in both the 'adapted' and 11168CH isolates of C. jejuni NCTC 11168. This implies that chitosan exposure to pre-grown biofilms can allow for subsequent formation. Absolute biofilm eradication was not observed in any of the strain/isolates tested after 24 hours chitosan exposure of pre-formed biofilms.

Two properties are thought to be attributable to surface-attached bacteria, namely an increased synthesis of EPSs (extracellular polymeric substances) and the development of antibiotic resistance. These are believed to assist in the production of a protective environment, which accounts for issues in terms of biofilm eradication (O'Toole et al., 2000). It has been shown previously that microorganisms can become resistant to even the highest deliverable concentrations of antibiotics whilst growing under biofilm conditions (Vergeres and Blaser, 1992).

Whilst few regulatory networks governing biofilm formation are exclusively devoted to this process (Ghigo, 2003), several global regulators, two-components regulatory systems and quorum sensing regulators may be responsible for biofilm formation and increases in general stress defence in both Gram negative and Gram positive bacteria (Davies et al., 1998; Davies et al., 1995; Jackson et al., 2002; Otto, 2004). The ability of Campylobacter jejuni to survive in unfavourable conditions, for instance outside the host, is vital for its survival; however the mechanisms which account for this are poorly defined (Theoret et al., 2012). Strategies which may allow for this enhanced survival conditions outside of the host are likely to exist through changes in aspects such as biofilm formation (Theoret et al., 2012).

A study conducted by Hanning and Slavik (2009) found that there was no significant correlation between the degree of biofilm formation in C. jejuni and passage through the chick gastrointestinal tract. However, because biofilm formation is recognised by some as a virulence factor in some bacteria Parsek and Singh (2003) and that passage through the gastrointestinal tract has illustrated an increase in subsequent virulence Sang et al. (1988), it would make theoretical sense that this would enhance biofilm formation.

With particular respect to biofilm formation, the second greatest biofilm former was seen to be the 11168CH isolate under control conditions and was seen to be comparable to the enhanced degree of biofilm formation under 0.004% (w/v) and 0.008% chitosan, in relation to the 'adapted' isolate of NCTC11168 (Figure 6.15). This suggests that previous passage through host environments for example chickens may allow for enhanced biofilm formation both in the absence and presence of stresses such as chitosan. This may also suggest a degree of cross protection which is particularly important for an increased survival potential.

With the increases in biofilm formation and motility observed with particular respect to the 'adapted' isolate when compared to that of the wild-type C. jejuni NCTC11168, it may therefore be more be feasible that these phenotypic alterations similar to that in the NCTC11168CH isolate may account for increases in survival, virulence and/or pathogenesis in a general sense. It is likely that with alterations in phenotype, lies an underlying difference in terms of transcriptional alterations, which could contribute to increased survival under stress inducing conditions.

To conclude, the results of this study show that C. jejuni is able to persist in an environment which is undesirable. Bacteria have the ability to undergo alterations in phenotype with regards to biofilm formation, often making them several times more resistance to a given antimicrobial (Costerton et al., 1995). The unexpected enhancement of biofilm formation at concentrations of chitosan which exceed planktonic MIC values illustrates that biofilm formation is an effective means of survival. Such aspects are likely to be fundamental with regards to overcoming numerous stresses encountered in a variety of environments. Further exploration into the possible transcriptional differences resultant from exposure to stresses such as chitosan, may provide additional information as to the precise mechanisms required for this enhanced survival of C. jejuni.

Chapter 7 – Transcriptional differences between wild-type and chitosan 'adapted' *C. jejuni* NCTC11168 isolates

7.1 Introduction

In order for bacteria to become more established under stressful conditions which do not allow for optimal growth, an increase in genetic variation can result in a selective advantage of a bacterial population. Bacteria species often possess several responses which provide mutational advantages in the way of an increase in mutation rates (Foster, 2005).

Stress hardening can also be developed by phenotypic alterations through changes in the expression of various genes thus contributing to an increased probability of survival. Bacteria are known to respond to stressful conditions by alterations in their patterns of gene expression. Responses can either be organised in coherent pathways in some cases, although functions in some cases, seen to overlap (Foster et al., 2005).

Environmental stress can be defined as the factors of an external nature which pose adverse effects on the physiological well-being of bacterial cells, resulting in either a reduction in the growth rate of the given microorganism or the complete inhibition, both in terms to individual and populations of cells (McMahon et al., 2007). There are numerous food preservation processes which have a purpose of extending the lag phase of bacterial growth in foodstuffs via the use of hurdle technology approaches whereby either one or using a hurdle

technology approach, involving multiple environmental stressors accounting for a desired slowing or prevention of bacterial growth (McMahon et al., 2007).

The absence of growth during stress does not always invoke an absence of metabolic or genetic activity, as bacterial cells can reduce the influence of environmental stresses via changes in genotypic adaptations which can ultimately lead to variation of phenotype (Storz and Hengge-Aronis, 2000).

Modifications in key components, for example protective shock proteins can provide a resistance to not only subsequent exposure to the initial stressor but other potentially unforeseen environmental stresses through cross-protection (Katzif et al., 2003; Rowan, 1999; McMahon et al., 2007). The ability of C. jejuni to respond to a wide variety of environmental stimuli via alterations in gene expression is paramount for the degree of pathogenesis, commensalism and thus survival outside the host organism (Grabowska et al., 2011).

Previous experiments have revealed numerous transcriptional changes as a result of changes in environmental stimuli. Alterations resultant from differences in temperature (Reid et al., 2008), iron concentration (Holmes et al., 2005), pH of culture medium (Stintzi et al., 2005), oxygen limitation and also sodium deoxycholate concentration (Woodall et al., 2005; Malik-Kale et al., 2008), have been previously documented.

In order to survive a given stress, C. jejuni must be adaptable and respond appropriately (Fields and Thompson, 2008). However, unanswered questions still remain with regards to increased survival through molecular mechanisms, accounting for enhanced pathogenesis virulence properties of Campylobacter

(Gundogdu et al., 2011; Fields and Thompson, 2008). A increased understanding in the response mechanisms which help combat the stresses encountered are needed to assist with applicable interventions to reduce the problems arising from C. jejuni-associated disease (Pittman et al., 2007).

Survival mechanisms in the genome sequenced C. jejuni NCTC11168 strain provide limited clues, as compared to other enteric bacteria including Salmonella spp. and Escherichia coli, the capacity for regulation of gene expression appear to be rather limited (Park, 2002). Many key regulators involved in stress defence which are not present in C. jejuni, include examples such as SoxRS and OxyR, involved in oxidative stress, GbsAB and BetAB involved in osmoprotection, a major cold-shock protein (CsPA) and RpoH which regulates heat-shock response, all of which are present in E. coli. However, they do possess two-component regulatory systems that are not usually found in other bacteria species, thought to be involved in stress defence (Murphy et al., 2006). Two-component regulators are thought to respond to alterations in environmental conditions via the regulation of set of genes, increasing the survival potential (Park, 2002). Two-components signal transduction systems (TCS) encompass a sensory histidine kinase, located within the cytoplasm and a response regulator positioned in the cytoplasmic membrane (Stock et al., 2000). According to Parkhill et al. (2000), there are said to be five putative TCSs with accompanying histidine kinase and response regulator within the C. jejuni NCTC11168 genome.

Such survival mechanisms may be paramount for the enhanced phenotype in the previously generated 'adapted' isolate of C. jejuni NCTC11168. Thus, it was deemed appropriate to investigate potential transcriptional which may be responsible for the alterations in phenotype in relation to the 'adapted' isolate

and wild-type C. jejuni NCTC11168 both in the presence and absence of low molecular weight chitosan.

7.2 Aims and Objectives

7.2.1 Aim

This chapter aims to elucidate the transcriptional alterations caused by low molecular weight chitosan exposure in relation to both wild-type and the chitosan "adapted" isolate of C. jejuni NCTC11168.

7.2.3 Objectives

- *To identify transcriptional changes which allow for differences in growth characteristics when exposed to a sub lethal concentration of low molecular weight chitosan between the two different isolates of C. jejuni NCTC11168.*

- *To propose transcriptional mechanisms accounting for the differences accounted for in the phenotype and gene expression with regards to the C. jejuni NCTC isolates.*

7.3 Materials and methods

7.3.1 Growth Inhibition Study

Several colonies of respective C. jejuni cultures were inoculated into 75 ml of Mueller Hinton broth and were incubated for 24 hours (early stationary phase) under microaerobic conditions at 42°C.

After these overnight cultures of C. jejuni had reached this stage, aliquots were then taken and diluted to a final concentration of approximately $3x10^6$ CFU/ml in a final volume of 40ml consisting of MH broth (adjusted to pH 6.0) and a volume low molecular weight chitosan added (pH 6.0) resulting in 0.0008% (w/v) or 0.0016% (w/v).

At various time intervals, aliquots were taken from samples, serially diluted and subsequently plated at 42°C for a maximum of 24 hours under microaerobic conditions. Counts were then enumerated as described previously.

7.3.2 RNA extraction and microarray analysis

In order to compare the potential transcriptional differences which are accountable for the alteration in the phenotype when comparing the wild-type and adapted isolates of C. jejuni NCTC 11168, microarray analysis was performed. The RNA of cells following sub-lethal exposure to a pre-defined concentration of low molecular weight chitosan for 16 hours at 42°C under microaerobic conditions was utilised.

After late exponential phase was reached in the respective tubes containing the wild-type and adapted isolates of C. jejuni NCTC 11168 with no chitosan present, an aliquot was taken from these broths and transferred into fresh MH broth (pH 6.0) containing either MH broth without low molecular weight chitosan (pH 6.0) or a final concentration of 0.0008% (w/v) low molecular weight chitosan and were subsequently incubated as described previously for 16 hours.

Immediately upon reaching the 16 hour time interval aliquots (mid exponential phase) were taken from both the control and treated samples of both the wild-type and adapted C. jejuni cells. These aliquots were then transferred into centrifuge respective centrifuge tubes containing a 1/10 volume of ice-cold (10%/90%) phenol/ethanol stop solution (Konkel et al., 2007). Contents were then centrifuged at 4°C at 8000 x g for 10 minutes and the supernatant discarded carefully into an appropriate waste container. The remaining procedure was then carried by the using the RNeasy Mini Kit (Qiagen, UK) according to manufacturer's instructions. Optional steps included the use of DNase to reduce the likelihood of DNA contamination. Modifications included extra washes with RPE buffer, increases in centrifugation times for various steps and drying of opened columns prior to eluting RNA with RNase free water (30µl volume). Quantification of RNA was achieved with a Nanodrop 2000c instrument.

When necessary, ethanol precipitation was performed in order to improve 260/280 and in particular 260/230 ratios. Here, 1/10th the volume of total sample volume of 3M sodium chloride was added along with 1µl RNase / DNase free glycogen and 2 volumes of 100% molecular grade ethanol followed by mixing via pipette. The contents were then stored at -80°C for a minimum of 2 hours after which the contents were centrifuged for 20 minutes at 13,400 x g.

the supernatant was removed and 100µl of 70% ethanol was added and centrifuged once more for 5 minutes at 13,400 x g. The supernatant was then removed and the tubes were left to air-dry for 5 minutes after which 30µl of RNase free water was added to re-suspend the tube contents. RNA samples were stored at -80°C until required.

The transcriptional changes when comparing the different isolates of C. jejuni NCTC11168 were assessed using a custom Campylobacter specific microarray design, kindly provided by the Bacterial Microarray Group at St George's University (London) - BµG@S. The microarrays with this design were purchased from Agilent (Agilent Technologies UK Ltd, Berkshire, UK) and were sent to the Functional Genomics, Proteomics and Metabolomics Facility (The University of Birmingham) with extracted samples for subsequent processing.

Before labelling and hybridisation, the RNA integrity of samples was assessed using an Agilent Bioanalyser RNA 6000 Nano Kit (Agilent Technologies UK Ltd, Berkshire, UK). Samples which had an RNA Integrity Number (RIN) value of 7 or greater were deemed appropriate for microarray analysis due to sample quality purity.

Five nanograms of good quality RNA (260/230 and 260/280 ratio ≥1.8) of each sample was subsequently labelled with an Agilent Low Input Quick Amp WT kit, One Colour, according to manufacturer's instructions. The efficiency and yield following labelling were assessed using a nanodrop device and samples yielding 600ng cRNA with a labelling efficiency of 6pmol/µgRNA were used for hybridisation.

Labelled RNA was hybridised to the microarray slides using the Agilent hybridisation Kit in accordance with manufacturer's guidelines. Hybridisations were achieved using an Agilent oven following baking for 17 hours at 65°C. Microarray slides were then washed with an Agilent Gene Expression Wash Buffer kit. These slides were then scanned with an Agilent scanner utilising the one-colour HD setting for each of the 8x15k arrays characteristic of a scan resolution of 5µm, PMT 100%, 10%, 16 bit. The resulting image for each array was then imported into Agilent feature extraction software. Raw data were normalised and p values were adjusted by a False Discovery Rate of 5% and differential expression was confirmed when $p<0.05$.

Upon receiving data output from the Functional Genomics, Proteomics and Metabolomics Facility, inverse log calculations were undertaken using the normalised log2 data, which was then utilised to establish the fold changes. The following comparisons were deduced from these data: wild-type control vs. wild-type treated, wild-type control vs. adapted control, wild-type treated vs. adapted treated and adapted control vs. adapted treated cells. Significant differences were assessed by means of relative fold changes in the gene targets, which illustrated a 2 fold or greater change in gene expression. In order to deduce the names of the genes which were seen to occupy significant transcriptional changes in the comparisons mentioned above, the respective probe IDs were inputted into the Bugsbase database (http://bugs.sgul.ac.uk/bugsbase/tabs/search.php).

The frequently used two-fold change threshold in order to determine significant changes is based upon an arbitrary figure (Bilban et al., 2002). Fold changes measured by microarray and/or qPCR which were greater or equal to a two-fold

change have previously been deemed a valid result (Rajeevan et al., 2001; Morey et al., 2006). Thus, in order to ensure that differentially expressed genes were biologically relevant, a two-fold or greater change in gene expression was utilised in the microarray analysis in this chapter. Another requirement was also adopted, namely t test results having a probability P value of ≤0.05 (Woodall et al., 2005). Additionally, the rationale for using the arbitrary two-fold change threshold is reinforced from its common use regarding microarray analysis, with examples concerning the transcriptional differences regarding C. jejuni in numerous other studies (Reid et al., 2008, Woodall et al., 2005, Palyada et al., 2009, Xia et al., 2013, Guo et al., 2008).

7.3.3 cDNA synthesis

Aliquots of extracted RNA (500ng) were reverse transcribed to cDNA utilising a High Capacity RNA -cDNA kit (Applied Biosystems). Individual test samples (9µl) were added to 10µl of 2 x RT buffer and either a final 1µl of either molecular grade water (negative control) or 20 x RT enzyme mix .

Reaction tubes were added to a thermal cycler (Techne) and underwent reverse transcription involving 60 minutes incubation at 37°C and inactivation at 95°C. Resulting cDNA was then stored at -20°C for long term storage.

7.3.4 Validation of Microarray analysis using real-time PCR

The microarray findings were validated by running quantitative real-time PCR on the cDNA originating from the same RNA samples used in the microarray experiment. Several target genes were selected based upon fold changes,

representative of the general findings. Briefly, two upregulated genes, two down regulated genes and a neutral gene target were chosen concerning comparison between the control and treated cells for each isolate type (wild-type and adapted). Additionally, the GapA (GAPDH) gene target was used as a housekeeping gene, theoretically possessing constant expression regardless of chitosan exposure or not.

These various targets were analysed using the method described previously and the resulting expression was compared to that of the fold changes observed in the microarray analysis. A correlation was then made with regards to the related target genes generated form the microarray data and the quantitative real-time PCR data. The differences in gene transcript levels of the sub-lethally treated cells were compared against the respective calibrators (control samples).

