Campylobacter jejuni: Biofilm Formation and Oxidative Stress

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Abstract

Campylobacter jejuni (*C. jejuni*) is a zoonotic pathogen most often associated with poultry and the leading cause of bacterial gastroenteritis in the developed world. It is an obligate microaerobe, requiring low levels of oxygen and increased levels of carbon dioxide for growth. Attempts to understand how this sensitive pathogen survives the variety of environmental challenges it faces have led to the suggestion that it may find protection in biofilms. This study investigates the effect of oxygen concentration on the formation of *C. jejuni* biofilms and their subsequent resistance to oxidative stress. In order to determine their ability to develop biofilms when exposed to oxidative stress, three strains (NCTC 11168 V1, NCTC 11168 V26 and 16-2R) of *C. jejuni* biofilm cells were grown on glass fibre filters in different atmospheres: MA – microaerobic (5 % O_2, 10% CO_2, 85% N); AIR – aerobic (20% O_2, 0.05% CO_2, 78% N) and ACO2 – aerobic enhanced with 5% CO_2 (19% O_2, 5% CO_2, 74% N). Biofilm formation was assessed using plate counts, biovolume analysis and scanning electron microscopy (SEM). Tolerance to oxidative stress was determined by exposure of biofilm cells to the exogenous oxidant hydrogen peroxide (H_2O_2).

Plate count data indicated no significant difference between biofilm cells for MA (9.6 to 9.8 $\log_{10}$ CFU/g) and ACO2 (9.6 to 9.9 $\log_{10}$ CFU/g) ($p > 0.05$). Biofilm cells grown in AIR (4.0 to 4.3 $\log_{10}$ CFU/g) were significantly lower than MA or ACO2 for all three strains ($p<0.001$). These results were supported by the SEM images. Biofilm development was more extensive in both MA and ACO2 than in AIR. SEM images indicated some strain variation with respect to biofilm development. Values for biovolumes also supported the trends observed in the plate count data. Total biovolume values for AIR (7.74 to 8.34 $\log_{10}$ $\mu m^3/g$) were at most 15% of the

corresponding values for MA (8.82 to 9.22 $\log_{10}$ $\mu m^3/g$). Strains NCTC 11168 V1 and 16-2R showed a slight preference for ACO2 (9.00 and 9.43 $\log_{10}$ $\mu m^3/g$) over MA (8.82 and 9.22 $\log_{10}$ $\mu m^3/g$). The NCTC 11168 V26 strain had very similar values for MA and ACO2 (9.15 and 9.16 $\log_{10}$ $\mu m^3/g$).

Biofilm cells grown in ACO2 showed higher tolerance to the exogenous oxidant H_2O_2 (2.70 $\log_{10}$ colony forming units/g reduction) than those from the MA condition (up to 4.57 $\log_{10}$ CFU/g reduction) for *C. jejuni* 16-2R. Growth condition appeared to have no effect on subsequent tolerance to oxidative stress for the other 2 strains (NCTC 11168 V1 and NCTC 11168 V26). However, the original clinical isolate, NCTC 11168 V1, exhibited more than twice the tolerance to H_2O_2 than the lab -passaged variant, NCTC 11168 V26.

C. jejuni formed biofilms equally well in both MA (10% CO_2 and 5%O_2) and ACO2 (5% CO_2 and 19% O_2), but only very poorly in AIR (0.05% CO_2 and 20% O_2). This suggests that it is not the concentration of oxygen, but rather the concentration of CO_2 which influences the development of biofilm, for the three strains investigated. Furthermore, tolerance to oxidative stress was subject to strain variation.

1 Literature Review

1.1 Introduction

Campylobacter jejuni is the leading cause of foodborne bacterial gastroenteritis and diarrhea in the developed world. Since it was recognized in the late 1970's as a significant pathogen, much research has been directed at the paradox of how this fastidious, delicate organism survives harsh environments. The connection to serious sequelae such as the neurological disorder Guillian Barré Syndrome and Reactive Arthritis, although rare, provide further justification for the development of methods to prevent cases of campylobacteriosis. Determining the mechanisms *C. jejuni* uses to survive in the environment may lead to the development of approaches for the elimination and/or control of this organism in food processing situations.

1.2 History

The first recorded isolation of *Campylobacter jejuni* was in 1886 by Theodor Escherich in the stools of infants who had died of what he called 'cholera infantum' (Skirrow and Butzler 2000). He was unable to culture these organisms and concluded that they were not the causative agent (Skirrow and Butzler 2000). The next historical encounter was in 1906. Organisms which resembled *Vibrio* ssp. were shown to be the causative agent for abortion in sheep (Sebald and Véron 1963). They were originally called *Vibrio fetus,* but with the advent of DNA typing and revised classification, the name was changed to *Campylobacter fetus* (Sebald and Véron 1963). In 1931, *Campylobacter* spp. were linked to enteric infections in cattle. Organisms similar to *Vibrio fetus* were isolated from scrapings of the jejunal mucosa of infected

animals and although they were originally referred to as *Vibrio jejuni*, they are now recognized to be *Campylobacter jejuni* (Jones et al 1931). In the late 1950's, Elizabeth King, investigating human isolates from blood, was the first to recognize that *C. jejuni* could be separated from *C. fetus* by preferential growth at 42°C (Skirrow and Butzler 2000). It wasn't until the 1970's that *Campylobacter* was linked to gastroenteritis in humans. This was due to the collaboration of Dr. Dekeyser from the National Institute for Veterinary Research and Dr. Butzler at St. Pierre University Hospital. Butzler had isolated a 'vibrio' from the blood of a young woman and using culture methods suggested by Dekeyser which included selective filtration, he was able to isolate and identify *Campylobacter* from these preserved feces (Skirrow and Butzler 2000).

1.3 Basic physiology and description of *Campylobacter jejuni*

Campylobacter jejuni is a Gram negative, non-sporeforming, curved or spiral rod. It ranges in length from 0.5 to 5 μm. It has been shown to form coccoid or spherical bodies in old cultures or under prolonged exposure to air which may indicate entry into a viable but non-culturable (VBNC) state (Harvey and Leach 1998). It has polar flagella at one or both ends and exhibits a corkscrew-like motility (Carrillo et al 2004, Fernando et al 2007). Growth temperature ranges from 30°C to 47°C, but optimum growth occurs at 42°C (Alter and Scherer 2006, Stintzi 2003) and the organism is easily destroyed by heating (Moore and Madden 2000). *C. jejuni* has a highly branched respiratory chain and is capable of both aerobic and anaerobic respiration, using a variety of alternative terminal electron acceptors. It is chemoorganotrophic and obtains energy from amino acids or tricarboxylic acid (TCA) cycle intermediates, but not from carbohydrates which are neither fermented nor oxidized by this bacterium (Nachamkin

2000). *C. jejuni* is considered to be an obligate microaerobe, requiring low levels of oxygen for growth (Kelly 2001, Kaakoush et al 2007).

1.4 Campylobacteriosis

Campylobacteriosis is a self-limiting gastroenteritis characterized by severe abdominal cramping and diarrhea, with or without blood. The average incubation period is 3.2 days and the initial symptoms include headache, fever and chills. Nausea is common, but vomiting is only seen in 15% of cases (Skirrow and Blaser 2000). The infective dose is relatively low, with only 500 to 1000 cells required to cause disease. A duration of 3 to 10 days accompanied by a weight loss of up to 5 kg is common. Relapses occur in 5 to 10% of untreated cases. Campylobacteriosis can be treated with antibiotics, but this is generally only done in severe cases, to prevent the development of antibiotic resistant strains. Sequelae associated with campylobacteriosis include Guillain Barré Syndrome (GBS) and Reactive Arthritis. Approximately 1 in 1000 cases of Campylobacteriosis leads to GBS (Skirrow and Blaser 2000). Approximately 1.7% of cases show some degree of Reactive Arthritis. Skirrow and Blaser (2000) have shown that individuals who possess the HLA B27 tissue antigen have a predisposition to suffer from Reactive Arthritis after a *C. jejuni* infection.

1.5 Epidemiology

C. jejuni is the leading bacterial cause of food-borne gastroenteritis and diarrhea in the developed world (Allos 2001, Ketley 1997, Tauxe 1992). Most infections occur as sporadic cases which are frequently linked to the consumption or handling of contaminated poultry

(Berndtson et al 1996, Friedman et al 2000, Hanninen and Hannula 2007, Olsen et al 2001, Pearson et al 1993, Zimmer et al 2003).

The second group includes larger outbreaks which were linked to the consumption of unpasteurized or contaminated milk, and contaminated or untreated surface water (Alary and Nadeau 1990, Friedman et al 2000, Petersen 2003, Schmid et al 1987). During the mid 1980's water and unpasteurized milk accounted for more than half of outbreak-related cases in the United States (Friedman et al 2000). More recently, foods other than milk and water were linked to 83% of outbreak-related cases in the United States (Friedman et al 2000). Transmission from pets and other animals is also recognized as a source of infection. Especially young animals or those suffering from diarrhea may carry and shed *Campylobacters* (Deming et al 1987, Friedman et al 2000).

1.6 Reservoirs

Campylobacteriosis is a zoonosis, a disease that is transmitted from animals to humans. Human to human transmission does not appear to occur (Friedman et al 2000). *Campylobacter jejuni* has been isolated from cattle, pigs, sheep, horses, rabbits, rodents, wild birds and domestic pets (Blaser et al 1979, Fitzgerald et al 2001, Neilsen et al 1997). The most significant reservoirs with respect to human infection are poultry and poultry processing environments.

C. jejuni is ideally suited to growth in the avian intestinal tract. The avian body temperature of 42°C and the low oxygen conditions in the intestinal tract enable *C. jejuni* to readily colonize avian intestines forming a commensal relationship with the host. It is found predominantly in the caecum and small intestine, but has been isolated from the spleen and other areas of the gut (Biswas et al 2006).

Altekruse et al (1994) reported that 60% to 80% of retail chickens were contaminated with counts of up to 10^6 CFU per carcass for fresh chicken and 10^4 CFU per carcass for frozen. Willis and Murray (1997) found that 69% of chickens in one US supermarket were contaminated with counts between 10^2 and 10^5 CFU per carcass. Another US study showed that there was seasonal variation, with 87% to 97% of retail poultry found to be contaminated during the summer months as opposed to only 7% to 32% during the winter (Willis et al 2000). A recent comprehensive review of contamination studies worldwide indicated that for all 73 studies included, *Campylobacter* prevalence in retail poultry was 58% worldwide, which was only slightly higher than the Canadian average of 57.7% which was based on 1477 samples from 3 studies (Suzuki et al 2009).

C. jejuni has been isolated from untreated surface water, such as lakes and streams (Taylor et al 1983, Terzieva and McFeters 1991). Although it will not grow in water, it can survive for extended periods (Buswell et al 1998). Contamination of surface water is most likely caused by runoff from the feces of animal carriers. *C. jejuni* is sensitive to chlorination and outbreaks associated with water are generally due to a breakdown in treatment or some form of post-treatment contamination. Some studies found that chlorination of poultry drinking water was associated with reduced infection rates in flocks (Kapperud et al 1993, Pearson et al 1993). It has also been shown that survival in water is extended by growth within a biofilm (Buswell et al 1998, Lehtola et al 2006, Joshua et al 2006).

1.7 Strain variation and genome plasticity

A bacterial species is defined as a group of strains sharing 70% DNA-DNA relatedness (Wayne et al 2000). Strain variation in *C. jejuni* is more complex than in many other bacteria due to the plasticity of its genome (Parkhill et al 2000). A study by Poly et al (2004) compared the strain ATCC 43431 with the sequenced strain NCTC 11168. DNA unique to the first strain was found to have a guanine and cytosine content of 26% as compared to the entire genome of the sequenced strain with a guanine and cytosine content of 30.6%. It was suggested that *C. jejuni* ATCC 43431 may have acquired new genes via horizontal gene transfer and that some of the unique genes show similarity to a possible pathogenicity island from *Helicobacter hepaticus* (Poly et al 2004). Dorrell et al (2001) found in a comparison of 11 *C. jejuni* strains that 21% of genes in the sequenced strain were absent or highly divergent in one or more of the isolates tested. Genes of known function that were conserved included those related to metabolic, biosynthetic, cellular and regulatory processes, but it was noted that many virulence determinants were also highly conserved (Dorrell et al 2001). Strain variable genes included those for iron acquisition, DNA restriction or modification and sialylation (Dorrell et al 2001). In a subsequent article, Dorell et al (2002) also reported that genes for the biosynthesis of surface structures, including flagella, lipo-oligosaccharide and capsule varied among strains. These structures are often associated with antigenic properties of pathogenic organisms. In a similar study, Pearson et al (2003) found that 16.3% of genes in 18 strains were divergent from the NCTC 11168 sequenced strain. Seven hypervariable plasticity regions with clusters of variable genes were identified. Genes in these regions included those related to the use of alternative electron acceptors for respiration and genes related to antigenic surface structures. This led the

authors to suggest that genetic diversity with respect to surface structures may play a role in the avoidance of both innate and adaptive immune response in the host and that variability in genes for the use of alternative electron acceptors for respiration may provide some strains with an advantage in restricted oxygen environments (Pearson et al 2003).

A study comparing the genetic diversity of clinical isolates with those from retail chicken carcasses in Scotland found that the degree of diversity among the strains was not significant in 2001, but by 2006 there were significant differences in the clonal complex and allele levels between clinical and retail-chicken isolates (Gormley et al 2008). Al-Mahmeed et al (2006) characterized 96 *C. jejuni* strains of clinical isolates in Bahrain showing that there was genetic diversity with respect to virulence in this population. AbuOun et al (2005) reported that 4 of 24 strains tested were lacking in cytolethal distending toxin (CDT) activity. Of these four strains, two were isolated from the blood of independent campylobacteriosis patients, one was from the stools of one of these patients and the last one was from a chicken carcass (AbuOun et al 2005). Cytolethal distending toxin has been shown to induce cell cycle arrest and interleukin -8 secretion from intestinal epithelial cells by a process which correlated with adherence and/or bacterial invasion. Also, *C. jejuni* 81-176 was found to be capable of replication within human monocytic cell vacuoles and inducing apoptotic death via CDT. (Hickey et al 2000). Gaynor et al (2004) found that a laboratory adapted variant of *C. jejuni* NCTC 11168 and the original clinical isolate differ phenotypically, but molecular genotyping procedures indicated no observable genetic differences which suggested that they may be clonal (Gaynor et al 2004). Carrillo et al (2004) compared genomic DNA of the NCTC 11168 V1 (original isolate) to that of the laboratory adapted NCTC11168 V26 and found that no gene deletions occurred between them, but a

comparison of the transcript profiles indicated yet unexplained differences in the expression of flagellar structural proteins (Carrillo et al 2004). A study by Lee et al (2005) showed that of 5 *C. jejuni* strains isolated from retail chickens, only one strain, *C. jejuni* IC21, grew well in aerobic conditions, leading the author to suggest that it was an aero-adaptive strain.

So, not only do *C. jejuni* strains show remarkable diversity in their actual genomes, but also clonal strains show significant phenotypic variation. This genome plasticity needs to be taken into account when comparing results from different studies. Conclusions for one strain may not hold true for other strains or even for lab-passaged variants of the strain itself.

1.8 Response to stress

1.8.1 Review of stress responses

Bacteria differ in their growth requirements and also vary in their ability to survive deviations from these requirements. Various environmental conditions such as pH, temperature, atmospheric conditions, moisture levels and nutrient availability influence growth and survival. Many bacteria have systems for dealing with stressful changes in environmental conditions. *Campylobacter jejuni* lacks many of the stress response systems found in other enteric bacteria.

For many bacteria, entry into stationary phase is typically accompanied by physiological changes that enhance resistance to heat shock, oxidative, osmotic and acid stresses (Kelly et al 2001). For a diverse range of bacterial species, which include most Enterobacteriaceae, *Pseudomonas* species and *Vibrio* species, these changes are mediated by the global stationary response factor, RpoS (Park 2002). This sigma factor has no homologue in the *C. jejuni* NCTC

11168 genome and it has been shown that *C. jejuni* is more sensitive to stresses during stationary phase than many other enteric bacteria (Martinez-Rodriguez 2005, Parkhill et al 2000, Kelly et al 2001).

Other stress response factors lacking in *C. jejuni* include the oxidative stress response factors, SoxRS and OxyR; the heat shock sigma factor, RpoH; the major cold shock protein, CspA and the global regulator of metabolism, Lrp (Park 2002). Campylobacters also have no known cold shock proteins and will not grow below 30°C, although they remain metabolically active and motile at temperatures as low as 4°C (Park 2002).

C. jejuni is sensitive to heat and is easily destroyed by pasteurization and standard cooking practices. It has been suggested that the RacRS (reduced ability to colonize) regulon plays a role in heat shock response in *C. jejuni* (Park 2002). The RacRS regulon is a two component regulatory system, required for the differential expression of proteins at 37 and 42°C. Heat shock proteins present in the *C. jejuni* genome include GroEL, DnaJ, DnaK, Lon protease, HrcA, GrpE, and HspR (Konkel et al 1998, Parkhill et al 2000).

Campylobacters are very sensitive to osmotic stress. This could be attributed to the absence of mechanisms for the synthesis and transport of compatible solutes which have been shown to play a role in osmotic stress resistance in other bacteria (Park 2002). *C. jejuni* does produce LuxS, the autoinducer 2 synthesis protein. This protein is involved in a quorum-sensing system similar to one in *Helicobacter pylori* where it is believed to play a role in limiting growth in certain environments (Elvers and Park 2002). *C. jejuni* stress defenses specific to oxidative stress are presented in section 1.9.3.

1.8.2 Viable but non-culturable (VBNC) state

Certain bacteria respond to stresses by entering a viable but non-culturable state (VBNC) where they remain infectious, but can no longer be cultured by conventional means. Studies regarding a VBNC state for *C. jejuni* have led to conflicting results. Cultures exposed to stress and older cultures of *C. jejuni* exhibit morphological changes from a spiral to a coccoid shape (Kelly et al 2001). This is similar to changes in other bacteria that relate to entry into the VBNC state (Kelly et al 2001). Chemical indicators of viability (Cappelier et al 1997, Tholozan et al 1999) and the measure of the longevity of certain macromolecules following loss of culturability (Lazaro et al 1999) have been used as indicators of the VBNC state for *C. jejuni*. Some studies suggest that the coccoid morphology represents a degenerate form of the cell which may retain metabolic activity, since the transition to the coccoid state is not prevented by the inhibition of protein synthesis or DNA replication. This suggests that this phenotype is not an active response to stress (Boucher 1994, Hazeleger 1995).

Animal models have been used to confirm that the cells are actually viable and capable of causing infection. The reversion of VBNC cells to the active type have had mixed results. Certain studies have been successful at detecting *C. jejuni* in the faecal matter (Lee, 2004, Saha 1991, Jones et al 1991) while others have not (Beumer 1992, Medema 1992). Research by Tholozan et al (1999) demonstrated that strain variation may account for the conflicting conclusions regarding the ability of *C. jejuni* to enter a VBNC state.

1.9 Oxidative stress

1.9.1 Reactive oxygen species (ROS)

Oxidative stress is the result of the cells' inability to sufficiently eliminate damaging reactive oxygen species (ROS). ROS occur as byproducts of aerobic metabolism and are also part of host cell defense. They include hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-) and the hydroxyl radical (·OH) (Guetens et al 2002). Reactive oxygen species cause DNA mutations, protein oxidation and lipid peroxidation, all of which can lead to cell death (Guetens et al 2002). Oxygen metabolism involves the generation of ATP via oxidative phophorylation which usually ends with the final electron in the electron transport chain reducing oxygen to produce water. Occasionally, the oxygen is reduced to form the superoxide radical. The superoxide radical requires an electron to make it stable and will remove electrons from DNA, proteins or other nearby molecules thereby causing damage to the cell (Guetens et al 2002).

In response to oxidative stress, many cells produce the enzyme superoxide dismutase (SOD) which catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen (Guetens et al 2002). Although this reduces the potential for cell damage by superoxide, the newly formed H_2O_2 is also capable of causing damage and can readily transform into a hydroxyl radical which is considered the most damaging of the ROS (Guetens et al 2002). The hydroxyl radical has a half-life of approximately 10^{-9} seconds in vivo and has a high reactivity (Yan et al 2005). The short half-life prevents the hydroxyl radical from being eliminated by an enzymatic reaction since diffusion to the enzyme's active site would be slower than the half-life of the molecule. Cells which produce catalase are able to convert H_2O_2 to water and oxygen, thereby reducing the potential for formation of the hydroxyl radical.

1.9.2 *Campylobacter jejuni* is an obligate microaerobe

There are conflicting conclusions about the sensitivity of *C. jejuni* to aerobic stress. Klançnik et al (2006) found aerobic stress to be detrimental, reducing survival and accelerating the conversion to a VBNC state. A comparison of survival of planktonic *C. jejuni* in three different gas mixtures (70/30% O_2/CO_2, 70/30% N_2/CO_2 and 100% N_2), indicated that survival was significantly reduced in the aerobic condition (Boysen et al 2007). However, other studies found that exposure to high levels of oxygen were beneficial, increasing the bacterium's ability to adhere to and invade host epithelial cells (Mihaljevic et al 2007, Pogačar et al 2009).

Numerous authors suggest that *C. jejuni* shows optimum growth in a microaerobic environment which is composed of 85% nitrogen, 10% carbon dioxide and 5% oxygen (Garenaux et al 2009, Hodge et al 1994, Joshua et al 2006, Juven and Rosenthal 1985, Seal et al 2007, St. Maurice et al 2007, Verhoeff-Bakkenes 2008). However, it has also been shown that certain strains will grow in anaerobic or CO_2 enhanced aerobic conditions (Kelly 2001).

C. jejuni has a respiratory metabolism and can use oxygen as well as alternative terminal electron acceptors. Mohammed et al (2005) found that the ability of *C. jejuni* to oxidize various substrates and thus grow anaerobically was strain dependant. Sellers et al (2002) reported that although *Campylobacter jejuni* encodes reductases which would allow the use of alternative electron acceptors to oxygen, it grew very poorly in anaerobic conditions. They concluded that anaerobiosis is a stress condition for *C. jejuni* (Sellers et al 2002). More recent findings by Kelly (2005) indicated that oxygen was required for DNA synthesis due to the use of an oxygen dependant ribonucleotide reductase. Mendz et al (2000) proposed that *C. jejuni* is an obligate

microaerobe requiring oxygen for growth, but at reduced tensions, and cannot grow or will grow only very poorly under fully oxic conditions (21% O_2).

Kaakoush et al (2007) examined the oxygen requirements and tolerance for 4 strains of *C. jejuni* and confirmed that *C. jejuni* is an obligate microaerobe requiring oxygen for growth. Their study looked at anoxic, microoxic and oxic atmospheres, all enriched with 10% CO_2. Cell density was found to be a significant factor. When the initial cell density was greater than 10^7 CFU/ml, the bacterium grew better aerobically (with 10% CO_2) than microaerobically. At cell densities between 10^5 and 10^6 CFU/ml, there was similar growth for both microaerobic and aerobic conditions. At cell densities below 10^5 CFU/ml, growth was better in the microaerobic than in the aerobic condition. No growth was observed in the aerobic condition for densities below 10^4 CFU/ml (Kaakoush et al 2007). Oxygen tolerance differed among strains which led the authors to suggest that strain variation may cause discrepancies in the results of different studies. In a recent article, Gareneaux et al (2008) showed that for 13 strains of planktonic *C. jejuni,* oxidative stress sensitivity was temperature dependent. Exposure to ambient levels of oxygen and the exogenous oxidant paraquat showed little reduction in survival at 4°C for three representative strains. Their results also indicated that sensitivity to oxygen varied among strains at both 25°C and 42°C.

Studies which reported growth at high levels of oxygen (up to 21%) often included CO_2 in the growth atmosphere (Fraser et al 1992, Hodge and Krieg 1994, Kaakoush et al 2007). Also, Mihowich et al (1998) showed that the addition of an endogenous CO_2 source ($KHCO_3$, $NaCO_3$ or $CaCO_3$) to the culture media allowed *C. jejuni* to grow in ambient atmospheric conditions.

It may be that the increased level of CO_2 plays a role in the cells' ability to alleviate oxidative stress, either by allowing for the use of alternative metabolic pathways which may not use oxygen and hence does not lead to the formation of ROS, or by aiding in the removal of the damaging ROS.

Kaakoush et al (2007) also speculated on the molecular mechanisms responsible for microaerophily. They suggest that a high susceptibility to ROS, a strong inhibition of certain respiratory enzymes by O_2 and/or substantial metabolic generation of ROS may all be contributing factors (Kaakoush et al 2007). This is supported by results from the study by Hodge and Krieg (1994) in which the addition of antioxidants to growth media enhanced growth of *C. jejuni* under aerobic conditions suggesting that it has a higher susceptibility to free radicals than aerotolerant bacteria. *C. jejuni* does express oxygen-sensitive proteins, such as L-serine dehydratase and rubredoxin oxidoreductase (Yamasaki et al 2004). A study by Verhoeff-Bakkenes et al (2008) showed that the inclusion of pyruvate in the media allowed *C. jejuni* to grow in a broad range of oxygen tensions. The cells in this study had reduced levels of catalase activity indicating that there was a reduction in the level of H_2O_2 in the cells (Verhoeff-Bakkenes et al 2008).

1.9.3 Oxidative stress defense mechanisms in *Campylobacter jejuni*

Although *Campylobacter jejuni* has limited defense mechanisms compared to many other bacteria, there are various *C. jejuni* enzymes which play a role in defense against oxidative stress. Superoxide dismutase (SodB) catalyses the dismutation of the superoxide free radical to H_2O_2 and oxygen (Purdy 1999). *C. jejuni* also produces the enzyme catalase (KatA) which degrades H_2O_2 to water and oxygen (Grant and Park 1995) and the iron-regulated alkyl

hydroperoxide reductase (AhpC) which can destroy toxic hydroperoxide intermediates and repair molecules which have been peroxidized (Baillon 1999).

