ELONGATION OF *ESCHERICHIA COLI* BY COLD OR CINNAMALDEHYDE EXPOSURE AND TRANSCRIPTOMIC CHANGES DURING CINNAMALDEHYDE DISSIMILATION

by

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THESIS FORMAT

The thesis has nine chapters of which, Chapters 3 to 7 are manuscripts at different stages of publication in peer-reviewed scientific journals. The style of each manuscript is modified for the purpose of standardized presentation of this thesis. An overall introduction to the study and a comprehensive review of literature are presented in Chapters 1 and 2, respectively. Chapter 8 provides an overall discussion to this study with suggestions for future studies. The overall conclusions are presented in Chapter 9 which is followed by bibliographical references.

Chapter 3 entitled "The viabilities of cells in cultures of *Escherichia coli* growing with formation of filaments at 6 °C" by J. Visvalingam, C.O. Gill, R.A. Holley, 2012 was originally published in the International Journal of Food Microbiology, 153, 129-134.

Chapter 4 entitled "Morphological and viability changes in *Escherichia coli* and *E. coli* O157:H7 cells upon rapid shift from 6 ºC to 37 °C" by J. Visvalingam, C.O. Gill, R.A. Holley, 2012 was originally submitted for publication in the journal Food Microbiology and was accepted for publication on 30/11/2012.

Chapter 5 entitled "Adherence of cold-adapted *Escherichia coli* O157:H7 to stainless steel and glass surfaces by J. Visvalingam, R.A. Holley, 2013 was originally published in the journal Food Control, 30, 575-579.

Chapter 6 entitled "Temperature-dependent effect of sub-lethal levels of cinnamaldehyde on viability and morphology of *Escherichia coli*" by J. Visvalingam, R.A. Holley, 2012 was originally published in the Journal of Applied Microbiology, 113, 591-600.

Chapter 7 entitled "Examination of the genome-wide transcriptional response of *Escherichia coli* O157:H7 to cinnamaldehyde exposure" by J. Visvalingam, J.D. Hernandez-Doria, R.A. Holley, 2012 was originally submitted for publication in the journal Applied and Environmental Microbiology and was accepted for publication on 20/11/2012.

Table of Contents

List of Tables

List of Figures

ABSTRACT

Refrigeration has been found to cause cell elongation in mesophilic enteric organisms like commensal *Escherichia coli* and *E. coli* O157:H7. As elongated cells may divide into multiple daughter cells, they may result in underestimation of pathogen numbers in foods when plate counts are used. When *E. coli* cultures were incubated at 6°C for ≤10 days, cells grew by elongation, did not divide, and lost viability (LIVE/DEAD vitality stain) at similar rates. Substantial fractions of cells in cultures elongating at 6°C were inactivated by an abrupt shift to 37°C. Direct microscopic observation of cells transferred to 37ºC after 5 days at 6°C showed that few or no cells of normal size (≤4μm) divided, while elongated cells (>4 μm) formed multiple daughter cells. Thus the threat from mesophilic pathogens with a low infective dose may be underestimated in refrigerated foods. It was also found that *E. coli* O157:H7 cultures containing elongated cells prepared at 6 or 15 °C have greater potential to attach to food contact surfaces than those grown at higher temperatures. Interestingly, at 6°C cell elongation was inhibited by ≥ 100 mg/l cinnamaldehyde and ≥ 200 mg/l cinnamaldehyde was lethal. In contrast, at 37°C 200 mg/l cinnamaldehyde initially delayed multiplication of *E. coli* cells by causing cell elongation, but from 2 to 4 h, growth resumed and cells reverted to normal length. To understand this transient behaviour, genome-wide transcriptional analysis of *E. coli* O157:H7 was performed at 2 and 4 h exposure to cinnamaldehyde in conjunction with reverse phase-high performance liquid chromatography analysis for cinnamaldehyde and other cinnamic compounds. At 2 h exposure, cinnamaldehyde induced expression of many oxidative stress-related genes, reduced expression of genes involved in DNA replication, synthesis of protein, O-antigen and fimbriae. At 4 h many repressive effects

of cinnamaldehyde on *E. coli* O157:H7 gene expression were reversed. Data indicated that by 4 h, *E. coli* O157:H7 was able to convert cinnamaldehyde into the less toxic cinnamic alcohol using alcohol dehydrogenase or aldehyde reductase enzymes (YqhD and DkgA). The results also showed that the antimicrobial activity of cinnamaldehyde was likely attributable to its carbonyl aldehyde group.

Chapter 1

Introduction

Refrigeration is proven technology which not only extends the safety and shelf life of perishable foods in the home but also reduces losses during processing, storage, transport and sale. Improved operation of the industrial cold chain has been accompanied by increased consumer access to affordable domestic refrigeration. Household use of refrigerators doubled in the 12 years prior to 2008, with numbers of units reported to be about 1 billion world-wide (Coulomb, 2008). Furthermore, increased consumer demand for convenience foods requiring minimal preparation has resulted in a new generation of minimally processed and ready-to-eat refrigerated foods with extended shelf life in developed countries (Juneja, 2003). While the combination of milder preservation methods like modified atmosphere packaging and refrigeration can extend the shelf life of minimally processed and ready-to-eat refrigerated foods, they cannot control the growth of psychrotrophic pathogens such as *Listeria monocytogenes* and *Yersinia enterocolitica* (Smid and Gorris 2007). Even foodborne illness outbreaks of mesophlic enteric pathogens like *Escherichia coli* O157:H7 have been linked with ready-to-eat refrigerated foods (Salmon, 2005; Bolduc et al., 2004; Doyle et al., 2006; Olaimat and Holley, 2012). Although the minimum temperature for sustained growth of *E. coli* O157:H7 is $\geq$ 7ºC, it can survive well under normal refrigeration conditions (Huang 2010, Keeling et al., 2009).

Commensal *E. coli* (i.e. non pathogenic to human) is an indicator organism for enteric mesophilic pathogens and is used as a surrogate for *E. coli* O157:H7 (Jay et al., 2005; Keeling et al., 2009). Interestingly, a cold adapted exponential phase *E. coli* strain was found

to produce filamentous cells at temperatures close to 7ºC without significant changes in viable numbers for several days before losing their viability (Jones et al., 2002; 2003). However, when temperatures periodically fluctuated above 7ºC, both sustained growth and filament formation apparently continued indefinitely at ≥ 2 to 7ºC (Jones et al. 2004). The extent and length of filamentous cells formed and viability lost by individual strains varied greatly at constant refrigeration temperatures (Gill et al., 2007). However, it was not clear whether these losses in viable numbers were attributable to elongating or non-elongating cells.