Table 7.1: Primer sequences used for microarray validation via qRT-PCR.

Target Gene	*Forward sequence (Tm - °C)*	*Reverse sequence (Tm - °C)*	*Product length (bp)*
GapA	*TTCTACAGGCGCAGCAAAAG (59)*	*TGGCACACGCATACTTTGTC (59)*	*82*
DapB	*AGGTACAACAGGGCTTGATGAG (60)*	*CGGCATCACTTCACTAGCAC (59)*	*64*
KpsM	*AGTTGGGGAGCCTATGAGTATTG (59)*	*TTGCCTGAGTTCCATTCATAAGC (59)*	*179*
Cj0025c	*GGGGTACCTGGAAGTGCAAC (59)*	*AGTGCCGAAGTCATAGCTCC (60)*	*158*
Cj1586	*ATGGCGATTTTAATGGCGGC (59)*	*AGGCAAGCTCCAACTATAGGAT (58)*	*131*
Cj1358c	*ATACATGGCAGGACCACACTC (59)*	*TTGCTCTTGGTTCGCCTTTG (59)*	*50*
Cj0731	*GGGGTGTAAGTGTTTTGCCTG (59)*	*TACTGCCCACACAAACAAGC (58)*	*114*
Cj1487c	*AGTGTTCGGCTTGTCATGGA (59)*	*GCAGCACCAAGCATTTCTCC (60)*	*151*
Cj1489c	*AGGACCTGATCTTGCTCGTG (59)*	*TTCATGCCAATCAGCCGTTC (59)*	*52*
Cj1560	*CAAAGCGCTCACAATCCTAGC (59)*	*GCACCGATAAAAACTGCCAC (58)*	*170*

7.3.5 Quantitative Real-time PCR

In order to determine the required input needed for real-time PCR analysis, the Stock I method was utilised (Gallup and Ackermann, 2008). Essentially, two stock mixes of cDNA were prepared; here equal volumes of wild-type control and wild-type treated cDNA were pooled to give a representative wild-type stock. The same was carried out with respect to the adapted samples (adapted control and treated cDNA was pooled in a separate tube).

Serial dilutions (1:10) were then performed for each stock mix with molecular biology grade water as the diluent. The concentrations tested ranged from 20ng

– 0.002ng total cDNA for each of the mixes for each of gene targets. The efficiency of the real-time PCR using the different concentrations was generated using a standard curve for each of the target genes. Only concentrations which fell within the standard curve range were deemed suitable for subsequent qualitative analysis.

Quantitative RT-PCR was undertaken in 20µl reactions for each well used within the 96-well plates used. The Fast SYBR green mastermix (Life Technologies UK Ltd) was used for respective reactions according to manufacturer's guidelines, using 5ng of respective cDNA for each reaction alongside forward and reverse primer depending on the sample type at a final concentration of 200nM.

Relative expression was evaluated by comparing the wild-type control versus the wild-type treated samples and also the adapted control versus the adapted treated samples using the comparative CT method which subsequent analysis following the method described in Pfaffl (2001). The gene encoding GAPDH was used as the reference target genes for all comparisons. Furthermore no template controls, where molecular biology grade water was added as oppose to cDNA template and reverse transcription negative controls (no reverse transcriptase added) were added to rule out potential background contaminants such as gDNA within the samples used. Melt curve analysis was also carried for each primer set to detect the absence of primer dimer formation and primer specificity.

In order to establish the statistical significance of changes in transcript levels between the respective control versus treated cells, t-tests were performed by comparing ΔCt values (reference Ct – target Ct), as described in Yuan et al. (2006).

7.4 Results

7.4.1 Determination of growth inhibition parameters for microarray analysis

In order to determine the concentration of chitosan and the exposure time parameters, prior to RNA extraction and subsequent microarray analytical purposes, both the wild-type and previously generated 'adapted' cells of C. jejuni NCTC 11168 were challenged to 0.0008% (w/v) or 0.0016% (w/v) chitosan.

The concentration of chitosan required for use in the transcriptional experimentation was based on a noticeable statistically significant difference between the wild-type and 'adapted' treated cells (0.0008% (w/v)). In addition, the time point used for RNA extraction purposes would require the control cells for each isolate of C. jejuni NCTC 11168 to not enter stationary phase, as any differences in gene expression may be based partially upon the comparison of the difference in growth phases. Also, the cell concentration was deemed important, as previous RNA extractions utilising less than 1 x 10^7 CFU/ml was seen to result in a low yield of RNA. Thus, the RNA extracted form samples would ideally need to be 1 x 10^7 CFU/ml or above.

Furthermore, the decision to utilise the one concentration of chitosan for both the wild-type and 'adapted' cell exposure was undertaken, as a direct comparison could then be made between the two isolates of C. jejuni NCTC11168.

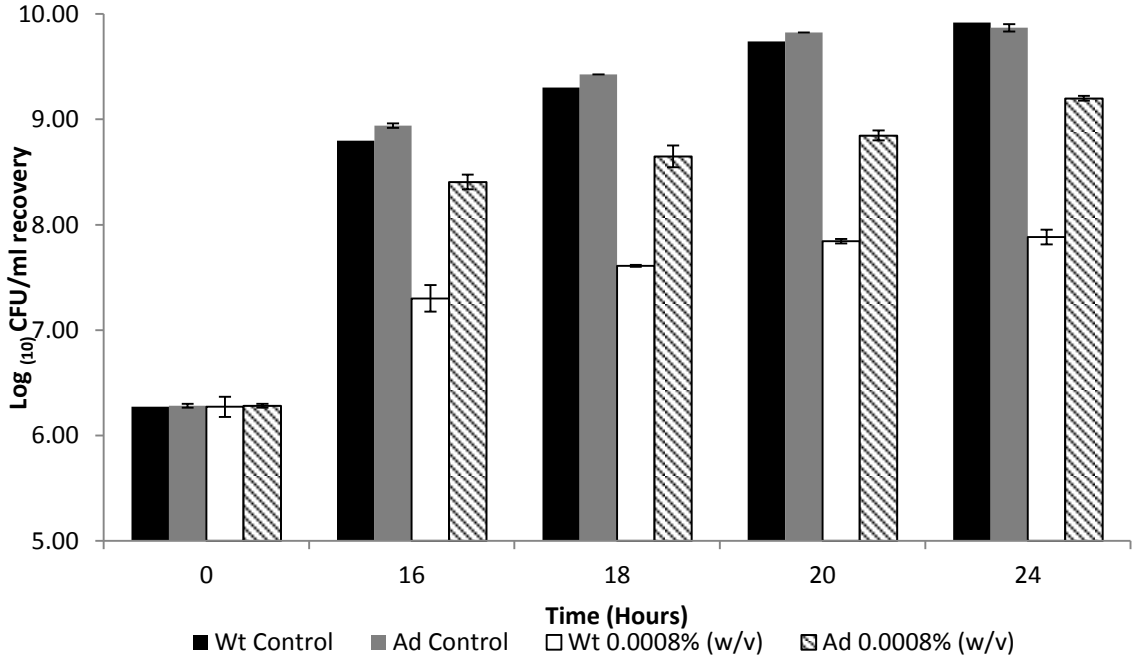

Figure 7.1: Growth inhibition of wild-type and 'adapted' C. jejuni NCTC 11168 during exposure to 0.0008% (w/v) low molecular weight chitosan (pH 6.0). Data represents mean values from experiments which were conducted n = 4, plated in triplicate. Error bars indicate SEOM.

Upon exposure to 0.0008% (w/v) low molecular weight chitosan, statistically significant differences were observed with regards to the wild-type and 'adapted' cells of C. jejuni NCTC 11168, as indicated via one way ANOVA analysis ($p < 0.05$). Post hoc analysis revealed significant differences which were apparent when comparing the difference between the treated wild-type and 'adapted' cells at 16, 18, 20 and 24 hours exposure time ($F(7, 88) = 962.9$, $p < 0.0001$). These differences were found to be 1.11, 0.86, 1, 1.12 and 1.32 log CFU/ml difference with respect to 16, 18, 20 and 24 hours, respectively (Figure 7.1).

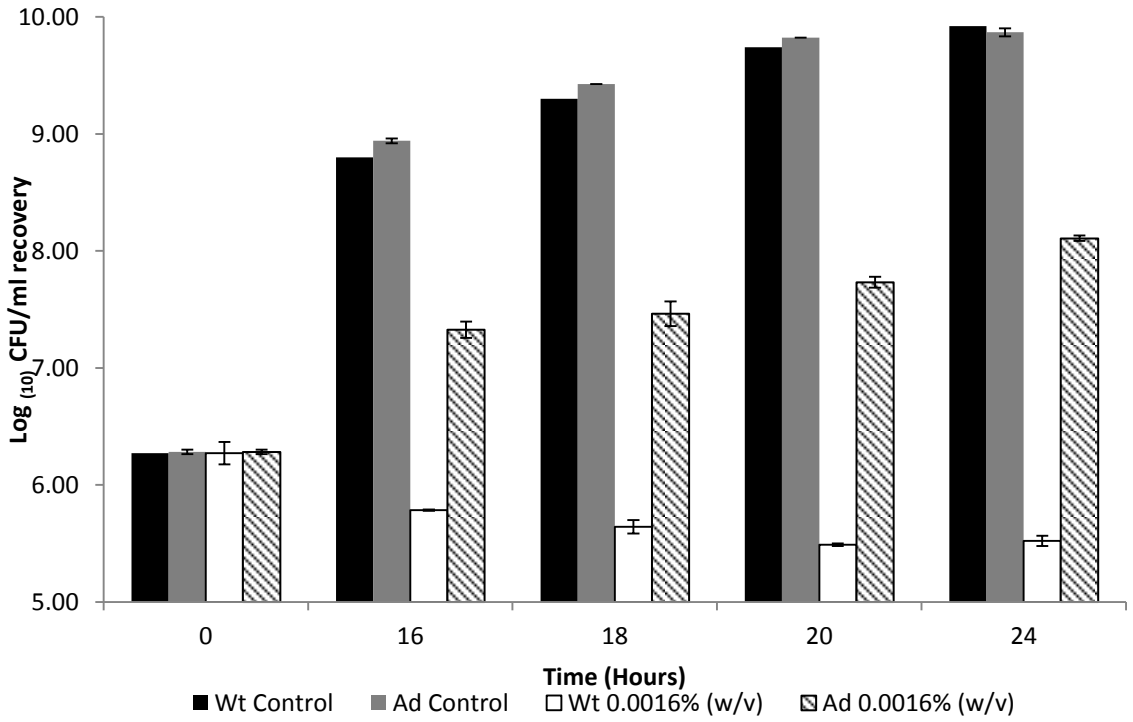

Figure 7.2: Growth inhibition of wild-type and 'adapted' C. jejuni NCTC 11168 during exposure to 0.0016% (w/v) low molecular weight chitosan (pH 6.0). Data represents mean values from experiments which were conducted n = 4, plated in triplicate. Error bars indicate SEOM.

As expected, with an increase in chitosan concentration (0.0016% w/v), statistically significant differences were found when comparing the wild-type and 'adapted' cells, following one way ANOVA analysis ($p < 0.05$). Post hoc analysis indicated significant differences when comparing the difference between the two isolate types after 16, 18, 20 and 24 hours exposure ($F(7, 88) = 3749$, p

<0.0001). Differences of 1.54, 1.82, 2.24, 2.40 and 2.59 log CFU/ml reduction were seen between the wild-type and the 'adapted' cells (Figure 7.2).

It was decided that a chitosan concentration of 0.0008% (w/v) and 16 hours exposure would be the most suitable conditions to extract RNA from, as the largest log CFU/ml difference was observed. Although greatest differences in terms of log CFU/ml were observed upon 20 and 24 hours exposure, they were deemed inappropriate time intervals to extract RNA from, owning to the control samples appearing to enter stationary phase, whilst the respective chitosan treated cells were still at exponential phase of growth.

Another set of parameters which may have been used to extract RNA from included the 16 hours exposure at 0.0016% (w/v) chitosan, as a difference between the treated cells of the wild-type and 'adapted' cells of just over 1.5 log CFU/ml. However, this time interval at this concentration of chitosan was not used, due to the low cell count expressed in the wild-type cells (5.78 log CFU/ml). Additionally, the wild-type cells exposed to 0.0016% (w/v) chitosan did not seem to show accumulative growth over the course of exposure. Therefore, due to the interest in the inhibition of growth, as oppose to lethal killing of cells, this concentration was not chosen for use.

7.4.2 Determination of target input range and qRT-PCR efficiency

When undertaking qRT-PCR analysis, it is essential to consider the efficiency of PCR reactions (Pfaffl, 2001). A standard curve was produced from each set of primers. In an attempt to establish the PCR efficiency of each primer set used to

amplify products, the stock I method was adopted (typical of pooled cDNA), which can be used to determine the input range of cDNA needed for comparable testing (Gallup and Ackermann, 2008).

Figure 7.3 illustrates the standard curve prepared from a ten-fold dilution series of pooled cDNA of both wild-type and pooled cDNA of the 'adapted' samples. These pooled cDNA samples originate from reverse transcribed RNA from respective control and treated samples. These Ct values at the given concentrations of pooled cDNA represent the housekeeper gene, GapA (encoding a GAPDH protein). All gene targets were assessed in terms of the generated R^2 values and percentage efficiency. cDNA input quantity was chosen based on an input range found to be within the linear standard curve.

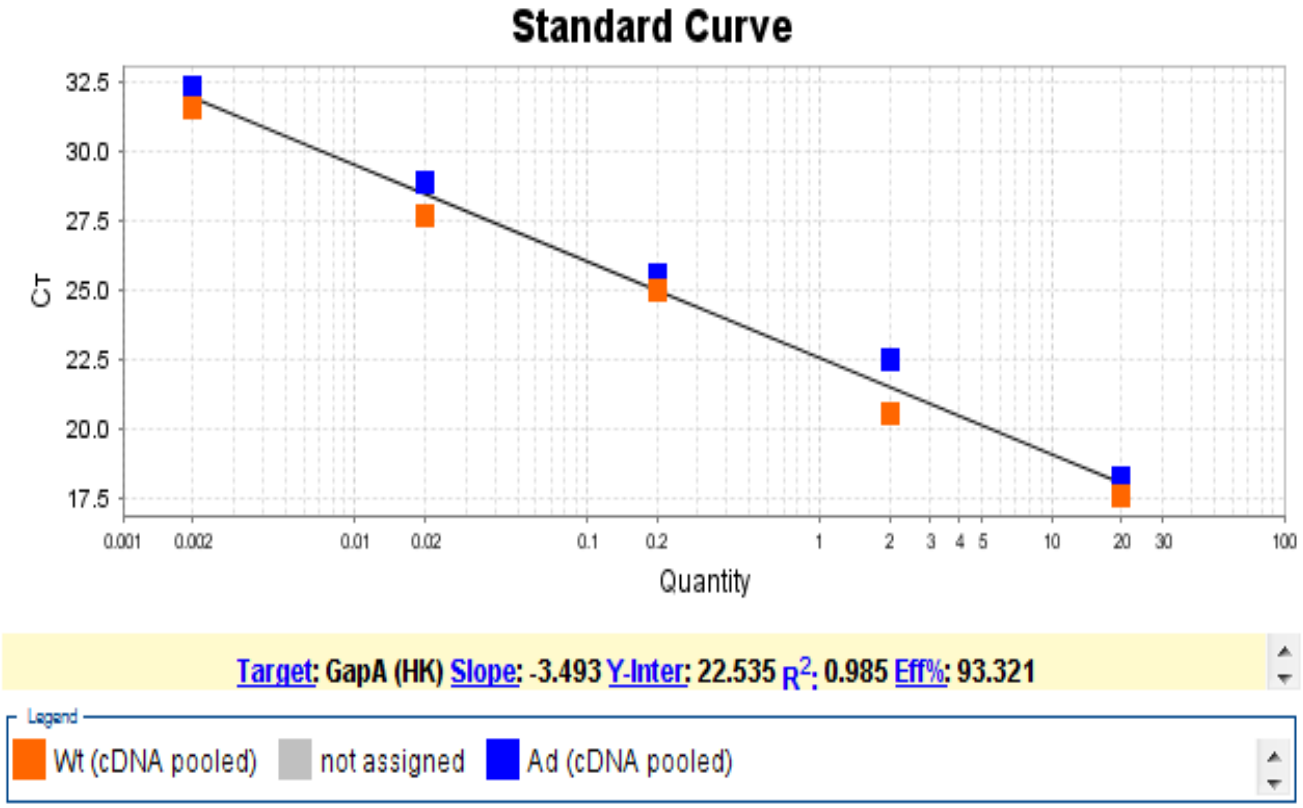

Figure 7.3: Representative standard curve of the housekeeper target gene, GapA and represent pooled cDNA versus Ct value.