In contrast to the limited range of protective enzymes described above, *E. coli* has been shown to possess three known superoxide dismutases: SodB, SodA and SodF as well as three catalases: KatA, KatE, KatG, and AhpC (Park 2002). Regulation of oxidative stress response in *E. coli* is mediated by SoxRS and OxyR which have no known homologues in *C. jejuni* (Parkhill et al 2000). It has been suggested that the hydrogen peroxide-sensing regulator, PerR, may play a role in regulating the oxidative stress response in *C. jejuni*, as it has been shown to regulate the expression of both KatA and AhpC (van Vliet 1999).

Another important regulator expressed by *C. jejuni* is the ferric uptake regulator (Fur). Iron homeostasis is critical, as iron is required for growth, but can also lead to increased levels of the highly reactive hydroxyl radical (·OH) via the Fenton reaction (Holmes et al 2005, Palyada et al 2004). It has been suggested that the constitutively expressed *dps* gene of *C. jejuni* which codes for a protein that binds iron could play a role in oxidative stress defense by preventing the formation of this radical (Ishikawa 2003).

The *spo*T gene in *H. pylori* plays a role in response to aerobic shock and acid exposure (Mouery et al 2006). This gene has been shown to regulate a stringent response in *C. jejuni* aiding in survival of oxidative stress (Gaynor et al 2005).

A study by Fields and Thompson (2008) found that a *C. jejuni csr*A (carbon starvation regulator) mutant exhibited changes in several virulence-related properties including, motility, adherence, invasion as well as oxidative stress resistance. Thus, the carbon response regulator also plays a role in oxidative stress defense.

Although not technically a defense mechanism, it is important to recognize that there is strain variation with respect to the ability of *C. jejuni* to survive or grow aerobically. Numerous authors have reported significantly different responses to aerobic or oxidative stress by various strains (Garenaux et al 2008, Lee et al 2005, Yaakoush et al 2007). The study by Lee et al (2005) mentioned earlier, found significant variation among the 5 *C. jejuni* chicken isolates tested with respect to their response to aerobic stress. Of the 5 strains, only *C. jejuni* IC21 grew well in milk under aerobic conditions. This strain also showed higher levels of oxidoreductase activities upon aerobic exposure with 11.8 times higher KatA activity, 4.4 times higher SodB and 2.0 times higher NADH-oxidase activities (Lee et al 2005).

1.9.4 Methods to assess oxidative stress

Various approaches have been taken to investigate the oxidative stress mechanisms of *C. jejuni*. Numerous authors employed mutant strains to determine the roles of particular genes with respect to growth and/or survival under different oxygen tensions (Kalmokoff et al 2006, Joshua et al 2006). Other studies simply monitored growth and survival under different conditions (Boysen et al 2007). Wainwright et al (2005) monitored oxygen consumption using an oxygen electrode and Lee et al (2005) analyzed the oxygen tolerances of various strains of *C. jejuni* using cell surface hydrophobicity, cell fatty acid composition and oxidoreductase activities. A study with the polychlorinated-biphenyl-degrading bacteria, *Pseudomonas* sp. strain B4, used enzymatic methods to directly measure levels of ROS in live cells (Chávez et al 2004). Cells grown under different conditions were incubated with the oxidative stress sensitive probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This probe is readily taken up by live cells. The acyl groups of the probe are removed by membrane esterases during

internalization, leaving the compound 2',7'-dichlorodihydrofluorescein (DCFH) which is sensitive to ROS and is oxidized to the highly fluorescent compound, 2', 7'-dichlorofluorescein. Although, DCFH can be oxidized by several reactive oxygen species, including RO_2, RO·, OH·, HOCl·, and $ONOO^-$, only the longer lived ROS actually contribute to the increase in fluorescence (Chávez et al 2004).

1.10 Biofilm formation

1.10.1 Biofilms

Biofilm formation occurs as bacteria attach to a surface and form a matrix of extracellular polymeric substances (EPS) also referred to as extracellular polysaccharides (Park 2005) or exopolysaccharides (Sutherland 2001, McLennan et al 2008). Extracellular polymeric substances are important components of the biofilm matrix. Their structures and synthesis mechanisms can differ significantly between bacteria (Donlan 2000) and even between strains of the same bacteria (Branda et al 2005). Biofilms are a natural mode of growth for many bacteria and have been recognized as a critical lifestyle stage for many bacteria. They are ubiquitous in nature and can be found in rivers and streams, in water distribution systems and in food processing facilities. Biofilm formation can be divided into 5 stages (Sampathkumar et al 2006). There is initial attachment which is reversible. This is followed by irreversible attachment. A form of quorum sensing occurs and once a threshold number of bacteria is present they begin to secrete EPS. In the fourth stage, there is sufficient growth and a mature biofilm has formed. The fifth stage is characterized by a second form of quorum sensing. The community has grown beyond a sustainable size and detachment occurs. Bacteria are released from the biofilm in a planktonic state and may now attach to other surfaces allowing the

process to begin again. Early studies of extracellular polysaccharides recognized that these polymers significantly impact bacterial virulence (Branda et al 2005) and biofilms have been recognized as important for survival in many environments, including those associated with pathogenicity (Carrillo et al 2004, McLennan et al 2008). This can be a concern in areas where biofilms may protect pathogens from sanitation.

1.10.2 *Campylobacter jejuni* biofilms

Studies conducted to determine the various factors involved in the formation of biofilms by *C. jejuni* have generally examined gene expression, protein expression profiles and effects of gene mutations (Joshua et al 2006, Kalmokoff et al 2006, McLennan et al 2008, Seal et al 2007, Sampathkumar et al 2006, Trachoo et al 2002). It has been proposed that *C. jejuni* biofilms are important for *in vivo* colonization and transmission as well as *ex vivo* survival (McLennon et al 2008).

Joshua et al (2006) showed that *C. jejuni* can exist in three forms of monospecies biofilm: surface attached, pellicle and flocs. Comparison of SEM images indicated a visual similarity among the three forms. Of the 9 strains and 8 mutants tested, 3 wild type strains did not form flocs and there was reduced floc formation in mutants for putative flagellar protein (*FliS*) and phosphate acetyltransferase (Cj0688). Floc formation was not affected in mutants for capsular polysaccharide (*kpsM*), flagella (*maf5*), protein glycosylation (*pglH*), and lipo-oligosaccharide (*neuB1*). Only the aflagellate mutant *maf5* showed no ability to attach to glass or form a pellicle (Joshua et al 2006).

Carrillo et al (2007) also investigated differences in gene expression for the different forms of immobilized growth of *C. jejuni*. Genes encoding proteins involved in iron uptake were

upregulated in pellicles and on plates, while higher levels of transcripts for genes encoding proteins involved in the respiratory chain and in flagellar biosynthesis were observed in biofilms (Carrillo 2007). Their study also confirmed earlier results which showed that genes which encode proteins involved in the motility complex and in the general stress response have higher transcript levels in biofilm-grown cells than in planktonic cells.

Kalmokoff et al (2006) demonstated that *C. jejuni* can form biofilms on a variety of surfaces. This study also found differences in protein expression profiles between planktonic and biofilm associated cells. Proteins that were found at higher levels in biofilms included those involved in the motility complex, including flagellins (FlaA, FlaB), filament cap (FliD) and basal body (FlgG, FlgG2); chemotactic protein (CheA), general stress response proteins (GroEL, GroES), oxidative stress response proteins (Tpx, AhpC), adhesins (Peb1, FlaC), and proteins involved in biosynthesis, energy generation and catabolism. It was also found that an *flhA* mutant lost the ability to form a biofilm or pellicle and also that mutants for any of the motility complex proteins were less able to form a pellicle. This indicates that the flagellar motility complex plays a crucial role in initial attachment as well as in the cell to cell interactions required for pellicle formation (Kalmokoff 2006). These authors also point out that the continued expression of the motility complex in mature biofilms is unusual and suggest that the flagellar apparatus may play a role in the biofilm phenotype (Kalmokoff 2006).

Sampathkumar et al (2006) examined the transcriptional and translational expression profiles to look at cellular mechanisms that facilitate a surface associated lifestyle. Results indicated that the immobilized bacteria shift away from metabolic, motility, and protein synthesis capabilities toward iron uptake, oxidative stress defense and membrane transport.

The authors suggest that this expression profile has similarities with the one for colonization as well as for biofilm formation in other species of bacteria.

Asakura et al (2007) in their investigation of two cell-binding proteins, Peb1A (CBF1) and Peb4 (CBF2) which are involved in adherence to host cells, found that Peb1A and Peb4 mutants had a reduced ability to form biofilm in times of stress. Further analysis indicated that proteins involved in adhesion, transport and motility which are required for biofilm formation were expressed at reduced levels in the mutant as compared to the wild type.

Svensson (2007) generated a mutant for a two-component regulatory system (cj1226c/cj1227c) which showed a 30% increase in autoagglutination in broth as well as enhanced biofilm formation. The mutant showed a 5-fold increase in long-term intracellular survival as well as higher levels of chick colonization. It was also shown that the two-component system was upregulated in the presence of host cells (Svensson 2007).

Sanders et al (2007) showed that *C. jejuni* biofilm formation on stainless steel increased by 50% from 24 to 48 hours, remained level to 96 hours and decreased by 88% after 168 hours. In mixed bacterial biofilms *C. jejuni* showed greater growth at 24 hours, but was approximately the same at 48 hours (Sanders 2007).

McLennon et al (2008) point out that biofilms and surface polysaccharides participate in stress survival, transmission, and virulence of *C. jejuni* and explain that the study of novel genes involved in these processes could lead to a better understanding of pathogenesis. Their work with a *C. jejuni* ΔSpoT mutant, revealed that it was the first stringent response mutant to be shown to up-regulate biofilm production (McLennon et al 2008).

In summary, *C. jejuni* is capable of forming biofilms and can also exist as part of a mixed culture biofilm. *C. jejuni* biofilms can attach to a variety of different surfaces and can also exist as flocs or pellicles. Gene expression in *C. jejuni* biofilm cells differs from that in planktonic cells with there being an up-regulation of genes related to iron-uptake, membrane transport, motility, the respiratory chain, flagellar biosynthesis, general stress response and more specifically oxidative stress response.

1.10.3 Biofilms provide protection from external stresses

Biofilms have been shown to provide protection from various environmental stresses. The biofilm matrix consists of approximately 97% water providing protection from desiccation. The concentration of oxygen has been shown to vary within a biofilm providing a range of oxygen concentrations between the channels and the interior of the microcolonies (Kim et al 2006). Numerous authors have suggested that biofilms may provide *C. jejuni* with protection from environmental stresses (Joshua et al 2006, Kalmokoff et al 2006, Seal et al 2007, Sampathkumar et al 2006, Trachoo and Frank 2005). In one study, biofilms were shown to confer protection against environmental stresses, with biofilm cells surviving 24 hours at ambient temperature and atmosphere as opposed to only 12 hours for planktonic cells (Joshua 2006).

Studies of *C. jejuni* survival in water showed that cells survive longer in biofilms, especially in mixed culture biofilms (Buswell et al 1998, Lehtola 2006). It was also found that standard culture methods may seriously underestimate *C. jejuni* counts in water and in biofilms (Buswell et al 1998, Lehtola 2006). *C. jejuni* in water was detectable by standard culture methods for only 1 day after a spiking, whereas bacteria from biofilms grew on plates for at

least 1 week after spiking and could be detected from outlet water of the reactor for 3 weeks when using fluoresecent in situ hybridization(FISH) (Lehtola 2006). Buswell et al (1998) reported that persistence within biofilms was significantly longer when determined by detection methods not involving culturing (Buswell et al 1998).

Trachoo et al (2002) suggested that biofilms may be a source of *C. jejuni* in poultry house water systems. They investigated this by growing three *Pseudomonas* isolates (P1 - isolate from a meat processing plant; Y1 and W1 – isolates swabbed from nipple drinkers in a commercial chicken house) for 2 days on PVC (polyvinyl chloride) and incubating the resulting biofilms with *C. jejuni*. Biofilm formation of *C. jejuni* was enhanced in these mixed culture biofilms, compared to single culture *C. jejuni* biofilms. Viable *C. jejuni* decreased with time and the greatest reduction occurred on surfaces without a preexisting biofilm. The number of viable *C. jejuni* determined by direct viable count (DVC) was greater than by cultural methods indicating that *C. jejuni* may enter a VBNC state within the biofilm (Trachoo et al 2002). In a subsequent study, Trachoo and Frank (2005) reported that the W1 biofilms, which had reduced oxygen tension showed greater enhancement of *C. jejuni* survival. Also, survival was better in the mixed biofilms, grown with W1 and Y1, which were thicker and had a more complex morphology than biofilms grown with P1, a *Pseudomonas* isolated from a meat plant (Trachoo and Frank 2005).

Reeser et al (2007) also investigated biofilm formation by *C. jejuni*. They found that high ambient temperatures, aerobic conditions and nutrient-rich conditions inhibited biofilm formation and suggested that conditions within watering system were conducive to biofilm formation (Reeser et al 2007). The same study also found that both quorum sensing and flagella

were required for maximal biofilm formation and that mutants for *flaAB* and *luxS* had reduced ability to form biofilms (Reeser et al 2007).

1.10.4 Methods used to study and measure biofilm formation

As more studies have shown that biofilms provide *C. jejuni* with protection from external stresses, the mechanisms involved in this phenotype have begun to be studied more extensively. Studies have included comparing growth on various surfaces (Kalmokoff et al 2006, Reeser et al 2007); enhanced survival over planktonic growth (Joshua et al 2006) and gene expression profiling (Kalmokoff et al 2006, Carrillo et al 2007). Studies of bacterial biofilm formation often use mutant phenotypes to determine the mechanisms and genes involved in biofilm formation.

An examination of the methods used to study *C. jejuni* biofilms indicates that often different techniques are employed, making comparisons difficult and possibly leading to discrepancies in the results from different studies. Although there may be advantages to these different approaches, a common methodology would allow authors to compare results more easily.

Joshua et al (2006) inoculated Mueller Hinton broth with a 10 µl loop of *C. jejuni* taken from a Columbia blood agar plate grown for 2 days under microaerobic conditions and incubated the broth at 37°C with 50 rpm shaking under microaerobic conditions. Aggregate and planktonic bacteria were grown for 3 days in vented 50 ml tissue flasks. For growth of pellicles and attached biofilms, 10ml of Brucella broth was inoculated with cultures from Columbia blood plates to an OD_{600} of 0.1 to 0.2 in glass test tubes incubated without shaking under microaerobic conditions at 37°C for five days.

Kalmokoff et al (2006) grew biofilms on sterile nitrocellulose membranes, food grade stainless steel coupons and glass fibre filters. Test surfaces were placed upright in 7 ml of Mueller Hinton (MH) broth contained within 12-well polystyrene tissue culture plates with each well inoculated with 10 µl from an overnight liquid culture. The plates were incubated at 37°C with reduced oxygen, without shaking. Test surfaces were aseptically transferred into fresh media on three consecutive days, after approximately 24 hours of growth at 37°C. Following completion of growth, test surfaces were rinsed by repeated immersion of the test surface in sterile phosphate buffered saline (PBS). Planktonic cells were collected from the wells used for incubation of the test surfaces from the final transfer (24 h).

Reeser et al (2007) inoculated the wells of a 24-well polystyrene plate containing 1ml Mueller- Hinton broth (MHB) with overnight cultures of *C. jejuni* isolates to an OD_{600} of 0.025 (~2.5×10^7 CFU). Plates were incubated at 37°C or 25°C in a 10% CO_2 atmosphere or aerobically for 24, 48 or 72 hours. Following incubation the medium was removed and the wells were dried for 30 minutes at 55°C and 1ml of 0.1% crystal violet (CV) was added for 5 minutes at room temperature. The unbound CV was removed and the wells were washed with H_2O. The wells were dried for 15 minutes at 55°C and bound CV was decolorized with 80% ethanol /20% acetone solution. One hundred µl of this solution was removed from the wells and placed in a 96-well plate and the absorbance at 570 nm was determined using a microplate reader to determine biofilm formation. Biofilm formation on abiotic surfaces was achieved by placing test surfaces (acrylonitrile butadiene styrene plastic, polyvinyl chloride plastic, polystyrene or copper) in 15 ml polypropylene tubes with 5 ml Mueller-Hinton broth such that the test surfaces were completely submerged. The tubes were inoculated with *C. jejuni* to an OD_{600} of

0.025 and incubated for 24 h at 37°C and 10% CO_2. The coupons were aseptically removed and placed in sterile 15-ml tubes with 2 ml of 0.1 M phosphate-buffered saline, pH 7.3, and 20- by 4-mm sterile glass beads. The bacteria were detached by vortex mixing on full speed for 1 min, which did not affect cell viability. Viable bacteria were enumerated by dilution plating on Mueller-Hinton agar supplemented with 5% blood.

Plate counting may underestimate the actual number of bacteria that grew in the biofilm. In order to investigate the potential of VBNC cells to affect the plate count values, a stain such as the LIVE/DEAD® *Bac*Light™ stain can be used in conjunction with confocal scanning laser microscopy and biovolume analysis.

The LIVE/DEAD *Bac*Light Bacterial Viability Kit employs two nucleic acid stains: the green-fluorescent SYTO® 9 stain and the red-fluorescent propidium iodide stain. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 stain labels both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing or quenching SYTO 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria and those with damaged membranes fluoresce red.

1.11 Colonization

1.11.1 Colonization sources of *C. jejuni*

Both wild and domestic animals which carry *C. jejuni* could act as sources for colonization of poultry. Once a source is available, transmission within infected flocks is rapid and the proportion of birds colonized is often close to 100% (Pearson et al 1993, Corry and Atabay 2001). Vertical transmission has been investigated and results suggest that transmission

of *C. jejuni* via eggs is rare and unlikely to play a significant role in the colonization of a flock (Snelling et al 2005). Horizontal transmission is a more significant source of colonization, as broilers readily pick up *C. jejuni* from the environment (Snelling et al 2005). Organic and free-range flocks have a higher rate of colonization than intensively reared flocks (Newell and Fearnly 2003). The presence of other farm animals, e.g. cattle and sheep, increases the risk of infection (Cardinale et al 2004). Other environmental sources that may contribute to flock colonization include lack of hygiene barriers, uncemented poultry house floors and contaminated air, dust, litter and insects (Snelling et al 2005). Also, transportation of birds prior to processing significantly increases colonization and carcass contamination (Stern et al 1995).

Colonization implies ingestion of a contaminated source. Studies have shown that the moisture content of feeds and litter is too low to support survival of *C. jejuni* which is very sensitive to desiccation (Pearson et al 1993). Drinking water has on various occasions been implicated as a risk and potential source of infection (Kapperud 1993, Pearson et al 1993). A study by Stern et al (2002) showed that chlorination of poultry drinking water was ineffective at preventing colonization of broiler flocks, while a similar study by Kapperud et al (1993) showed that chlorination was effective at reducing the risks of colonization. It was suggested that *C. jejuni* which is known to be sensitive to chlorine may be protected within water-borne protozoa (Snelling et al 2005). Other studies have suggested that *C. jejuni* may find protection from such environmental stresses in poultry house water systems by existing in biofilms (Trachoo et al 2002, Trachoo and Frank 2005).

1.11.2 Colonization of poultry

Natural colonization of chicks usually occurs around 3 weeks of age (Snelling et al 2005, Evans 1992, Fields and Swerdlow 1999, Boyd 2005). Newly hatched chicks can become colonized by a single oral dose, cloacal inoculation or by repeated low level inoculation through drinking water (Evans 1992, Alterkruse 1998). Experimental challenge of 1 day old chicks with as few as 30 colony forming units (CFU) of *C. jejuni* achieved caecal colonization with levels up to 10^{10} CFU/g of caecal content within 3 days (Newell 2001). Intestinal contents of colonized birds are often in the range of 10^5 to 10^9 CFU/g and fecal samples may contain up to 10^7 CFU/g (Newell 2001, Pearson et al 1993).

Colonized birds usually show the highest level of *C. jejuni* in the large intestine, caecum and cloaca (Snelling et al 2005, Park 2002). *C. jejuni* is found in the intestinal mucous layer in the crypts of the intestinal epithelium (Beery et al 1988). *C. jejuni* is well adapted to this environment. They possess chemotactic mechanisms that attract them towards the fucose in the mucin of the intestinal mucous and fully motile, but non-chemotactic mutants do not colonize animal models (Hugdahl et al 1988, Takata et al 1992). Also, the corkscrew-type motility allows *C. jejuni* to remain motile in the highly viscous environment which rapidly paralyzes other motile rod-shaped bacteria (Ferrero and Lee 1988, Shigematsu et al 1998). Another factor that enables *C. jejuni* to compete successfully in this niche is their ability to acquire iron from both the host and other gastrointestinal bacteria (Park et al 2002). These features added to the fact that the avian body temperature of 42°C is the ideal growth temperature for *C. jejuni* and the low oxygen conditions in the intestinal tract are well suited to

the microaerophilic nature of this organism contribute to the explanation of the high rate of colonization in poultry.

In order to reach the intestinal tract *C. jejuni* must pass through the stomach and survive the harsh conditions there. Murphy et al (2003) found that an Adaptive Tolerance Response (ATR) could be induced in *C. jejuni* in these conditions, but that the response varied between strains and with the type of media used. Studies have shown that *C. jejuni* also has mechanisms to deal with bile. Raphael et al (2005) performed mutation studies and determined that the gene Cj0643, which they designated *cbrR* (*Campylobacter b*ile *r*esistance *r*egulator) was important to bile resistance and that mutants for this gene had reduced colonization ability. Fox et al (2007) found that exposure of *C. jejuni* to bile induced the expression of a variety of genes including some related to virulence.

1.11.3 Colonization gene expression profiles

Numerous studies indicate the importance of motility to *C. jejuni's* ability to colonize animal models. Carrillo et al (2004) found that in a comparison of 2 strains that reduced ability to colonize chicks was related to the flagellar regulatory system. Gaynor et al (2004) compared *C. jejuni* NCTC 11168-O, the original clinical isolate with the genome sequenced clonal isolate, *C. jejuni* NCTC 11168-GS and found that the genome sequenced strain had undergone vertical evolution and was less able to colonize chicks than the original clinical isolate. Microarray transcriptional profiles of the two variants revealed significant differences in genes and operons relating to respiration and metabolism as well as those relating to flagella and motility. Targeted sequencing of sigma factors revealed specific DNA differences which may account for

the phenotypic differences in these clonal isolates. It was suggested that *C. jejuni's* ability to adapt to various environmental conditions may involve genetic evolution (Gaynor et al 2004).

Guerry, (2007), in her review of *Campylobacter* flagella, points out that flagellar glycans mediate autoagglutination. Mutants for a flagellar glycan were shown to adhere to INT407 (eukaryotic intestinal epithelial) cells as single cells as opposed to the wild type which were found to adhere as groups indicating that the number of bacteria associated with the eukaryotic cells increased based on the ability of the cells to autoagglutinate (Guerry 2007). This also has implications for biofilm formation which begins with the formation of microcolonies similar to those found in the study and has also been shown to be diminished in flagellar mutants (Kalmokoff 2006).

Woodall et al (2005), like Carrillo et al (2004), reported that *C. jejuni* regulates electron transport and metabolic pathways to adapt to the conditions in the avian intestinal tract (Woodall et al 2005). Biswas et al (2007) found that the *Campylobacter* invasion antigen (Cia) proteins secreted by certain strains of *C. jejuni* may influence colonization of chicks. The genome sequenced strain, NCTC 11168 V26 which was characterized as a poor colonizer of chicks compared to the other strains tested (NCTC 11168 V1, 81–176, *fla*A– mutant of NCTC11168 V1) did not secrete Cia proteins. It was suggested that presence of a minimum flagellar apparatus sufficient for secretion of these proteins may be required for colonization (Biswas et al 2007).

In another study, mutational analysis of *fliA, rpoN* and *flgK* was done to determine the role of the two sigma factors and the flagellar hook filament junction protein in regulating colonization (Fernando et al 2007). The results indicated that *fliA* mutants were able to secrete

Cia proteins, but had reduced motility compared to the wild type and were unable to colonize chicks. The *rpoN* mutants showed less reduced motility but did not secrete Cia proteins and were also completely attenuated for colonization. The *flgK* mutants showed a colonization capacity 100,000-fold lower than the wild type (Fernando et al 2007).

Biswas et al (2006) also investigated the role of cytolethal distending toxin (CDT) in colonization of the chicken intestine. Mutants for CDT were able to colonize birds either directly or by horizontal transfer and were also able to adhere to HD-11 (chicken macrophage) cells as well as the wild type, indicating that CDT does not play a role in colonization.

1.11.4 Methods used to assess colonization ability of *C. jejuni* strains

1.11.4.1 Cell line assays

Biswas et al (2006) used HD-11 cells to determine the effects of *C. jejuni* CDT mutants on adherence and invasion. Bacterial suspensions (100 µl with approximately 10^7 cells) in minimal essential medium (MEM) with 1% fetal bovine serum was inoculated into duplicate wells of a 24-well tissue culture plate containing a semi-confluent monolayer of HD-11 cells (10^5 cells/well). The culture plate was incubated for 3 hours at 37°C in a 5% CO_2-humidified atmosphere to allow bacteria to adhere to the cells. To measure adhesion, the infected monolayers were washed 3 times with MEM without FBS, lysed with a solution of 1% Triton X-100, and the number of viable bacteria was determined by counting resulting colonies on Meuller Hinton agar plates.

Asakura et al (2007) measured adherence of wild-type and *peb*4 mutant *C. jejuni* to INT407 cells. Bacteria were incubated with the cell monolayer for 2 h at 37°C in 5% CO_2/95%

air. The cells were washed with Hank's balanced salt and removed from the dish with 0.01% Triton X-100. The total bacteria were enumerated by plate count on Mueller-Hinton agar.

1.11.4.2 Chick assays

Carrillo et al (2004), Biswas et al (2006), and Fernando et al (2007) performed colonization studies using 1-day-old chicks which were randomly assigned to groups of 20 to 25 birds provided with feed and water *ad libitum.* Five birds in each group were tested for *C. jejuni* prior to the challenge. All birds in the group were orally challenged with indicated doses of *C. jejuni* in 0.5 ml of normal saline. Viable cell counts were obtained by plating serial dilutions on Mueller-Hinton agar. Colonization of birds was determined by culturing cloacal swabs on Karmali agar. Birds were euthanized by cervical dislocation and ceca were aseptically collected for qualitative and quantitative assessment. To assess the ability of *C. jejuni* to colonize unchallenged birds that were placed in contact with orally challenged birds, 25% of birds were orally challenged and comingled with unchallenged birds.