E. coli O157:H7 has the ability to modify the type and extent of proteins expressed and its cytoplasmic membrane composition during extended exposure to cold (Russell, 2002; Jones et al., 2006; Vidovic et al., 2011). These modifications can alter the surface hydrophobicity, the surface charge of bacteria and affect their ability to attach and form biofilms on food contact surfaces (Hood and Zottola, 1995). Furthermore, altered morphology during extended exposure to cool temperatures may also affect the ability of *E. coli* O157:H7 to attach to food contact surfaces. Since biofilms in food processing facilities continue to be persistent sources of contamination for otherwise unadulterated food products (Pennington, 2010; Sofos and Georgnaras, 2010; Van Houdt and Michiels, 2010), it is essential to evaluate the effects of cold adaptation and cold-induced cell elongation upon the adherence of *E. coli* O157:H7 to food contact surfaces.

Studies have shown that moving *E. coli* cultures containing elongating cells from 6 to 12ºC yielded a rapid increase in colony numbers and the return of normal morphology (Jones et al., 2002). Warming of filamentous *Salmonella* cells formed at 8 to 37ºC also resulted in

rapid division of filamentous cells and return to normal morphology (Mattick et al., 2003a). A single cell regardless of its size develops into a single colony during standard plate counting. If filamentous cells divide simultaneously into more than two daughter cells when contaminated refrigerated foods are exposed to warm temperature, then the number of organisms estimated by direct plate counting soon after removal of food from chill storage may underestimate the microbiological risk of mesophilic pathogens like *E. coli* O157:H7.

The majority of studies on refrigeration temperature-induced filamentation of *E. coli* have been conducted with commensal *E. coli* strains. However, it has been reported that some *E. coli* O157:H7 strains formed a larger proportion of filamentous cells than commensal *E. coli* strains at 8ºC (Mattick et al., 2003b). Additionally, it is not known whether filamentous cells of *E. coli* O157:H7 maintain their viability and multiply at warm temperatures. Therefore, further studies are needed to obtain a good understanding of the behaviours of elongating cells and to assess the risks from mesophilic pathogens like *E. coli* O157:H7 in refrigerated foods. Furthermore, additional strategies may be required for either control of *E. coli* O157:H7 cell elongation or to inactivate this pathogen in refrigerated foods.

While conventional food preservation techniques like heat treatment or chemical preservatives may effectively control *E. coli* O157:H7, negative consumer perception towards these techniques and demand for fresh foods or foods preserved with natural preservatives has fueled the search for alternatives like natural antimicrobials to control foodborne pathogens. Plant based natural antimicrobial compounds like cinnamaldehyde, thymol, and carvacrol have been reported to be effective against many foodborne pathogens including *E. coli* O157:H7 (Holley and Patel, 2005). Cinnamaldehyde has been shown to

improve the safety of many perishable foods by inactivating *E. coli* O157:H7 and other foodborne pathogens (Juneja and Friedman, 2008; Obaidat and Frank, 2009a; Amalaradjou et al., 2010; Baskaran et al., 2010; Ayari et al., 2012; Yossa et al., 2012). Despite earlier reports indicated that cinnamaldehyde treatment inhibited cell separation and caused cell elongation in *Bacillus cereus* and *E. coli* (Kown at al., 2003; Domadia et al., 2007) there is not much information available on cinnamaldehyde-induced cell elongation of *E. coli* O157:H7. As recent research has promoted cinnamaldehyde as an effective agent against *E. coli* O157:H7 and as many foods are reported to contain this compound, it is important to understand how cinnamaldehyde exposure affects the morphology, especially cell length, of *E. coli* O157:H7.

Therefore this work was carried out to achieve following objectives:

i) To assess how cold temperature and cinnamaldehyde, alone or in combination, affect the morphology and viability of commensal *E. coli* as well as *E. coli* O157:H7

ii) To assess the food safety risk associated with elongated or filamentous *E. coli* O157:H7 cells formed at refrigeration temperature by examining the multiplication pattern of filamentous and non-filamentous cells at the individual level

iii) To evaluate the effects of cold adaptation and cold-induced cell elongation upon the adherence of *E. coli* O157:H7 to food contact surfaces and

iv) To understand the antimicrobial action of cinnamaldehyde and the molecular mechanisms underlying cinnamaldehyde-mediated cell elongation of *E. coli* O157:H7.

Chapter 2

Review of Literature

2.1 Food refrigeration

2.1.1 Significance of food refrigeration

Changes in food consumption patterns have occurred over time. The factors that have driven these changes are higher income, urbanization, demographic shifts, improved transportation, and consumer perceptions regarding nutritional quality and safety (Regmi 2001). A major portion of consumer food spending (about 60 % of total food expenditure) goes towards perishable foods, which includes fresh produce, dairy, fresh meat, and seafood (Regmi, 2001; AAFC, 2007; AAFC, 2011). Thirty to fifty years ago, consumers relied on thermally processed canned or dehydrated fruits, vegetables and other seasonal foods during the off seasons. Now produce and previously seasonal perishable foods are available throughout the year as a result of improved infrastructure and world agricultural trade (Regmi, 2001). Furthermore, increased consumer demand for convenience foods requiring minimal preparation has resulted in a new generation of minimally processed and ready-to-eat refrigerated foods with extended shelf life in developed countries (Juneja, 2003). Unlike canned or dehydrated foods, which are stable at room temperature for extended period of time, perishable and minimally processed foods have shorter shelf life. Refrigeration plays an important role in improving the safety and shelf life of perishable foods and is extensively used by the food industry and consumers. As perishable food production and processing are largely centralized in only a few establishments, the safe distribution of raw or processed

perishable food is dependent upon the effective operation of the cold supply chain (Montanari, 2008).

2.1.2 Temperature control in the cold chain

Temperature is one of the major factors that determines microbial growth in perishable foods. Growth of foodborne pathogens, especially mesophilic enteric organisms at low temperatures, is much slower than at higher temperatures and has an extended lag period. The minimum temperature for sustained growth of *E. coli* and related enteric pathogens is about 7ºC (Mackey et al., 1980; Smith, 1985; Huang, 2010). Thus, food safety authorities uniformly recommend that perishable foods be held at about 4 to 5°C or below to maintain their shelf life and safety (Kosa et al., 2007; James et al., 2008). Proper temperature control must be observed from the point of production, processing, through to storage at the household or restaurant. Although maintenance of proper temperature during transport is key to achieve this goal, it is hard to assure temperature control as it is complex, often involving unloading and storage at several different points before food reaches consumers (Montanari, 2008).