Melt curve analysis was also performed following qRT-PCR reactions, assessing the PCR product specificity. Specific binding and an absence of abnormalities such as primer-dimer formation was indicative of multiple uniform peaks for each primer set. Figures 7.4 and 7.5 illustrate examples of specific primer binding following qRT-PCR reactions with respect to microarray validation analysis.

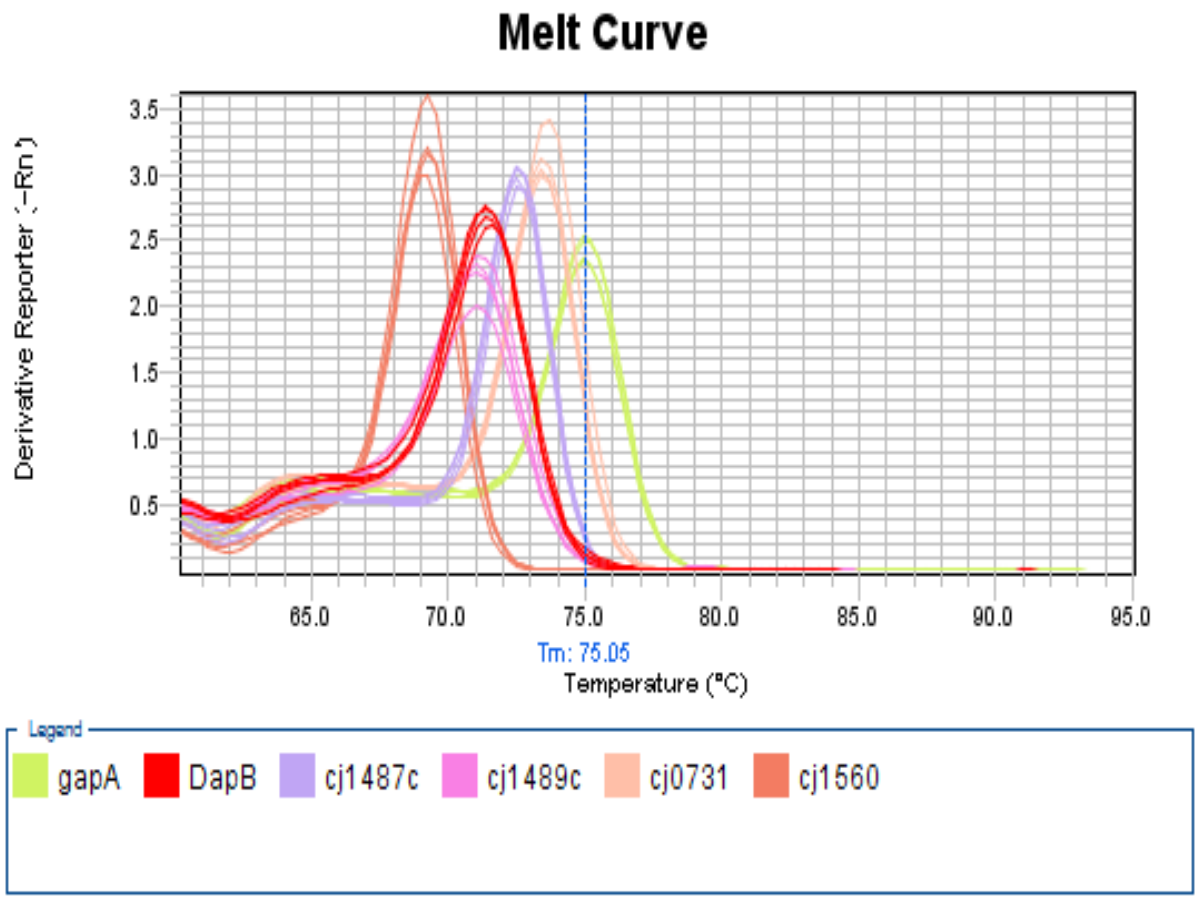

Figure 7.4: Melt curve of various primer pairs used for qRT-PCR microarray validation. Peaks are indicative of target genes required for wild-type treated vs. wild-type control comparison.

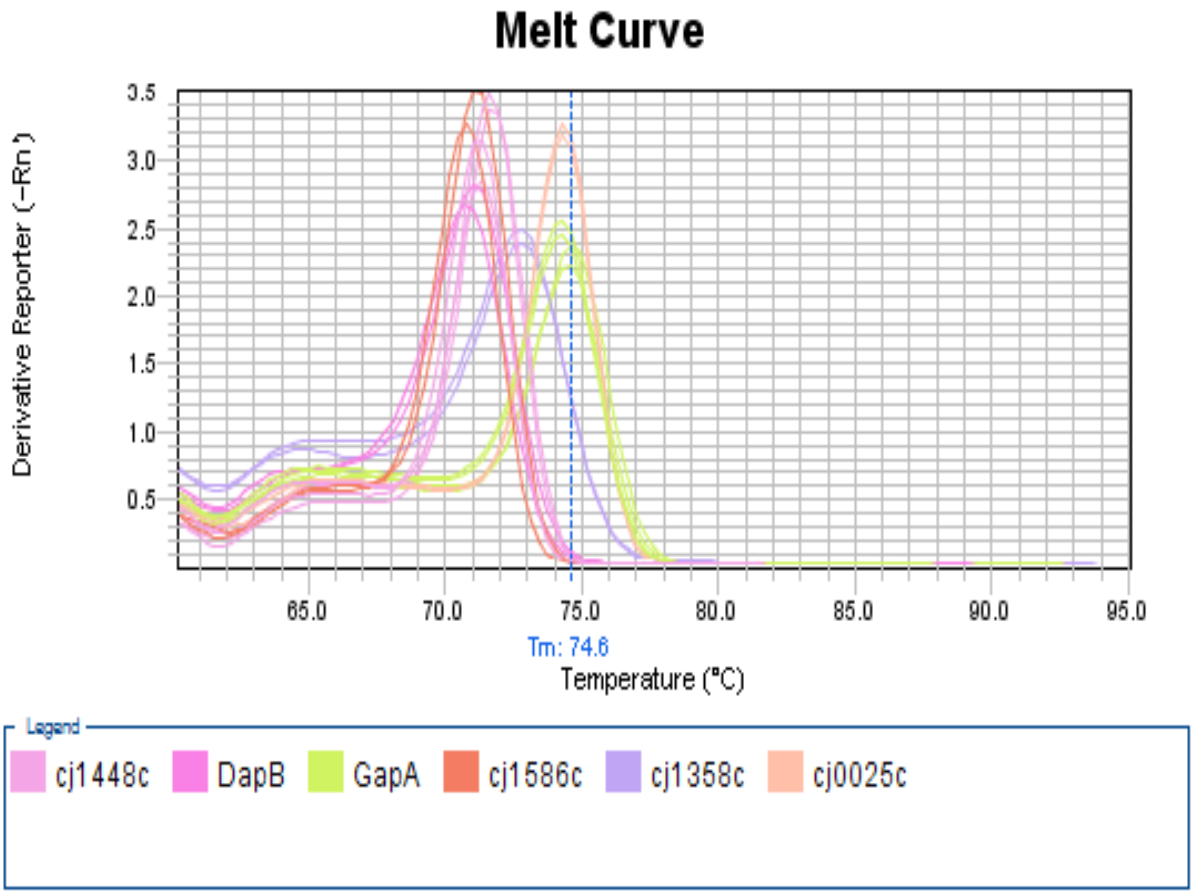

Figure 7.5: Melt curve concerning primer pairs used for qRT-PCR microarray validation. Peaks are indicative of target genes required for 'adapted' treated vs. 'adapted' control comparison.

7.4.3 RNA extractions

In order to investigate any transcriptional differences between control (untreated) and chitosan exposure cells of C. jejuni, RNA extraction was required. The findings upon undertaking mock RNA extractions reiterate the initial concern with using a cell concentration less than 1 x 10^7 CFU/ml with especially in the case of RNA extracted from chitosan exposed cells of C. jejuni NCTC11168.

The yield and subsequent A260/280 and A260/230 ratios with respect to control cells were not found to be an issue. In the example below, when utilising 10ml of culture, adequate yield and quality was achieved.

However, when attempting to extract RNA from wild-type cells which had undergone chitosan exposure for 16 hours under microaerobic conditions at 42°C, a very low yield along with inadequate A260/280 and A260/230 ratio, was observed (Table 7.2).

Table 7.2: RNA Profiles following exposure to low molecular weight chitosan (0.0008% w/v) at pH 6 after 16 hours under standard conditions (n = 1).

Sample ID	*Nucleic Acid Conc.*	*A260/280*	*A260/230*
Wt 1 Control 16hrs	*772.2*	*2.2*	*2.51*
Wt 1 Treated 16hrs	*29*	*1.87*	*1.31*

Table 7.3: Representative CFU/ml concerning treated and non-treated to low molecular weight chitosan for 16 hours (n = 1).

Sample type	*Av. CFU/ml (0hrs)*	*Av. CFU/ml (16hrs)*
Wt 1 Control 16hrs	*4.60E+06*	*7.17E+08*
Wt 1 Treated 16hrs	*4.56E+06*	*2.25E+07*

Notably, chitosan exposure was seen to hinder both RNA yield and quality (data not shown). Thus, in order to combat this problem, an increase the yield as well an increase in the A260/280 and A260/230 ratios, the amount of culture was increased accordingly under the same 16 hours exposure.

When using 80ml of culture per samples of the wild-type treated cells of C. jejuni, a small increase in RNA yield was apparent. However, the A260/230 ratios indicated the RNA quality was not suitable. A decrease in the A260/230 ratio, indicative of salt contamination, was seen to be a regular occurrence, especially upon an increase in culture volume (Table 7.4).

Table 7.4: RNA profiles following exposure to low molecular weight chitosan (0.0008% w/v) at pH 6 after 16 hours under standard conditions (n = 1).

Sample ID	*Nucleic Acid Conc.*	*A260/280*	*A260/230*
Wt 1 Control 16hrs	*753.1*	*2.2*	*2.43*
Wt 1 Treated 16hrs	*37.8*	*2.05*	*1.32*

Table 7.5: Representative CFU/ml data samples both treated and non-treated to low molecular weight chitosan for 16 hours (n = 1).

Sample type	*Av. CFU/ml (0hrs)*	*Av. CFU/ml (16hrs)*
Wt 1 Control 16hrs	*4.20E+06*	*6.00E+08*
Wt 1 Treated 16hrs	*4.08E+06*	*2.33E+07*

An increase in culture volume filtered through the spin columns accounted for a build-up of residue on the silica membrane, assumed to be resultant from increases in chitosan and media. This phenomenon was absent in RNA extracted using a lesser volume of culture volume (I.e.10ml for the control samples). This may have affected both the amount of RNA which could bind to the silica membrane and also the decrease in A260/230 ratio. Guanidine salt found in the

RLT and RW1 buffers may be less efficiently removed from the column via the RPE washing step in the event of membrane residue. In order to rectify the on-column residue, an RNase free pipette tip was carefully used to retrieve any residue which had collected on parts of the silica membrane. Additional washes with RPE were also undertaken, including inversion of the columns after closing the caps.

In the event of increasing the culture volume to 160ml, including the additional step mentioned above, the following RNA extractions were carried out (Table 7.6).

Table 7.6: RNA profiles regarding exposure to low molecular weight chitosan (0.0008% w/v) at pH 6 after 16 hours under standard conditions (n = 1).

Sample ID	*Nucleic Acid Conc.*	*A260/280*	*A260/230*
Wt 1 Control 16hrs	*390*	*2.18*	*2.34*
Wt 1 Treated 16hrs (240ml)	*129.3*	*2.08*	*2.08*

Table 7.7: Representative CFU/ml data concerning samples both treated and non-treated to low molecular weight chitosan for 16 hours (n = 1).

Sample type	*Av. CFU/ml (0hrs)*	*Av. CFU/ml (16hrs)*
Wt 1 Control 16hrs	*4.22E+06*	*5.00E+08*
Wt 1 Treated 16hrs	*4.20E+06*	*3.25E+07*

The additional steps and increase in the amount of culture utilised appeared to rectify the issues faced with extraction of wild-type RNA from chitosan treated cells. Moreover, the yield and corresponding A260/280 and A260/230 ratios were found to be appropriate for downstream application.

RNA was then extracted for subsequent use in microarray analysis, employing the additional steps with particular attention to the wild-type treated cells. Ten millilitres of culture was used to obtain RNA via extraction for both the wild-type and 'adapted' cells after 16 hours exposure, as above. Culture volumes of 40ml were used to extract RNA per sample.

The first set of RNA extraction along with respective cell counts can be seen below:

Table 7.8: RNA profiles regarding exposure to low molecular weight chitosan (0.0008% w/v) at pH 6 after 16 hours under standard conditions (n = 2).

Sample ID	*RNA yield (ng/µl)*	*A260/280*	*A260/230*
Wt 1 Control 16hrs	*281.3*	*2.18*	*2.14*
Wt 2 Control 16hrs	*274.7*	*2.15*	*1.96*
Wt 1 Treated 16hrs	*179.3*	*2.08*	*2.08*
Wt 2 Treated 16hrs	*181.9*	*2.11*	*2.01*
Ad 1 Control 16hrs	*1079.5*	*2.21*	*2.46*
Ad 2 Control 16hrs	*1110.2*	*2.21*	*2.43*
Ad 1 Treated 16hrs	*214.7*	*2.17*	*2.06*
Ad 2 Treated 16hrs	*232.7*	*2.17*	*2.21*

Table 7.9: Representative CFU/ml data concerning samples both treated and non-treated to low molecular weight chitosan for 16 hours (n = 2).

Sample type	*Av. CFU/ml (0hrs)*	*Av. CFU/ml (16hrs)*
Wt Control 1	*4.00E+06*	*7.33E+08*
Wt Control 2	*4.33E+06*	*6.83E+08*
Ad Control 1	*3.32E+06*	*7.50E+08*
Ad Control 2	*3.50E+06*	*6.50E+08*
Wt Treated 1	*4.12E+06*	*1.33E+07*
Wt Treated 2	*4.20E+06*	*2.88E+07*
Ad Treated 1	*2.56E+06*	*2.25E+08*
Ad Treated 2	*3.15E+06*	*2.92E+08*

A second set of samples were then subject to RNA extraction:

Table 7.10: RNA profiles regarding exposure to low molecular weight chitosan (0.0008% w/v) at pH 6 after 16 hours under standard conditions (n = 2).

Sample ID	*RNA yield (ng/µl)*	*A260/280*	*A260/230*
Wt 1 Control 16hrs	*575*	*2.22*	*2.57*
Wt 2 Control 16hrs	*582*	*2.11*	*2.36*
Ad 1 Control 16hrs	*545*	*2.21*	*2.49*
Ad 2 Control 16hrs	*547.8*	*2.22*	*2.55*
Wt 1 Treated 16hrs	*132*	*2.03*	*2.00*
Wt 2 Treated 16hrs	*146.3*	*2.04*	*2.01*
Ad 1 Treated 16hrs	*154.6*	*2.14*	*1.86*
Ad 2 Treated 16hrs	*191.1*	*2.17*	*1.87*

As highlighted in the above, the yield appeared to be very low with respect to the wild-type treated samples and consequently the 260/230 ratios suffered.