Gaynor et al (2004) obtained eggs from specific pathogen-free chickens. These were hatched in isolators. Groups of 10 chicks were kept in separate isolators and provided with unlimited food and water. One day old chicks were orally dosed by gavage with 10^2 to 10^9 CFU of *C. jejuni* in 0.1 ml of phosphate buffered saline. Birds were killed after 5 days and bacterial colonization levels were determined by plating serial dilutions of caecal contents.

Asakura et al (2007) measured the colonization of mice with the wild-type and a *peb*4 mutant of *C.jejuni* NCTC 11168. Thirty female BALB/c mice aged 6 weeks (obtained from Japan SLC) were used. Thirty minutes before the infection, the animals were given 0.5mL sodium bicarbonate solution orally, to neutralize gastric acidity, and then inoculated with a 0.5mL oral

dose of Mueller-Hinton broth (MHB) containing c. 2.0 x 10^7 CFU of *C. jejuni* cells grown in MHB at 37°C. Control mice received 0.5mL of MHB alone. Fecal excretion of *C. jejuni* was monitored over time by culturing a fecal homogenate [10% in phosphate-buffered saline] on modified CCDA agar supplemented with cefoperazone, vancomycin, and amphotericin B (Merck). The selected *Campylobacter* colonies were confirmed by examining their morphology and by standard oxidase test.

1.12 Relationship between colonization and biofilm formation

Recent studies have revealed that there may be a link between gene expression during colonization and that during biofilm formation. Seal et al (2007) in a comparative proteomic analysis of a robust colonizing strain (A74C) with a poor colonizer (NCTC 11168-PMSRU) revealed that the protein profile for the robust colonizing strain was similar to biofilm gene expression patterns recently identified by Kolmokoff et al (2006) and Sampathkumar et al (2006). Colonization factors included *sodB, racR, pglH* and *cadF* as well as flagellin genes and those related to iron uptake and metabolism (Seal et al 2007). Biofilm related proteins include those involved with motility (FlaA and FlaB), oxidative stress (AhpC, HtrA) and certain adhesins (Peb1 and FlaC). The authors suggest that robust colonizing strains of *C. jejuni* may have genetic expression patterns that are similar to isolates in biofilms.

Asakura et al (2007) investigated C. *jejuni* mutants for the cell-binding protein Peb4 with respect to cell adherence and the ability to colonize mouse intestine. They found that adherence of the mutant strain to INT407 cells was 1-2% that of the wild type and that mouse challenge experiments showed a reduced level and duration of colonization. Also, fewer of the

mutant cells responded to stress by forming a biofilm. Further analysis revealed that the mutants had reduced expression of a variety of proteins required for biofilm formation.

1.13 Objectives of this study

Campylobacter jejuni is recognized as a sensitive and fastidious pathogen, but remains the leading cause of foodborne gastroenteritis in the developed world. *C. jejuni* is recognized as an obligate microaerobe, but growth in high levels of oxygen occurs and remains unexplained. *C. jejuni* has fewer stress defense mechanisms than other enteric pathogens and it has been suggested that survival in stressful situations may be aided by growth within biofilms. Studies have shown that *C. jejuni* is capable of forming biofilms and that biofilms provide protection from both environmental stresses and sanitation and disinfection procedures. However, very few studies have been done to determine the conditions conducive to biofilm formation for this pathogen.

The objectives of this study are to determine if the level of carbon dioxide in the growth atmosphere plays a role in the ability of *C. jejuni* to develop biofilms in conditions of aerobic stress and, if biofilms cells grown with higher levels of oxygen show increased tolerance to exogenous oxidative stress.

Three strains of *C. jejuni* will be assessed for their ability to form biofilms under different atmospheric conditions. The strains include *C. jejuni* NCTC 11168 V1, which has been shown to be an effective colonizer, *C. jejuni* NCTC 11168 V26 which has been shown to be a poor colonizer and *C. jejuni* 16-2R, a poultry isolate.

Assessment of biofilm development will be based on plate counting, biovolume analysis and biofilm structural differences, which will be explored using the SEM. In order to determine

if the cells grown with higher levels of oxygen show increased tolerance to further oxidative stress, biofilm cells grown with different amounts of oxygen will be exposed to the exogenous oxidant, hydrogen peroxide and tolerance will be measured by plate counts and by direct measure of reactive oxygen species (ROS) in the cells.

2 Biofilm development of three strains of *Campylobacter jejuni* in three conditions on glass fibre

2.1 Introduction

Campylobacter jejuni is presently recognized as the leading cause of bacterial gastroenteritis in Canada and the developed world (Kalmokoff et al 2006). *C. jejuni* is a zoonotic pathogen and resides asymptomatically in the gastrointestinal tract of many domestic animals and birds. The avian gastrointestinal tract provides ideal growth conditions for this organism and human infections are most commonly caused by the consumption or handling of contaminated poultry (Kalmokoff 2006, Joshua et al 2006, Pearson et al 1996). A recent review of contamination studies revealed that on average 57.7% of retail poultry meats in Canada were contaminated (Suzuki and Yamamoto 2009)

C. jejuni is an obligate microaerobe and requires low levels of oxygen for growth (Kelly 2005). This requirement for oxygen has been linked to the use of an oxygen-dependent ribonucleotide reductase for DNA synthesis (Kelly 2005). Microaerobes are recognized as a metabolically diverse group specialized for growth in O_2-limited environments (Kaakoush et al 2007, Ludwig 2004). Microaerobic conditions are typically described as 5% O_2, 10% CO_2 and 85% N_2. However, various authors report different optimal atmospheric conditions for growth of *C. jejuni*. Svensson et al (2008) claim that the optimal growth environment includes 6% O_2 and 12% CO_2. Kaakoush et al (2007) suggest partial oxygen tension of 2-10% for best growth and incorporate 10% CO_2 in each assay. Verhoeff-Bakkenes et al (2008) state that oxygen concentrations of 3-15% and CO_2 concentrations of 3-5% are sufficient for satisfactory growth.

It is often suggested that microaerophiles are unable to grow or grow only poorly in atmospheres with elevated levels of oxygen (Alter 2006, Gaynor et al 2005, Park 2002, Skirrow 1991). However, numerous studies report *in vitro* growth of *C. jejuni*, in atmospheres with 15 to 21% oxygen (Bolton and Coates 1983, Hodge and Krieg 1994, Hoffman et al 1979, Juven and Rosenthal 1985, Kaakoush et al 2007, Mihaljevic et al 2007, Pogačar et al 2009). It has also been noted that oxygen tolerance varies by strain (Garenaux et al 2008, Kaakoush et al 2007).

The molecular mechanisms responsible for microaerophily are not fully understood. Attempts to elucidate the role oxygen plays in micoaerophily have led to categorizing oxygen-related enzymes as: a) protective against harmful reactive oxygen species; b) oxygen-sensitive; c) active in maintaining intracellular redox status, and d) requiring oxygen for activity (Kaakoush et al 2007). Various studies confirm that *C. jejuni* possess enzymes from each category (Baillon 1999, Yamasaki et al 2004, St Maurice 2007, Weerakoon 2008, Kelly 2005). Wainwright et al (2005) identified another type of oxygen-related enzyme. The *C. jejuni* truncated haemoglobin (Ctb) was found to be involved in moderating O_2 flux and the authors suggested that, in the host, where O_2 concentrations are lower than optimal, such high affinity O_2 binding and reduction mechanisms could facilitate O_2 uptake and transfer to the appropriate metabolic enzymes (Wainwright et al 2005).

Studies involving the metabolism of *C. jejuni* also provide some explanation for atmospheric growth requirements (St. Maurice et al 2007, Ludwig 2004). St Maurice et al (2007) explain that the microaerophilic nature of these bacteria is based on the inactivation of oxygen-sensitive metabolic enzymes such as pyruvate:ferredoxin oxidoreductase (PFOR), iron sulfur proteins and other respiratory components. Ludwig (2004) suggests that microaerobes

possess *oxidative metabolic gearing* which allows microaerophilic bacteria to respond to changes in physiological O_2 relatively rapidly, conferring a selective advantage in limited oxygen environments over aerobic, anaerobic, or facultative microorganisms whose metabolic rates slow in response to physiological O_2 limitation (Ludwig 2004). Although it has been recognized that *C. jejuni* is capnophilic (Gaynor et al 2005, Park 2002, Skirrow 1991), little information is available on the role carbon dioxide plays in growth and biofilm development.

Campylobacter jejuni is capable of forming biofilms, both in the environment (Trachoo et al 2002) and in laboratory settings (Joshua et al 2006). Investigations of biofilm development in *C. jejuni* have shown that it is capable of forming biofilms on various surfaces (Kalmokoff et al 2006) and can form pellicles, flocs or surface-attached biofilms (Joshua et al 2006). Gene expression for immobilized bacteria on agar or in biofilms differs from planktonic cells and shows similarities to gene expression during host colonization (Carrillo et al 2007, Kalmokoff et al 2006, Sampathkumar et al 2006, Seal et al 2007). Immobilized cells show increased expression of genes related to iron uptake, general stress response, oxidative stress defense, membrane transport and motility (Sampathkumar et al 2006, Carrillo et al 2007, Kalmokoff et al 2006). The ability to form biofilms varies among strains and it has been suggested that survival in the environment may correlate with a strain's ability to form biofilms (Joshua et al 2006, Seals et al 2007).

Although there are studies which confirm that existing biofilms provide *C. jejuni* with protection from environmental stresses (Alter 2006, Buswell et al 1998, Joshua et al 2006, Kalmokoff et al 2006, Trachoo and Frank 2005), as well as from disinfection and sanitation procedures (Chantarapanont et al 2004, Wirtanen and Mattila-Sandholm 1992, Zottola and

Sasahara 1994), there are few investigations which examine the ability of *C. jejuni* to form biofilms under stressful conditions. Only one such study has been done to date. This study, by Reeser et al (2006), found that biofilm formation for the human clinical isolate, *C. jejuni* M129 was significantly higher in a 10% CO_2 enhanced atmosphere than under ambient aerobic conditions.

C. jejuni has been shown to have significant strain variation indicating a need for a more thorough investigation of the effect of atmospheric conditions on biofilm development.

The aim of the present study was to investigate the development of biofilm by three strains of *C. jejuni* under three different atmospheric conditions in order to determine how exposure to different levels of oxygen and carbon dioxide affected biofilm development.

2.2 Material and Methods

2.2.1 Bacterial strains and culture conditions

The sequenced strain, *Campylobacter jejuni* NCTC 11168 V1 was purchased from the ATCC and is representative of the original clinical isolate which was isolated from a case of human enteritis in 1977 (Ahmed 2002). *C. jejuni* NCTC 11168 V26 (Carrillo et al 2004), the laboratory passaged version of V1, was kindly donated by Dr. Brenda Allan from the Vaccine Infectious Diseases Organization (VIDO) in Saskatoon. *C. jejuni* 16-2R, an isolate from poultry meat, was kindly donated by Dr. Joseph Odumeru, Laboratory Services Division, University of Guelph.

For each strain, stock cultures were prepared by mixing equal parts of *C. jejuni* grown on Mueller Hinton agar (Oxoid) resuspended in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) with 50% glycerol. All 3 strains

were maintained at -80°C in an ultra freezer (Thermo Electron). Cells from stock cultures were resuscitated on Mueller Hinton agar (MHA) by incubating at 42°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) for 24 h. Resuscitated cells were then transferred by a polyester tipped sterile swab (Fisherbrand®) onto fresh MHA and incubated at 37°C under microaerobic conditions for 24 h prior to preparation of inocula.

2.2.2 Preparation of biofilm cells

For each experiment 0.1g of glass fibre filters (Whatman GF/F) were placed in 250ml glass bottles (Pyrex) which were then autoclaved at 121°C for 20 min. Glass fibres provide extensive surface area and are amenable to the removal of cells. Bottles were cooled overnight and 20 ml of sterile Mueller Hinton broth (MHB) was added to each bottle. Inoculum was prepared fresh, from frozen stock, for each experiment in order to avoid the transcriptional variation inherent in these bacteria which are known to exhibit genome plasticity. Cells were transferred from the subculture to sterile phosphate buffered saline (PBS) using a polyester tipped sterile swab (Fisherbrand®). Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK), inocula were standardized to an OD_{600} of 0.3 ± 0.015, which was equivalent to approximately 10^8 CFU/ml as confirmed by plate counting. Each bottle was inoculated with 1.0 ml of standardized inoculum. Bottles were placed in Ziploc™ freezer bags, flushed with either a microaerobic gas (5% O_2, 10 % CO_2, 85% N), a 5% CO_2 enhanced gas (19% O_2, 5% CO_2, 76% N) or room air (20 % O_2, 0.05 % CO_2, 78% N) and then incubated at 37°C with gentle agitation (25 rpm) in an incubator shaker (New Brunswick Scientific , *Innova*™ 4430) for 24h.

2.2.3 Harvesting of biofilm cells

After 24 hours of growth, bottles were removed from the Ziploc ™ freezer bags and placed on ice. The broth containing planktonic cells was aseptically removed and the glass fibre filters were washed 3 times with 25ml of cold PBS. Filters were aseptically transferred to sterile 100ml glass bottles containing 5g of glass beads (SEPHEX, 450-600µm) and 10ml of PBS and vortexed vigorously for 2 minutes using a Fisherbrand vortex set at 10. The supernatant was then filtered through sterile paper filters to remove excess glass fibre and the removed biofilm cells were collected in sterile 1.5 ml microcentrifuge tubes.

2.2.4 Enumeration of biofilm cells by standard plate count

Serial dilutions of the removed biofilm cells were prepared using sterile PBS. Five 10µl drops of each dilution were plated on MHA and incubated microaerobically at 42°C. Colonies were counted at 24 and 48 hours and CFU/g of glass fibre was determined for each sample.

2.2.5 Estimation of the total biovolume of biofilm cells by nucleic acid staining in conjunction with confocal scanning laser microscopy (CSLM) and PHLIP analysis.

Suspensions of the removed biofilm cells were combined with 4% formaldehyde at 4°C for 24 to 48 hours in order to kill the cells prior to staining with propidium iodide (Molecular Probes). Propidium iodide is an intercalating agent which only enters cells with compromised membrane integrity. Specifically, 0.5 ml of removed biofilm suspension was combined with 0.5ml of 4% formaldehyde and placed at 4°C for 24 to 48 hours in 1.5 ml microcentrifuge tubes. Tubes were then centrifuged at 3000xg for 30 minutes at 4°C and the pellets were resuspended in 1 ml of PBS. Propidium iodide was added to each tube with a final concentration of 0.1µg/ml

and placed in the dark at room temperature for 15 minutes. Tubes were then centrifuged again at 3000xg for 30 minutes (4°C) and pellets were resuspended in 1 ml of PBS. Samples were filtered onto 0.2µm black membrane filters (Isopore™ membrane filters GTBP02500, Millipore) using a millipore vacuum filtration unit. The filters were placed on glass slides. Mounting oil (Millipore) or ddH2O was placed on the filters and coverslips were sealed with nail polish. Slides were immediately taken for viewing and image capture using the Laser Scanning Microscope Fluoview, version 4.3 FV300 (Olympus FV300 CSLM) and a 60x PlanApo NA 1.4 oil immersion lens. Propidium iodide has excitation/emission maxima of 535/617nm. A HeNe Green (1mW, 543nm) laser was used to excite the samples. Barrier filters were used to limit the range of wavelengths reaching the channel 2 PMT. The bandpass filter BA660IF (<660nm) was used in conjunction with the dichroic mirror which allowed the range of 570nm to 660nm. Biovolumes for each condition and strain are averages of 3 trials with 5 random fields of view for each sample. Images were analyzed for total biovolume using the biofilm image analysis program PHLIP (Phobia Lasers Image Processing Software - The New Laser Scanning Microscope Image Processing Package) (Mueller et al., 2004).

2.2.6 Estimation of biofilm cell biovolumes by nucleic acid staining using LIVE/DEAD® *Bac*Light™ stain in conjunction with CSLM and PHLIP analysis

C. jejuni biofilm cells were grown as described in section 2.2.1. Biofilm cells collected for staining with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Invitrogen), were removed into 10 ml of 0.85% NaCl as suggested by the manufacturer in order to avoid phosphates from interfering with staining efficiency. Samples of biofilm cells from each condition were then stained with acridine orange (Sigma) in order to determine the appropriate

dilution to be used with the *Bac*Light™ stain. Dilutions were then prepared in 0.85% NaCl (either 1:10 or 1:100 depending on cell density). The fluorescent dyes propidium iodide(PI) (20mM in DMSO) and SYTO 9 (3.34mM in DMSO) were initially mixed in a 1:1 ratio as suggested by the manufacturer. This was found to cause excessive background on channel 1 which interfered with PHLIP analysis. A 2:1 ratio (PI:SYTO 9) provided better image results and was used for all subsequent trials. The 2:1 dye mixture was prepared in advance and stored at 4°C. Diluted samples (0.5ml) were incubated with 1.5µl of dye mixture at 24°C in the dark for 20 minutes. Samples were then immediately filtered onto 0.2µm black membrane filters (Isopore™ membrane filters GTBP02500, Millipore) and slides were prepared for viewing on the CSLM as described above. Images were acquired with a 60x PlanApo NA 1.4 oil immersion lens, using sequential scanning. SYTO 9 has excitation/emission maxima of 480/500nm. Argon (10 mW, force air cooled, blue 488nm) and HeNe Green (1 mW, 543 nm) lasers were used with FVX-BA 510 -530 band pass emission. Biovolumes for each condition and strain are averages of 3 trials with 10 random fields of view for each sample. For each strain and condition, a one-way analysis of variance was used to determine if biofilm development differed significantly between strains or conditions (alpha value of 0.05). Due to the difficulty in locating cells from the AIR condition during the imaging with propidium Iodide, this condition was not included in the LIVE/DEAD® *Bac*Light™ assay.

Cell counts were done manually for a selection of the images for each strain and values were converted to cells/g glass fibre. For *C. jejuni* V1 cell counts were obtained for 3 images allowing the calculation of averages and standard deviations. Plate count data were obtained for the same samples in order to do a direct comparison.

2.2.7 Visualization of biofilm on glass fibre filters by scanning electron microscope

Biofilms were grown on glass fibre filters as stated above and washed 3 times with 25ml of PBS. Standard procedures for imaging cells on the scanning electron microscope (SEM) which include fixing with glutaraldehyde, followed by an ethanol dilution series and critical point drying eliminate EPS (Harrison et al 2006). Air drying may affect cellular morphology, but prevents loss of EPS and was chosen in order to keep the biofilm as close to its original state as possible. In order to visualize biofilms, filters were processed as described by Harrison et al (2006). Specifically, filters were air dried in a ventilated covered container and coated with gold using an Ernest F. Fullen gold target sputter coater. Samples were then visualized using a JOEL 5900 LV scanning electron microscope at an accelerating voltage of 13 kV. Spot size ranged from 23 to 26. Images of each sample were taken at 500x, 1000x, 2500x, 5000x and 10000x magnification. In some cases a 20000x magnification was also taken.

2.2.8 Statistical analysis

Assessment of significant differences between conditions and strains was performed using t-tests and one-way analysis of variance (ANOVA) with an alpha value of 0.05 with the Statistical Package for the Social Sciences (SPSS Statistics 17.0). T-tests were used for plate count comparisons between strains. ANOVA was used for comparing plate count values.for all strains and all conditions. ANOVA was also used to compare biovolume values for all strains and conditions. Plate count values are averages of at least 13 independent biological replicates done in triplicate. When cell counts were below the detection limit (3.6 $\log_{10}$ CFU/g), one-half of the detection limit value was used for statistical analyses (Clarke, 1998).

Biovolume values from the PI assay were averages of 3 trials with 5 random fields of view each sample. Biovolume values from the *Bac*Light ™ assay were averages of 3 trials with 10 random fields of view for each sample. All error bars represent standard deviation from the mean.

2.3 Results

2.3.1 Evaluation of biofilm development of three strains of *C. jejuni* in different atmospheric conditions by plate counting

The development of culturable biofilm cells over 24h in each condition for each strain is shown in Figure 2.1. A one-way analysis of variance of all three strains in the microaerobic (MA) and 5% CO_2 enhanced (ACO2) conditions indicated that there were no significant differences between plate count values for any of these strains or conditions ($p<0.05$). There was however, a significant reduction in values for culturable biofilm cells in the aerobic (AIR) condition when compared to either MA or ACO2 for all three strains included in this study (Table 2.2 $p<0.05$).

Biofilm formation in AIR was not significantly different between the 3 strains, ranging from 3.59 $\log_{10}$ CFU/g glass fibre for V26 to 4.20 $\log_{10}$ CFU/g glass fibre for 16-2R ($p>0.05$). Growth in the microaerobic condition varied from 9.72 $\log_{10}$ CFU/g glass fibre for strain 16-2R to 9.82 $\log_{10}$ CFU/g glass fibre for strain V26, while in the CO_2 enhanced condition the range was from 9.58 $\log_{10}$ CFU/g glass fibre for V1 to 10.01 $\log_{10}$ CFU/g glass fibre for 16-2R.

For approximately 60% of trials, growth in AIR was below the detection limit of 3.6 $\log_{10}$ CFU/g glass fibre and as described by Clarke (1998) half the detection limit value (3.3 $\log_{10}$ CFU/g glass fibre) was used for plotting and statistical analysis.

In order to determine if the 2 minute vortex period sufficiently removed the biofilm cells, filters were vortexed for an additional 2 minute period. Results indicated that over 90% of the cells removed in the 4 minutes were from the first 2 minute period (data not shown).

2.3.2 Evaluation of biofilm development of three strains of *C. jejuni* in different atmospheric conditions by CSLM in conjunction with PHLIP analysis

Total biovolume values were acquired by killing removed cells with 4% formaldehyde prior to staining with the fluorescent dye propidium iodide (PI) which only enters dead or damaged cells. The confocal scanning laser microscope (CSLM) in conjunction with image analysis was used to determine biovolumes of biofilm cells (Fig.2.2).

A one-way analysis of variance including both MA and ACO2 values for all three strains showed no significant differences (Table 2.2 $p=0.385$). Strain differences for growth in AIR were not significantly different, but had a p value of 0.051 suggesting that strain variation had a slight effect on growth in AIR.

There was some difficulty in locating biofilm cells in AIR for each strain which is reflected in both the images (Fig. 2.4) and the lower total biovolumes for each strain in this condition (Fig. 2.2). Total biovolume values for AIR (7.09 to 7.80 $\log_{10}$ $\mu m^3/g$) ranged from 5 - 21% of the corresponding values for MA (8.53 to 8.68 $\log_{10}$ $\mu m^3/g$) (Fig. 2.2).

Both V1 and V26 showed significantly reduced biofilm formation in AIR (7.80 and 7.71 $\log_{10}$ $\mu m^3/g$ respectively) when compared with corresponding values for ACO2 (8.78 and 8.93 $\log_{10}$ $\mu m^3/g$ respectively) ($p<0.05$). Values in AIR were lower than for MA (8.68 and 8.53 $\log_{10}$ $\mu m^3/g$ respectively), but this difference was not significant ($p>0.05$). For 16-2R the biovolume

value for growth in AIR (7.09) was significantly lower than the values for both ACO2 (9.05) and MA (8.56) ($p<0.05$).

2.3.3 Evaluation of biofilm development for three strains of *C. jejuni* biofilm by nucleic acid staining using LIVE/DEAD® *Bac*Light™ stain in conjunction with CSLM and PHLIP analysis

The *Bac*Light™ assay involved staining samples with a mixture of the fluorescent dyes SYTO 9 which stains cells green (G) and propidium iodide (PI) which stains cells red (R). The SYTO 9 indicates cells which are healthy while PI only stains cells which are dead or membrane-damaged. A selection of images of *Bac*Light™ stained *C. jejuni* biofilms cells from each condition and strain are presented in Figure 2.5. Biovolume values obtained using dual staining are subject to colocalization; areas where both dyes are present. In this case, adding the biovolumes from each stain individually results in a value greater than the total biovolume given by PHLIP. The difference, or overlap in the values was found to be less than 3% of the total for all trials in this study and therefore not included in the analysis.

A comparison of values from each condition obtained using *Bac*Light™ (Fig. 2.3.1) indicated that, with the exception of V1 MA G (8.17 $\log_{10}$ $\mu m^3/g$) being significantly lower than V1 ACO2 G (9.31 $\log_{10}$ $\mu m^3/g$) ($p<0.05$), there were no significant differences between conditions for any of the other values ($p>0.05$). Total biovolume values were significantly higher for 16-2R (ACO2 10.74, MA 10.70 $\log_{10}$ $\mu m^3/g$) than V1 (ACO2 10.00, MA 10.02 $\log_{10}$ $\mu m^3/g$) or V26 (ACO2 10.14, MA 10.02 $\log_{10}$ $\mu m^3/g$) for both conditions (Table 2.2 $p<0.05$). All three strains had higher values for dead and damaged than for healthy cells after 24 hours of growth at 37°C. For strains V1 and 16-2R the difference was significant in both ACO2 (9.88 and 9.31 $\log_{10}$ $\mu m^3/g$ for V1, 10.68 and 9.36 $\log_{10}$ $\mu m^3/g$ for 16-2R) and MA (9.89 and 8.17 $\log_{10}$ $\mu m^3/g$ for V1, 10.59

and 9.32 $\log_{10}$ $\mu m^3/g$ for 16-2R) ($p<0.05$). For V26 this difference was not significant with 9.92 and 9.54 $\log_{10}$ $\mu m^3/g$ in ACO2 and 9.80 and 9.36 $\log_{10}$ $\mu m^3/g$ in MA.

An analysis of the percent of healthy cells (10.76 to 35.87%) compared to dead or damaged (Fig. 2.3.2) indicated that the percent of healthy cells was significantly lower than the percent of dead or membrane-damaged cells for all three strains (Table 2.2). The values for healthy cells did not differ significantly between conditions for all three strains ($p>0.05$).