On the other hand, operational temperatures of retail display or household refrigerators can be controlled and monitored without much difficulty. However, temperatures of refrigerated cabinets do not always comply with the recommended $\leq$ 4ºC and are often found to fluctuate from a minimum of 2ºC to a maximum of 12ºC (Olsson, 1990; Greer et al., 1994). Similarly, studies of consumer food handling revealed that 30 to 70 % of household refrigerators were operated at > 4°C with an average temperature ranging from 5 to 8ºC (Laguerre et al., 2002; Marklinder et al., 2004; Kosa et al., 2007; James et al., 2008;

Lagendijk et al., 2008).

2.1.3 Outbreaks of *E. coli* O157:H7 linked to refrigerated foods

E. coli is a facultatively anaerobic Gram-negative bacterium that commonly colonizes the mucous layer of the colon of warm blooded animals, including humans (Garmendia et al., 2005). Although most *E. coli* strains are harmless (commensal) to humans some serotypes, especially verotoxigenic *E. coli* (VTEC), are pathogenic to humans and have been linked with many foodborne illness outbreaks. *E. coli* serotypes are generally classified based on their O- (the polysaccharide portion of the outer membrane lipopolysaccharide) and H- (flagellar) antigens (Gill and Gill, 2010). The enhanced virulence of VTEC strains is attributed to the acquisition of novel virulence genes and genetic islands by horizontal gene transfer (Garmendia et al., 2005). The defining characteristic of VTEC strains is the presence of one or more genes that express(es) verotoxins. The term verotoxin is used because of their toxicity to cultured vero cells. Based on differences in homology with the Shiga toxin of *Shigella dysenteriae* and in the target receptors, 6 variants of verotoxins, (VT1, VT2, VT2c, VT2d, VT2e) have been identified to date. Additional virulence factors that contribute to the pathogenicity of VTEC are genes belonging to the locus of enterocyte effacement (LEE) pathogenicity island and hemolysin (HlyA). VTEC infections generally lead to self-limiting episodes of bloody diarrhoea, however, about 5 to 15 % of cases may develop into life-threatening haemolytic-uremic syndrome (HUS) (Gill and Gill, 2010).

The first foodborne illness caused by VTEC serotype O157:H7 (*E. coli* O157:H7) was reported in 1982. Since then, *E. coli* O157:H7 has become an important public health concern worldwide and has been the focus of regulatory action and surveillance in Canada

and the USA (Gill and Gill, 2010). There have been more than 207 *E. coli* O157:H7 illness outbreaks reported internationally in the scientific literature. Because of underreporting, the actual number of outbreaks/ illness could be considerably higher (Doyle et al., 2006; Olaimat and Holley, 2012). Many of the outbreaks have involved contaminated perishable foods including those minimally processed and ready-to-eat with extended shelf life (Table 2.1). As these foods are only mildly heat-processed (if at all), refrigeration plays a vital role in controlling their safety and shelf life (Marth, 1998). Although the minimum temperature for sustained growth of *E. coli* O157:H7 is $\geq 7^{o}$C, it can survive well under normal refrigeration conditions, and resume growth when temperatures exceed 7ºC (Sumner and Krist, 2002; Wang, 2004; Huang 2010). Furthermore, *E. coli* O157:H7 infections represent a considerable burden in terms of morbidity and stress on the healthcare system. In the USA, the estimated total healthcare cost associated with *E. coli* O157:H7 infection was $993 million US (Scharff, 2010). Another US study showed that the estimated average cost per person for a hospitalized *E. coli* O157:H7 patient without any complications like haemolytic uremic syndrome was $6,922 US (Buzby and Roberts, 2009). In Canada, the estimated mean annual cost for a case of gastrointestinal illness was $1,342.57 CAN (Henson et al., 2008). These estimates include costs related to medical care, death and lost productivity but do not include costs related to food recalls. If these values were included with healthcare costs, the total economic burden would be even higher than currently estimated.

Recent findings showed that non-O157 VTEC may cause 30 to 50 % of VTEC illnesses in North America and these VTEC are important foodborne pathogens in many other countries. In 2008, the USDA announced its intension to test foods for the presence of

an additional 5 VTEC serogroups (O26, O45, O103, O111, and O145) (Gill and Gill, 2010).

2.1.4 Effect of refrigeration on physiology of *E. coli*

Exposure of microorganisms to temperatures below the optimum for growth can cause several physiological changes. Reduced enzyme activity and defects in temperature-sensitive metabolic regulatory processes lead to changes in metabolic products, metabolic imbalance and eventual growth cessation at low temperatures (Beales, 2004). In order to survive or to maintain growth at low temperatures, microorganisms have to sense changes in temperature and adapt their metabolic processes accordingly. Along with the cytoplamic membrane, proteins, nucleic acids and ribosomes have been suggested to function as sensors of temperature change (Phadtare et al., 2000).

The cytoplamic membrane plays a key role in bacterial survival/viability by providing a selective barrier between the cytoplasm and the environment. The physical state of lipid bilayers of the cytoplamic membrane determines the barrier function and regular cellular activity requires fluid lipid bilayers. The fluidity of the membrane bilayer is optimal at the physiologically optimum temperature of the organism, where it is in a liquid crystalline state. Temperature downshift reversibly changes the membrane from a fluid to nonfluid, gel state. As a way to maintain the membrane fluidity, microorganisms have developed the ability to change the lipid bilayer composition (Yamanaka, 1999; Mansilla et al., 2004).

The membranes of *E. coli* contain three major phospholipids including phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The three major fatty acids present the *E. coli* membranes are palmitic (saturated), palmitoleic (unsaturated; UFA) and

cis-vaccenic (UFA) acids (Cronan and Rock, 1996). At low temperatures *E. coli* modifies its membrane composition by increasing the incorporation of *cis*-vaccenic acid into phospholipids and decreasing palmitic acid content (Marr and Ingraham, 1962). The enzymes β-hydroxydecanoyl dehydrase, the product of the *fabA* gene, is responsible for introduction of a *cis* double bond into a 10-carbon fatty acid intermediate (Mansilla et al., 2004). Then, this intermediate is converted into UFAs found in the cytoplamic membrane via addition of carbon units by beta-ketoacyl-acyl carrier protein synthase (KAS) I and II (Garwin et al., 1980). The activity of KAS I, a product of the *fabB* gene which is involved in the synthesis of *cis*-vaccenic acid, is unaffected by a downshift in temperature. On the other hand, KAS II, a product of the *fabF* gene, is solely responsible for temperature-regulated synthesis of *cis*-vaccenic acid and the maintenance of membrane fluidity at low temperatures (de Mendoza et al., 1983). The increased synthesis of *cis*-vaccenic acid at low temperatures is due to the elevated activity of KAS II, rather than increased synthesis of this enzyme by cold shock (Garwin and Cronan, 1980). Periodic temperature fluctuations from low to high or the reverse can lead to the retention of the membrane fatty acid profile at higher temperature similar to that observed at low temperature (Ivancic et al., 2009). In addition, *E. coli* adjusts the composition of lipid A (a component of lipopolysaccharide) by increasing palmitoleic acid and decreasing lauric acid content when shifted to low temperature (Carty et al., 1999).