The actual counts for the above data can be seen in Table 7.11, below.

Table 7.11: Representative CFU/ml data concerning samples both treated and non-treated to low molecular weight chitosan for 16 hours (n = 2).

Sample type	*Av. CFU/ml (0hrs)*	*Av. CFU/ml (16hrs)*
wt 1 C 16hrs	*4.21E+06*	*6.15E+08*
wt 2 C 16hrs	*4.42E+06*	*5.70E+08*
ad 1 C 16hrs	*3.48E+06*	*6.12E+08*
ad 2 C 16hrs	*3.50E+06*	*6.53E+08*
Wt 1 Treated 16hrs	*4.11E+06*	*2.17E+07*
Wt 1 Treated 16hrs	*4.30E+06*	*3.44E+07*
Ad 1 Treated 16hrs	*3.00E+06*	*2.25E+08*
Ad 2 Treated 16hrs	*3.00E+06*	*1.50E+08*

A summary of RNA profiles is shown below which were sent to the Functional Genomics, Proteomics and Metabolomics Facility (University of Birmingham).

Table 7.12: Summary of RNA samples used for microarray analysis, with respective ratios.

Tube ID	*Description of contents*	*RNA yield (ng/µl)*	*A260/280 ratio*	*A260/230 ratio*
JW 1	*Wild-type control 1*	*281.3*	*2.18*	*2.14*
JW 2	*Wild-type control 2*	*274.7*	*2.15*	*1.96*
JW 3	*Wild-type control 3*	*575*	*2.22*	*2.57*
JW 4	*Wild-type control 4*	*582*	*2.11*	*2.36*
JW 5	*Adapted control 1*	*1079.5*	*2.21*	*2.46*
JW 6	*Adapted control 2*	*1110.2*	*2.21*	*2.43*
JW 7	*Adapted control 3*	*545*	*2.21*	*2.49*
JW 8	*Adapted control 4*	*547.8*	*2.22*	*2.55*
JW 9	*Wild-type treated 1*	*179.3*	*2.08*	*2.08*
JW 10	*Wild-type treated 2*	*181.9*	*2.11*	*2.01*
JW 11	*Wild-type treated 3*	*132*	*2.03*	*2*
JW 12	*Wild-type treated 4*	*146.3*	*2.04*	*2.01*
JW 13	*Adapted treated 1*	*214.7*	*2.17*	*2.06*
JW 14	*Adapted treated 2*	*232.7*	*2.17*	*2.21*
JW 15	*Adapted treated 3*	*154.6*	*2.14*	*1.86*
JW 16	*Adapted treated 4*	*191.1*	*2.17*	*1.87*

7.4 Microarray results

7.4.1 Transcriptional comparison of 'adapted' control versus wild-type control C. jejuni NCTC 11168 cells

Several genes were seen to have differences in expression when comparing the wild-type and chitosan 'adapted' isolates of C. jejuni NCTC 11168 in the absence of low molecular weight chitosan ($p < 0.05$). Five genes were seen to show at least 2 fold increases in expression in the 'adapted' isolate, as opposed to the wild-type C. jejuni NCTC 11168 cells when incubated for 16 hours at pH 6.0, under microaerobic conditions (42°C). These genes included Cj1500, encoding an inner membrane protein, Cj0289c (PEB3) encoding an antigenic peptide, Cj0017c a gene involved in disulphide bond formation, kpsM believed to encode a protein functioning as an inner membrane protein involving exporting capsule polysaccharides and Cj0294 thought to encode a probable MoeB/ThiF family protein (Table 7.13).

Additionally, four genes were down regulated in the 'adapted' isolate under the same conditions as mentioned above. These included Cj0025c, thought to encode a sodium-dicarboxylate family transmembrane symporter, a two-component regulator (Cj1608), CjaA a gene believed to encode an amino acid transporter substrate-binding protein and Cj1666c, encoding a putative periplasmic protein. Of these down regulated genes, the greatest decreases were observed in Cj0025c and Cj1608, with 9.2 fold and 6.6 fold decreases, respectively.

Table 7.13: Genes significantly up/down regulated (2 fold threshold) when comparing 'adapted' control relative to wild-type control samples of C. jejuni NCTC11168, following 16hrs growth at 42°C under microaerobic conditions (pH 6.0).

Gene name	*Product function*	*Fold change*
kpsM	*capsule polysaccharide export system inner membrane protein*	*+ 2.2*
Cj0289c (PEB3)	*major antigenic peptide*	*+ 2.4*
Cj1500	*inner membrane protein*	*+ 2.2*
Cj0294	*Putative MoeB/ThiF family protein*	*+ 2.4*
Cj0017c	*Disulphide bond formation protein (dsbI)*	*+ 2.0*
Cj1666c	*putative periplasmic protein*	*- 2.0*
Cj0982c (cjaA)	*Probable amino acid transporter substrate-binding protein*	*- 3.6*
Cj1608	*two-component regulator*	*- 6.6*
Cj0025c	*putative sodium-dicarboxylate family transmembrane symporter*	*- 9.2*

7.4.2 Transcriptional comparison of 'adapted' treated versus wild-type treated versus C. jejuni NCTC11168 cells

Following exposure to low molecular weight chitosan at a concentration of 0.0008% (w/v) for 16 hours under the same microaerobic conditions, differences in expression level of various genes were also apparent between wild-type and chitosan 'adapted' isolates of C. jejuni NCTC 11168.

Up regulation of 8 different genes was observed regarding the 'adapted' isolate compared to the wild-type cells upon chitosan challenge. The genes included PEB3, encoding for a major antigenic peptide, CmeR, a gene involving a protein responsible for regulation of transcription, Cj0294, thought to encode a protein with a similar function to members of the HesA/MoeB/ThiF family, CjE1107 thought to encode a protein with functionality of a TraG-like protein, a probable periplasmic protein encoding gene (Cj0420), Cj0088 (dcuA) encoding an anaerobic C4-dicarboxylate transporter, Cj0017c (dsbI) involved in disulphite bond formation or ATP/GTP binding protein and a C8J_0913 which is unknown in terms of function (Table 7.14).

The greatest degree of up regulation of gene expression was noted for the Cj0289c and Cj0294 when comparing the 'adapted' treated cells to the wild-type C. jejuni NCTC 11168 cells, representative of 2.8 fold and 2.5 fold increases, respectively (Table 7.14).

Conversely, 18 genes were typical of a twofold or greater down-regulation with respect to the chitosan 'adapted' isolate when compared to the wild-type C. jejuni NCTC 11168. These genes included Cj1167 (ldh), Cj0873c, Cj0876c,

Cj17683-01, Cj0263 (ZupT), Cj0171 Cj0753c (TonB3), Cj0874c, Cj8486_1775c, Cj1584c, transcriptionally coupled; Cj1628 (exbB2) and Cj1629 (exbD2), CJJ81176_0884, Cj1659, Cj1358c (nrfH), Cj0982c (cjaA), Cj1608 and Cj0025c (Table 7.14). Similarly to the substantial down regulation of Cj1608 and Cj0025c in Table 13, these genes were also found to show the greatest decrease in terms of gene expression, totalling a 3.6 and 6.2 fold decreases, respectively.

Table 7.14: Genes significantly up/down regulated (2 fold threshold) when comparing 'adapted' relative to wild-type treated (0.0008% w/v) samples of C. jejuni NCTC11168, following 16hrs growth at 42°C, under microaerobic conditions (pH 6.0).

Gene name	*Product function*	*Fold change*	*Other notes*
Cj0289c (PEB3)	*major antigenic peptide*	*+ 2.8*	
CmeR	*transcriptional regulator*	*+ 2.1*	
Cj0294	*Unknown (similar to members of the hesA/moeB/thiF family)*	*+ 2.5*	
CJE1107	*TraG-like protein*	*+ 2.2*	
Cj0420	*putative periplasmic protein*	*+ 2.0*	
Cj0088 (dcuA)	*anaerobic C4-dicarboxylate transporter*	*+ 2.1*	
Cj0017c (dsbI)	*disulphide bond formation protein (probable ATP/GTP binding protein)*	*+ 2.0*	
C8J_0913	*hypothetical protein*	*+ 1.9*	
Cj1167 (ldh)	*L-lactate dehydrogenase*	*- 2.0*	
Cj0873c	*unknown*	*- 2.0*	
Cj0876c	*putative periplasmic protein*	*- 2.0*	
Cj17683-01	*Unknown*	*- 2.0*	
Cj0263 (ZupT)	*zinc transporter*	*- 2.0*	
Cj0171	*Unknown*	*- 2.2*	
Cj0753c (TonB3)	*TonB transport protein*	*- 2.2*	
Cj0874c	*putative cytochrome C*	*- 2.2*	
Cj8486_1775c	*similar to Campylobacter coli RM2228 YeeE/YedE family protein family (CCO0026)*	*- 2.2*	
Cj1584c	*peptide ABC transporter substrate-binding protein*	*- 2.2*	
Cj1628 (exbB2)	*ExbB/TolQ family transport protein*	*- 2.9*	
CJJ81176_0884	*cytochrome c family protein, degenerate*	*- 2.2*	
Cj1659	*Putative a high-affinity iron transporter*	*- 2.2*	*Proposed by Chan et al. (2010)*
Cj1358c (nrfH)	*Cytochrome C nitrite reductase*	*- 2.6*	
Cj1629 (exbD2)	*ExbD/TolR family transport protein*	*- 3.1*	

Cj0982c (cjaA)	*Probable amino acid transporter substrate-binding protein*	*- 3.0*
Cj1608	*two-component regulator*	*- 3.6*
Cj0025c	*sodium:dicarboxylate family transmembrane symporter*	*- 6.2*

7.4.3 Transcriptional comparison of wild-type exposed versus wild-type control C. jejuni NCTC11168 cells

Nevertheless, numerous genes were found to be up regulated in the wild-type cells exposed to 0.0008% (w/v) chitosan in contrast to the wild-type control samples (no exposure to chitosan) after 16 hours incubation under the conditions mentioned previously ($p < 0.05$). Twenty four genes were observed as having a twofold or higher gene expression, these were Cj0046, Cj1211, Cj0731, Cj1560, Cj0730, Cj0967, Cj0444, Cj0179 (exbB1), Cj1158c, Cj0301c (modB), Cj0035c , Cj0676, Cj0563, Cj1539c, Cj0742, Cj1395, CJE1049, Cj0291c, Cj0967, Cj0874c, Cj1038, Cj0379c (YedY), Cj1530 (coaE) and Cj0201c.

Two genes were found to be equally down regulated in the wild-type C. jejuni NCTC 11168 cells exposed to low molecular weight chitosan compared to the non-chitosan exposed control samples, these were Cj1497c (ccoP) and Cj1489c (ccoO), encoding cytochrome C oxidase subunits (Table 7.15).

Table 7.15: Genes significantly up/down regulated (2 fold threshold) when comparing wild-type treated (0.0008% w/v) and wild-type control (no chitosan exposure) samples of C. jejuni NCTC11168, following 16hrs growth at 42°C under microaerobic conditions (pH 6.0).

Gene name	*Product function*	*Fold change*	*Other notes*
Cj0046	*Pseudogene (probable transport protein)*	*+ 2.4*	*putative sodium:sulphate transmembrane transport protein*
Cj1211	*putative competence family protein*	*+ 2.5*	
Cj0731	*ABC transporter permease*	*+ 2.3*	
Cj1560	*Putative permease*	*+ 2.6*	
Cj0730	*putative ABC transport system permease*	*+ 2.3*	*Part of a binding-protein-dependent transport system. Probably responsible for the translocation of the substrate across the membrane*
Cj0967	*putative periplasmic protein*	*+ 2.4*	
Cj0444	*pseudogene*	*+ 2.8*	*putative TonB-dependent outer membrane receptor*
Cj0179 (exbB1)	*biopolymer transport protein*	*+ 2.2*	
Cj1158c	*small hydrophobic protein*	*+ 2.5*	
Cj0301c (modB)	*molybdenum transport system permease*	*+ 2.2*	
Cj0035c	*putative efflux protein*	*+ 2.2*	*Antibiotic resistance (Ge et al., 2005)*
Cj0676	*pseudogene (KdpA)*	*+ 2.6*	*potassium transporting ATPase A chain*
Cj0563	*hypothetical protein*	*+ 2.2*	
Cj1539c	*putative anion-uptake ABC-transport system permease protein*	*+ 2.2*	*Part of a binding-protein-dependent transport system. Probably responsible for the translocation of the substrate across the membrane*
Cj0742	*pseudogene*	*+ 2.1*	
Cj1395	*pseudogene*	*+ 2.0*	*putative MmgE/PrpD family protein*

CJE1049	*hypothetical protein*	*+ 2.1*	
Cj0291c	*glycerol-3-phosphate transporter*	*+ 2.0*	*probable pseudogene*
Cj0874c	*putative cytochrome C*	*+ 2.0*	
Cj1038	*putative cell division/peptidoglycan biosynthesis protein*	*+ 2.0*	
Cj0379c (YedY)	*putative sulphite oxidase subunit*	*+ 2.4*	
Cj1530 (coaE)	*dephospho-CoA kinase*	*+ 2.0*	
Cj0201c	*putative integral membrane protein*	*+ 2.0*	
Cj1487c (ccoP)	*cb-type cytochrome C oxidase subunit III*	*- 2.0*	
Cj1489c (ccoO)	*cbb3-type cytochrome c oxidase subunit II*	*- 2.0*	

7.4.4 Transcriptional comparison of 'adapted' chitosan treated versus 'adapted' control exposed C. jejuni NCTC11168 cells

When comparing the gene expression between the chitosan 'adapted' isolate exposed C. jejuni cells against those which were grown in chitosan free conditions, 17 genes were seen to be upregulated, all of which were typical of a twofold or higher increase ($p < 0.05$). These genes associated with an up regulation were found to be Cj0025c, C8J_0988, Cj8486_1111, C8J_1082, Cj0240c (iscS), Cj0939c, Cj0239c (NifU), Cj0379c (YedY), Cj1665, Cj0539, Cj1448c (kpsM), Cj1663, Cj1637c, Cj1375, Cj0779 (tpx), Cj1280c and Cj1104. Of these genes, the highest fold increase was apparent with respect to the Cj0025c (5.2 fold), which was the opposite in comparison the wild-type control cells versus those wild-type cells which were exposed to chitosan (Table 7.16).

Sixteen genes were also seen to be down regulated in the chitosan treated 'adapted' isolate in comparison to that of the control samples which indicative of a twofold or greater decrease in expression ($p < 0.05$). The greatest down regulation was typical of the Cj1358c (5.5 fold), Cj0465c (8.4 fold), Cj1586 (20.3 fold) genes. Other genes showing statistically significant twofold or higher decreases in gene expression included Cj0783, Cj0782 (napH), Cj8486_0800, Cj0438 (sdhB), Cj0453 (ThiC), Cj1357c, Cj0439, Cj0437, CJJ81176_0069, Cj1500, CJE0515, Cj0358 and Cj0761.

Table 7.16: Genes significantly up/down regulated (2 fold threshold) when comparing and 'adapted' treated (0.0008% w/v chitosan) relative to 'adapted' control (no chitosan exposure) samples of C. jejuni NCTC 11168, following 16hrs growth at 42°C under microaerobic conditions (pH 6.0).