Cell counts obtained by directly counting cells in the images from the LIVE/DEAD® *Bac*Light™ assay were consistently lower than those from the corresponding plate counts (Fig. 2.3.3). For V1, cell counts for ACO2 (6.17 $\log_{10}$ cells/g) were not significantly different from corresponding plate counts (7.31 $\log_{10}$ CFU/g) ($p<0.05$). Cell count values for MA (6.58 $\log_{10}$ cells/g) were significantly lower than the corresponding plate counts (8.02 $\log_{10}$ CFU/g) ($p<0.05$). However the differences between cell counts and plate counts were very similar for both ACO2 (1.14 $\log_{10}$ CFU/g) and MA (1.44 $\log_{10}$ CFU/g). Results for V26 and 16-2R showed similar trends (data not shown).

2.3.4 Evaluation of biofilm development of three strains of *Campylobacter jejuni* in different atmospheric conditions by scanning electron microscope

Biofilm development was visualized using the SEM. The 10,000X magnification was chosen in order to compare strains and conditions (Fig. 2.6). All magnifications are presented in figures 2.7 to 2.17. A qualitative description of the appearance of biofilm for each strain in each condition is presented in Table 2.1. Biofilms were described in terms of biofilm quantity, surface area coverage, and biofilm surface depth.

Comparison with controls (Fig. 2.7 and Fig. 2.8) indicated that samples from the aerobic (AIR) condition provided no evidence of biofilm formation for both the V1 and V26 strains (Fig. 2.9 and Fig. 2.10), but there was some evidence of biofilm formation in AIR for 16-2R (Fig. 2.11, see arrow).

Biofilm appearance was very similar for strains V1 and V26. Biofilms grown in MA showed excessive (luscious) growth, thickly coating the glass fibre filters (Fig. 2.12, Fig. 2.13) Biofilms grown in ACO2 showed more moderate growth (Fig. 2.6). Here the glass fibres had a decorated appearance and the biofilm appeared to be more internalized, with the surface of the glass fibre filter visible above the layer of biofilm. The only notable difference between these 2 strains occurred in the ACO2 condition, where the images of V26 (Fig. 2.16) showed slightly more dense biofilm than V1 (Fig. 2.15). Biofilm appearance for the 16-2R strain was similar to V1 and V26 in MA, with the glass fibre being coated with excessive biofilm (Fig. 2.14). A notable difference occurred in ACO2, where the 16-2R biofilm showed more excessive biofilm than the other strains (Fig. 2.17). Although biofilm growth for the 16-2R strain in ACO2 was excessive and the surface of the glass fibre was coated with biofilm, the biofilm appeared to be thinner than the one grown microaerobically.

2.4 Discussion

The microaerophilic nature of *C. jejuni* implies that these bacteria are sensitive to atmospheric conditions. Biofilms have been shown to provide *C. jejuni* with protection from such external stresses; allowing extended survival in the environment. Yet, very little work has been done to determine the atmospheric conditions conducive to biofilm formation for this

prevalent pathogen. In order to assess the effects of aerobic stress on biofilm development, biofilm formation was examined for three strains of *C. jejuni*, in three atmospheric conditions. The microaerobic condition (MA) with 5% oxygen provided minimal oxidative stress while the aerobic (AIR) and the 5% CO_2 enhanced (ACO2) condition provided similarly high levels of oxygen (20% and 19% respectively). The 5% CO_2 enhanced condition provided the bacteria with higher levels of CO_2 (5%) than the aerobic condition (0.05%). The investigation included three components. Plate counting was used to determine the number of culturable cells in each condition. Nucleic acid staining of biofilm cells, combined with CSLM and PHLIP analysis provided an estimate of biofilm cell biovolumes in each condition. Finally, SEM imaging was used to visualize the biofilms.

The first relevant finding of this study was that *C. jejuni* did not readily form biofilms in ambient aerobic conditions. The plate count data presented in Figure 2.1 indicate that there was a significant 5 to 6 log reduction in biofilm growth in AIR (with CO_2 levels below 1%) as compared to either MA or ACO2 for all three strains ($p<0.05$). Plate counts for aerobic growth were not only significantly lower than the other conditions but were the result of multiple attempts. For all three strains approximately 60% of attempts showed no growth in AIR at the detection limit of 3.6 CFU/g glass fibre.

C. jejuni cells have been shown to enter a VBNC state under stressful conditions (Gaynor et al 2005, Klançnik et al 2008, Tholozan et al 1999). There was a concern that plate counts would underestimate the total number of viable cells, as they do not account for these VBNC cells. Biovolumes of the dislodged biofilm cells were obtained in order to determine if the plate count data accurately reflected the amount of biofilm that grew in each condition. Initially the

biofilms were fixed in 4% formaldehyde and stained with PI. Later, biofilm cells were stained directly after removal, using the LIVE/DEAD® *Bac*Light™ assay allowing for assessment of healthy, as well as dead or membrane-compromised cells. Total biovolume values obtained using *Bac*Light™ (Fig 2.3.a) were consistently higher than those obtained from the PI assay (Fig. 2.2). These differences could be due to the fact that the protocol for the *Bac*Light™ assay included improved techniques for dispersing bacteria on the filters, diluting samples to prevent crowding (crowding may have led to underestimated values by PHLIP), and increasing the number of fields of view from 5 to 10 for the samples processed with the *Bac*Light™ kit. Also, processing the cells with 4% formaldehyde required pelleting and washing of samples which was not needed for the *Bac*Light™ assay. The PI assay was performed on cells from all three conditions but, due to difficulty in locating cells in AIR, only cells from MA and ACO2 were processed with the *Bac*Light™ assay. As a result, only total biovolume values are available for AIR. However, if results could be obtained for AIR, it would be interesting to determine the relative level of healthy and damaged cells.

The biovolume data confirmed the results from the plate counting. The total biovolume values (Fig. 2.2) obtained with PI for AIR ranged from 5 – 21% of corresponding values from MA. Although values for AIR were consistently lower, the PI assay was subject to variability and these differences were not always statistically significant (Fig. 2.2).

Scanning electron microscope images also supported the plate count data. There was no evidence of biofilm formation in AIR for either V1 or V26 and only scant amounts for 16-2R (Figs. 2.9- 2.11). This strain variation was also present in the plate count data, where the value for 16-2R in AIR was slightly higher than for V1 or V26.

These findings confirmed the results of other studies which have shown that under ambient aerobic conditions, *C. jejuni* demonstrated reduced growth planktonically (Boysen et al 2007, Kaakoush et al 2007) as well as reduced biofilm formation (Reeser 2007). The apparent lack of biofilm formation in the aerobic condition could be a reflection of the difficulty *C. jejuni* has with aerobic stress. *C. jejuni* has a limited arsenal of oxidative stress defense genes suggesting that the reduced growth may be due to an inability to adequately inactivate the reactive oxygen species (ROS) that would likely occur at elevated levels in the aerobic condition. Also oxygen-sensitive proteins in *C. jejuni* such as pyruvate ferredoxin oxidoreductase (PFOR), iron sulfur proteins and other respiratory components (St. Maurice et al 2007), and the Rrc protein which is homologous to rubredoxin oxidoreductase/rubrerythrin (Yamasaki et al 2004) would find such high levels of oxygen detrimental.

The second and more interesting finding from this study was that biofilm formation did not vary between the microaerobic (MA) and 5% CO_2 enhanced aerobic (ACO2) conditions. Results from plate counting indicated that the level of culturable cells (Fig. 2.1) was not significantly different between MA (5% O_2, 10% CO_2) and ACO2 (19% O_2, 5%O_2), where the bacteria are exposed to levels of oxygen that are similar to AIR (20% O_2, 0.05% CO_2), but have additional CO_2 at their disposal, for any of the 3 strains ($p>0.05$).

Both sets of biovolume data supported these plate count values. Biovolume data using killed cells, stained with PI and biovolume data acquired with the *Bac*Light™ kit both confirmed that total biovolumes were not significantly different between MA and ACO2 for all three strains ($p>0.05$). Values from the *Bac*Light™ assay indicated significantly more growth for 16-2R for both MA and ACO2 when compared to the other strains ($p<0.05$). This may be a result of the

improved sensitivity provided by the *Bac*Light™ assay. Also, since no dilutions were done for any of the samples in the PI assay, biovolume values for both MA and ACO2 are likely underestimated due to overcrowding (Fig. 2.4).

Analysis of the *Bac*Light™ data (Fig. 2.3.a) indicated that total biovolumes did not significantly differ between MA and ACO2 for each strain and values for healthy cells and corresponding values for dead or damaged cells were also not significantly different ($p<0.05$). A percent analysis (Fig 2.3.b) of these data indicated that the percent of dead and damaged cells did not differ significantly between V1 and 16-2R, but was significantly lower for V26 ($p<0.05$).

An analysis of cell counts obtained by direct counting from the *Bac*Light™ images indicated that cell counts were consistently lower than the corresponding plate count values, but showed the same trends of values not being significantly different between the MA and ACO2 conditions. The lower values from the direct counting could be due to cell clumping which made it difficult to distinguish individual cells.

Although there appeared to be excessive biofilm in both MA and ACO2 SEM images (Fig. 2.6), there was some variation in biofilm morphology between these conditions. The biofilm appeared to be embedded in the filters exposed to ACO2 (Fig. 2.15 -2.17), while those exposed to MA (Fig. 2.12-2.14) were more thickly coated.

The fact that biofilm formation was just as prevalent in ACO2 as in MA, but was significantly reduced in AIR, suggests that the increased level of CO_2 may play a role in the cells' ability to alleviate oxidative stress, either by allowing for the use of alternative metabolic pathways which don't use oxygen and hence don't lead to the creation of ROS, or by aiding in the removal of the damaging ROS.

Few studies have been done to examine the effect of atmosphere on biofilm formation. Reeser et al (2007) found that biofilm formation of *C. jejuni* M129 at 37°C was significantly lower in the straight aerobic condition than in the 10% CO_2 enhanced condition which agrees with the findings for AIR in the present study. No studies were found which compared biofilm formation in microaerobic (5% O_2, 10% CO_2) with CO_2 enhanced aerobic (5-10% CO_2 and >10% O_2) conditions.

There have been, however, several studies which examined the effects of atmosphere on planktonic growth. Kaakoush et al (2007) found that the ability to grow with elevated oxygen was dependent on cell densities and varied by strain. For inocula with low cell densities ($<5x10^5$ CFU/ml), growth was only observed with microaerobic conditions while at cell densities similar to those in the present study (~10^8CFU/ml), growth was found to be significantly greater in oxic (10% CO_2, 19%O_2) than the microoxic (10%CO_2, 6%O_2) condition. Although this does not directly reflect the results of the present study, the differences could be attributed to the differences in the conditions used or the difference between planktonic and biofilm associated growth. What is relevant is that they also observed excessive growth in conditions with high oxygen concentrations and high levels of CO_2. A comparison of strains indicated that NCTC 11168 and RM1221 both required low levels of oxygen to grow, but NCTC 11168 was significantly more tolerant to high levels of oxygen (19%) than RM 1221 (Kaakoush et al 2007). Further investigation revealed that these strain differences could be due to genes which are present only in 11168. Most likely candidates include genes proposed to be responsible for a small alternative respiratory pathway and/or genes for putative oxidoreductases which are implicated in oxygen-related metabolism and protection against ROS (Kaakoush et al 2007).

Other studies on the physiology and metabolism of *C. jejuni* provide some explanation for the requirement of elevated levels of CO_2. *C. jejuni* has a complex, highly branched respiratory chain allowing both aerobic and anaerobic respiration with a variety of alternative electron acceptors. These bacteria are unable to metabolize glucose or other carbohydrates, as they lack the key enzyme, phosphofructokinase (Kelly 2001). They do possess other glycolytic enzymes and use gluconeogenic pathways to provide intermediates for biosynthesis of cell wall materials, vitamins and nucleic acids (St. Maurice et al. 2007). The main sources of carbon and energy are organic acids and amino acids. St. Maurice et al. (2007) found that *C. jejuni* can incorporate CO_2 into biomass, forming pyruvate via the bidirectional PFOR:FldA:FqrB pathway. *C. jejuni* also uses CO_2 in anapleurotic reactions to replace tricarboxylic acid cycle intermediates and it has been suggested that the need for CO_2 may be related to a reliance on phosphoenolpyruvate carboxykinase as a mechanism to generate ATP (Kelly 2001).

Park (2005) has suggested that the mucus layer in the avian gastrointestinal (GI) tract is microaerobic and that the atmosphere in the colonic lumen is anaerobic. According to the Canadian Society of Intestinal Research (CSIR), the average person generates 1 to 3 pints of gas per day, 90% of which is ingested air (oxygen, nitrogen and carbon dioxide) and the other 10% (hydrogen, methane and carbon dioxide) is produced by colonic bacteria. Although these values represent the human GI tract, it is likely that similar gases are also present in the avian GI tract.

Results from the present study suggest that the concentration of CO_2 is more relevant to biofilm development than the aerobic stress from elevated levels of oxygen. The concentration of CO_2 in the gastrointestinal tract may affect the ability of *C. jejuni* to colonize this niche and may also play a role in host cell infection.

Although *C. jejuni* often shows significant strain variation, the 3 strains in the present study showed very little strain variation with respect to biofilm formation in the different atmospheres. 16-2R had significantly more biovolume in MA and ACO2 than the other strains (*Bac*Light™ assay) and SEM images indicated more excessive biofilm formation for this strain. However, these differences had no bearing on the conclusions regarding the effects of atmosphere, as this strain followed the same trends as the others.

One significant implication of this finding is in the regulation of modified atmosphere packaging (MAP) where the high levels of CO_2 used to delay spoilage and deter other pathogens, could potentially lead to increased growth of *C. jejuni* (Phebus et al 1991, Phillips 1996).

In summary, *C. jejuni* is capable of developing biofilm in conditions with elevated levels of oxygen as long as there is sufficient CO_2 present. The levels of CO_2 in ambient air do not appear to support biofilm development of *C. jejuni*. Preliminary work suggests that CO_2 may be involved in the metabolism of these bacteria, but further studies are required to confirm the role it plays and also to investigate the effects of CO_2 concentration on the colonization and virulence properties of these bacteria.

Table 2.1. Visual comparison of three strains of *Campylobacter jejuni* biofilms in three growth conditions based on SEM images. AIR – room air; CO2 – 95% room air enhanced with 5% CO_2; MA – microaerobic.

Strain and condition	Biofilm quantity	Surface area coverage	Depth of biofilm surface
V1 AIR	Absent	N/A	N/A
V26 AIR	Absent	N/A	N/A
16-2R AIR	Low	Poor	N/A
V1 MA	High	Continuous	Thickly coated
V26 MA	High	Continuous	Thickly coated
16-2R MA	High	Continuous	Thickly coated
V1 CO2	Moderate	Fair	Decorated
V26 CO2	Moderate to high	Good	Decorated
16-2R CO2	High	Excellent	Thinly coated

N/A – not applicable.

Table 2.2 Summary of Statistical Analyses

Value	Program	Test performed	Homogeneous subset	P value
Plate counts (Fig. 2.1)	SPSS/Excel	One-way ANOVA	All values	$3.7x10^{-102}$
Plate counts (Fig. 2.1)	SPSS	One-way ANOVA - Tukey Test	V1 AIR, V26 AIR, 16-2R AIR	0.239
			V1 MA, V1 ACO2, V26 MA, V26 ACO2, 16-2R MA, 16-2R ACO2	0.704
Plate counts (Fig. 2.1)	Excel	T-test	V1 AIR vs V26 AIR	0.106
Plate counts (Fig. 2.1)	Excel	T-test	V1 AIR vs 16-2R AIR	0.172
Plate counts (Fig. 2.1)	Excel	T-test	V26 AIR vs 16-2R AIR	0.797
Total biovolumes (Fig. 2.2)	SPSS/Excel	One-way ANOVA - Tukey Test	V1 MA, V1 ACO2, V26 MA, V26 ACO2, 16-2R MA, 16-2R ACO2	0.385
Total biovolumes (Fig. 2.2)	SPSS/Excel	One-way ANOVA - Tukey Test	V1 AIR, V26 AIR, 16-2R AIR	0.052
LIVE/DEAD biovolumes (Fig.2.3.1)	SPSS	One-way ANOVA - Tukey Test	MAGV1	1.000
			ACO2GV1, MAG16-2R, ACO2G16-2R, MAGV26, ACO2GV26	0.940
			ACO2G16-2R, MAGV26, ACO2GV26, MARV26	0.057
			ACO2GV26, MARV26,ACO2RV1, MARV1, ACO2RV26	0.208
			MARV26,ACO2RV1, MARV1, ACO2RV26, ACO2TV1, MATV1, MATV26, ACO2TV26,	0.406
			MAR16-2R, ACO2R16-2R, MAT16-2R,ACO2T16-2R	1.000
LIVE/DEAD biovolumes (Fig.2.3.2)	SPSS	One-way ANOVA - Tukey Test	ACO2G16-2R, MAGV1, MAG16-2R, ACO2GV1	0.354
			MAG16-2R, ACO2GV1, MAGV26	0.099
			ACO2GV1, MAGV26, ACO2GV26	0.432
			ACO2RV26, MARV26, ACO2RV1	0.366
			MARV26, ACO2RV1, MAR16-2R	0.105
			ACO2RV1, MAR16-2R, MARV1, ACO2R16-2R	0.367

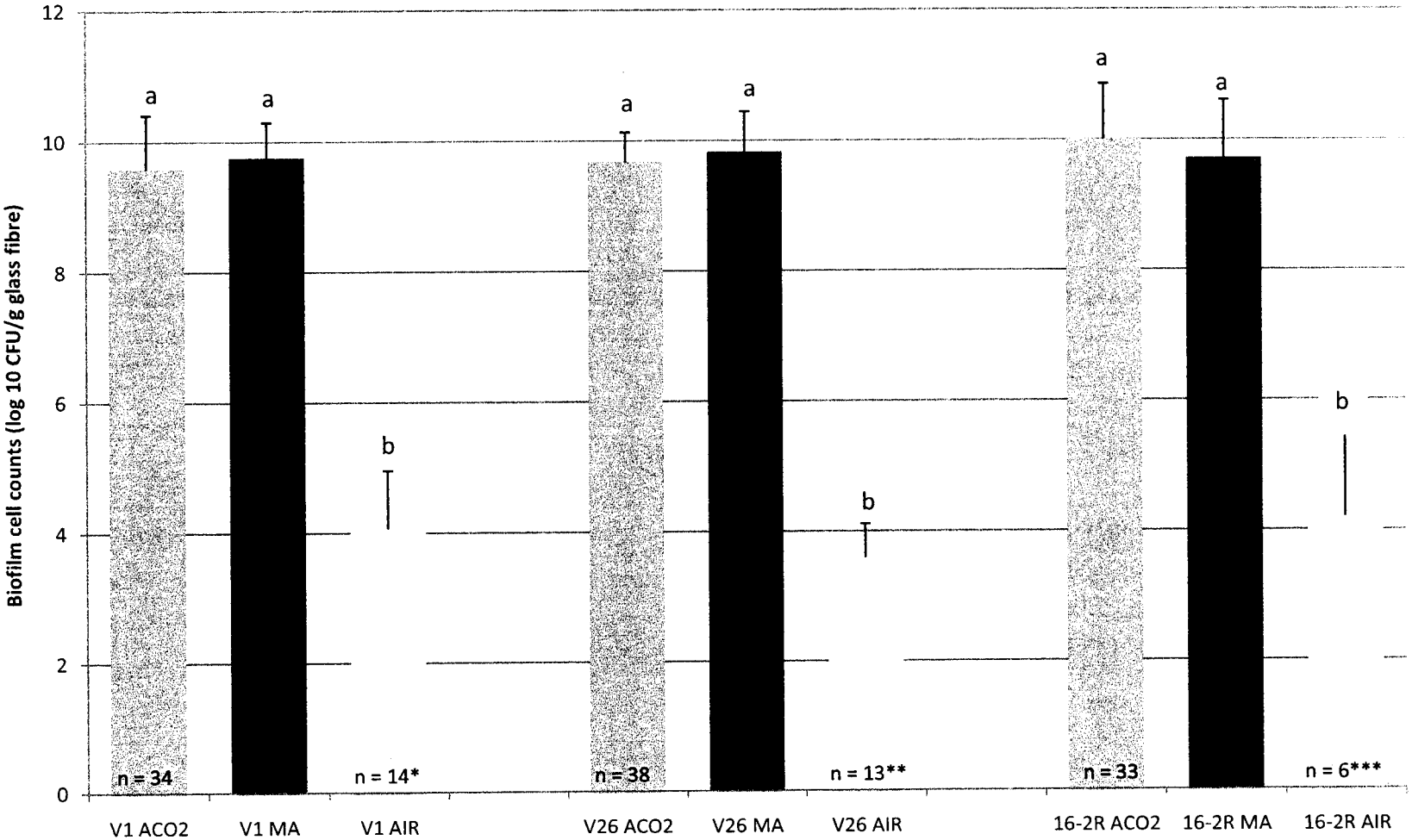

Figure 2.1. Biofilm cell counts for *Campylobacter jejuni* strains grown on glass fibre filters for 24 hours under different atmospheric conditions. MA - microaerobic; CO_2 - 5% CO_2 with 95% room air; AIR - 100% room air. Error bars represent standard deviation from the mean. Values were below the detection limit for * 6 of 14 samples, ** For 8 of 13 samples, and for *** 3 of 6 samples analyzed. For values below detection limit, half of the detection limit was used for plotting and statistical purposes. (detection limit - of 3.6 $\log_{10}$ CFU/g glass fibres). V1 - *C. jejuni* NCTC 11168 V1, V26 - *C. jejuni* NCTC 11168 V26; 16-2R - *C. jejuni* 16-2R (meat isolate). Small letters indicate values that are significantly different ($p<0.05$).

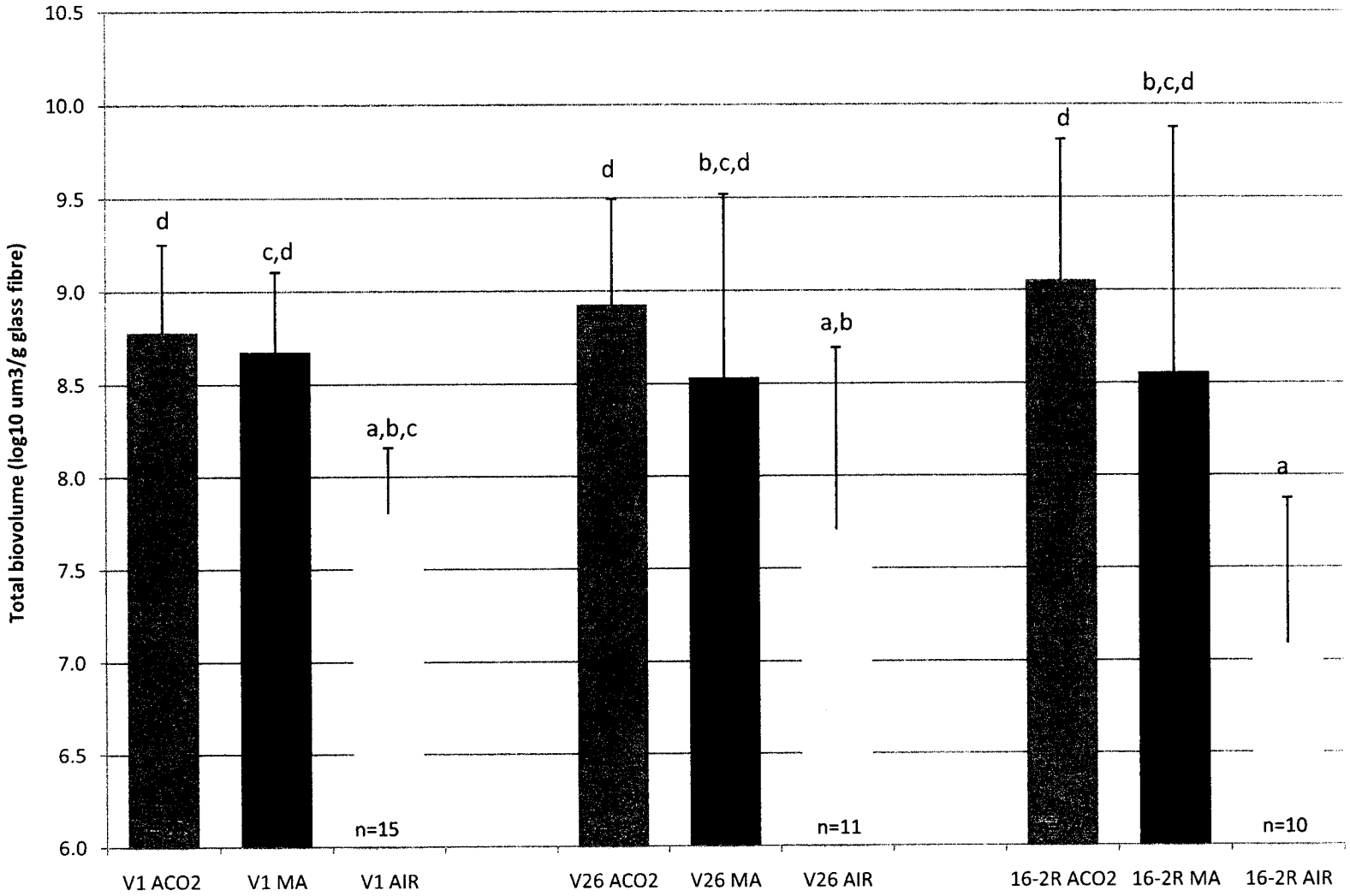

Figure 2.2. Total biovolumes for each strain of *Campylobacter jejuni* biofilm cells grown in each condition, exposed to 4% formaldehyde for 24 hours and then washed and stained with propidium iodide. MA-microaerobic; ACO2 - 5% CO_2 enhanced; AIR - ambient room air. V1 - *C. jejuni* NCTC 11168 V1, V26 - *C. jejuni* NCTC 11168 V26; 16-2R - *C. jejuni* 16-2R (meat isolate). Small letters indicate values that are significantly different ($p<0.05$).