Proteins have been suggested to act as thermosensors in *E. coli*. For example, the unmethylated protein, aspartate chemoreceptor, serves as a warm thermosensor in *E. coli*, while the reversibly methylated aspartate chemoreceptor senses temperature downshift (Nishiyama et al., 1999). Several proteins are transiently induced in *E. coli* as a response to

sudden shift to low temperatures and these proteins are called cold shock proteins (Yamanaka, 1999; Phadtare et al., 2000). The initial identification of the cold shock response was made by Jones et al. (1987) and it is described as expression of a specific pattern of genes in response to temperature downshift, which includes induction of cold shock proteins, persistent synthesis of transcriptional and translational proteins, and repression of heat shock proteins during the lag period. Generally a temperature downshift of 13ºC or more induces the cold shock response and the greater the magnitude of the temperature downshift, the greater is the induction of a response (Jones et al., 1992). However, the expression of cold shock proteins ceases and returns to a basal level at the end of the lag period and subsequently, resumption of growth and synthesis of non-cold shock proteins are observed. Thus, the induction of a cold shock response can be considered to be an adaptive response (Jones et al., 1987; Jones and Inouye, 1994).

Based on the expression pattern, cold shock proteins are categorized into two types; class I and class II. The class I proteins include CspA, CspB, CspG, CspI, CsdA, RbfA, NusA, and PNP, are expressed at a very low level at 37°C, but increase to very high levels after a temperature downshift. While the class II proteins including RecA, IF-2, H-NS, the α subunit of DNA gyrase, Hsc66, HscB, trigger factor (TF), dihydrolipoamide acetyltransferase and pyruvate dehydrogenase are expressed at 37°C and moderately increased after cold shock (Yamanaka, 1999; Phadtare et al., 2000). The functions of these proteins vary from acting as molecular chaperones to contributing in energy generation. CspA, CspB, CspG and CspI have been suggested to act as RNA/DNA chaperones. Secondary structures formed in RNAs at low temperature hinder disintegration of RNA by RNase and make RNAs incompatible for

translation. CspA destabilizes the secondary structures and keeps RNA in a linear form, which is essential for translation initiation. CspA may also play an essential role during initiation of transcription by interacting with DNA (Jones and Inouye, 1994; Phadtare et al., 2000). With the exception of translation of mRNAs for cold shock proteins, translation of other cellular mRNAs is transiently blocked upon cold shock as a result of nonfunctional non-cold adapted ribosomes. Ribosomal factors RbfA and CsdA convert non-cold adapted ribosomes to cold-adapted ribosomes during the lag period. Translation initiation factor, IF-2, allows the initiation tRNA binding to the 30S ribosomal subunit. These changes lead to translation of cellular mRNAs (protein synthesis) and resumption of growth. The trigger factor has been proposed to function as protein molecular chaperones, which facilitate proper folding and the refolding of cold shock damaged proteins (Jones and Inouye, 1994; Yamanaka, 1999; Phadtare et al., 2000).

Maintenance of DNA-related functions such as replication, transcription, and recombination depends on DNA supercoiling, and negative supercoiling of DNA has been proposed as a sensor for temperature changes. The enzyme DNA gyrase regulates the extent of the negative supercoiling of DNA in *E. coli*. Exposure of *E. coli* to cold shock increases the synthesis of the cold shock-inducible α subunit of DNA gyrase and negative supercoiling of DNA. H-NS, a DNA-binding cold shock-inducible protein plays a role in structuring the chromosomal DNA and mutations in H-NS increase the cold sensitivity of *E. coli*. Another cold shock-inducible protein RecA has roles in recombination and in response to DNA damage (the SOS response). These observations indicate that maintenance of DNA structure and functions rely on several cold shock proteins. Therefore, it is possible these proteins,

which regulate DNA supercoiling and topology, may function as sensors of temperature change (Jones and Inouye, 1994; Yamanaka, 1999; Phadtare et al., 2000).

Unlike the heat shock response, which is regulated by sigma factor RpoH, no cold specific sigma factor has been identified thus far (Phadtare et al., 2000). Sigma S (RpoS) has been known to provide protection against a variety of stresses. A recent study conducted by Vidovic et al. (2011) highlights the possibility that the cold shock response of commensal *E. coli* and *E. coli* O157:H7 may differ and that some cold adaptive responses such as membrane fatty acid modification, energy generation and membrane transport of *E. coli* O157:H7 may be regulated by RpoS. Exposure to low temperature may also change the virulence properties of *E. coli* O157:H7 (Carey et al., 2009).

Accumulation of organic compounds called osmolytes or compatible solutes plays a significant role in bacterial adaptation during exposure to harsh environments involving high osmolarity or low temperature. Compatible solutes are highly soluble, low molecular weight organic compounds like betaine, glycine betaine, carnitine and trehalose, which can be accumulated at very high concentrations in the cytoplasm of bacteria in response to high osmolarity without affecting their physiological functions (Kandror et al., 2002; Angelidis and Smith, 2003; Hoffmann and Bremer, 2011). For example, uptake of glycine betaine occurs 15-fold faster at 7ºC than at 30ºC in *L. monocytogenes* and this leads to enhanced growth of this organisms at cold temperatures (Ko et al., 1994). Active transport of glycine betaine is mainly mediated by glycine betaine porter II (Gbu) at low temperature. In addition to glycine betaine, uptake of carnitine via the carnitine transporter OpuC can also provide protection against cold stress and can enhance growth at low temperature (Angelidis

and Smith, 2003). Increased accumulation of trehalose, through *de novo* synthesis using trehalose synthase (OtsAB) enzymes, facilitates the survival of *E. coli* at low temperatures. However, this process does not enhance the growth of *E. coli* at cold, growth permissive temperature (16ºC) (Kandror et al., 2002).