Gene name	Product function	Fold Change
Cj0025c	Putative sodium:dicarboxylate family transmembrane symporter	+ 5.2
C8J_0988	Hypothetical protein	+ 3.1
Cj8486_1111	similar to Campylobacter jejuni RM1221 hypothetical protein CJE1052	+ 3.0
C8J_1082	hypothetical protein	+ 2.1
Cj0240c (iscS)	cysteine desulfurase	+ 3.2
Cj0939c	hypothetical protein	+ 2.3
Cj0239c (NifU)	Nitrogen fixation protein	+ 2.3
Cj0379c (YedY)	putative sulfite oxidase subunit	+ 2.3
Cj1665	putative lipoprotein thioredoxin	+ 2.3
Cj0539	hypothetical protein	+ 2.2
Cj1448c (kpsM)	capsule polysaccharide export system inner membrane protein	+ 2.4
Cj1663	putative ABC transport system ATP-binding protein	+ 2.2
Cj1637c	putative periplasmic protein	+ 2.1
Cj1375	probable multidrug efflux transporter	+ 2.3
Cj0779 (tpx)	thiol peroxidase	+ 2.1
Cj1280c	putative ribosomal pseudouridine synthase	+ 2.0
Cj1104	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	+ 2.1
Cj0783	periplasmic nitrate reductase small subunit (cytochrome C-type protein)	- 2.1
Cj0782 (napH)	quinol dehydrogenase membrane subunit	- 2.1
Cj8486_0800	similar to Campylobacter jejuni NCTC 11168 putative ferredoxin - Cj0782	- 2.0
Cj0438 (sdhB)	putative succinate dehydrogenase iron-sulfur protein	- 2.2
Cj0453 (ThiC)	thiamine biosynthesis protein	- 2.4

Cj1357c	*putative periplasmic cytochrome C*	*- 3.2*
Cj0439	*putative succinate dehydrogenase subunit C*	*- 2.2*
Cj0437	*succinate dehydrogenase flavoprotein subunit*	*- 2.3*
CJJ81176_0069	*hypothetical protein*	*- 2.0*
Cj1500	*putative inner membrane protein*	*- 2.4*
CJE0515	*hypothetical protein*	*- 2.3*
Cj0358	*putative cytochrome C551 peroxidase*	*- 2.7*
Cj1358c (nrfH)	*cytochrome c nitrite reductase*	*- 5.5*
Cj0761	*unknown protein*	*- 4.0*
Cj0465c (ctb)	*group III truncated haemoglobin*	*- 8.4*
Cj1586	*single domain haemoglobin*	*-20.3*

Several genes were also found to overlap between the initial comparisons tested (Table 7.17). For instance, the PEB3 gene was found to be significantly over expressed in the 'adapted' isolate of C. jejuni with respective control and chitosan exposure cells of the wild-type C. jejuni NCTC11168. An increase in expression was also found in the Cj0017c and Cj0294 in the 'adapted' C. jejuni cells control and chitosan treated cells in relation to the respective cells wild-type C. jejuni.

In contrast, similarities were found in Cj0025c, Cj1608 and Cj0982c genes which were consistently down regulated in the comparison between 'adapted' control and chitosan treated cells, relative to the wild-type C. jejuni cells which exposed under the respective conditions. In the case of the Cj0025c gene, an over expression in relation to the 'adapted' treated versus control cells was found. With respect to the Cj0379c gene, a consistent over expression in both the wild-type and 'adapted' isolates which were treated with chitosan was identified, relative to their respective controls.

Table 7.17: Summary of overlapping genes found to be significantly up/down regulated in more than one initial comparison.

Gene name	*Ad control vs Wt control*	*Ad treated vs Wt treated*	*Wt treated vs Wt control*	*Ad treated vs Ad control*
PEB3	*+2.4*	*+2.8*	*-*	*-*
Cj0294	*+2.4*	*+2.5*		
Cj0017c	*+2.0*	*+2.0*	*-*	*-*
Cj0025c	*-7.6*	*-5.3*	*-*	*+5.9*
Cj1608	*-6.6*	*-4.9*	*-*	*-*
Cj0379c	*-*	*-*	*+2.4*	*+2.3*
Cj0874c	*-*	*-2.2*	*+2.0*	*-*
Cj1358c	*-*	*-2.6*	*-*	*-5.5*
Cj0982c	*-3.6*	*-3.0*	*-*	*-*
KpsM	*+2.2*	*-*	*-*	*+2.4*
Cj1500	*+2.2*	*-*	*-*	*-2.4*

Wt = wild-type C. jejuni NCTC11168, Ad = 'adapted' isolate of C. jejuni NCTC11168

7.4.5 Microarray Validation via qRT-PCR

As a means of validating the transcriptional differences observed by microarray analysis, qRT-PCR was conducted on cDNA samples from the original RNA yielded for the microarray experimentation (Figure 7.6).

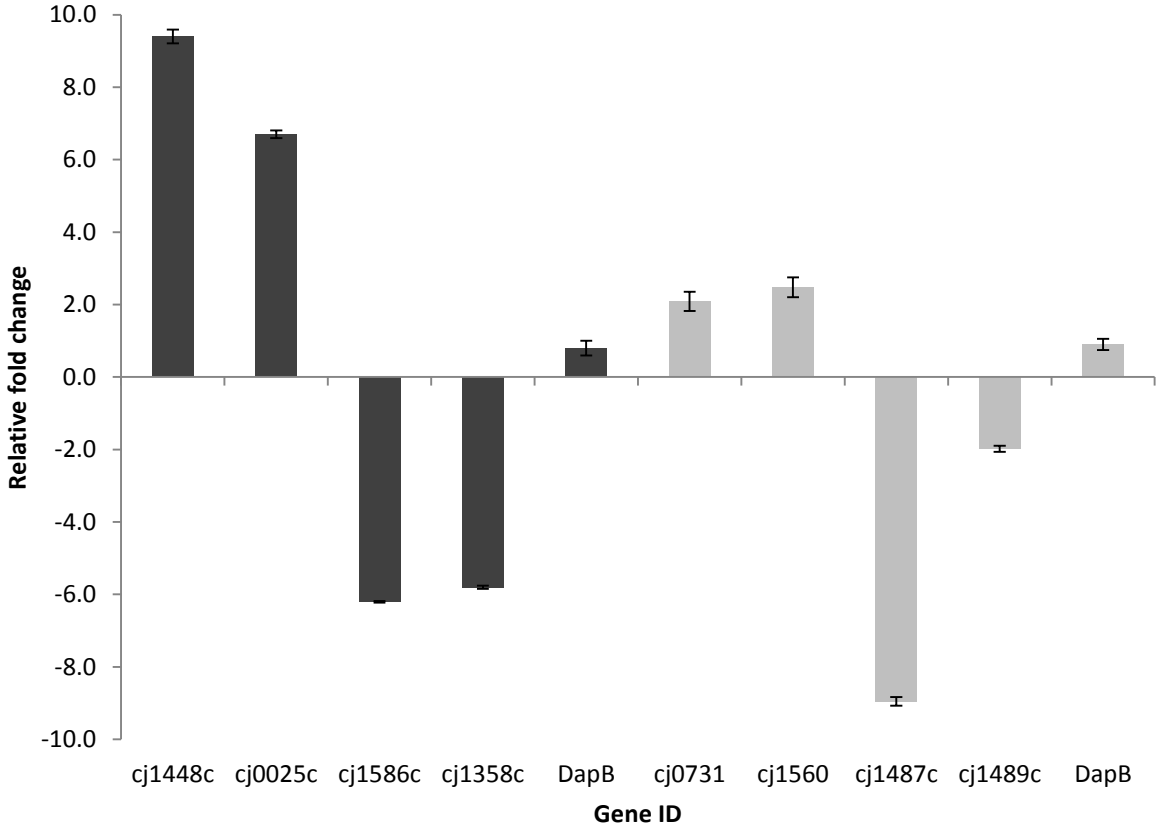

Figure 7.6: Relative fold changes in selected target genes with regards to microarray validation. Data values represent means, error bars indicate standard deviation (n = 4). Darker bars indicate fold changes in target genes regarding the wild-type treated vs. control samples, whereas lighter grey bars indicate targets validating 'adapted' treated vs. 'adapted' control samples.

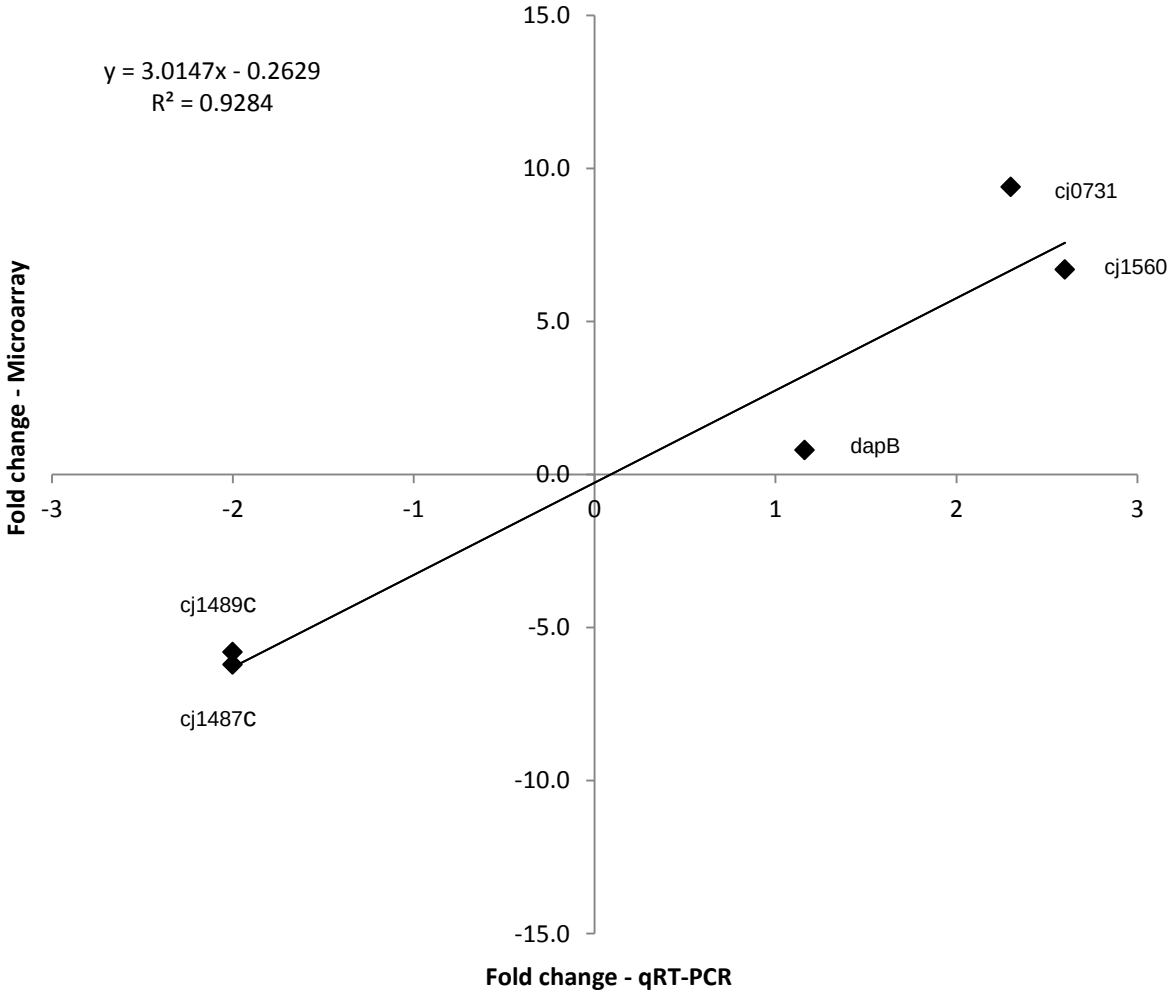

Figure 7.7: Microarray fold change vs. qRT-PCR fold change regarding wild-type control vs wild-type treated C. jejuni following 16 hours exposure to 0.0008% chitosan. Data points represent the mean of 4 biological replicates.

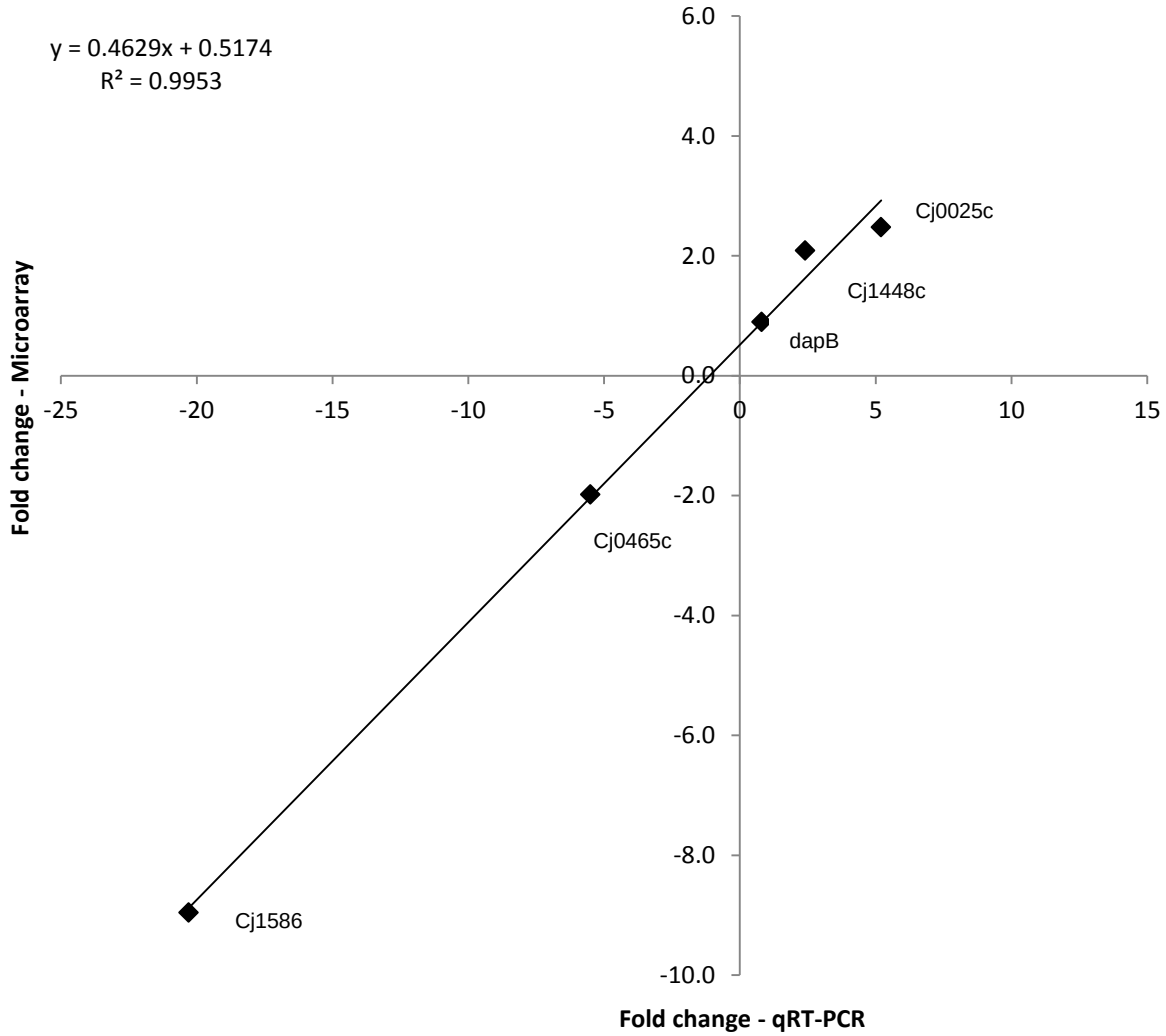

Figure 7.8: Microarray fold change vs. qRT-PCR fold change regarding adapted control vs adapted treated C. jejuni following 16 hours exposure to 0.0008% chitosan. Data points represent the mean of 4 biological replicates.

When comparing the genes up/down regulated in both the microarray and the qRT-PCR analysis, a strong correlation was apparent between the selected genes chosen for the wild-type control versus the wild-type treated samples (Figure 7.7) and those involving the control versus the treated chitosan 'adapted' samples (Figure 7.8). Respective R^2 values of 0.9284 and 0.9953 were seen to show a strong correlation (Woodall et al., 2005). Thus, the qRT-PCR was seen to validate the microarray data in the gene tested, meaning that all findings in the microarray were therefore accepted to be true.