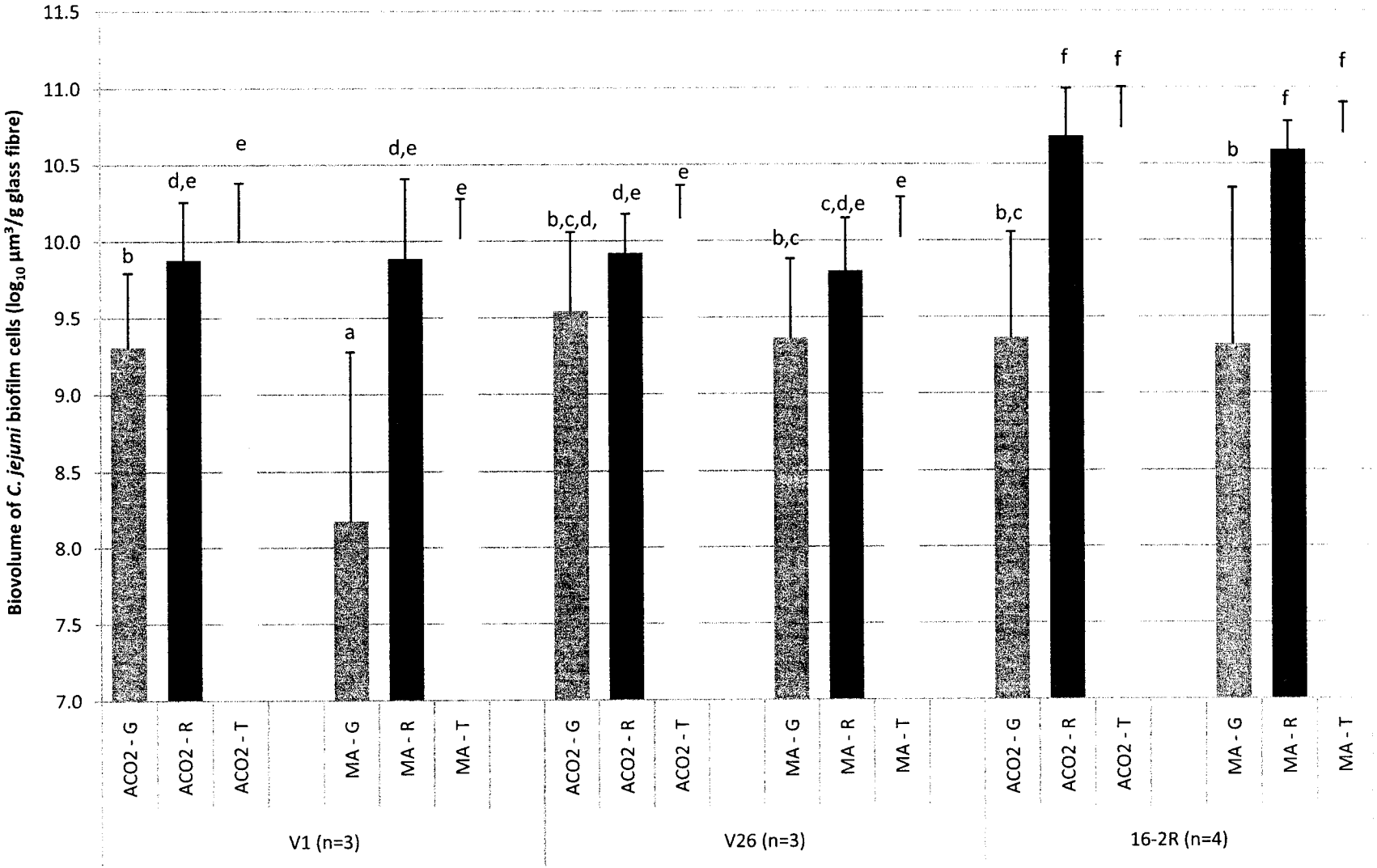

Figure 2.3.1. Comparison of log transformed values for biovolumes of healthy (G) , membrane-damaged (R) and total (T) *C. jejuni* biofilm cells. MA- microaerobic, ACO2 - 5% CO2 enhanced, G - green, R- red, T -total, s- significant, ns - not significant. Small letters indicate values which are significantly different ($p<0.05$).

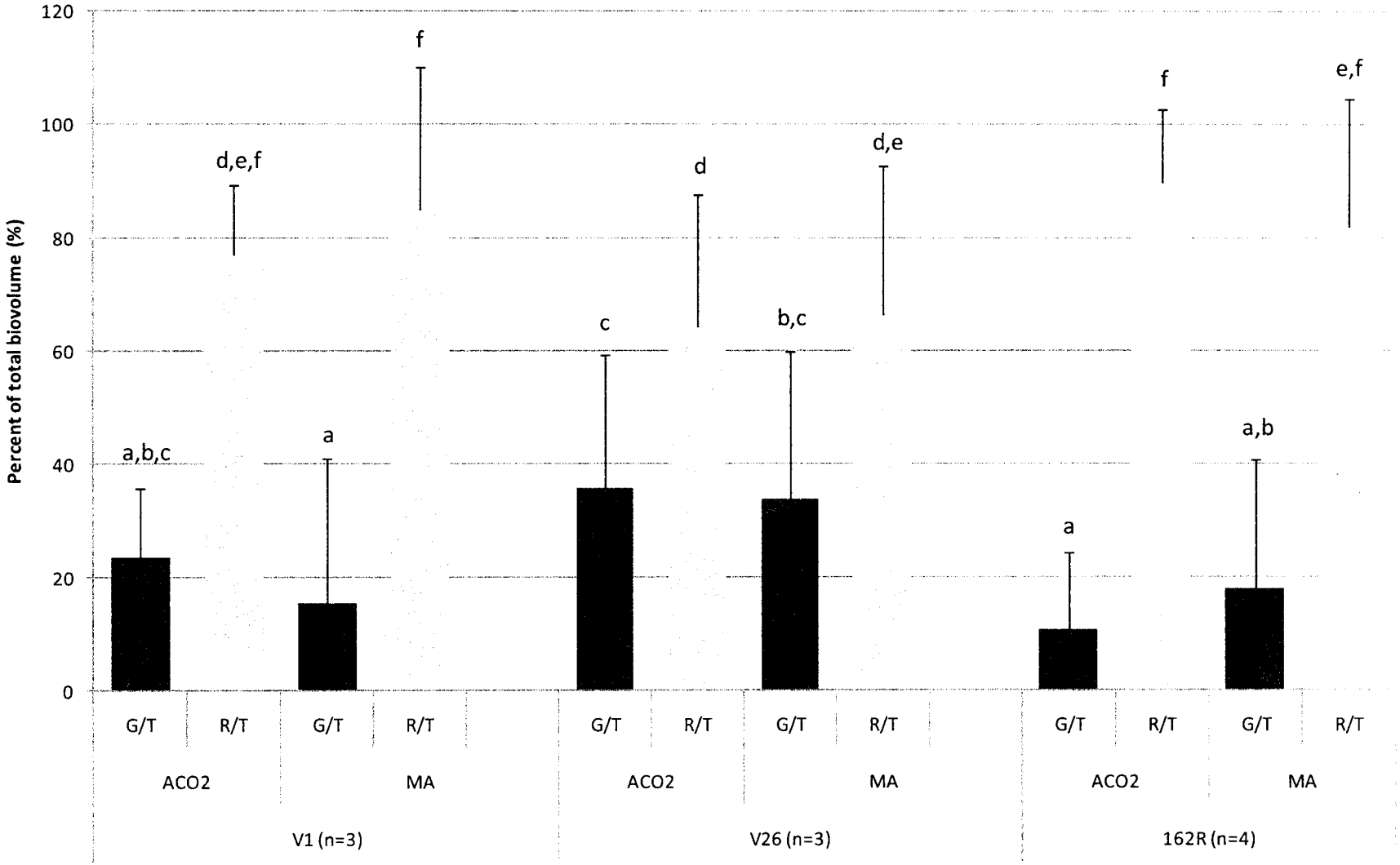

Figure 2.3.2. Comparison of biovolumes of healthy (G) and membrane-damaged (R) *C. jejuni* biofilm cells as percent of total biovolume. MA- microaerobic, ACO2 - 5% CO_2 enhanced, G/T - green as percent of total, R/T - red as percent of total. Small letters indicate values which are significantly different ($p<0.05$).

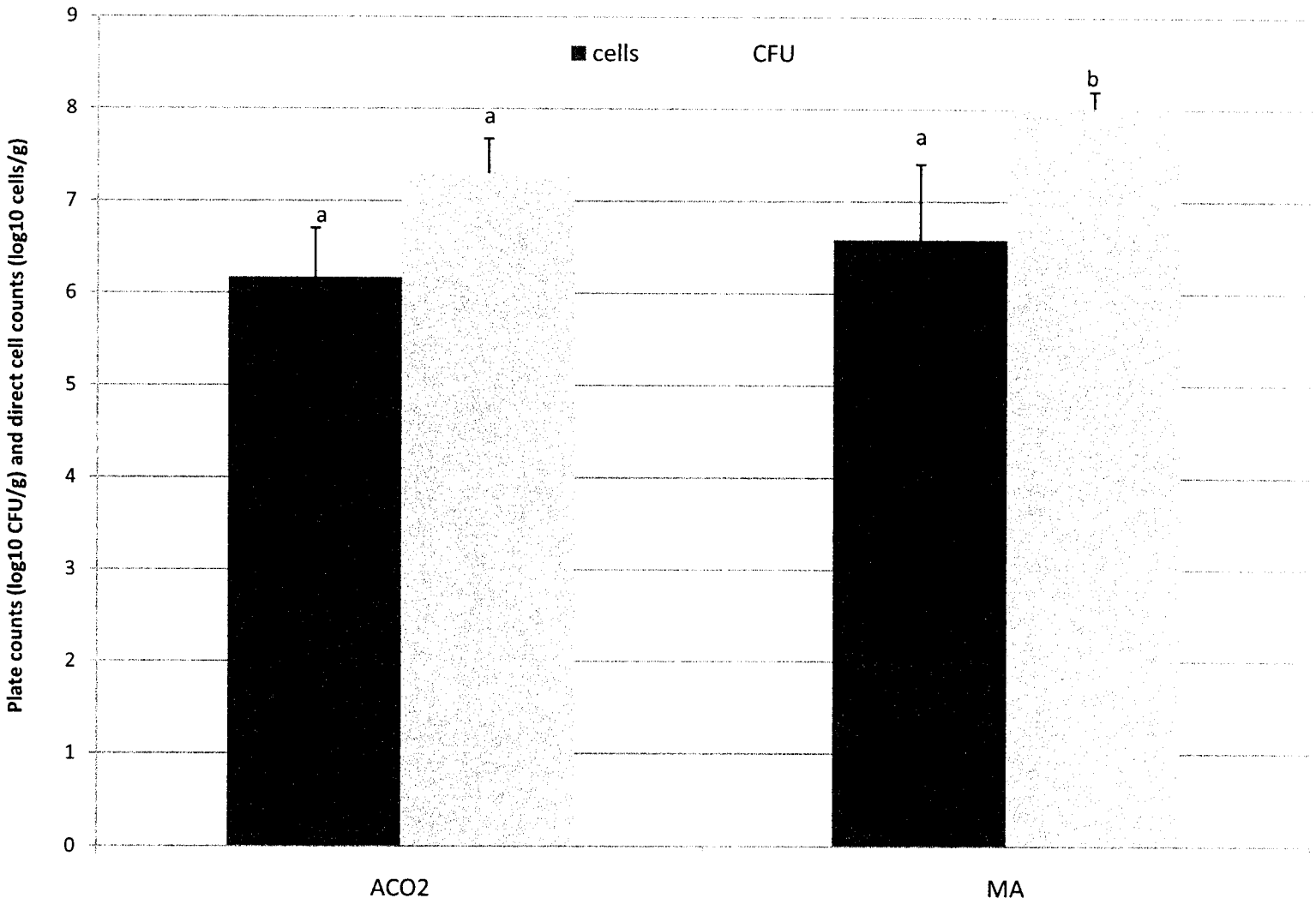

Figure 2.3.3. Comparison of plate count data(log_{10} CFU/g) for *C. jejuni* V1 with cell counts (log_{10} counted cells/g) obtained for the same samples using the LIVE/DEAD® *Bac*Light™ assay . Error bars represent standard deviation from the mean (n=3). MA - microaerobic, ACO2 - 5% CO_2 enhanced aerobic. Small letters indicate values which are significantly different ($p<0.05$).

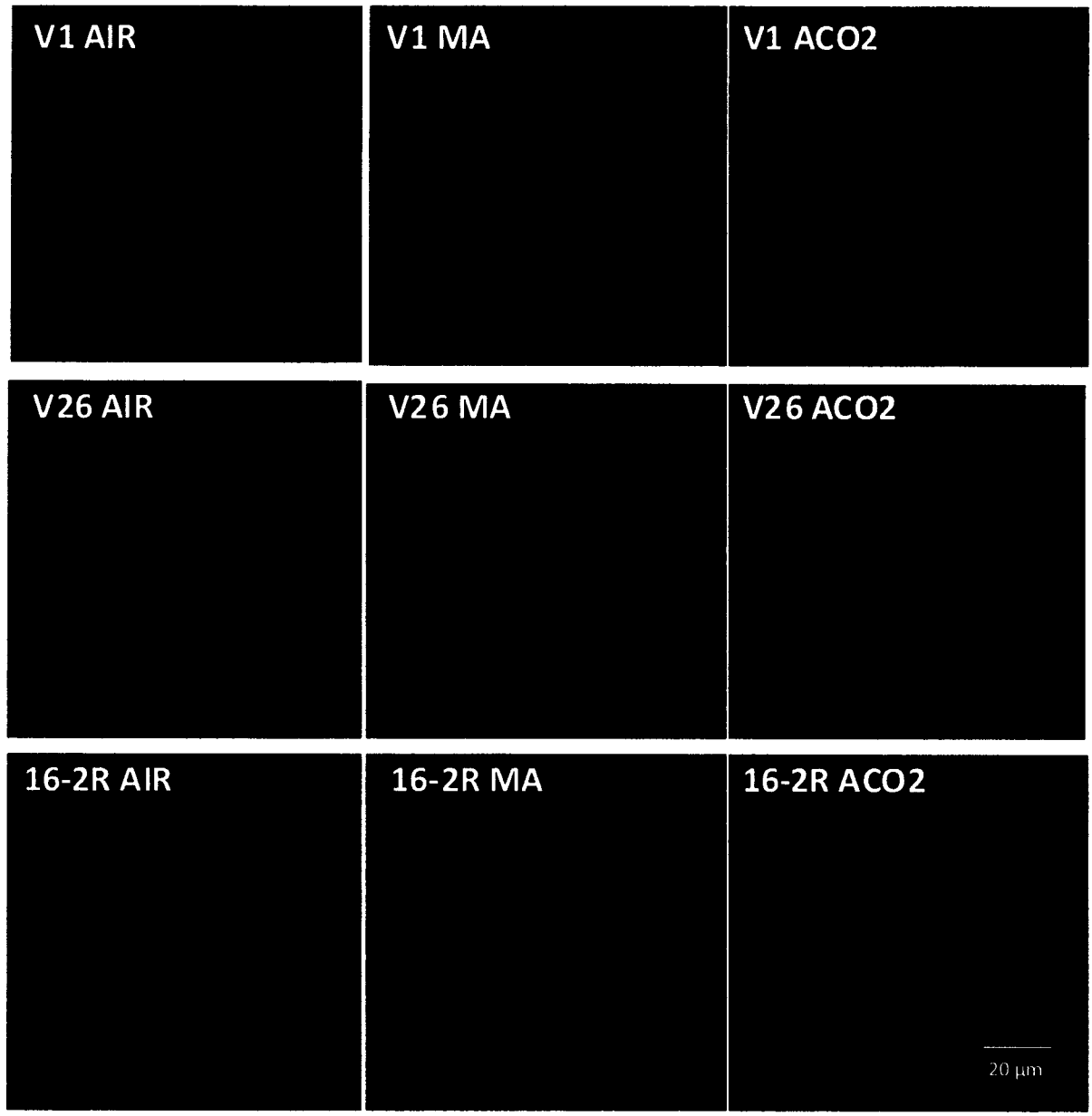

Figure 2.4. Confocal images of *C. jejuni* biofilm cells killed with 4% formaldehyde and stained with propidium iodide. AIR – ambient room air, MA – microaerobic, ACO2 – 5% CO_2 enhanced air.

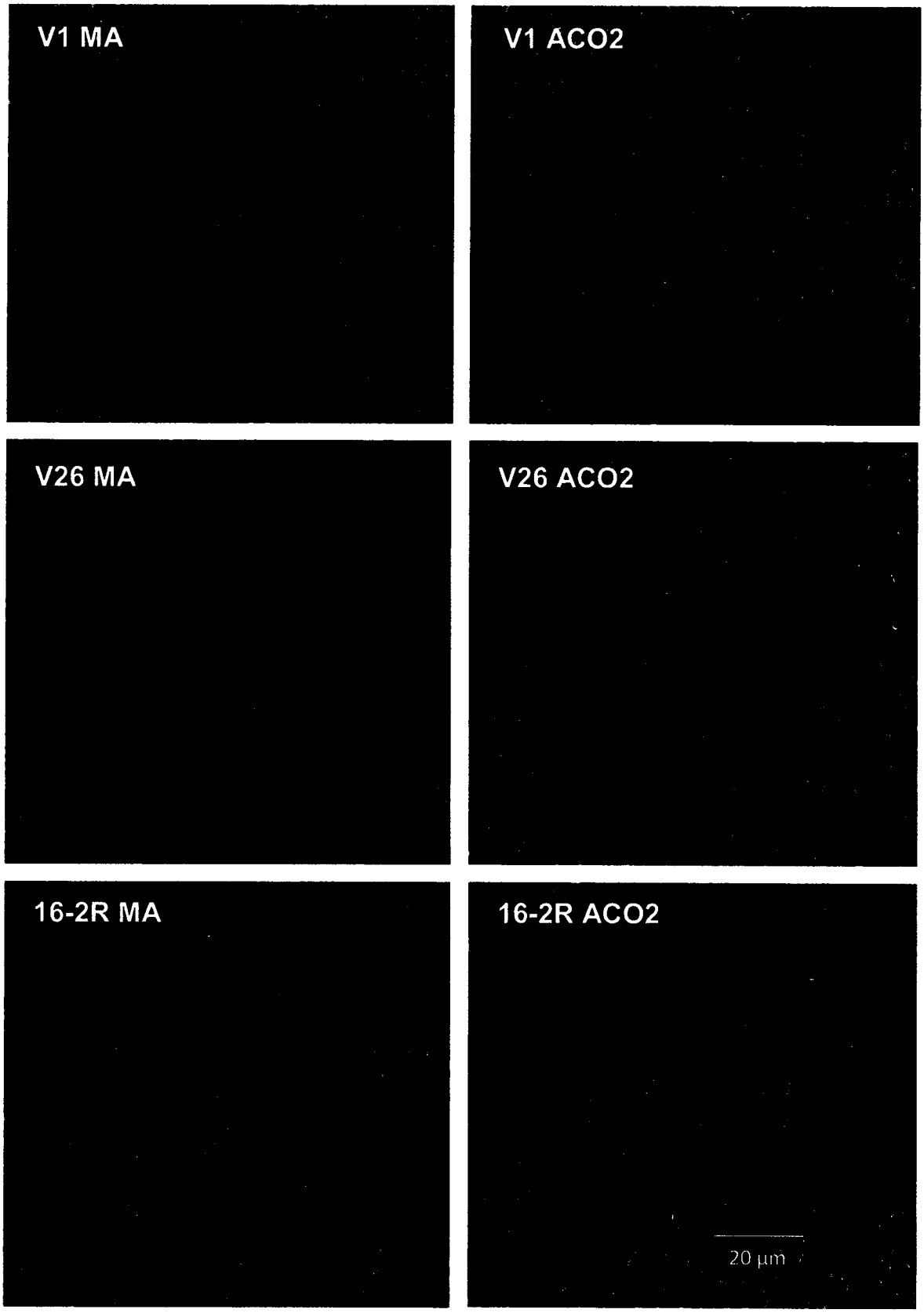

Figure 2.5. Confocal images of *C. jejuni* bioflm cells stained with LIVE /DEAD® *Bac*Light™. MA – microaerobic, ACO2 – 5% CO_2 enhanced air.

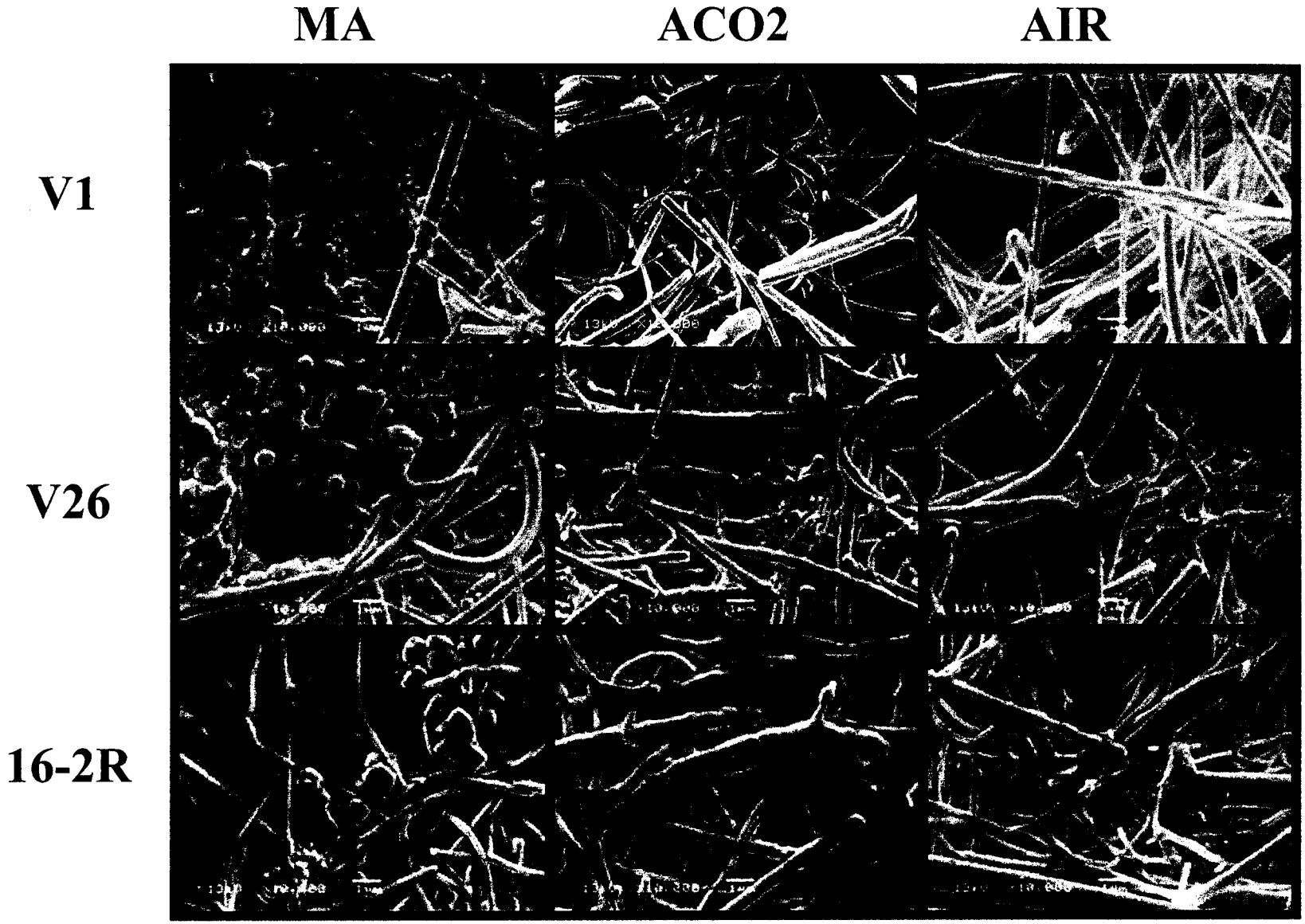

Figure 2.6. SEM visualization of three strains of *C. jejuni* biofilms in three conditions. MA-microaerobic, ACO2 - 5% CO_2 enhanced air, AIR – 100% air; V1:*C. jejuni* 11168 V1, V26: *C. jejuni* 11168 V26, 16-2R – *C. jejuni* 16-2R.