2.1.5 Effect of refrigeration on morphology of *E. coli* and foodborne pathogens

Shaw (1968) first reported that prolonged exposure of *E. coli* to temperatures below the minimum for growth causes cell elongation. Despite this report there has not been much interest on this matter over 30 years. As food refrigeration has become more common, understanding refrigeration-induced cell elongation of *E. coli* and related mesophilic pathogens and its significance to food safety is essential. Recently Jones et al. (2002, 2003, 2004) conducted series of studies that examined cell elongation of *E. coli* at various refrigeration temperatures (Table 2.2). Incubation of *E. coli* at constant temperatures between 6 and 10°C causes cell elongation, but the extent of elongation and the maximum mean length reached during the exposure varied depending on the temperature. At temperatures ≥ 9°C cells elongated for a shorter duration, and eventually returning to normal length as they resumed growth (Jones et al., 2003; Gill et al., 2007) while at ≤ 7°C cells continued to elongate for longer periods. Viable numbers of *E. coli* did not change for ≤ 5 d at ≤ 7°C and remained unchanged or decreased to varying extents, depending on the *E. coli* strain. Absorbance at 600 (A_{600}), which measures changes in cell mass, continued to increase as cells become elongated at ≤7°C (Jones et al., 2002, 2003; Gill et al., 2007). The mean length, extent of cell elongation and A_{600} values reached by different *E. coli* strains varied greatly at ≤8°C (Gill et al., 2007). Shifting elongating *E. coli* cells from 6 to 12ºC resulted in a rapid

increase in viable numbers and this suggested that elongated cells may have divided into multiple daughter cells when exposed to growth permissive temperatures (Jones et al., 2002).

Studies conducted by Mattick et al. (2003b) with several commesal *E. coli* and *E. coli* O157 strains suggested that generally, the extent of cell elongation was greater with *E. coli* O157. Since different research groups arbitrarily used different cut-off limits to define elongated or filamentous cells it is difficult to make comparisons of the extent of cell elongation observed by these groups (Gill et al., 2007; Mattick et al., 2003b).

Although incubation at 2ºC or 4°C does not cause cell elongation in *E. coli*, periodic temperature fluctuations from 2°C or 4°C to 10°C at 6 h intervals causes elongation (Jones et al., 2004). Temperature fluctuations from 6°C to 10°C at 12 h intervals extended cell elongation for several days and subsequently cells divided into normal-sized cells (Jones et al., 2004). This suggests that storing foods in refrigerators with poor temperature control or even in display cabinets having daily defrosting cycles may enhance the chances of cell elongation in *E. coli*.

In addition, induction of cell elongation at refrigeration temperatures is not limited to *E. coli*. *Salmonella* Entertitidis, *S.* Typhimurium, *Clostridium perfringens* and *Aeromonas hydrophila* also elongate extensively at low temperatures (Table 2.2). As with *E. coli*, the extent of elongation varies among *Salmonella* strains. It appears that the extent of cell elongation can also be influenced by the growth medium used. Commercial laboratory media as well as chicken broth have been found to enhance cell elongation in *Salmonella* at 8ºC to a greater extent than skim milk. Furthermore, a single elongated *Salmonella* cell was able to produce multiple daughter cells when exposed to 37°C and each elongated cell had the

potential to cause a 95 to 210-fold increase in viable numbers within a short period (≤ 4 h) (Mattick at al., 2003a). This observation combined with results from an earlier growth study with *E. coli* conducted by Jones et al. (2002) indicate that risk assessments made based on viable number estimations using plate count techniques may potentially underestimate the risk associated with these pathogens.

Overall, these observations provide evidence that extended exposure of mesophilic enteric pathogens to refrigeration temperatures may cause cell elongation, which cannot be detected by regular plate counting techniques. However, the mechanism that induces cell elongation at refrigeration temperatures has not been well understood. Studies conducted with mutant RpoS S*almonella* strains at 8°C showed that RpoS is not responsible for cell elongation (Mattick et al., 2003a). Expression patterns of proteins in *E. coli* cells elongating at 6°C for ≤ 8 d showed that proteins involved in the TCA cycle as well as in electron transport increased substantially while expression of proteins involved in protein synthesis decreased (Jones et al., 2006). Based on this observation Jones et al. (2006) proposed that these changes were indicators of energy deprivation. This condition leads to the development of a stringent response and conversion of GTP to guanosine pentaphosphate or tetraphosphate, (p)ppGpp. As GTP is essential for polymerization of cell division protein FtsZ, the reduced level of GTP could have hindered FtsZ polymerization, subsequent septal ring assembly and caused cell elongation. Further studies are needed to confirm this hypothesis.

2.2 Bacterial cell elongation under other marginal conditions

As optimal growth of many foodborne pathogens occurs at a water activity (a_w) of

0.99, reducing a_w by drying or addition of salt or sugar has been conventionally used to extend the shelf life of food. However, many foodborne pathogens like *Salmonella* and *E. coli* O157:H7 can tolerate and survive at low a_w for extended periods (Gill et al., 1983; Chacon et al., 2006b). Exposure of commensal *E. coli*, *E. coli* O157 and *Salmonella* to an $a_w \leq 0.96$ for 5 or more days induced cell elongation (Mattick et al., 2000, 2003b). *Listeria monocytogenes* grows by elongation when treated with 5.84% (1M) or 8.76 % (1.5M) NaCl or a combination of 10% NaCl and pH 5 (Isom et al., 1995; Jorgensen et al., 1995; Bereksi et al., 2002). Exposure to 5 % NaCl causes cell elongation in *Bacillus cereus* (den Besten et al., 2009). Similarly, exposure of *L. monocytogenes* to pH 5 to 6 or ≥ pH 9; *Salmonella* and commensal *E. coli* and *E. coli* O157 to ≤ pH 4.4 also cause cell elongation (Isom et al., 1995; Mattick et al., 2003b; Giotis et al., 2007). As with cold-induced cell elongation of foodborne pathogens, the extent of cell elongation also greatly varies among bacterial strains (Mattick et al., 2003b). Segregated nucleoids observed along the lengths of elongated cells indicate that these marginal conditions do not inhibit chromosomal DNA replication (Mattick et al., 2000).

High hydrostatic pressure treatment has gained popularity because it can be applied to pre-packaged foods to control foodborne pathogens. Thus, re-contamination of processed food with pathogens can be eliminated. Nonetheless, many studies showed that application of high hydrostatic pressure between 40 and 75 MPa causes extensive cell elongation in *E. coli* without affecting chromosomal DNA replication and segregation (Tamura et al., 1996; Kawarai et al., 2004). Denaturation of the FtsZ protein by high hydrostatic pressure has been suggested as the reason for cell elongation (Kawarai et al., 2004).