7.5 Discussion

Generally, microarray analysis of the wild-type and chitosan 'adapted' isolates of C. jejuni NCTC11168 revealed multiple transcriptional differences in relation to cells exposed to low molecular weight chitosan and those which were not subject to chitosan.

7.5.1 Transcriptional differences between wild-type versus 'adapted' untreated cells

When assessing the differences between gene expression in control samples compared to the chitosan 'adapted' isolates against multiple genes were indicative of significant up/down regulation ($p < 0.05$). Of the five genes which happened to be up regulated in the chitosan 'adapted' control, a gene encoding a major antigenic peptide (PEB3) was found to have the most substantial difference relative to the wild-type control cells.

PEB3 is thought to be one of the most abundant glycoproteins in eukaryotes, functioning as a surface protein (Linton et al., 2002; Wacker et al., 2002). The structure of PEB3 may also lend itself to have functionality as a transportation protein and/or in adhesion, resembling PEB1a which transports both aspartic and glutamic acids (Leon-Kempis Mdel et al., 2006.)

The degree of adherence to intestinal epithelial cells may play an important role in C. jejuni resistance when in this environment. Other adhesins which are found in C. jejuni thought to mediate bacterial cell adherence to host cells include the antigens PEB1a and PEB4 (Pei et al., 1998; Asakura et al., 2007). Along with the role of adherence in of these two genes, their function seems to be also linked to

the in vivo colonisation (Jeon et al., 2010). There have been instances whereby mutations in PEB1a and PEB4 resulted in diminishing levels of colonisation in mice intestines (Pei et al., 1998; Asakura et al., 2007). A study undertaken by Asakura et al. (2007) also found a lower incidence of PEB4 mutant cells that undertook stress induced biofilm formation when compared to the wild-type cells of C. jejuni. Thus, increases in expression of PEB3 in the 'adapted' cells of C. jejuni NCTC 11168 as seen in the microarray analysis may raise questions as to whether an increased virulence potential can be associated compared to that of the wild-type, which typically has a lower expression of PEB3. The related genes which encode the PEB1a and PEB4 proteins mentioned above may suggest that the 'adapted' isolate is perhaps more able to colonise a given host through induction of PEB3. Additionally, it is possible that an up regulation of PEB3 may have implications with respect to an increased biofilm formation, if the true function is related to adherence.

The kpsM gene encoding an inner membrane protein was also found to be significantly up regulated in the 'adapted' isolate, compared to the wild-type cells of C. jejuni NCTC 11168 under control conditions. The kpsM encoding protein belongs to an ATP-binding (ABC) transporter superfamily, which in turn are thought to be important in terms of virulence, viability and pathogenicity of bacteria though the export of toxic compounds and mediation of numerous substrates including polysaccharides, ions, amino acids including others (Pigeon and Silver, 2006; Davidson et al., 2008). It has been seen previously that kpsM influences intestinal invasion of epithelial cells in vitro, approximately 10 fold decreases in C. jejuni 81-176 was observed with respect to a kpsM mutant when compared to that of the wild-type (Bacon et al., 2001). The influence of kpsM upon colonisation and subsequent virulence in C. jejuni NCTC11168 was

indicated in a study conducted by Jones et al. (2004), with a total absence of colonisation in chickens presented in a kpsM mutant in comparison to wild-type cells.

However, a study assessing biofilm formation with regards to an isogenic kpsM mutant was seen to have an increased pellicle formation in comparison to the wild-type C. jejuni strain (Joshua et al., 2006). As well as the observed up regulation of PEB3 in the chitosan 'adapted' cells of C. jejuni, simultaneous increases in kpsM may contribute the of an enhanced tolerance towards low molecular weight chitosan, generally observed in the 'adapted' cells. Interestingly, this coincides with a notable visual increase in pellicle formation upon conducting biofilm assays in relation to the 'adapted' isolate (data not shown).

The greater expression of these genes seen in the 'adapted' cells may also explain the enhanced degree of biofilm formation both in the presence and absence of chitosan, illustrated in the 'adapted' variant when compared to the wild-type C. jejuni NCTC11168 (chapter 6). Given that the PEB3 protein is thought to be involved in adherence and colonisation, this may potentially be linked to the visual increases in pellicle quantity observed in the biofilm formation and eradication studies (chapter 6).

In other bacteria, it has been observed that the extracellular matrix in formed biofilms can contain carbohydrate-containing molecules (Solano et al., 2002; Sherlock et al., 2005). Therefore, the increased expression of kpsM may be partially responsible for mediation of biofilm formation through subsequent changes in the extracellular matrix.

Gram negative bacteria have the ability to form disulphide bonds which is facilitated via the Dsb family of proteins, functioning within the periplasmic space. The oxidation pathway, in where some members of the family are related to, is responsible for the formation of disulphide bond in proteins which have recently undergone synthesis, upon crossing the cytoplasmic membrane (Grabowska et al., 2011). Despite expression of membrane-bound oxidoreductases such and dsbB and dsbI being dependant on iron concentration/availability and interaction with Fur proteins, involved in iron regulation, changes in dsb protein levels can lead to the regulation of extraplasmic virulence factors (substrates of the dsb system), which in turn is controlled by environmental conditions (Grabowska et al., 2011). The dsb family has also been associated with intestinal colonisation, as findings in experiments conducted by Lasica et al. (2010) found that mutants of both dsbI and dsbB illustrated decreased invasion and intracellular survival in human intestinal epithelial T84 cells. Also, double mutants of dsbB and dsbI were not recovered after 3 hours of invasion into these cells. This suggests that dsbI up regulation may contribute to the pathogenicity and survival of C. jejuni as a whole.

Of the genes which were seen to be down regulated in the untreated 'adapted' cells in comparison to the untreated wild-type C. jejuni cells, the greatest fold difference (9.2) was Cj0025c, believed to encode a protein encoding sodium-dicarboxylate transmembrane symporter.

Generally, divalent anion/sodium symporters (DASSs) are a family of secondary active transporters which utilise the energy from the movement of sodium ions across the electrochemical gradient, at the same time facilitating the transport of

decarboxylases and inorganic anions through the membrane (Pajor, 2006). Prokaryotic sodium:dicarboxylate symporter proteins within this DASS family have been characterised, one of these being SdcS, originally from Staphylococcus aureus (Hall and Pajor, 2005). According to Strickler et al. (2009), this symporter occupies similar transport properties to those symporter present in mammalian cells.

C. jejuni, similar to all prokaryotic cells can react to environmental stressors via utilisation of two-component regulatory systems or TCRSs, which involve regulators and sensor kinases responsible for the mediating gene expression (Raphael et al., 2005; Mikkelsen et al., 2011). Several TCRSs have currently been identified in terms of unique functionality, namely the reduced ability to colonise system or RacRS (Bras et al., 1999), Campylobacter planktonic growth regulation or CprRS (Svensson et al., 2009), diminished capability to colonise or DccRS (MacKichan et al., 2004), FlgRS involved with flagella motility (Wosten et al., 2004) and CbrR, linked to colonisation and resistance (Raphael et al., 2005). The amount of translated protein following changes in gene expression involving the TCRSs can be altered further by various posttranslational modifications, including glycosylation and disulphide bond formation, which in the modify structures impacting virulence and accounting for alterations in pathogenesis (Heras et al., 2009; Grabowska et al., 2011). With the above in mind, it can be suggested that although the exact function of the protein encoded by the Cj1608 gene has not been established, this two-component regulator may be important for an enhanced survival upon exposure to chitosan.

7.5.2 Transcriptional differences between wild-type treated versus chitosan 'adapted' treated cells

Interestingly, when comparing the gene expressional changes in the chitosan treated cells of the 'adapted' isolate, relative to the wild-type cells of C. jejuni NCTC 11168, the genes PEB3 and dbsI were seen to be up regulated, totalling a 2.8 fold and 2.0 fold change, respectively ($p < 0.05$). These encode a major antigenic peptide believed to involve glycosylation and disulphite bond formation, respectively. As these same two genes were seen to be up regulated in the untreated 'adapted' cells versus the wild-type untreated cells of C. jejuni (Table 7.14), this supports the potential importance of enhanced expression of these genes from a survival aspect. This may contribute to the enhanced growth of the 'adapted' cells under 0.0008% (w/v) chitosan in comparison to the wild-type cells over the course of 16 hours under microaerobic conditions (pH 6.0, 42°C).

A 2.5 fold increase in Cj0294 was also observed when comparing the chitosan 'adapted' cells versus the wild-type cells after having undergone chitosan exposure. Again, this proposes that the gene thought to encode MoeB/ThiF family proteins may be important in the higher overall tolerance typically observed in the generated 'adapted' isolate of C. jejuni NCTC11168.

A gene believed to encode a transcriptional regulator (CmeR) was found to be significantly upregulated in the 'adapted' cells, as oppose to the wild-type cells after chitosan treatment (2.1 fold increase). CmeR belongs to a family of transcriptional regulators (TetR) and is said to function as a repressor of CmeABC operon, which in turn functions as an efflux pump. The three components; CmeA, CmeB and CmeC have been shown to contribute to

resistance in Campylobacter spp. with respect to antimicrobials and bile components in a hosts intestinal tract (Lin et al., 2002; Lin et al., 2003, Guo et al., 2008). A Mutant generated resulting in CmeABC inactivation was found to demonstrate an inability to colonise chicken intestines in vivo (Lin et al., 2003).

It is therefore interesting that an up regulation in the 'adapted' treated cells was observed in comparison to the wild-type, as increases in CmeR would theoretically allow for a decrease in efflux. If during exposure, chitosan was able to enter the cells, it would be expected that transcriptional repressors such as CmeR would be down regulated so that subsequent increases in efflux pumps would be seen. However, it may be the case that due to the increase tolerance of the 'adapted' cells of C. jejuni, they may be more efficient at preventing chitosan influx, thus accounting for a lesser need for such mechanisms.

The intermediates in the tricarboxylic acid cycle fumarate, succinate and malate can be transported by several C4-dicarboxylate carriers, including DcuA and DcuB (Guccione et al., 2008). These carries are restricted to anaerobic and facultative anaerobic bacteria species, including C. jejuni (Hofreuter et al., 2006).

The DcuA and DcuB transporters may have important roles due to their essential aspect of electron donation with respect to fumarate and succinate metabolites within C. jejuni (Stahl et al., 2012). It is possible that DcuA may be also be involved in subsequent cytoplasmic-periplasmic membrane transport, as the succinate and fumarate required for this process is thought to be initiated by the DcuA and DcuB transporters (Guccione et al., 2010).

This may explain gene expression increases typical of 2.1 fold in Cj0088 (DcuA) and a putative periplasmic protein encoding gene (Cj0420), seen to be induced 2 fold in the chitosan exposed 'adapted' cells, compared to the wild-type C. jejuni NCTC 11168 cells. Campylobacter relies greatly upon the tricarboxylic acid cycle for the energy requirements (Stahl et al., 2012), hence it would make sense that the 'adapted' cells of C. jejuni would potentially occupy increased energy needs for a decreased susceptibility to such stressors as chitosan.

The greatest down regulations were equal to 6.2 and 3.6 fold for Cj0025c and Cj1608, respectively. Interestingly, the same trend was observed in the wild-type versus the 'adapted' untreated cells of C. jejuni. This reinforces the potential importance in the regulation of protein-coding genes which function as sodium:dicarboxylate transmembrane symporter and two-components regulators in the ability to increase survival. It may be possible that the upon exposure to 0.0008% (w/v) chitosan, up regulation in these genes may be required to enhance the capacity for growth, whereas the more tolerant 'adapted' isolate may theoretically rely less on these genes for survival, resulting in relative down regulation.

Additional genes which were found to be down regulated in the 'adapted' cells in relation to the wild-type included Cj1629 (3.1 fold) and Cj0982c or cjaA (-3.0 fold). These genes encode proteins perceived to be related to ExbD/TolR family transport and amino acid transport mediated through substrate binding, respectively. Down regulation in other genes thought to be have similar functionality were also apparent, including Cj0753 (TonB3) encoding a TonB transport protein and Cj1628 (exbB2) encoding an ExbB/TolQ family transport protein.

Genes believed to be involved in energy transduction (exbB1–exbD1, exbB2–exbD2, tonB1, tonB2 and tonB3) are thought to have some bearing upon iron acquisition, as they are generally greater expressed under iron limiting conditions (Holmes et al., 2005; Miller et al., 2009). Therefore, a down regulation in such genes present in the 'adapted' cells versus the wild-type cells whilst under chitosan exposure (down regulation in tonB3 gene of 2.2 fold) may imply that the wild-type cells require more iron for metabolic purposes, in an attempt to combat the chitosan stress. Alternatively, the 'adapted' isolate upon exposure to chitosan, may down regulate various genes related to iron transport/acquisition in order to conserve energy needed for other unknown processes, accounting for an increased survival potential. Iron uptake in C. jejuni is well regulated to avoid reactive oxygen species (ROS) formation through combination of iron and oxygen (Masse and Arguin, 2005).

Generally, Gram negative microorganisms such as C. jejuni have iron acquisition systems which comprise of outer-membrane receptors, transport via TonB/ExbB/ExbD complexes, periplasmic proteins and inner-membrane ABC transporters (Holmes et al., 2005). This may clarify simultaneous down regulation is other genes encoding periplasmic proteins and inner-membrane ABC transporters, namely Cj1358c (putative periplasmic cytochrome c), Cj0876c (probable periplasmic protein) and Cj1584c (peptide ABC transporter-substrate binding protein). It has been proposed that a fraction of iron uptake could be facilitated via the ZupT gene (Cj0263), encoding a transporter predominantly responsible for uptake of zinc (Stahl et al., 2012). In E.coli, a ZupT homolog has illustrated substrate affinity for various cations, including ferrous ions; however the exact function of this gene has not been confirmed in C. jejuni (Grass et al.,

2005). The gene Cj1659, also found to be down regulated in the 'adapted' treated cells compare to the wild-type treated cells is a periplasmic membrane protein which is potentially involved in iron transport (Holmes et al., 2005).

7.5.3 Transcriptional differences between wild-type control versus wild-type chitosan treated cells

Comparing expressional differences between the wild-type untreated (control) cells versus the those treated with 0.0008% (w/v) chitosan, revealed several genes which were significantly up regulated following chitosan exposure, in relation to control cells ($p < 0.05$).

Several of these genes were thought to be related to transportation of ions/substrates. These included Cj0731 (ABC transporter permease), Cj1560 (probable permease), Cj0730 (putative ABC transport permease), Cj0967 (putative periplasmic protein) and Cj1539c, thought to be involved in an anion-uptake ABC-transport system permease protein (Table 7.15). It would make logical sense that upon exposure to antimicrobials including chitosan may enhance genes involved in transport to be better able to survive in a less than ideal environment.

Other genes perceived to be related to transport, were up regulated in terms of the wild-type treated versus respective controls. These included the pseudogene Cj0046 (probable transport protein), exbB1 (biopolymer transport) and modB, encoding a protein molybdenum transport system permease.

Interestingly, a putative efflux protein encoding gene (Cj0035c) was also seen to be up regulated in the treated cells of the wild-type C. jejuni cells compared to those of which were subject to no chitosan challenge (2.2 fold). It is possible that C. jejuni may up regulate such genes in order to remove chitosan which may have entered the cell through membrane perturbation, a potential mode of action proposed by Helander et al. (2001); Zakrzewska et al., (2005) and Je and Kim (2006).

Additional up regulation of both Cj0291c, encoding a glycerol-3-phosphate transporter and Cj0379c, possibly involving a sulphite oxidase subunit was observed in the treated versus non-treated wild-type cells. C. jejuni is known to convert glycerol-3-phosphate to glyceraldehyde-3-phosphate, which can then be utilised in the glycolysis pathway. An enhanced ability in the uptake of glycerol-3-phophate, as seen in the chitosan treated cells of the wild-type when compared to the untreated cells, may result in a metabolic advantage (Hofreuter et al., 2006).