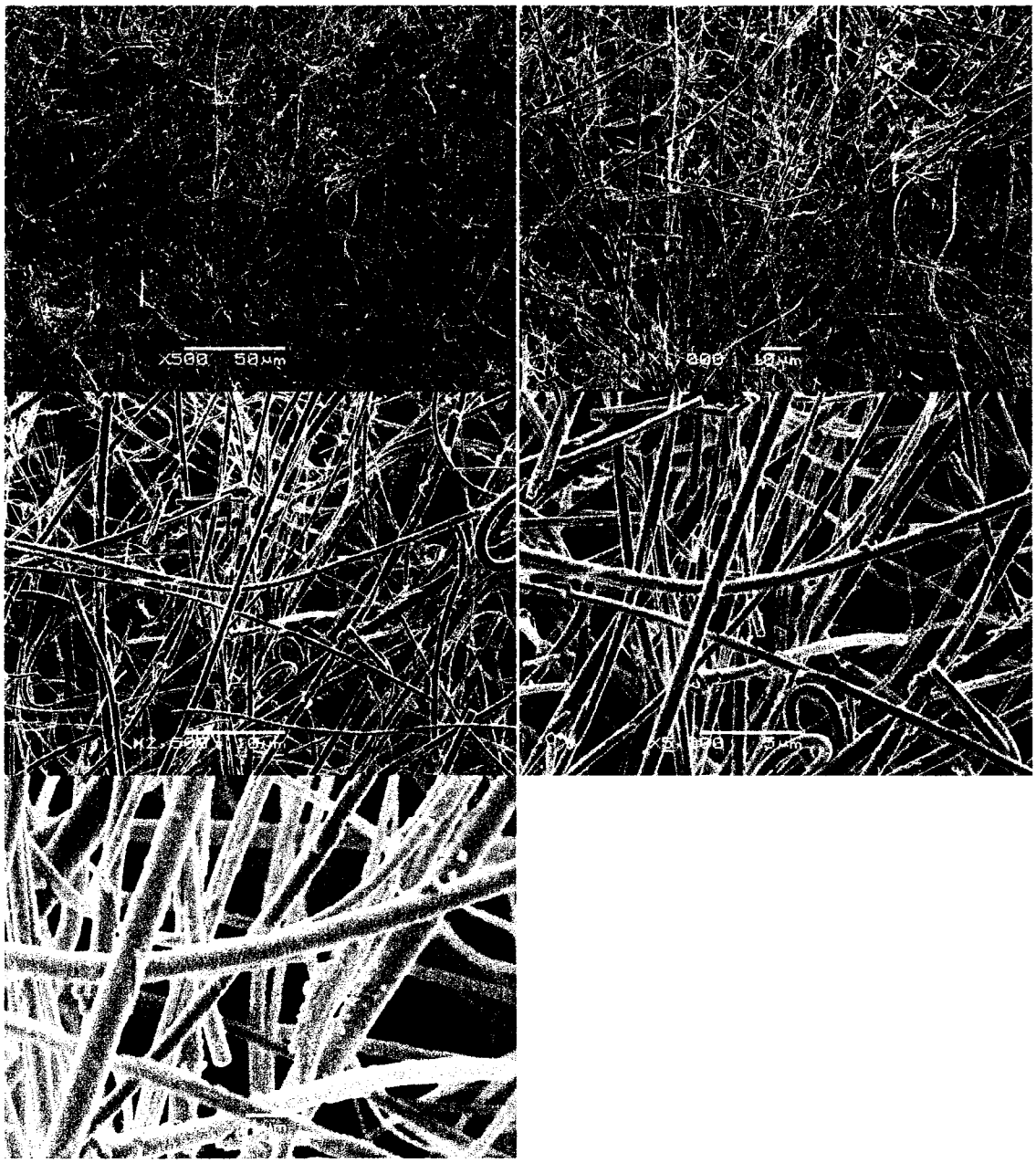

Figure 2.7. SEM image of the glass fibre filter control (smooth side) Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

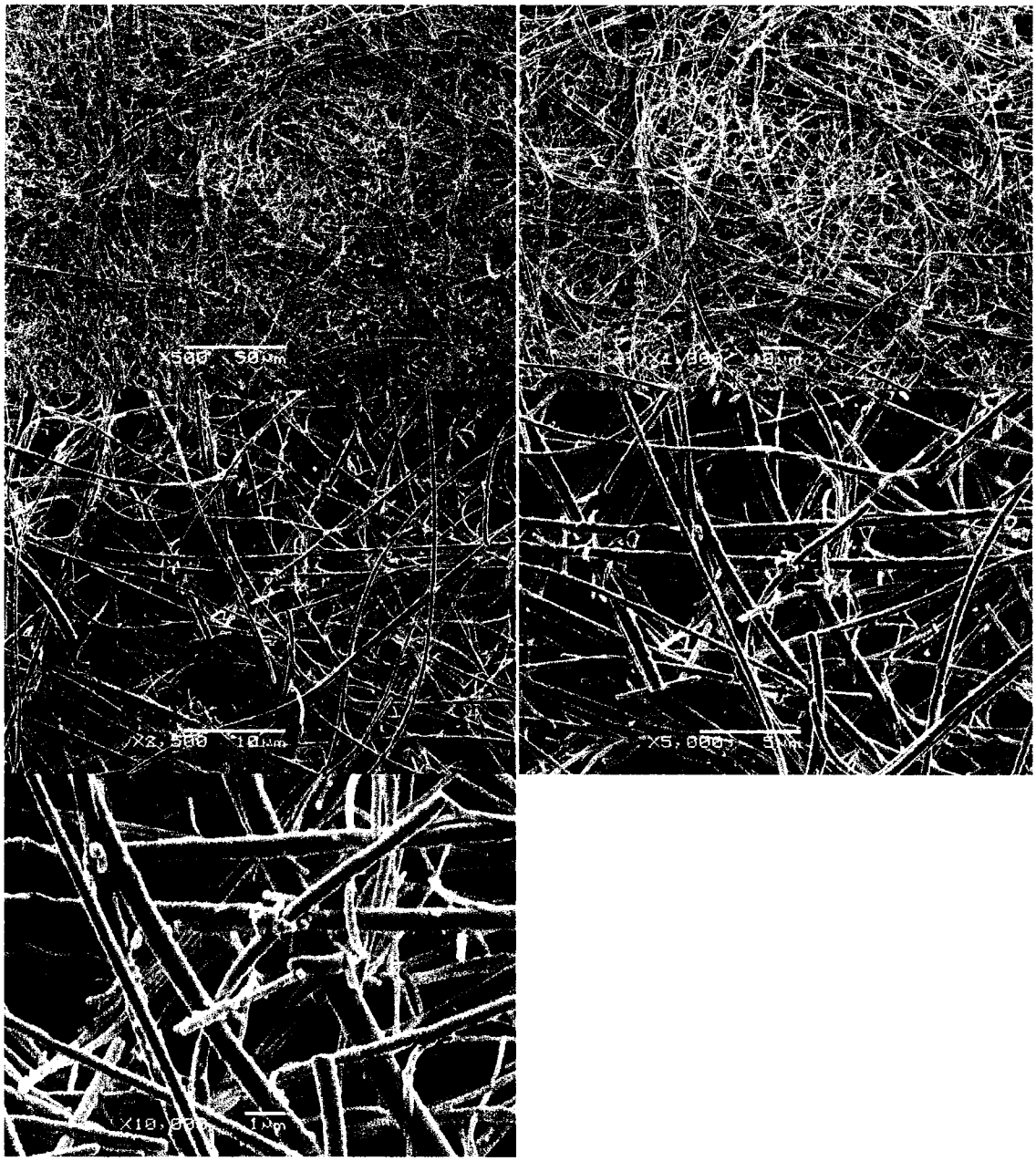

Figure 2.8. SEM image of the glass fibre filter control (rough side) Images are taken from the same field of view at increasing magnifications of 500x 1000x, 2500x, 5000x and 10000x.

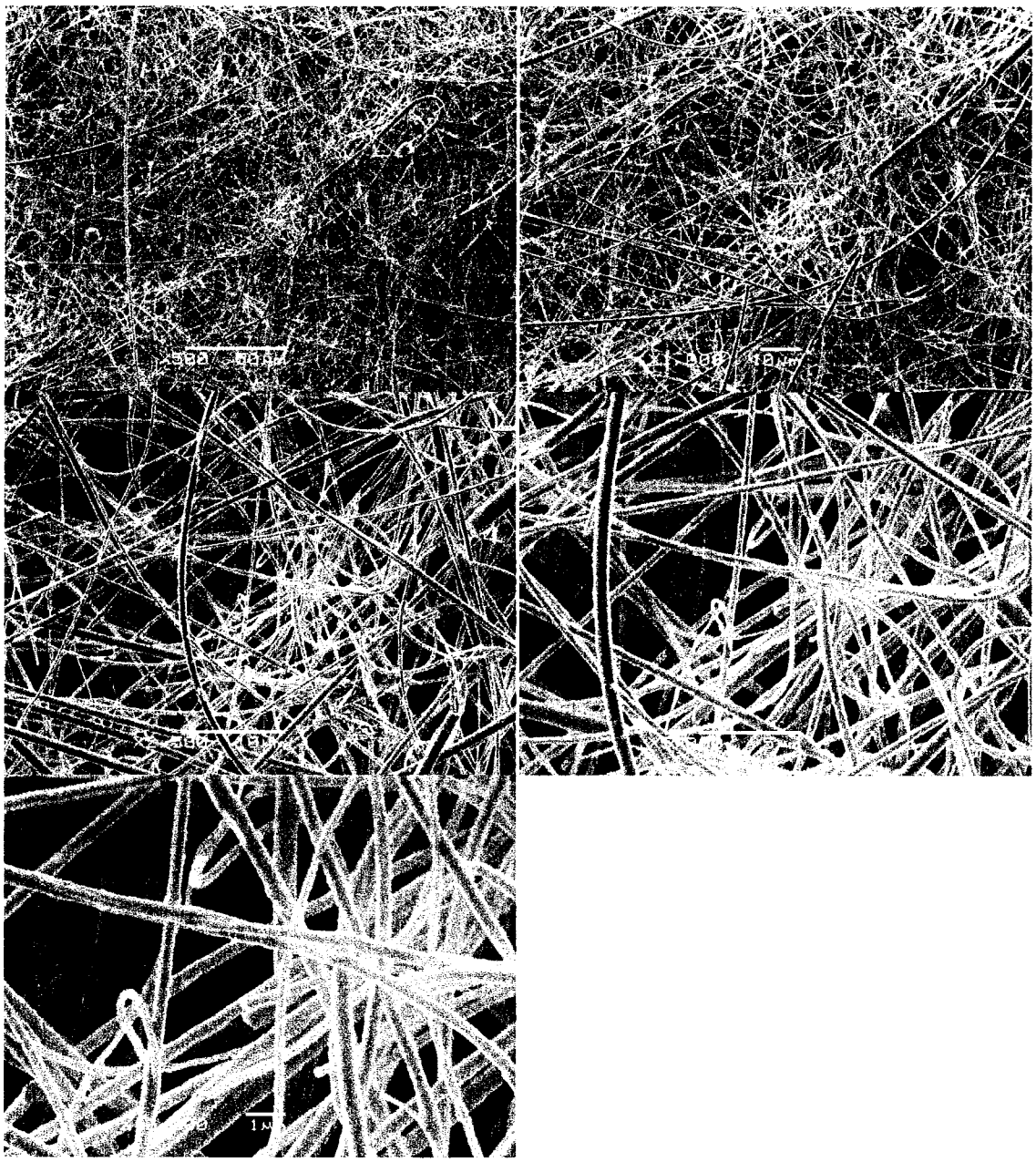

Figure 2.9. SEM images of *C. jejuni* V1 AIR. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

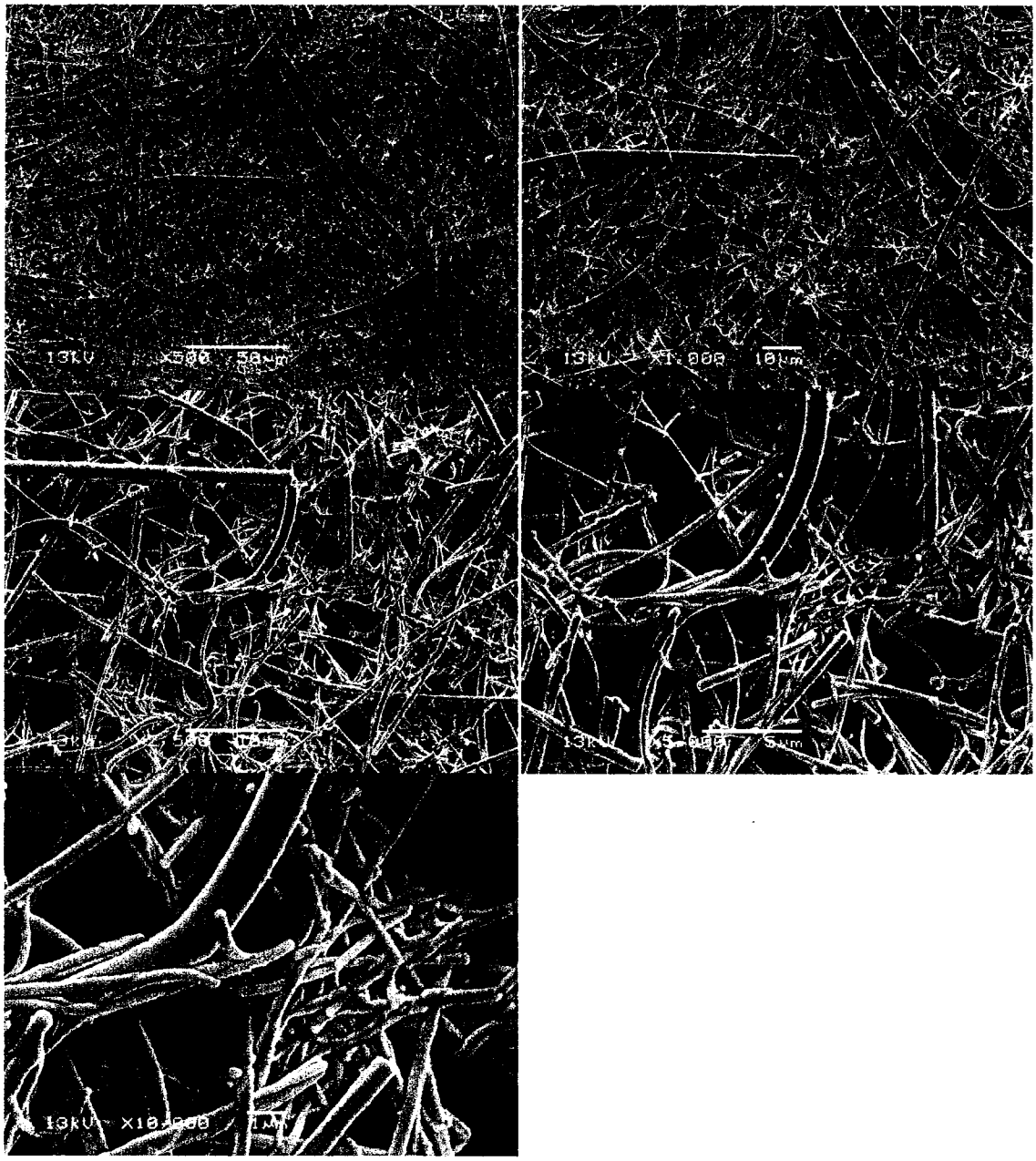

Figure 2.10. SEM images of *C. jejuni* V26 AIR. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

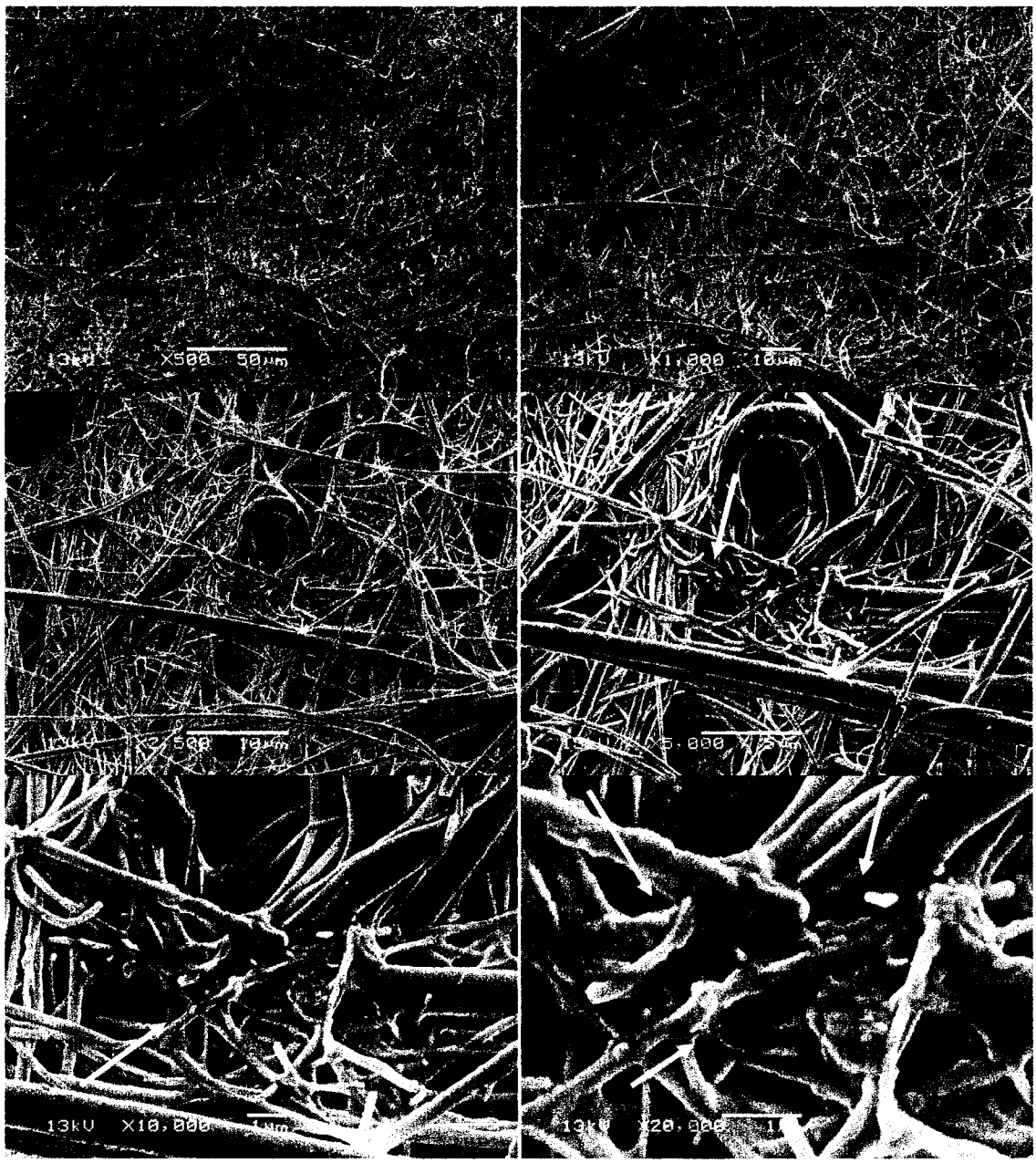

Figure 2.11. SEM images of *C.jejuni* 16-2R AIR . Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x, 10000x and 20000x. Arrows indicate areas with EPS.

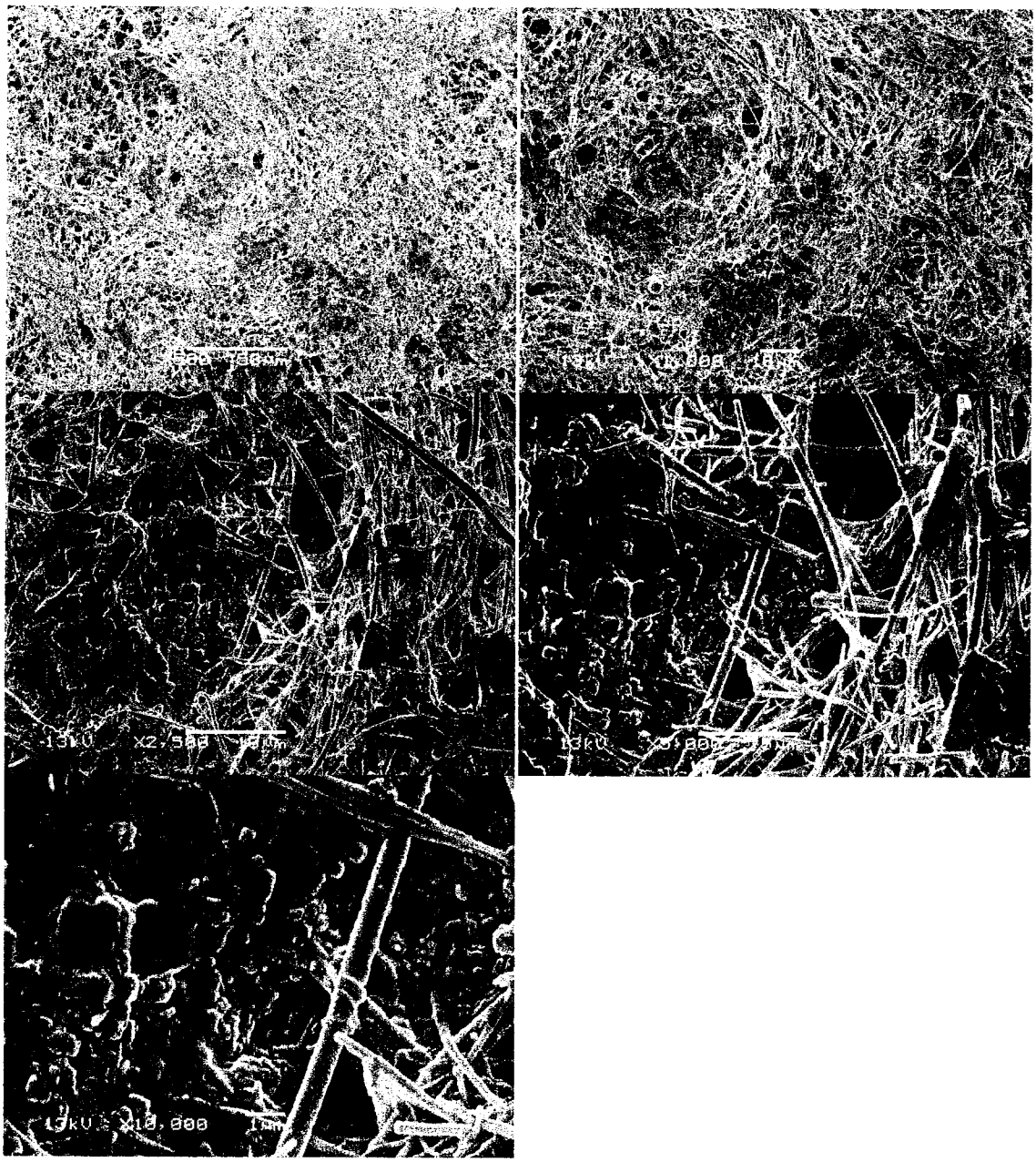

Figure 2.12. SEM images of *C. jejuni* V1 MA. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

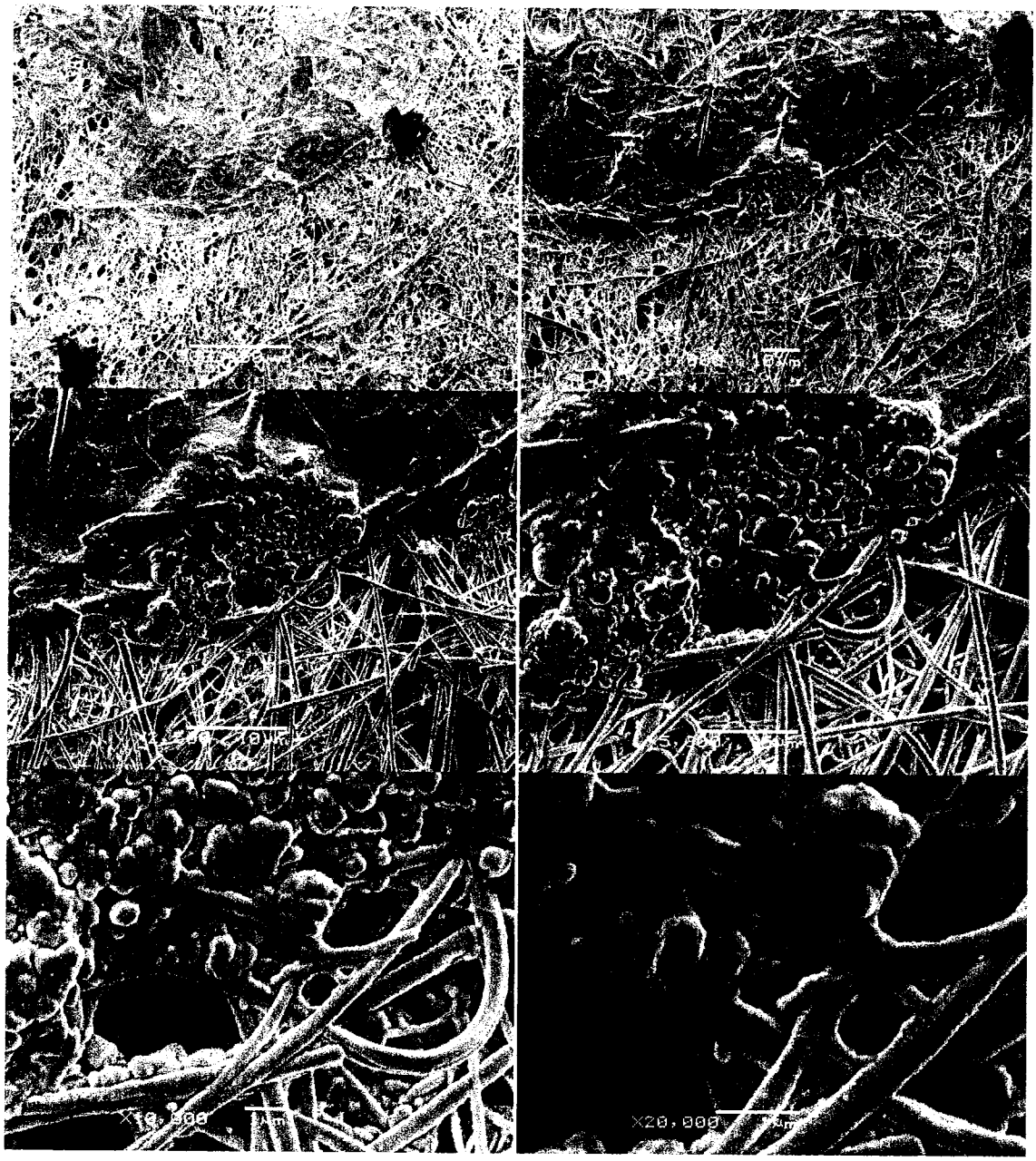

Figure 2.13. SEM images of *C. jejuni* V26 MA. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x, 10000x and 20000x.