2.4 Application of natural antimicrobials to control foodborne pathogens in food

Despite the fact that refrigeration can extend the shelf life of perishable foods, it cannot inactivate mesophilic enteric pathogens like *E. coli* O157:H7. In fact refrigeration can induce cell elongation in *E. coli* O157:H7 and elongated cells may divide to form multiple daughter cells when exposed to growth permitting temperatures. It is understood that psychrotrophic pathogens such as *L. monocytogenes* and *Yersinia enterocolitica* can grow at refrigeration temperature (Smid and Gorris, 2007). As it is clear from the existing literature, marginal conditions created by low a_w, low or high pH, or high hydrostatic pressure can cause cell elongation in many foodborne pathogens. Along with these issues, increasing consumer demand for foods which are processed with mild preservation methods and free of artificial chemicals highlight the need for alternative preservation methods. Plants, animals and microorganisms produce several natural antimicrobial compounds as part of their defence against microbial infections. Lysozyme, lactoferrin, ovotransferrin, chitosan, pleurocidin, protamine and defensins are natural antimicrobials of animal origin that have been shown to be effective against foodborne pathogens and spoilage organisms. Likewise antimicrobials of microbial origin such as bacteriocins and reuterin, and also plant-based products like essential oils, cathechins and glucosinolates have been demonstrated to be effective against foodborne pathogens. However, with the exception of lactoferrin, nisin, and some plant essential oils, application of most of these natural antimicrobials is still in the research and development stage and further research as well as regulatory approval are needed (Juneja et al., 2012a).

2.4.1. Application of bacteriocins

Bacteriocins are the antimicrobial proteins or peptides produced by bacteria which kill closely related bacteria. Although several bacteria including *E. coli* can produce bacteriocins, only those produced by lactic acid bacteria are widely tested for food application. Bacteriocins such as nisin, pediocin, plantaricin, sakacin, enterocin and leucocins or their producer strains can be used alone or in combination as biopreservatives with other hurdle technologies to control foodborne pathogens (Cleveland et al., 2000; Aymerich et al., 2006). The Joint Food and Agricultural Organization and World Health Organization expert committee on Food Additives and the FDA have approved nisin as a preservative for foods (Aymerich et al., 2006). Generally Gram-positive bacteria are more sensitive to nisin than Gram-negative bacteria. This is because the outer membrane of Gram-negative bacteria acts as a permeability barrier and protects cells by excluding nisin (Stevens et al., 1991). The effectiveness of nisin against *L. monocytogenes* and *E. coli* O157:H7 can be increased by combining it with lysozyme and EDTA (Gill and Holley, 2000ab). Nisin treatment at 50 µg/g and 100 µg/g has been shown to decrease *L. monocytogenes* numbers by 6 log cfu/g in dry fermented sausages at < 25 days (Hampikyan and Ugur, 2007). The combined treatment of 0.6% thyme essential oils (EO) and 500 or 1000 IU/g nisin treatment reduced *L. monocytogenes* population from 4 log cfu/g to below 1 log cfu/g in ground beef within 2 d storage at 4ºC and the level was maintained during 12 d storage at 4ºC (Solomakos et al. 2008). Despite its promising effect against Gram-positive bacteria, ineffectiveness of nisin against Gram-negative pathogens restricts its use as a broad-spectrum natural antimicrobial (Juneja et al., 2012a).

2.4.2 Application of lactoferrin

Lactoferrin is a naturally occurring bioactive protein in milk and other animal fluids like saliva, tears, and mucus. The antimicrobial activity of lactoferrin results from its ability to bind two Fe^{3+} ions with high affinity in cooperation with two bicarbonate ions (Naidu, 2002). Lactoferrin has been approved for application on beef in the United States (Juneja et al., 2012a). Several studies reported that lactoferrin can be used as an antimicrobial against foodborne pathogens and spoilage bacteria in meat and meat products (Al-Nabulsi and Holley, 2006, 2007; Al-Nabulsi et al., 2006). Incorporation of microencapsulated lactoferrin with or without sodium carbonate and EDTA in dry fermented sausage batter decreased numbers of *E. coli* O157:H7 by 4.2 log cfu/g at 28 d. However, the observed reduction in pathogen numbers appeared to be the result of cell injury caused by lactoferrin rather than its lethality. This finding limits the use of lactoferrin application in fermented meat products (Al-Nabulsi and Holley, 2007)

2.4.3 Application of plant-based antimicrobials

Several plant parts and their extracts, especially EOs, have been used for flavour, aroma, perfume and medical purposes from prehistoric times and their antimicrobial activity against wide range of microorganisms is well estabilished. Most of the EOs consist of phenol-based compounds such as carvacrol, thymol, geraniol, eugenol, trans-cinnamaldehyde, p-cymene and γ-terpinene with few exceptions like mustard and horseradish EO which contains allyl isothiocyanate (Burt, 2004; Holley and Patel, 2005). *In vitro*, the antibacterial potency of EOs and their components vary significantly. Generally higher concentrations of crude essential oils are required to control foodborne pathogens than that of

their components (Burt, 2004). Many plant essential oils have been considered generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA, 2012).

C. pefringens is a spore-forming Gram-positive organism. *C. pefringens* is ubiquitously present in a wide variety of environments including the animal gut. It grows rapidly in meat and meat products which are subjected to temperature abuse. Juneja et al. (2006) showed that incorporation 0.5% cinnamaldehyde in ground beef gave > 3 log cfu/g reduction in spore germination compared with a non-treated control during 21 h abusive chilling, while carvacrol, thymol, or oregano oil gave similar reductions at only a 2% level. Similar reductions in *C. pefringens* spore germination were observed during abusive chilling of ground turkey as well, when the same compounds were applied. Unlike cinnamaldehyde (0.5%), concentrations of 0.5% and 1% of carvacrol, thymol, or oregano oil failed to control *C. pefringens* spore germination during > 15 h cooling (Juneja and Friedman, 2007). In a later study, it was found that incorporation of 2% green tea extract containing 697 mg total catechins/g extract, to cooked ground beef, chicken and pork inhibited *C. perfringens* spore germination significantly during 21 h abusive chilling and the inhibition was > 3 log cfu/g compared with non-treated controls (Juneja et al. 2007).