The metabolic alterations which seem to be resultant occur in an attempt to combat such chitosan exposure may also be associated with the overexpression of genes thought to encode cytochrome C proteins (Cj0874c), which facilitate transfer of electrons in the electron transport chain. Likewise, this links to other genes, for instance the up regulated Cj1530, involving the enzyme dephospho-CoA kinase, a phosphotransferase which involves catalysis of the de-phosphorylation of ATP in the tricarboxylic acid cycle.

Interestingly, only two genes were found to be significantly down regulated in the chitosan treated wild-type cells compared to those cells which were grown in

the absence of chitosan, encoding a cbb3-type cytochrome C oxidase subunit II and a cb-type cytochrome C oxidase subunit III (ccoO and ccoP, respectively).

7.5.4 Transcriptional differences between 'adapted' treated versus 'adapted' untreated cells

When assessing the differences in gene expression with regards to the 'adapted' chitosan treated cells against those which did not undergo exposure to chitosan during the 16 hours of growth, the greatest increase in gene expression was typical of the Cj0025c, encoding a putative sodium:dicarboxylate family transmembrane symporter (5.2 fold increase). This is contrary to what was observed in the comparison of the 'adapted' treated cells versus the wild-type treated cells and adapted untreated versus wild-type untreated cells. Thus, these data suggest an important role of this gene in terms of the degree to which C. jejuni is able to survive. Unfortunately, as there is no definitive function of this gene in Campylobacter, it is difficult to establish to exact mechanism of action of the gene product.

Iron is known to be an essential nutrient for organisms in general due to its involvement in enzymes as a cofactor as well as having a prominent role in electron transport and redox reactions (Earhart, 1996). Bacteria such as C. jejuni have the ability to transfer iron complexes across the outer membrane via high affinity receptors. Transportation of the iron complexes over the cytoplasmic membrane is mediated by ABC transporters (van Vliet et al., 2002).

Uncontrolled iron uptake may cause iron toxicity and oxidative stress, leading to impaired growth characteristics (Litwin and Calderwood, 1993). Thus, the

maintenance of iron homeostasis seems to be paramount to survival of microorganisms, this regulation of iron is usually controlled via a balance in the uptake and storage (van Vliet et al., 2002). Iron homeostasis is associated with various mechanisms to protect against oxidative stress and also the formation of toxic oxygen intermediates, which in turn is related to iron metabolism (Touati, 2000; van Vliet et al., 2002).

Although C. jejuni is equipped with an array of enzymes which may facilitate oxygen-independent respiration, it has an inability to grow in strictly anaerobic conditions (Veron et al., 1981). Thus the pre-requisite for oxygen indicative of the microaerobic requirements, brings a disadvantage in that electron transfer of a non-specific nature in the respiratory chain can be transferred to oxygen molecules, ultimately leading to ROS generation, namely superoxide and hydrogen peroxide (Cabiscol et al., 2000; Hofreuter et al., 2014). However, C. jejuni possesses a number enzymes involved in ROS-detoxification, thiol peroxidase (Tpx) being one of these (Atack et al., 2008).

As study conducted by Atack et al., (2008) revealed that Tpx plays important roles in the resistance to a multitude of oxidative and nitrosative agents, including molecular oxygen. Studies indicated that Tpx functions as a hydrogen peroxide reductase. Moreover, a gene encoding a Tpx protein (Cj0779) was found to be up regulated in the treated cells of the 'adapted' isolate, as oppose to the untreated cells. The build-up of highly reactive species can account for damage to vital components within C. jejuni; these include proteins, nucleic acids and cell membranes (Atack et al., 2008). The ability of C. jejuni to overcome such compounds may contribute to an enhanced survival and potentially an increased pathogenesis/virulence (Imlay, 2003).

Bacteria have mechanisms to counter such stress, which include cytochrome c peroxidases and peroxiredoxins (Atack and Kelly, 2006; Wood and Kelly, 2006). Up regulation with regards to the chitosan exposed 'adapted' cells was observed in the Cj1665 gene, encoding a putative lipoprotein thioredoxin was observed. This may suggest that the 'adapted' cells which upon exposure to chitosan may occupy a greater ability to deal with inevitable ROS toxicity. Excess Iron which is not utilised immediately needs to be stored in a non-reactive state, minimising the hydroxyl radical formation and thus preventing iron toxicity (van Vliet et al., 2002). Another advantage of storing iron is that it is available during iron shortage in cells. Two classes of bacterial iron storage proteins are known to exist, ferritin and bacterioferritins (Andrews, 1998).

Genes which encode bacterioferritins are usually said to be located in the genes which code small ferredoxins. A protein associated with E.coli (bacterioferritins-associated ferredoxin, Bfd) has been shown to have similarity to a region of the NifU protein. This NifU protein is encoded for in a number of genes which are thought to be responsible for the biosynthesis of iron-sulphur [Fe-S] centres in terms of the nitrogen-fixation proteins (Crichton, 2001).

Additionally, homologs of nitrogen-fixation protein, for example IscS homolog of NifS, encodes a cysteine desulphurase, involves the catalysis of sulphur from cysteine, allowing for [Fe-S] cluster synthesis (Zheng al.,1998; Agar et al., 2000). Genes of this nature were found to up regulated in the 'adapted' treated cells when compared to those which were untreated included Cj0240c (IscS) and Cj0239c (NifU), encoding proteins cysteine desulphurase and nitrogen-fixation, respectively.

Thus up regulations of a putative ABC transporter (Cj1663) and gene encoding a periplasmic protein (Cj1637c) with regards to the 'adapted' treated cells over those not subjected to chitosan addition may well contribute to iron acquisition. Iron acquisition may be an important attribute contributing to the increased survival following chitosan treatment. The greatest decreases in gene expression with respect to the 'adapted' treated cells compared to the non-treated controls were present in Cj1586, encoding a single domain haemoglobin (Cgb) and Cj0465c, encoding a truncated haemoglobin protein (Ctb).

C. jejuni is often exposed to nitric oxide (NO), which is produced by the host upon transmission. C. jejuni is thought to express two haemoglobins which may account for an increase tolerance of such nitrosative stress conditions (Tinajero-Trejo and Poole, 2012). Surprisingly, these two haemoglobins were those found to be down regulated, as indicated by the microarray analysis. The Cgb haemoglobin is believed to be involved in the detoxification of NO, whilst the Ctb has been associated with cytochrome c peroxidase catalysis and also oxygen delivery (Lu et al., 2007; Wainwright et al., 2006), as oppose to aspect of NO protection (Tinajero-Trejo and Poole, 2012).

In a study conducted by Monk et al. (2008) a Ctb mutant was not compromised in its ability to tolerant nitrosative stress related agents, implying that the Ctb protein is not directly involved in combatting nitrosative stress. However, since the functions of the Ctb and Cgb haemoglobins are thought to be intimately related somewhat, this may explain previously observed co-regulation (Wainwright et al., 2005).

The down regulation in the bacterial haemoglobin related genes mentioned above may link to the down regulation in genes which are speculated to encode a cytochrome c peroxidase (Cj0358) and also two genes, Cj1358c (nrfH) and Cj0783, encoding a putative cytochrome c nitrite reductase and a cytochrome c-type protein periplasmic nitrate reductase small subunit, respectively. It can be suggested that with increases in expression of thioredoxin functionality (Cj1665) and thiol peroxidase activity (Cj0779, Tpx), may facilitate the down regulation in the cytochrome c peroxidases as this involves the catalysis of hydrogen peroxide to water. Thus, if increases in genes related to reducing ROS are accounted for, down regulation in cytochrome c peroxidases may follow potentially due to a decreased likelihood ROS development, such as hydrogen peroxide. This may also account for decreases in genes responsible for nitrosative stress (e.g. Cj1358c and Cj0783) due to co-regulation between these types of stresses commonly faced by C .jejuni. This in turn may explain down regulation in the Ctb gene, if its role in mediation of oxygen is correct. The observed down regulation in the Ctb gene present in the treated 'adapted' cells as opposed to the untreated cells may be present to reduce oxygen flux into the cytoplasm. It is known that iron, along with oxygen molecules can form ROS such as hydrogen peroxide (Holmes et al., 2005). A decrease in oxygen intake could theoretically limit the possibility of subsequent ROS generation. This especially links to the apparent iron acquisition and storage in which genes thought to be related to this were up regulated in the 'adapted' treated cells compared to those cells which were untreated. As co-regulation was apparent in terms of the genes involved in oxidative and nitrosative stress, it is not surprising that co-regulation in the two bacterial haemoglobins was observed.

A previous study found an over expression of genes encoding enzymes involved in oxidative stress are often in accordance with expression of iron transport systems, presumably owing to the anticipation of iron influx into the cell (Holmes et al., 2005). Transcript levels in non-essential iron-containing proteins have been shown to decrease in relation to iron depletion, seemingly as a mechanism to allow for iron to become more readily available for more vital processes within the cell. Genes encoding non-haem protein (Cj0012c), ferredoxin (Cj0333c), cytochrome c peroxidase (Cj1358) and members of the succinate dehydrogenase operon (Cj0437-0438) were all found to show a down regulation (Holmes et al., 2005).

This is somewhat in accordance to findings highlighted in Table 7.17, with down regulations being apparent in Cj1358, Cj1358c (nrfH), Cj0437, Cj0438 and Cj0439 (a putative succinate dehydrogenase subunit C). In this case, it may be that the genes Cj0465c and Cj1586 (encoding the bacterial haemoglobins), are subject to a related down regulated in chitosan treated 'adapted' isolate when compared to the control, potentially resultant from a greater need for iron acquisition in more important cell activities elsewhere.

7.5.5 Overlapping genes between comparisons tested

Of the genes which we seen to overlap between initial comparisons (Table 7.18), it is clear that protein-coding PEB3 and Cj0017c were both over expressed with regards to the 'adapted' isolate in relation to the wild-type, both in response to and in the absence of chitosan. As TCRSs can be altered by posttranslational modifications, namely glycosylation and disulphide bond formation, this may

suggest a close relationship between an up regulation in PEB3 and Cj0017c, believed to encode protein functioning in glycosylation and disulphide bond formation, respectively, with a simultaneous down regulation in the putative two-component regulator (Cj1608) observed in both instances (table 7.18).

7.5.6 Conclusions

To date, there appears to be no previously documented data regarding the transcriptional differences following C. jejuni exposure to chitosan. The results in this chapter highlight differences in gene expression not only in response to chitosan treatment but also between C. jejuni isolates.

Generally, genes encoding proteins believed to be associated with energy metabolism, solute/ion uptake/acquisition and efflux systems, were seen to be differentially regulated when growth in the presence of low molecular weight chitosan. Differences were also present when comparing the wild-type and 'adapted' cells of C. jejuni when grown in the presence of chitosan and also in the absence of chitosan challenge.

A number of genes were found to be of particular interest, an example includes the observed co-expression of PEB3 and Cj0017c, exclusively found to be over expressed in the 'adapted' isolate under in the presence and absence of chitosan, potentially highlighting major roles towards development an enhanced phenotype, established in previous findings.

Consistent over expression observed in the 'adapted' control versus the wild-type control and 'adapted' chitosan treated relative to the control in the protein-coding gene KpsM, thought to involved capsule polysaccharide export, may contribute to aspects such as enhanced biofilm formation phenotype, in the 'adapted' isolate, compared to the wild-type C. jejuni cells (chapter 6).

An increased expression in the genes, encoding a putative efflux protein (Cj0035c) and a probable multidrug efflux transporter (Cj1375) in response to chitosan treatment, seen in the wild-type treated and 'adapted' isolates when compared to their respective untreated controls, may also highlight potential mechanisms involved in efflux, are likely to be important in combatting the antimicrobial effects of chitosan.

Findings also strongly suggest that the mediation of the putative two-component regulator (Cj1608) and/or sodium:dicarboxylate symporter (Cj0025c) may be important for mediation of numerous processes concerning transport, following chitosan exposure. For instance, the wild-type treated cells accounted for an up regulation in protein-encoding genes relating to amino acid, iron and ion transportation. It is possible that because of the greater susceptibility to chitosan with regards to the 'adapted' isolate, increases in a number of these genes, alongside co-expression in CJ1608 and Cj0025c, may facilitate such up regulations.

With particular respect to the observed increases in expression in these efflux related genes, it can be suggested that exposure to chitosan may disrupt the bacterial membrane to some extent. In the possibility of chitosan being able to interfere with cell permeability and membrane function, cell death could be

prevented by metabolic imbalances and alterations in ionic homeostasis (Raafat et al., 2008). It has also been suggested that chitosan may bind to cell wall polymers, which could account for destabilisation and/or disruption of the bacterial membrane. This may consequently lead to cellular leakage of key components with the cells (Raafat et al., 2008).

There are limited studies which have investigated the effect of chitosan exposure from a transcriptional perspective. However, the findings presented in this chapter support some of the previous findings observed in a study undertaken by Mellegard et al. (2011). Results indicated numerous transcriptional differences were observed when comparing Bacillus cereus exposed to chitosan in relation to the non-treated cells. An up regulation in genes involved iron uptake and transport were typical with regards to the chitosan cells, as oppose to the control samples. Other genes also found to be upregulated included encoded proteins involved in alkali metal ion binding, potassium ion binding and ion transmembrane transportation (Mellegard et al., 2011). Related down regulations in genes involving nitrate metabolic pathways and oxidoreductase activity with respect to nitrogenous compounds were also observed, which is in accordance with findings in this chapter.

Some similarity to previous transcriptional analysis undertaken by Raafat et al. (2008) was also apparent. This study investigated the transcriptional alterations resulting from chitosan exposure with respect to S. aureus SG511 cells relative to untreated control cells. Chitosan treatment was speculated to interfere with cellular energy metabolism. This was based on an increased expression of genes which are normally preferentially expressed in response to oxygen depletion. Transcriptional increase in the chitosan treated cells, typical of genes encoding

proteins involved in nitrite reduction, anaerobic respiration and NADH dehydrogenase, were presumed by the authors to be a result of uncoupling of the electron transport chain, resulting in an impairment of oxygen consumption. This also may explain the seemingly odd results with respect to the 'adapted' chitosan treated cells compared to control cells, typical of a down regulation in two bacterial haemoglobin related genes.

Several possible modes of action have been proposed with respect to chitosan. The binding of chitosan to anionic compounds has been proposed as a mode of action, which has previously been observed on the surface of Gram negative bacteria (Helander et al., 2001). It has also been found that the presence of divalent cations has been shown to impair the antimicrobial effect of chitosan via binding to chitosan targets (Helander et al., 2001). This has been reinforced by the observed interaction between chitosan and negatively charged surface components of bacteria, namely phospholipids (Liu et al., 2004).

Moreover, the interaction of chitosan with negatively charged surface components may perhaps cause cell surface alterations, consequently impairing important bacterial activities and thus general functionality (Je and Kim, 2006). It has been found that Salmonella typhimurium mutants with a reduced overall negative cell surface charge in comparison the parental strain was less susceptible to the antimicrobial effects of chitosan (Helander et al., 2001). Another study assessing Staphylococcal mutants, which displayed different cell overall surface charges found that a highly negative cell surface enhanced the activity of chitosan (Raafat et al., 2008).

In addition, the Chelating ability of chitosan has also been speculated to be involved in terms of mode of action (Rabea et al. (2003), which may explain

observed over expression in acquisition/uptake related genes, as chitosan limit available metal ions which could be transported into the cell for metabolic purposes.

To conclude, although the exact mode of action of chitosan remains to be established definitively, the findings in this chapter support the theory that chitosan may affect metabolic processes, probably through initial membrane perturbation. It is also likely that the mechanism of action with regards to chitosan is multifactorial, with an occurrence of several events compared to a single molecular system. Especially in the case of the gene with putative function, there is a requirement for additional gene characterisation. Gene inactivation studies may also been required to reveal the association between the certain gene targets which are thought to be of particular importance and in an attempt to decipher their role in response to chitosan.