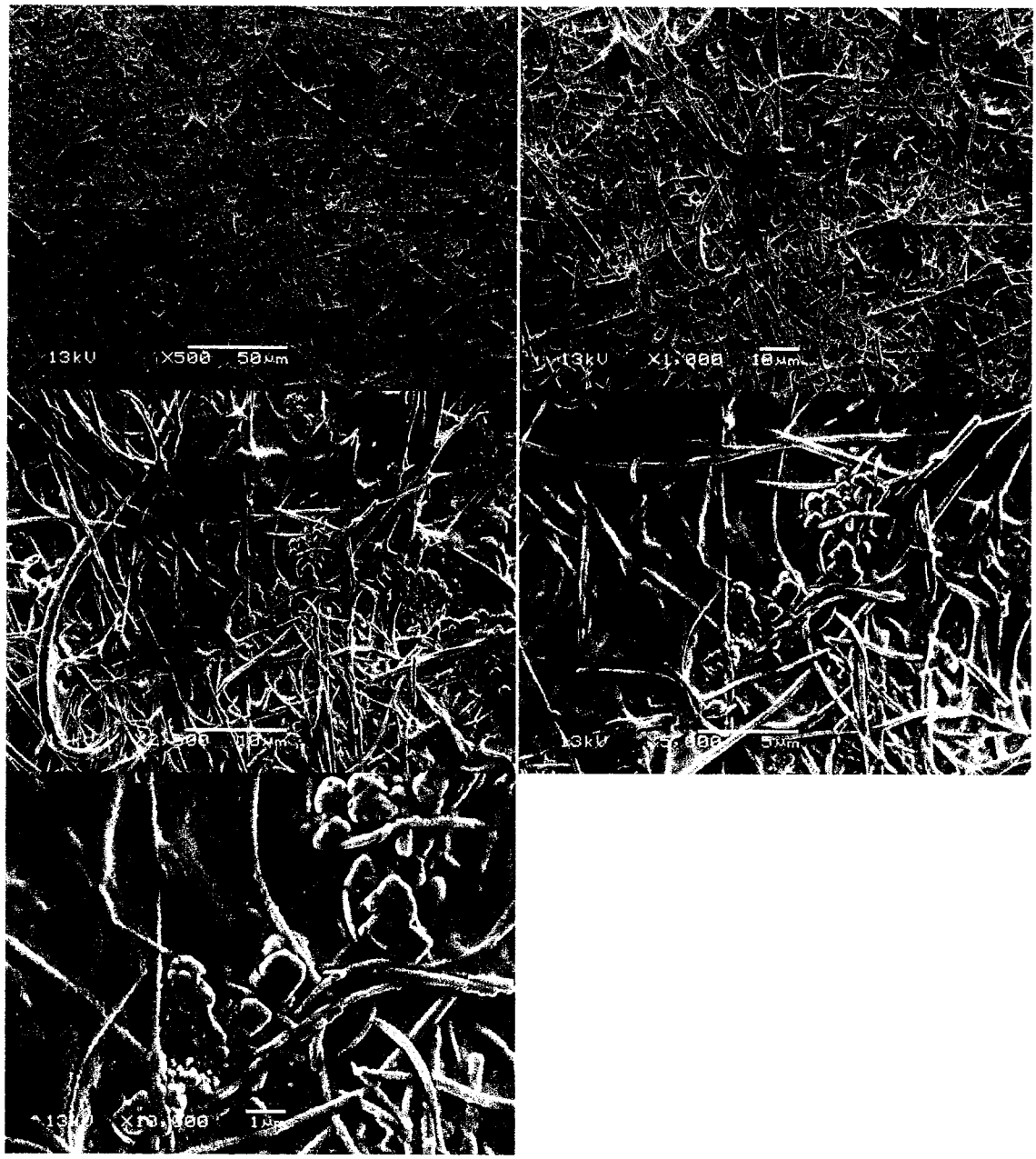

Figure 2.14. SEM images of *C. jejuni* 16-2R MA. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

Figure 2.15. SEM images of *C. jejuni* V1 ACO2. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x, 10000x and 20000x.

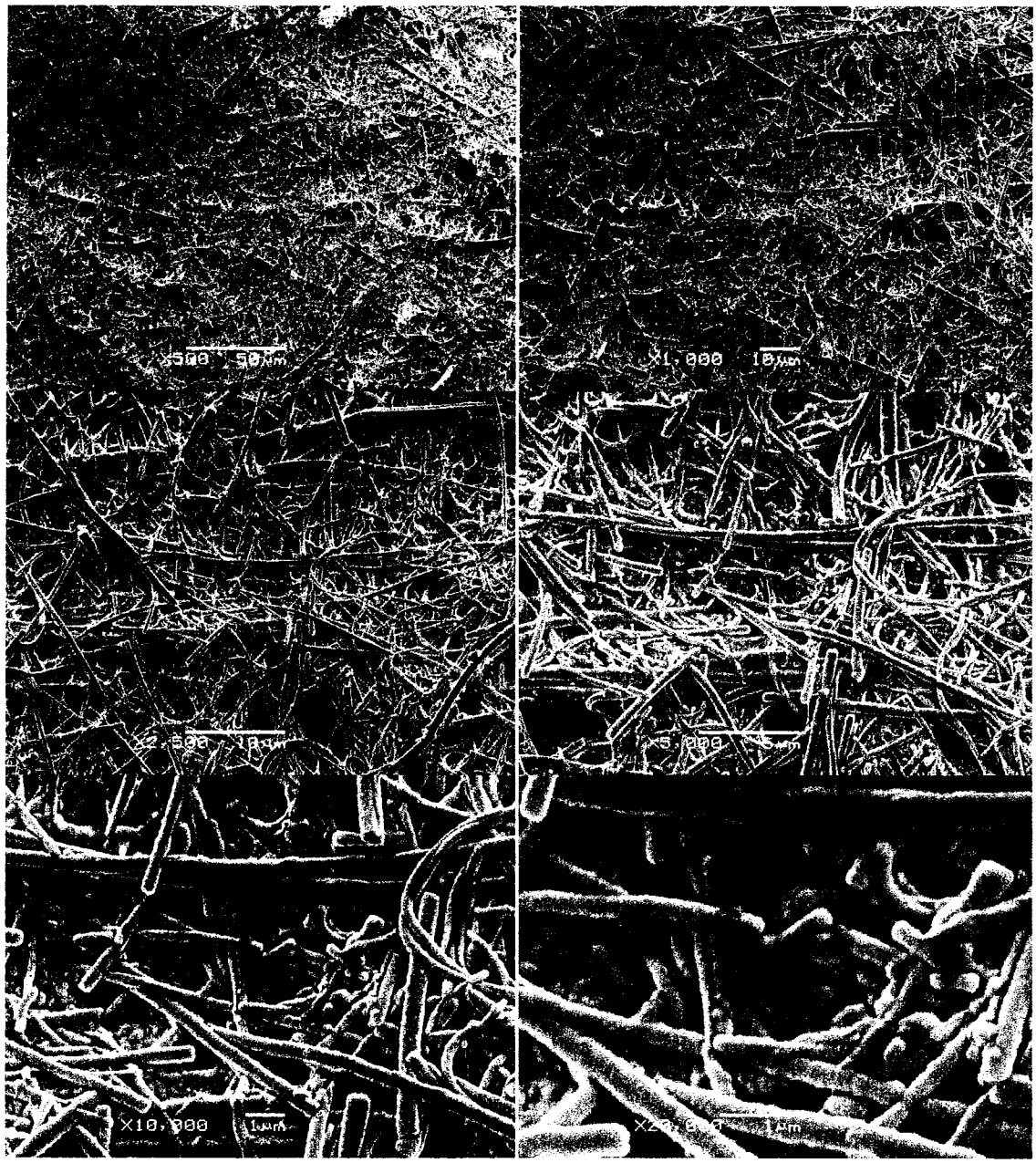

Figure 2.16. SEM images of *C. jejuni* V26 ACO2. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x, 10000x and 20000x.

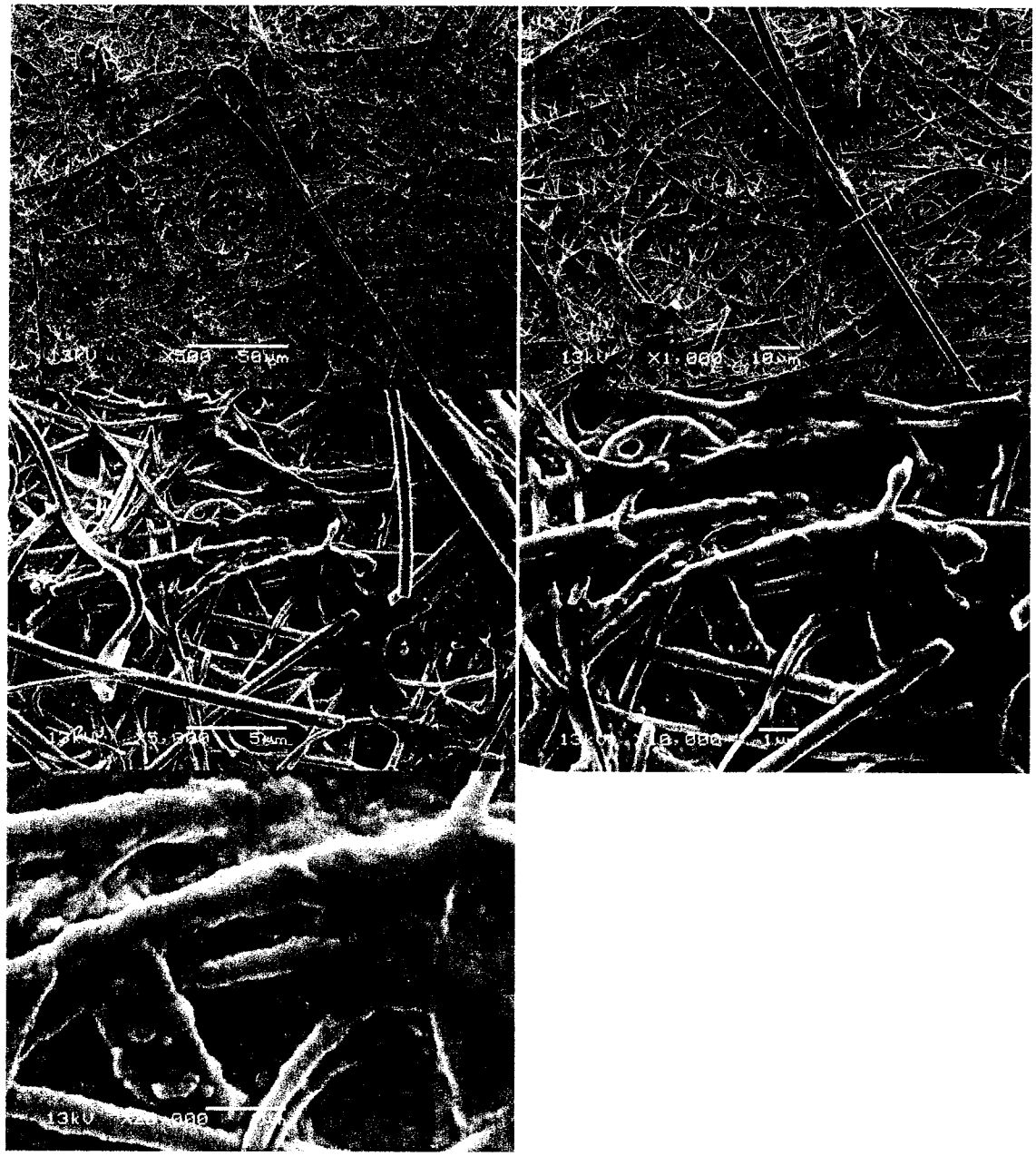

Figure 2.17. SEM images of *C. jejuni* 16-2R ACO2. Images are taken from the same field of view at increasing magnifications of 500x, 1000x, 2500x, 5000x and 10000x.

3 Investigation of oxidative stress tolerance and *in vivo* ROS levels for three strains of *Campylobacter jejuni* biofilm cells grown in three atmospheric conditions on glass fibres

3.1 Introduction

The microaerobic nature of the foodborne pathogen *Campylobacter jejuni* implies that it is sensitive to oxidative stress. Oxidative stress results when the level of reactive oxygen species (ROS) within the cell, exceeds the cell's capacity to neutralize these harmful oxygen species eventually leading to cell death. ROS includes the oxygen radicals: superoxide $(O_2\cdot)^-$, hydroxyl (OH·), peroxyl ($RO_2\cdot$), and alkoxyl (RO·) as well as certain nonradicals that are either oxidizing agents or easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite (ONOO–), singlet oxygen ($^1O_2$), and H_2O_2(Guetens et al 2002). It is known that H_2O_2 and $(O_2\cdot)^-$ are much less reactive and hence less damaging than OH·, but H_2O_2 can be converted to the highly reactive OH· via the Fenton reaction, thereby accelerating cell damage due to oxidative stress. ROS are capable of causing DNA damage, lipid peroxidation, and protein oxidation (Guetens et al 2002).

Campylobacter jejuni has a complex, highly branched respiratory chain which allows both aerobic and anaerobic respiration with a variety of alternative electron acceptors. *C. jejuni* would encounter ROS as a result of aerobic respiration or during host infection where H_2O_2 is produced by the mononuclear phagocyte system as part of host defense (Garenaux 2008, Yamasaki 2004).

Although *C. jejuni* has been shown to be limited in terms of oxidative stress defenses as compared to other enteric pathogens (Parkhill et al 2000), these bacteria possess the enzyme catalase (KatA) which catalyses the decomposition of H_2O_2 to O_2 and water (Purdy et al 1999).

Mutant studies have shown a role for KatA in H_2O_2 resistance and intramacrophage survival (Day et al 2000, Grant and Park 1995, Wassenaar 1997). Other genes involved in oxidative stress tolerance include superoxide dismutase (SodB) which catalyses the dismutation of the superoxide radical to hydrogen peroxide and oxygen; the iron-regulated alkyl hydroperoxide reductase (AhpC) which can destroy toxic hydroperoxide intermediates and repair molecules which have been peroxidized and the constitutively expressed *dps* gene which binds iron and may prevent formation of the hydroxyl radical via the Fenton reaction (Ishikawa 2003).

It has also been shown that biofilms confer increased stress tolerance to *C. jejuni* and recent studies suggest that they may also contribute to *in vivo* survival (Joshua et al 2006, Svensson et al 2008). Gene expression studies have indicated an overlap between expression in biofilm or sessile forms with colonization (Svensson et al 2008). Differences in gene expression when comparing biofilm or sessile with planktonic growth include downregulation of genes related to metabolism and upregulation of genes related to stress tolerance and uptake (Sampathkumar et al 2006). DeBeer et al (1994) measured oxygen content in various regions of a biofilm and found high levels in the water channels, but very low oxygen levels at the centre of the cellular microcolonies. They suggested that upregulation of oxidative stress genes in biofilms may be due to iron starvation (DeBeer et al 1994). The regulation of iron homeostasis and oxidative stress are both regulated by PerR in *C. jejuni*. Conditions with low levels of iron would signal cells to uptake iron leading to transiently high levels. This could lead to the formation of ROS via the Fenton reaction and hence oxidative stress.

Although one study found that *C. jejuni* biofilm cells were able to survive in conditions of aerobic stress for 24 days as compared to their planktonic counterparts, which only survived 12

days (Joshua et al 2006), most studies to date have investigated the effects of oxidative or aerobic stress on planktonic cells. Boysen et al (2007) who compared the survival of 6 strains of planktonic *C. jejuni* in three atmospheres found that all strains showed the greatest reduction in the atmosphere containing oxygen, confirming that these strains exhibit sensitivity to aerobic stress. Various factors were shown to be involved in oxygen tolerance for planktonic *C. jejuni*. Garenaux et al (2008) reported that for 13 strains of planktonic *C. jejuni*, oxygen sensitivity was temperature dependant and showed significant strain variation. Kaakoush et al (2007) examined the oxygen requirement and tolerance for 4 strains of *C. jejuni* by incubating planktonic cells in anoxic, microoxic and oxic atmospheres enriched with 10% CO_2. Oxygen tolerance was dependant on cell densities and exhibited strain variation (Kaakoush et al 2007). Vehoeff-Bakkenes et al (2008) found that planktonic *C. jejuni* NCTC 11168 was able to grow in a wide range of dissolved oxygen tensions (0.1 to 90%) when pyruvate was present in the growth media. Klançnik et al (2008) reported that starvation did not improve resistance to oxidative (10 minutes of exposure to 3mM H_2O_2) or aerobic stress (prolonged exposure to atmospheric oxygen concentrations) for planktonic *C. jejuni* K49/4C.

Gene expression and mutation studies have identified genes that may play a role in oxidative stress tolerance. Baillon et al (1999) found that planktonic *C. jejuni* 81116 mutants for alkyl hydroperoxide reductase (aphC) had less resistance to aerobic stress, but not to H_2O_2 than the wild type. Yamasaki et al (2004) identified an oxidative stress-sensitive protein (RrC) in the human clinical isolate *C. jejuni* 9131, which showed similar sensitivity to both H_2O_2 treatment and exposure to atmospheric oxygen. They speculated that the protein plays a role in oxidative stress protection based on iron-related oxidation-reduction reactions (Yamasaki et al 2004).

Barnes et al (2007) found that for planktonic *C.jejuni* 81116, gamma-glutamyl transpeptidase (GGT) mutants had increased resistance to H_2O_2 stress. Gamma-glutamyl transpeptidase plays a major role in the degradation of the antioxidant glutathione and loss of GGT has been shown to lead to increased levels of glutathione (Nakayama et al 1994). Bingham-Ramos et al (2008) examined the response of planktonic mutants for two putative cytochrome C peroxidases and found that neither contributed to H_2O_2 resistance which they found to be mainly attributed to the activity of catalase.

The present study investigated the tolerance of three strains of *C. jejuni* biofilm cells grown in microaerobic and CO_2 enhanced atmospheres to the exogenous oxidant hydrogen peroxide. Levels of reactive oxygen species were also measured for each strain and each condition.

3.2 Materials and Methods

3.2.1 Exposure of *Campylobacter jejuni* biofilm cells to 0.5mM hydrogen peroxide

C. jejuni biofilm cells from the microaerobic (MA) and CO_2 enhanced (ACO2) conditions were grown and collected as described in sections 2.2.1.-2.2.3. and then exposed to 0.5mM hydrogen peroxide in a manner similar to Barnes et al (2007). Biofilm cells from the microaerobic and CO_2 enhanced conditions were collected in 10 ml of PBS. An aliquot (0.5 ml) of each suspension was mixed with 1mM hydrogen peroxide to a final concentration of 0.5mM. Controls were mixed with PBS to maintain an equivalent dilution. All samples were maintained microaerobically at 42°C during the exposure period (up to 60 min.) and 100µl aliquots were removed and plate counts performed at as soon as possible after the treatment (~5 min.) and

then every 20 minutes up to 60 minutes (T0, T20, T40 and T60 respectively). The 120 minute time point was initially included, but plate counts were consistently below detection limits and it was eliminated from future trials. Biofilm cells from the aerobic condition were not included in the H_2O_2 tolerance assay due to low plate counts in the initial trials.

3.2.2 Detection and quantification of reactive oxygen species (ROS) by DCFH-DA

Reactive oxygen species were detected and quantified using DCFH-DA (*Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit*, Molecular Probes). *C. jejuni* biofilm cells from the microaerobic (MA) and CO_2 enhanced (ACO2) conditions were grown and collected as described in sections 2.2.1.-2.2.3. For the DCFH-DA assay, biofilm cells were removed by washing and vortexing the glass fibre filters in 0.85% NaCl as was suggested by the manufacturer. Cells were pelleted by centrifuging 2ml of each suspension at 3000xg for 10 minutes at 4°C. Pellets were resuspended in 400 µl of 0.85% NaCl and exposed to 100 µl of working solution (final concentration 5 µM) of the oxidative stress-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 minutes microaerobically at 42°C. All samples were handled to avoid light. Aliquots of each sample (20µl) were immediately loaded onto black 96 well plates (Costar 3915) and 0.085% NaCl was added to a final volume of 200 µl in each well. Fluorescence was measured (ex/em 485/520) on a microtitre plate reader (FLUOstar Optima, BMG Labtech). Gain was set at 90% and thus determined automatically by the instrument, based on a whole plate approach. Detection of ROS using the fluorescent probe DCFH-DA provided raw values for each sample. These values were normalized using total biovolume data for the same sample. Values for fluorescence per CFU were also calculated but were not used in the analysis as they did not include non-culturable cells which might be contributing to the level of ROS. Total

biovolume data was obtained as described in section 2.2.6, by staining each sample using the LIVE/DEAD® *Bac*Light™ assay prior to imaging on the CSLM. Biovolumes of healthy and membrane damaged biofilm cells were obtained using the image analysis software PHLIP as described in section 2.2.6. In order to obtain values that reflected the level of ROS/cell, each fluorescence value was divided by the biovolume value for the same sample. More specifically, values were arrived at by first taking the average of the fluorescence values (n=4) for each sample. This average fluorescence value (FU/ml) was then divided by the corresponding biovolume (μm^3/ml) for the same sample. These adjusted values then reflected the level of ROS per cell. Two separate experiments were done for detection and quantification of ROS levels in each strain and condition.

3.2.3 Statistical analysis

Data was analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). A one-way analysis of variance (ANOVA) with Tukey's post test was used to determine if there were significant changes over time for both controls and treated samples with significance level of 0.05. Mann Whitney tests were used to compare significant differences between treatments. Linear regression analyses were used to compare rates of reduction over time for each strain and condition. Analysis of the ROS data was done using T-tests in Microsoft Excel. When cell counts were below the detection limit (3.6 $\log_{10}$ CFU/g glass fibre), one half the detection limit value was used for plotting and statistical analyses (Clarke, 1998). Error bars represent standard deviation from the mean.

3.3 Results

3.3.1 Tolerance of *C. jejuni* biofilm cells to the exogenous oxidant hydrogen peroxide

Campylobacter jejuni biofilm cells from the MA condition showed a greater reduction in survival during 60 minutes of exposure to 0.5 mM hydrogen peroxide than those from the ACO2 condition (Fig.3.1 to Fig. 3.3). However, these differences were only significant in certain cases and also subject to strain variation (Table 3.2). Control values showed no significant changes over the 60 minutes duration of the experiment (Table 3.2 ANOVA $p>0.05$).

Tolerance to exogenous oxidative stress was significantly greater for *C. jejuni* 16-2R cells from the MA condition with a 4.57 ± 1.11 log reduction over 60 minutes, than for those from the ACO2 condition with a 2.70 ± 1.49 log reduction ($p<0.05$). Although total reductions in culturability for V1 MA (1.97 ± 0.06 $\log_{10}$CFU/g) were greater than those for V1 ACO2 (1.72 ± 0.38 $\log_{10}$CFU/g), this difference was not statistically significant. The total log reductions over the duration of the experiment for V26 were also not significantly different between MA (4.38 ± 1.98) and ACO2 (4.26 ± 2.58).

The 16-2R biofilm cells showed the greatest difference between conditions with a 1.87 $\log_{10}$CFU/g greater reduction for cells from the microaerobic condition than those grown in the CO_2 enhanced condition. For V1 this difference was 0.24 and for V26, it was 0.12.

Values for survival showed a statistically significant drop below controls for V1 in both conditions at T20 and T60 ($p<0.05$). V26 values did not fall significantly below controls for either condition at T20 but showed significant differences for both conditions at T40 and T60 ($p<0.05$). Values for 16-2R treated cells were also significantly lower than corresponding control values at both T40 and T60 for both conditions ($p<0.05$).

Linear regression analyses indicated that for both V1 and V26, growth condition had no significant effect on subsequent tolerance to oxidative stress ($p>0.05$). However, for 16-2R the regression analysis comparing the rate of reduction for cells grown in MA ($R^2= 0.997$) with that of cells grown in ACO2 ($R^2=0.973$), was almost significant with a p value of 0.069. Also, T-tests showed that the control values for 16-2R MA and ACO2 at T0 were not significantly different ($p=0.83$) while the corresponding values for treated cells were almost significantly different ($p=0.08$) after approximately 5 minutes of exposure. By T20 treated values differed significantly between conditions ($p=0.02$). Comparing treated values with controls at T0 indicated that there was already a greater effect on cells from the MA condition ($p=0.09$) than those from ACO2 ($p=0.88$) within minutes of exposure.

Strain variation was also evident between V1, the original clinical isolate, and V26 the lab-passaged version of V1. V1 showed greater than twice the tolerance to exogenous oxidative stress than V26 for the MA condition with a reduction of 1.97 $\log_{10}$CFU/g, as compared to 4.38 $\log_{10}$CFU/g. This trend was also evident in the ACO2 condition with a log reduction for V1 of 1.72 $\log_{10}$CFU/g, compared to 4.26 $\log_{10}$CFU/g for V26.

3.3.2 Evaluation of ROS levels in *C. jejuni* biofilm cells grown in 3 atmospheric conditions

Figure 3.4 shows both the biovolume values from the LIVE/DEAD® *Bac*Light™ assay and the values for ROS levels in biofilm cells for each strain and condition which have been normalized using the corresponding biovolume values. Biovolumes are presented according to stain colour with green representing healthy cells and red representing membrane-damaged or dead cells.

The adjusted values for levels of ROS for strains V1 or V26 did not differ significantly between conditions for both of the trials done. For 16-2R, values were significantly lower in the ACO2 condition than in the MA condition for 1 of the 2 trials ($p<0.05$) and not significantly different in the other. ROS values for V1 MA ranged from 97 to 100% of corresponding values in ACO2. For V26 this range was from 75 to 96% and for 16-2R it was from 59 to 96%.

Trends for plate count results for each trial, presented in Table 3.1 indicate that total plate count values show similar trends to biovolumes for healthy cells. The one exception was for V1 MAR 25 which had unusually high plate count values for this experiment.

3.4 Discussion

Biofilm cells from both conditions for all three *C. jejuni* strains showed a reduction in survival with exposure to 0.5mM H_2O_2. Hydrogen peroxide causes damage to nucleic acids, protein oxidation and lipid peroxidation, all of which lead to reduced survival. The Fenton reaction converts H_2O_2 to the highly reactive hydroxyl radical which is even more destructive within cells. *C. jejuni* does possess mechanisms to deal with excess H_2O_2, and oxidative stress only occurs when the level of ROS within the cell exceeds the ability of the cells to neutralize the ROS. Yamasaki et al (2004) showed that exposure of planktonic *C. jejuni* 9131 to 0.2mM H_2O_2 had no significant effect on survival, but that at the concentration used in the present study (0.5mM), H_2O_2 caused a significant reduction in CFU counts within 20 minutes.