Exposure of artificially inoculated oysters to 1 % cinnamaldehyde or carvacrol reduced *Salmonella* numbers by ≥ 5 log cfu/g within 3 d at 4ºC. Inactivation of *Salmonella* was also observed in celery by both of these compounds (Ravishankar et al., 2010). Application of cinnamaldehyde-Tween (800T) to spinach leaves was found to reduce *E. coli* O157:H7 by > 3 log cfu /g (Yossa et al., 2012). Interestingly, vapours of allyl isothiocyanate and cinnamaldehyde also effectively inactivated *Salmonella* and *E. coli* O157:H7 in

tomatoes, lettuce and spinach at 4 and 10°C (Obaidat and Frank, 2009ab).

Mytle et al. (2006) reported that application of 1 % or 2% clove oil on chicken frankfurters inhibited *L. monocytogenes* growth for 14 d at 5°C and for 7 d at 15ºC. The 2% clove oil treatment decreased *L. monocytogenes* numbers by > 2 log cfu/g when inoculation levels of either 2-3 log cfu/g or 4-6 log cfu/g were used. When the effect of oregano and nutmeg EOs against *Y. enterocolitica* and *L. monocytogenes* in broth culture and barbecued chicken were studied by Firouzi et al. (2007) they found that both EOs were effective in broth culture however, application of EOs at 1, 2 and 3 µl/g failed to control *Y. enterocolitica* and *L. monocytogenes* growth in barbecued chicken stored at 3, 8 and 20ºC for 72 h. Kamdem et al. (2007) evaluated partial replacement of nitrite with spices on the shelf life and safety of fresh sausage and they found that a spice mix containing predominantly cinnamon, clove, coriander, anis-seed, garlic, and black pepper significantly reduced *L. monocytogenes* growth to 2.80 log cfu/g when compared with an untreated control which contained 4 log cfu/g after 14 d at 10 ºC.

The Canadian Food Inspection Agency imposed strict regulations for fermented meat products, which require minimum of a 5 log cfu reduction in numbers of *E. coli* O157:H7 during their manufacturing (CFIA, 2006). The EOs from horseradish or mustard, which contain allyl isothiocyanate (AIT) as a major component has strong activity against *E. coli* O157:H7 and *Salmonella* (Ward et al., 1998; Lin et al., 2000). Several studies showed that incorporation of AIT or mustard powder in meat or fermented meat products reduced *E. coli* O157:H7 numbers by ≥ 5 log cfu in a shorter duration than in untreated controls (Nadarajah et al., 2005ab; Chacon et al., 2006ab; Graumann and Holley, 2008; Luciano et al., 2011;

Nilson and Holley, 2012). Oriental mustard, which is a source of AIT was also found to inactivate *L. monocytogenes* in bologna (Lara-Lledo et al., 2012).

EOs and EO components have been shown to increase radiosensitivity of foodborne pathogens. Cinnamon oil (major component; cinnamaldehyde), clove oil (major component; eugenol), winter savory (major component; carvacrol) and mustard essential oil (major component; AIT) increased the radiosensitivity of *E. coli* O157:H7 and *Salmonella* by ≥ 2.4-folds and reduced the radiation dose needed to inactivate these pathogens in ground beef (Turgis et al., 2008). The combination of carvacrol and modified atmosphere packaging (60% O_2, 30% CO_2, 10% N_2) was also found to increase the radiosensitivity of these pathogens in ground beef (Chiasson et al., 2005). When cinnamaldehyde, DL-menthol, eugenol, thymol were tested for radiosensitization of *B. cereus* spores in minced beef, cinnamaldehyde significantly increased the radiosensitivity of the spores and inhibited spore germination during refrigerated storage (Ayari et al., 2012).

Interestingly, cinnamaldehyde and carvacrol have been found to increase the thermal inactivation of *E. coli* O157:H7 in ground beef by increasing its thermal sensitivity (Juneja and Friedman, 2008). The decimal reduction time (D-value) *E. coli* O157:H7 in cinnamaldehyde-containing beef patties was 1.85 min at 60°C while it was 2.70 min for controls. Similarly, the addition of 0.3% cinnamaldehyde to beef patties decreased the D-value of *E. coli* O157:H7 from 0.29 min to 0.08 min at 65°C (Amalaradjou et al., 2010). Supplementation of chicken with 0.1 to 1.0% cinnamaldehyde or carvacrol was also found to aid thermal inactivation of *Salmonella* (Juneja et al., 2012b).

2.4.4 Mechanism of antimicrobial action of plant essential oil components

Generally Gram-positive bacteria are more sensitive to EOs than Gram-negative bacteria (Burt, 2004). However, the minimal inhibitory concentration of EO components is similar between Gram-positive and –negative bacteria (Kim et al., 1995). Greater hydrophobic cell surfaces and physico-chemical complexity of the double membrane-containing cell envelope of Gram-negative bacteria has been considered to be a contributing factor to their resistance to EOs as compared with the single membrane-peptidoglycan or β-glucan-based structure of Gram-positive bacteria and yeast, respectively (Holley and Patel, 2005). Among the Gram-positive bacteria, lactic acid bacteria showed greater resistance to EOs and essential oil components (Gill and Holley, 2006a; Holley and Patel 2005).

Although Burt (2004) and Holley and Patel (2005) described some possible mechanisms of antimicrobial action of EOs or their components against bacteria, the mechanism of antimicrobial action of EOs has not been well understood. The damage to the cytoplasmic membrane and reduction in intracellular pH have been postulated as possible modes of action of cinnamon oil against bacteria (Oussalah et al., 2006). At lethal levels, cinnamaldehyde, eugenol and carvacrol have been reported to cause membrane damage, decrease cellular ATP level, reduce glucose uptake, and inhibit membrane ATPase (Gill and Holley, 2004, 2006ab). Kwon et al. (2003) reported that cinnamaldehyde inhibited cell separation and caused cell elongation in *Bacillus cereus*. Cinnamaldehyde has been reported to have the ability to inhibit the guanosine-5'-triphosphatase (GTPase) activity of the cell septum-forming protein FtsZ in a concentration dependent manner. This inhibitory activity was found to be due to the binding of cinnamaldehyde with FtsZ to form an inactive complex

(Domadia et al., 2007).

Table 2.1- *E. coli* O157:H7 illness outbreaks linked to refrigerated foods.