Chapter 8 – General conclusions and future work

8.1 Conclusions

This study set out to establish the phenotypic and molecular mechanisms which facilitate C. jejuni to adapt and survive exposure to low molecular weight chitosan. C. jejuni is a leading cause of gastroenteritis, primarily resultant from the consumption of contaminated foodstuffs. Although C. jejuni is renowned for its fastidious nature, this microorganism continues to cause problems from a health perspective.

At present, there remains a high demand for novel and naturally derived antimicrobials which can be utilised to combat pathogens. This has been strengthened by consumer demands which detract from the more traditional preservation methods. Chitosan, a naturally derived from the deacetylation of chitin, has been seen to possess antimicrobial properties in a number of microorganisms, and has been shown to be particularly effective against C. jejuni (Ganan et al., 2009). This antimicrobial has received increasing interest due to the GRAS nature (FDA, 2001) and variety of potential applications.

Exposure of bacteria species to non-optimal environmental conditions can induce an adaptive tolerance response (ATR), which can aid the survival to further homologous and/or heterologous stress (Murphy et al., 2003; Kumar-Phillips et al., 2013). An adaptive response of C. jejuni towards repeated sub-lethal exposure through step-wise increases of commonly used biocides has been previously documented, resulting in a non-permanent tolerance (Mavri and

Možina, 2012). These phenomenon pose problems in terms of a public health risk, as unwanted persistence may result from this (Mavri and Možina, 2012).

It has been suggested that resistant sub-populations can also exist alongside wild-type populations and that repeated the same sub-lethal treatment to stressors can also lead to the potential for a proportion of cells which occupy an enhanced resistance (Rajkovic et al., 2009). Thus, the exploration of such stress responses is encouraged in an attempt to understand the mechanisms which can lead to this enhanced survival, which is also driven by industrial and safety concerns within the food industry (Scheyhing et al., 2004).

There appears to be no literature which has previously demonstrated the generation of adaptive response towards low molecular weight chitosan. Thus, chapter 4 aimed to address the effect of exposure to low molecular weight chitosan towards C. jejuni NCTC11168, and to establish if this strain of C. jejuni was able to become stress hardened following increasing sub-lethal concentrations of chitosan.

After defining the intensities required needed for at least a 1 log CFU/ml reduction in cell count, no decreases in susceptibility was apparent after repeated challenge to 0.02% (w/v) and 0.03% (w/v) chitosan, when applying methods modified from Rajkovic et al. (2009). This was indicated by the lack of deviation of recovered cells after each treatment over the course of 20 days. Several attempts were made in order to generate an adaptive sub-population through step-wise training typical of increasing increments of chitosan exposure. Despite the inability to produce adaptive responses following methods adapted from Mavri and Možina (2012) in MH broth, when applying an agar-based

alternative using similar step-wise incremental increases, an adaptive response was identified, possessing an increased tolerance to low molecular weight chitosan. Serial passaging onto non-chitosan containing media revealed a non-stable adaptation of the newly generated isolate, referred to as the chitosan 'adapted' C. jejuni NCTC11168. After 12-13 passages onto non-chitosan containing agar with simultaneous plating onto 0.044% (w/v) chitosan containing agar accounted for no cell recovery after incubation for 72 hours under conditions mentioned previous. This complies with the study conducted by Mavri and Možina (2012), which also found a non-permanent resistance in C. jejuni to several biocides.

Whilst a chitosan 'adapted' isolate of C. jejuni NCTC11168 was successfully generated, presenting an enhanced tolerance to chitosan, these findings are limited to this well characterised genome strain. It can be assumed that if C. jejuni NCTC11168 is able to present an adaptive tolerance response towards chitosan, other bacteria species which harbour more widespread mechanisms can also development of antimicrobial resistance.

This chapter presented what was assumed to be the first documented case of an ATR resultant from sub-lethal exposure to low molecular weight chitosan. Understanding how sub-lethal treatments to stressors may allow for enhanced survival properties of C. jejuni is necessary when evaluation current food preservation methods, as these may well have undesirable effects on aspects such as virulence and alteration in gene expression (Ma et al., 2009).

Chapter 5 aimed to establish the differences in terms of susceptibility of several C. jejuni strain/isolates towards low molecular weight chitosan under varying

conditions. Briefly, results indicated that the antimicrobial activity of chitosan was highly dependent on pH, with greater MIC and MBC values following an increase in pH in all C. jejuni strains/isolates tested. This is in accordance with other previous research, thought to be resulting from a higher solubility and protonation under more acidic conditions (Aider et al., 2010).

Overall, the wild-type C. jejuni NCTC11168 strain was found to harbour the greatest susceptibility towards chitosan when compared to the other C. jejuni strains/isolates tested. Whilst the previously developed 'adapted' isolate of C. jejuni NCTC11168 was found to possess the lowest susceptibility of all strains/isolates tested, as revealed by the MIC agar dilution results (pH 6.0).

Additionally, time kill assays also reinforced differences between C. jejuni strain/isolate susceptibility when challenged for a total of 6 hours under both nutrient rich (MH broth) and limited (PBS) conditions. Generally speaking, under nutrient limitation, subsequent recovery was found to be lower, which was dependant on both exposure time and chitosan concentration utilised. The wild-type NCTC11168 and RM1221 strains of C. jejuni were found to occupy the lowest degree of total recovery in nutrient rich conditions. A similar trend was observed in both strains with respect to the same concentrations of chitosan under nutrient depleted conditions. Whereas, in the chitosan 'adapted' isolate of C. jejuni NCTC11168, 81116 strain and NCTC11168CH isolate, cell viability was identified even after 6 hour exposure following exposure to 0.01% (w/v) in nutrient rich conditions.

Growth inhibition studies also complemented previous findings with particular respect to the wild-type versus the chitosan 'adapted' isolate of C. jejuni

NCTC11168. To summarise, growth inhibition was more apparent in the wild-type C. jejuni NCTC11168 compared to the 'adapted' isolate previously generated through step-wise exposure to chitosan. This was especially noticeable when cells were grown in the presence of 0.0016% (w/v) chitosan. The findings in chapter 5 highlight not only that C. jejuni strains/isolates tested are sensitive to low molecular weight chitosan, when compared to other species as previously illustrated by Ganan et al. (2009), but also express strain/isolate dependant antimicrobial efficacy. The enhanced tolerance found in the 'adapted' isolate of C. jejuni NCTC11168 in relation to the wild-type may explain how this microorganism, through step-wise training exposure to antimicrobial stresses, can lead to enhanced persistence which allow for phenotypic and genotypic alterations.

Aspects such as biofilm formation, motility and chemotaxis are all thought to provide C. jejuni with an improved capability in which to survival environmental stresses. Aspects such as motility of C. jejuni, for instance are said to have a direct influence on the ability for colonising the host through migration through gastrointestinal mucus (Lertsethtakarn et al., 2011). Additionally, it has been suggested that flagella may also be required in order for biofilm development and maturation (Reeser et al., 2007). The process of biofilm formation is known to be of great importance to survival particularly in unfavourable conditions. In the event of an enhanced biofilm formation occurring in vivo for instance, has seen suggested to not only account for an increased protection from high levels of bile in the gall bladder, but may also lead to a limitation in antibiotic effectiveness (Begley et al., 2005).

The goal of chapter 6 aimed to establish any phenotypical differences between the C. jejuni strains/isolates with particular attention to biofilm development, motility and chemotaxis. Overall, the degree of motility was found to be strain/isolate dependant and increased in all strains/isolates tested from 24-72 hours, as expected. After 72 hours incubation at 42°C under microaerobic conditions, the greatest degree of motility was observed in the RM1221, followed by the 'adapted' C. jejuni NCTC11168 cells. Conversely, the lowest degree of motility was typical of the wild-type C. jejuni NCTC11168. This implies that through the development of an 'adapted' isolate under a step-wise training regime, phenotypic alterations can result and may account for advantages in terms of survival.

All strain/isolates tested were able to form biofilms after 2 and 3 days incubation under control conditions (chapter 6).With respect to the 'adapted' isolate of C. jejuni NCTC11168, an increase in biofilm formation was apparent after 3 days incubation, when compared to the wild-type C. jejuni NCTC11168 strain. Biofilm formation was also evident under chitosan conditions. Therefore, although chitosan illustrated a strong antimicrobial activity towards C. jejuni in general, especially when compared to other bacterial species, as indicated in a study conducted by Ganan et al., (2009), the ability of C. jejuni to form biofilms even in concentrations which would not permit growth in a planktonic state, is likely to be of great also be of importance in terms of persistence. Findings also indicated not only that pre-formed biofilms appear to be less susceptible to chitosan exposure than planktonic cells, but that the 'adapted' isolate, with particular in particular relation to the wild-type C. jejuni NCTC11168 occupies an altered biofilm phenotype, which can facilitate survival and potentially virulence. It is interesting that the isolate previously subjected to passage through a chicken

was similarly able to tolerate chitosan in a pre-formed biofilm state. It can be speculated that previous entry into the gastrointestinal tract may contribute to an enhanced phenotype, which may impact biofilm formation. However, other assays involving cell adherence and invasion may establish to true extent of the 'adapted' isolates tolerance to host stress. These findings in chapter 6 suggest that step-wise training of C. jejuni leading to an adaptive tolerance response to chitosan may contribute to alterations in key aspects such as biofilm formation and motility.

As discussed previously, upon exposure to unfavourable stresses, microorganisms such as C. jejuni can adaptive responses which can alter the phenotype which can have implication towards enhanced survival. This was observed to be the case in terms of the 'adapted' isolate of C. jejuni NCTC11168, which exerted a decrease in susceptibility towards chitosan exposure.

In order to understand the transcriptional changes which may be responsible for these phenotypic changes in the 'adapted' isolate of C. jejuni NCTC 11168, relative to the wild-type strain, microarray analysis was conducted (chapter 7). Many genes were found to be altered in terms of gene expression in response to sub-lethal chitosan. To summarise, protein-coding genes associated with efflux systems, energy metabolism and solute/ion transport/acquisition were found to be differentially expressed following sub-inhibitory exposure to chitosan. Several genes were also found to overlap between the initial comparisons tested. Examples included significant up regulation of PEB3 (major antigenic peptide), Cj0017c (disulphide bond) and Cj0294 (potentially involved in thiamine biosynthesis) were found to be up regulated in 'adapted' C. jejuni cells both when compared to the wild-type both in the presence and absence of chitosan.

The exclusive over expression of these genes was therefore deemed important for an increased phenotype associated with the 'adapted' isolate. Overlapping down regulation was found in genes thought to be involved in a putative sodium:dicarboxylate symporter and two-component regulator (Cj1608) with respect to the same comparisons. It can also be speculated that these genes are therefore of some importance for dealing with chitosan challenge.

Owing to the wild-type isolate being notably more susceptible to chitosan exposure, any up regulation in genes may enable increased survival. Interestingly, the putative sodium:dicarboxylate symporter gene-encoding protein (Cj0025c) was also up regulated in the 'adapted' treated cells relative to the respective control (untreated). This also implies that this putative gene is important towards survival.

Perhaps the most surprising findings with regards to microarray analysis were apparent when cells of the 'adapted' isolate of C. jejuni NCTC11168 were exposed to chitosan, an observed down regulation of two bacterial haemoglobins, ctb and cgb, related genes, thought to be involved in oxygen metabolism and nitrosative stress respectively. A possible explanation for this surprising observation may be that upon chitosan treatment, there is a shift in metabolic activity, potentially due to an inhibition of oxygen consumption in an attempt to preserve energy needed for more important stress responses. This may be a mechanism which leads to an increased chance of survival potential. Dufour et al. (2010) found similar decreases in expression of genes thought to allow for an impaired oxygen consumption of C. jejuni when challenged to the naturally derived antimicrobial, benzyl isothiocyanate. This switching from oxygenic respiration in C. jejuni to an alternative electron transport pathway

may also prevent the amount of ROS formed by components of the electron transport chain. This may also explain the down regulation of two oxygen-accepting cytochrome C oxidase subunits (ccoP and ccoO) with respect to the wild-type when challenged with chitosan. These findings correlate to the probable electron transport chain uncoupling, suggested by Raafat et al. (2008) involving S. aureus challenged to chitosan. In this study, several genes usually preferentially expressed under oxygen depleted conditions, were upregulated upon treatment. Thus, it can be speculated that chitosan exposure can lead to oxygen impairment and interferes with cellular energy metabolism (Raafat et al., 2008).

In summary, this study has revealed some important phenotypic and transcriptional responses of C. jejuni in response to low molecular weight chitosan. This study has demonstrated that C. jejuni can illustrate an adaptive response through repeated exposure of increasing concentrations of chitosan, resulting in an isolate with an altered phenotype, through molecular mechanisms. In a general sense, these findings demonstrate that although C. jejuni is thought to be limited to an extent with regards to its limited growth conditions, specific nutrient requirements and some general stress response genes (Gaynor and Wells, 2005), this microorganism is able to efficiently adapt to the chitosan, which can have a profound effect on virulence potential.

From this study, it can be suggested that when using antimicrobials such as chitosan, there is a necessity to make logical decisions in terms of application such as to limit the virulence potential of the contaminating microorganisms. In controlling the given pathogen effectively through these means, there is a decreased the chance of future food-borne outbreaks, which can result through

persistence. Understanding how sub-lethal exposure to antimicrobials such as chitosan, may further promote the survival of C. jejuni, is required for evaluating food preservation strategies, as there may be unwanted effects on virulence through transcriptional changes (Ma et al., 2009).

8.2 Future Work

While the antimicrobial effects of chitosan have been demonstrated in this study, the effects in combination with additional hurdles such as low temperature and oxygen limitation which are encountered during food processing/storage and the host upon infection, may provide some additional information as to how C. jejuni is able to survive. With respect to the 'adapted' isolate, the assessment of cross protection to other antimicrobials through susceptibility assays could be undertaken to reveal whether this altered phenotype associated with this isolate may contribute to resistance on a grander scale.

The findings in this study highlight some interesting stress responses to chitosan, both at a phenotypic and transcriptional level. In order to provide more information as to the individual effects that certain genes such as KpsM and the two-component regulator (Cj1608), gene inactivation studies of these genes could be undertaken. This may establish why these genes are differentially regulated following chitosan exposure and what specific role they may have in combatting chitosan stress. If functions are effectively defined in candidate genes, this may reinforce current theories with respect to the aspects such as the mode of action(s) involving chitosan.

Additional gene expression studies on different strains of C. jejuni may also be worthwhile, as this study was limited to wild-type C. jejuni NCTC11168 and the subsequently generated 'adapted' isolate. Therefore, to generalise the findings in this study, the use of additional strains of C. jejuni, especially from a variety of sources of origin, would likely be necessary.

Other experiments could also include other aspects involved in virulence such as adherence, invasion and quorum sensing which could potentially allow for some interesting comparisons to be established between the strains/isolates tested.

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VIII Appendices

Publications associated with thesis:

Woolford, J., Allen, S. C. H. and Phillips, C. A. (2013) DGGE and qRT-PCR comparison of wild-type and chitosan "adapted" isolates of Campylobacter jejuni NCTC 11168. Poster presented to: 17th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO), Aberdeen, UK, 15-19 September 2013.

Woolford, J., Allen, S. C. H. and Phillips, C. A. (2012) Antimicrobial effects of chitosan against Campylobactor jejuni NCTC 11168. Poster presented to: 23rd International Committee on Food Microbiology and Hygiene of the IUMS (ICFMH) Symposium, FoodMicro 2012, Istanbul, Turkey, 03-07 September 2012.

Woolford, J., Allen, S. C. H. and Phillips, C. A. (2012) Antimicrobial efficiency of low molecular weight chitosan against Campylobacter jejuni NCTC 11168. Invited Presentation presented to: Society for Applied Microbiology (SFAM) Summer Conference 2012, Edinburgh, UK, 02-05 July 2012.