Biofilm cells from the ACO2 condition showed greater tolerance to treatment with H_2O_2 than their microaerobically grown counterparts for all 3 strains. Biofilm cells grown in the ACO2

condition, with the higher level of oxygen (19%) may already be expressing genes related to oxygen tolerance and/or those involved in protection from ROS and may respond more quickly to the added oxidative stress. The level of oxygen in the microaerobic condition is only 5% so it is likely that the biofilm cells from this condition are not expressing oxygen tolerance related genes to the same degree. In this case the addition of the exogenous oxidant H_2O_2 may cause an upregulation of these genes. Yamasaki et al (2004) found that planktonic cells (grown under microaerobic conditions in broth at 37°C) exposed to 0.5mM H_2O_2 had a greater than 3 log reduction in the first 20 minutes, but recovered to initial levels within 2 to 4 hours. They used scopoletin to measure the concentration of H_2O_2 in the culture and found that H_2O_2 was still detectable after 15 minutes (0.45mM). The significant recovery in the 0.5mM treatment after the initial drop was attributed to the recovery of unculturable cells, as *C. jejuni* is not capable of dividing quickly enough to account for the increased CFU/ml values obtained after 40 minutes. Since they originally grew the cells microaerobically, it is possible that the recovery could be attributed to upregulation of oxidative stress tolerance related genes.

Tolerance to H_2O_2 varied by strain. For both V1 and V26 the level of oxygen in the growth atmosphere had no significant effect on the subsequent tolerance to oxidative stress ($p<0.05$). However, 16-2R cells grown with higher levels of oxygen exhibited significantly greater tolerance to H_2O_2 ($p<0.05$).

Strain variation was also observed between the strains V1 and V26. V1 showed greater tolerance to oxidative stress with a 1.97 log reduction while V26 showed less tolerance with a 4.38 log reduction over the same period. The reduced tolerance of V26 could be due to changes in gene expression as a result of being subcultured repeatedly (Carrillo et al 2004).

Due to the automatic setting of the gain, values for ROS are not comparible between experiments or strains. In order to allow for comparisons, future experiments should be done using a set gain value. In spite of this limitation, it is still possible to analyze the trends in ROS values for each experiment. For both V1 and V26, the level of ROS did not differ significantly between conditions for both experiments. This is consistent with the results for the H_2O_2 tolerance assay, where prior exposure to oxygen had no significant effect on subsequent oxidative stress tolerance for either of these strains.

Results from one experiment for 16-2R indicated that the level of ROS was significantly lower for cells from the ACO2 condition than for those from the MA condition. This result is consistent with the results of the tolerance assay, where the tolerance to exogenous oxidative stress is significantly lower for cells from the MA condition than for those from ACO2 for 16-2R. It is possible that biofilm cells grown with the higher level of oxygen in the ACO2 condition are already expressing the genes for oxidative stress defense and are thus better able to reduce the level of ROS as compared to biofilm cells from the MA condition which were grown with lower levels of oxygen. The level of ROS in the second trial for 16-2R showed no significant difference between conditions.

C. jejuni NCTC 11168 V26 is a lab-passaged version of *C. jejuni* NCTC 11168 V1 and although the two strains have almost identical genomes, it has been shown that they have significantly different transcriptomes (Carrillo et al 2004). *C. jejuni* 16-2R is a poultry isolate and not directly related to V1 or V26. The phenotypic differences with respect to response to the exogenous oxidant H_2O_2 could be explained by differences in gene expression or actual genome differences for this strain.

In terms of strain origin, it appeared that for the meat isolate (16-2R) prior exposure to oxygen increased the tolerance to further oxidative stress, while for the clinical isolates, prior exposure showed no effect. The greatest tolerance to oxidative stress was observed for the V1 strain showing the lowest log reduction for biofilm cells grown in ACO2, over the 60 minutes of exposure (1.50). Kaakoush et al (2007) also found that the oxygen tolerance of the clinical isolate (11168) was greater than that of a poultry isolate (*C. jejuni* RM1221). An investigation by Gunther IV and Chen (2009) found that this poultry isolate, *C. jejuni* RM1221, was also less able to form biofilms on glass, stainless steel or plastic than *C. jejuni* 81-176. Garenaux et al (2008) found that sensitivity to oxidative stress was subject to strain variation. In their study, however, it was the poultry isolate, *C. jejuni* C356 that showed better survival to exogenous oxidative stress than reference strain 11168. A more comprehensive approach is needed to determine if there are any correlations between strain origin and response to oxidative stress.

This strain variation has implications for both the virulence and colonization potential of these bacteria. Strains with better tolerance to hydrogen peroxide will have better potential for both colonization and virulence as hydrogen peroxide is encountered in both of these situations. Studies have shown that V1 is more virulent and also better able to colonize the gastrointestinal tract than V26 (Carrillo et al 2004). The log reduction for *C. jejuni* 16-2R cells from the MA condition (4.57) was very similar to that for V26 (4.38), while the log reduction for 16-2R cells from the ACO2 condition (2.70) was more similar to that for V1 (1.97). These findings suggest that tolerance to oxidative stress varies among strains and could, for some strains be affected by prior growth conditions. Strain variation makes it difficult to generalize

about bacterial behavior at the species level and indicates a need for further investigation, characterizing phenotypes at the strain level.

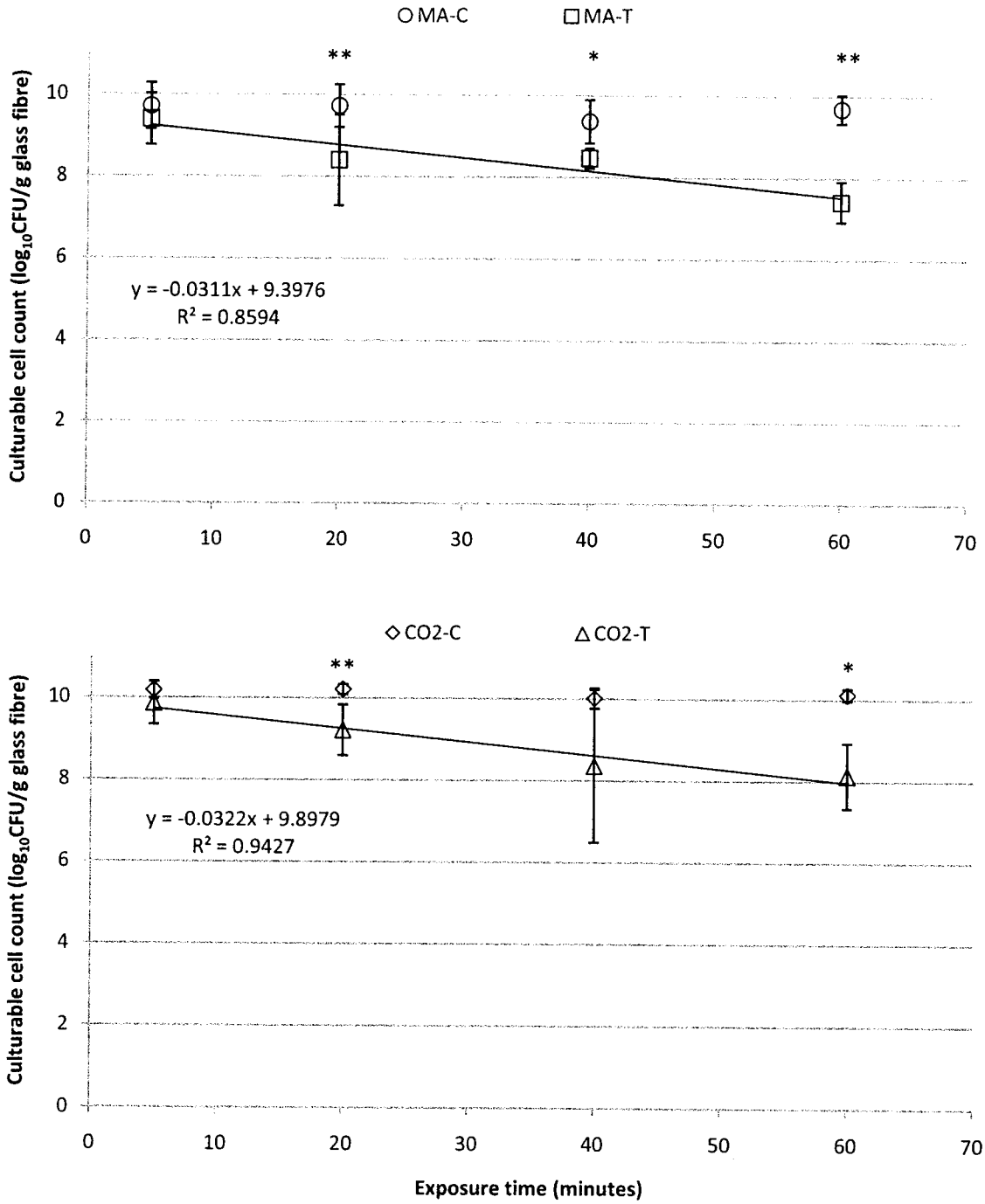

Figure 3.1. Hydrogen peroxide tolerance data for *C. jejuni* NCTC 11168 V1. Biofilm cells from the each condition were exposed to hydrogen peroxide at a final concentration of 0.5mM (treated) or PBS (control) and incubated at 42°C microaerobically. Plate counts were obtained at 20 minute intervals. C - control, T - treated. Error bars represent standard deviation from the mean. Values that are significantly different from control are indicated by : * $p<0.05$, ** $p<0.01$.

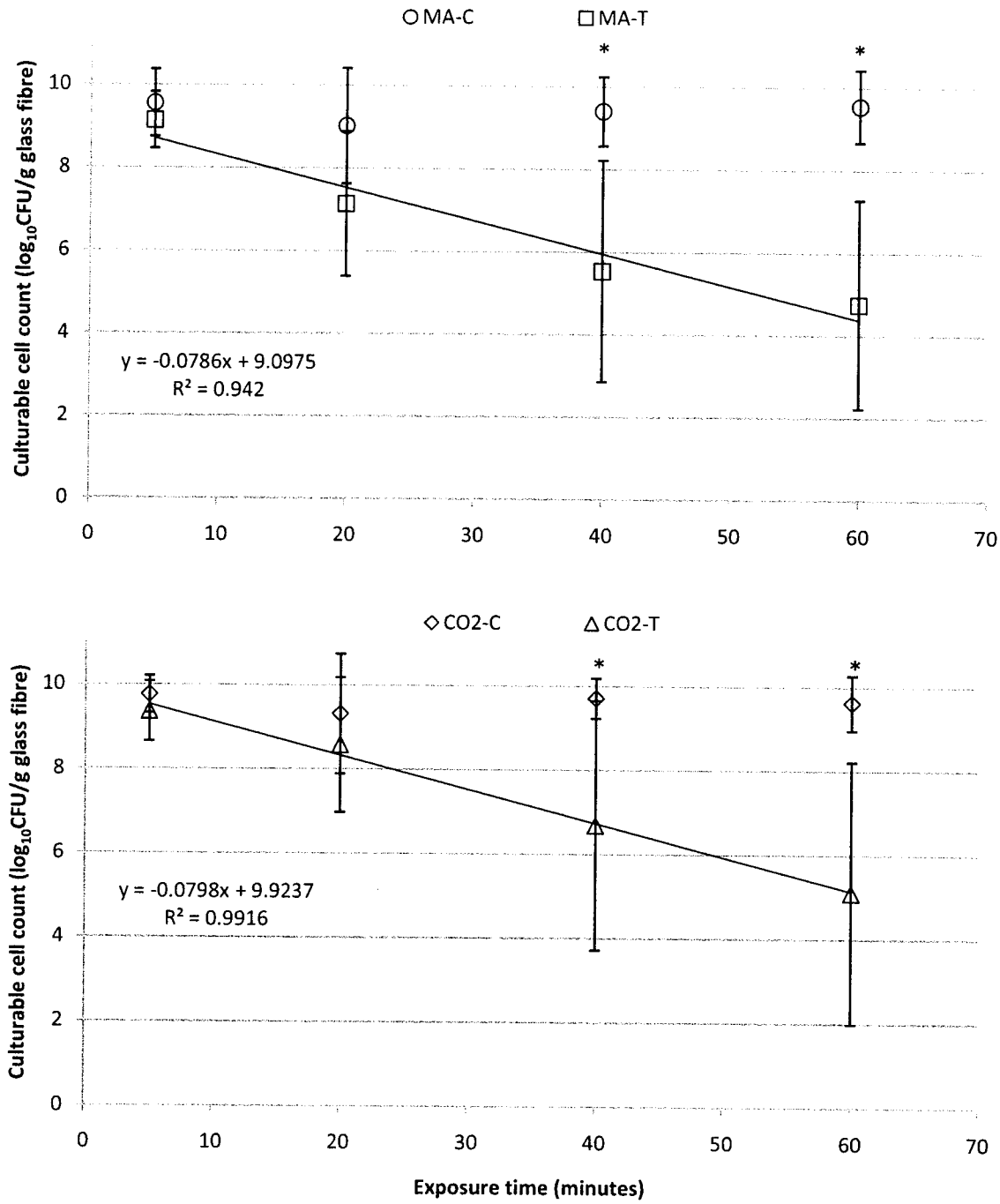

Figure 3.2. Hydrogen peroxide tolerance data for *C. jejuni* NCTC 11168 V26. Biofilm cells from each condition were exposed to hydrogen peroxide at a final concentration of 0.5mM (treated) or PBS (control) and incubated at 42°C microaerobically. Plate counts were obtained at 20 minute intervals. C - control, T - treated. Error bars represent standard deviation from the mean. Values that are significantly different from control are indicated by : * $p<0.05$, ** $p<0.01$

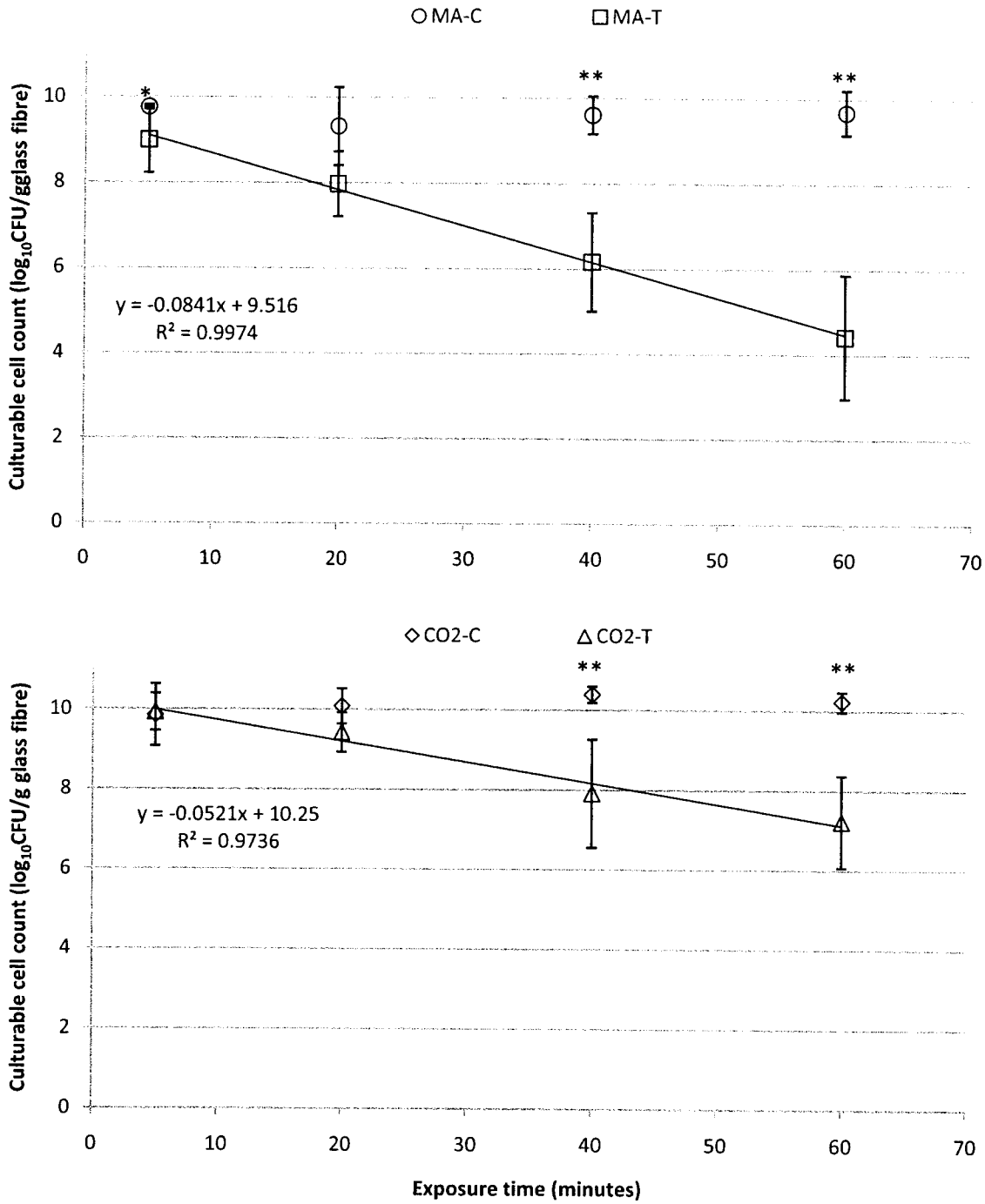

Figure 3.3. Hydrogen peroxide tolerance data for *C. jejuni* 16-2R. Biofilm cells from each condition were exposed to hydrogen peroxide at a final concentration of 0.5mM (treated) or PBS (control) and incubated at 42°C microaerobically. Plate counts were obtained at 20 minute intervals. C - control, T - treated. Error bars represent standard deviation from the mean. Values that are significantly different from control are indicated by : * $p<0.05$, ** $p<0.01$.

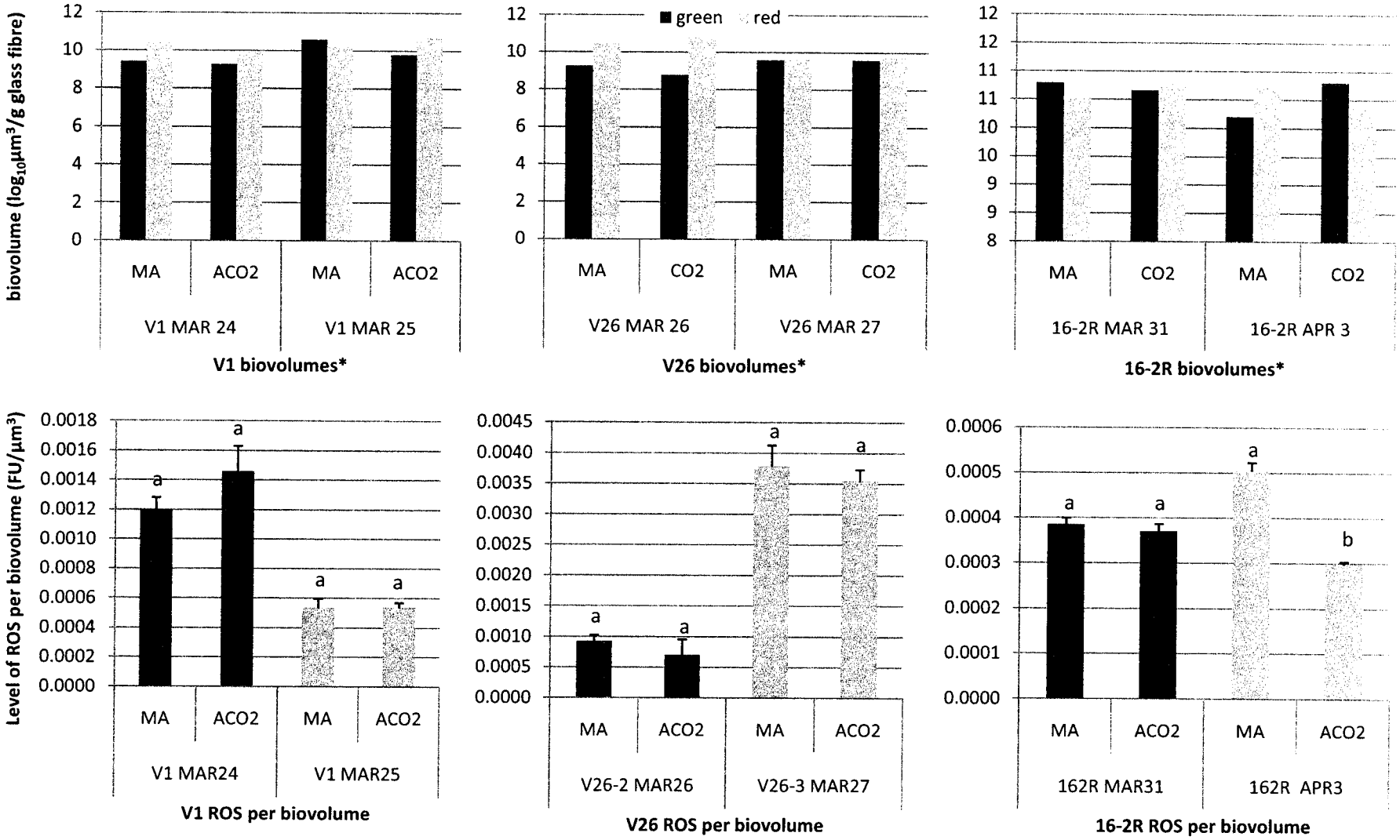

Figure 3.4. Levels of reactive oxygen species (ROS) in *C. jejuni* biofilm cells grown in microaerobic (MA) or 5%CO_2 enhanced conditions (ACO2) were adjusted using biovolume data for the same sample (shown above*). Biovolume values from the LIVE/DEAD° *Bac*Light™ assay are indicated by stain colour. For each experiment, small letters indicate significant differences between conditions ($p<0.05$).

Table 3.1. Plate count values corresponding to ROS values of *C. jejuni* biofilm cells grown in 2 conditions.

Strain	Trial	Condition	
		MA*	ACO2*
V1	MAR 24	10.20 ± 0.54	9.26 ± 0.82
	MAR 25	11.18 ± 0.54	11.18 ± 0.82
V26	MAR 26	9.66 ± 0.62	9.51 ± 0.46
	MAR 27	9.45 ± 0.62	9.48 ± 0.46
16-2R	MAR 31	10.52 ± 0.89	10.45 ± 0.84
	APR 3	10.35 ± 0.89	10.95 ± 0.84

* all values given in $\log_{10}$ CFU/g glass fibre, MA – microaerobic, ACO2 – 5% CO_2 enhanced aerobic.

Table 3.2 Significance values for H_2O_2 treated samples compared to corresponding control values* (Fig.3.1 – 3.3).

Treatment	Strain		
	V1	V26	16-2R
MA-0	ns (p=0.2086)	ns (p=0.1775)	* (p=0.0284)
MA-20	** (p=0.0081)	ns (p=0.1143)	ns (p=0.1143)
MA-40	* (p=0.0299)	* (p=0.0381)	** (p=0.0025)
MA-60	** (p=0.0061)	* (p=0.0167)	** (p=0.0010)
CO2-0	ns (p=0.1994)	ns (p=0.6095)	ns (p=0.8846)
CO2-20	** (p=0.0043)	ns (p=0.3810)	ns (p=0.1143)
CO2-40	ns (p=0.1143)	* (p=0.0167)	* (p=0.0286)
CO2-60	* (p=0.0238)	* (p=0.0333)	* (p=0.0286)

Significance values calculated using GraphPad Prism 5 - Mann Whitney tests. * p<0.05, ** p<0.01, ns=not significant

4 Conclusions and proposed future studies

In conclusion, the three strains of *C. jejuni* used in this study, readily formed biofilms in both the microaerobic (MA) and the CO_2 enhanced aerobic (ACO2) conditions, but only very poorly in the straight aerobic (AIR) condition. Tolerance to exogenous oxidative stress was significantly greater for biofilm cells grown with higher levels of oxygen for one strain, *C. jejuni* 16-2R, but not for V1 or V26. Also, V1 showed more than twice the resistance to H_2O_2 than V26, indicating that strain variation plays a role in tolerance to oxidative stress.

The fact that biofilm formation was just as prevalent in ACO2 as in MA, but was significantly reduced in AIR, suggests that the increased level of CO_2 is involved in the ability of *C. jejuni* to alleviate oxidative stress, either by allowing for the use of alternative metabolic pathways which don't use oxygen and hence don't lead to the creation of ROS, or by aiding in the removal of the damaging ROS. Studies on the metabolism and physiology of *C. jejuni* have indicated roles for CO_2 including, carbon assimilation via the pyruvate ferredoxin oxidoreductase (PFOR) pathway, synthesis of amino acids via anapleurotic reactions and generation of ATP related to a reliance on phosphoenolpyruvate (PEP) carboxykinase (Kelly 2001, St. Maurice et al 2007). Future studies could investigate the role of these pathways on the growth and biofilm development of *C. jejuni* as well as determining minimum levels of CO_2 required for growth in aerobic atmospheres.

The increased tolerance to oxidative stress for 16-2R biofilm cells which had prior exposure to high levels of oxygen could be due to the up-regulation of genes involved in stress tolerance. A comparison of gene expression for 16-2R biofilm cells from aerobic and microaerobic

conditions indicated a significant up-regulation of the oxidative stress genes, *ahp*C, *fdx*A and *sod*B and a non-significant upregulation of *kat*A for cells from the aerobic condition as compared to those from the microaerobic condition (Brookes 2007). Gene expression studies for V1 and V26 have indicated significant differences in gene expression for these two strains when grown in microaerobic conditions at 37°C (Carrillo et al 2004). In their study, V26 showed reduced motility, host cell invasion and colonization as compared to V1 (Carrillo et al 2004). Analysis of the biovolume data from the *Bac*Light™ assay in the present study, indicated that the ratio of healthy cells for V26 did not vary significantly between the MA and ACO2 conditions and that this strain was less affected by condition than either V1 or 16-2R.

In order to have a more comprehensive picture of oxidative stress response in *C. jejuni*, future studies could determine the level of healthy cells in the AIR condition for all three strains, as well as investigating gene expression of biofilm cells from all three conditions for V1 and V26.

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