Year	Vehicle	Location	Reference
1986	milk, unpasteurized	US, Canada	Doyle et al., 2006
1988	beef roast	US	Doyle et al., 2006
1991	yogurt	UK	Doyle et al., 2006
1991	apple cider	US	Doyle et al., 2006
1992-93	cheese, unpasteurized	France	Doyle et al., 2006
1993	salads	US	Doyle et al., 2006
1994	milk, unpasteurized	UK	Doyle et al., 2006
1994	sandwiches	US	Doyle et al., 2006
1995	goat milk, unpasteurized		Doyle et al., 2006
1995	lettuce	US, Canada	Doyle et al., 2006
1996	apple juice	US, Canada	Doyle et al., 2006
1996	radish sprouts	Japan	Doyle et al., 2006
1997	alfalfa sprouts	US	Doyle et al. 2006
1997	cake, cream filled	UK	Doyle et al., 2006
1998	cheese curds	US	Doyle et al., 2006
1998	cream, unpasteurized	US	Doyle et al., 2006
1999	milk, unpasteurized	UK	Doyle et al., 2006
2000	foods, deli	UK	Doyle et al. 2006
2002	salads	Canada	Doyle et al., 2006
2003	spinach	US	Doyle et al., 2006
2004	goats' cheese	France	Doyle et al., 2006
2005	lettuce	Sweden	Doyle et al., 2006
2006	spinach	US	Olaimat and Holley, 2012
2006	lettuce	US	Olaimat and Holley, 2012
2006	spinach	US	Olaimat and Holley, 2012
2009	cookie dough	US	Neil et al., 2012
2011	lettuce	US	Olaimat and Holley, 2012
2011	strawberries	US	Olaimat and Holley, 2012

Table 2.2- The mean length and proportion of elongated cells in cultures of *E. coli* and foodborne pathogens at low temperatures.

Organisms	Strain	Marginal condition	Elongation		Reference
			Mean length (µm)	Proportion of population (%)	
Escherichia coli	ML30	6 °C for ≤ 14 d	NR (300)[a]	NR	Shaw, 1968
	8WT	6 °C for 8 d	7	38	Jones et al., 2002
	8WT	7°C for 8 d	14	80	Jones et al., 2003
		8 °C for 4 d	10	NR	
		9 °C for 2 d	8	NR	
	A	8 °C for 4 d	NR	22	Mattick et al., 2003b
	B	8 °C for 7 d	NR	100	
	C	8 °C for 7 d	NR	68	
	D	8 °C for 7 d	NR	50	
	NCTC9001	8 °C for 7 d	NR	26	
	O157:H7 E107772	8 °C for 7 d	NR	81	
	O157:H7 E112586	8 °C for 7 d	NR	84	
	O157:H7 E107773	8 °C for 7 d	NR	80	
	O157:H7 30858	8 °C for 7 d	NR	80	
	O157:H7 510299	8 °C for 7 d	NR	69	
	O157:H7 E100793	8 °C for 7 d	NR	89	
	O157:H7 NCTC12900	8 °C for 7 d	NR	47	

Table 2.2- Continued

Organisms	Strain	Marginal condition	Elongation		Reference
			Mean length (µm)	Proportion of population (%)	
Escherichia coli	8WT	6 °C, fluctuated to 10 °C at 12 h interval	NR	NR	Jones et al., 2004
		2 or 4 °C , fluctuated to 10 °C at 6 h interval	NR	NR	
	8WT	10 °C ≤ 6 d	12.9 (86.3)	NR	Gill et al., 2007
	ATCC11229	10 °C ≤ 3 d	26.3 (122.5)	NR	
	ATCC11775	6 °C ≤ 3 d	3.8 (11.0)	NR	
	ATCC23739	8 °C ≤ 9 d	44 (145)	NR	
	ATCC33985	6 °C ≤ 9 d	10.4 (26.7)	NR	
	ATCC35327	8 °C ≤ 7 d	27.3 (116.1)	NR	
	ATCC35328	6 °C ≤ 10 d	4.8 (15.1)	NR	
	O157:H7ATCC43895	6 °C ≤ 5 d	3.8 (7.6)	NR	
Salmonella	PT4 E	4 °C ≤ 12 d	(150)	NR	Phillips et al., 1998
Entertitidis	PT4 I	4 °C ≤ 12 d	(35)	NR	
	PT4 E	8 °C ≤ 7 d	NR	72	Mattick et al., 2003ab
	PT4 LA5	8 °C ≤ 7 d	NR	80	
	PT EAV54	8 °C ≤ 4 d	NR	82	

Table 2.2- Continued

Organisms	Strain	Marginal condition	Elongation		Reference
			Mean length (µm)	Proportion of population (%)	
Salmonella	DT104 30	8 °C ≤ 4	(150)	95	Mattick et al., 2003a
Typhimurium	DT104 10	8 °C ≤ 4d	(150)	91	
Clostridium perfringens	WU90103	15 °C	NR	NR	de Jong et al., 2004
Aeromonas	NCIMB 9240	5 °C and 100 % CO_2 ≤ 28 d	5.8	NR	McMahon et al., 1998
hydrophila	NCIMB 84		5.8	NR	
	AM1h		5.8	NR	
	AM12h		5.8	NR	

[a]Values in parenthesis are the reported maximum lengths of the organism. NR- not reported.

Chapter 3

The viabilities of cells in cultures of *Escherichia coli* growing with formation of filaments at 6°C

3.1 Abstract

Cold adapted, exponential phase cells of three strains of *Escherichia coli* were incubated at 6°C for 10 d. Cells in all cultures grew by elongation, with formation of filaments to different extents, but with generally only small changes in the numbers of colony forming units (cfu). At later incubation times some cells of all lengths were identified as dead by LIVE/DEAD staining, with cell wall damage being apparent in some cells stained as dead but not in cells stained as live. When samples of cold-adapted cultures were incubated at 37°C for 2 h, the numbers (cfu) of two strains (ATCC 11775 and ATCC 23739) increased similarly and were not affected by the time of prior exposure to 6ºC. When incubated at 37°C, the numbers (cfu) of the other strain (8WT) increased less after incubation at 6°C for 1, 2 or 3 d than when transferred to 37 ºC from 15ºC. After > 3 d at 6ºC numbers of strain 8WT were reduced after incubation at 37°C for 2 h. The findings show that in cultures of *E. coli* elongating at 6ºC, elongating cells and filaments do not divide; and cells of all lengths lose viability at similar rates. Also, the findings indicate that substantial fractions of cells in cultures elongating at refrigerator temperatures are inactivated by an abrupt change of temperature to 37°C.

3.2 Introduction

The minimum temperature for growth of *Escherichia coli* is generally considered to be 7°C (Herbert and Sutherland, 2000). However, cold-adapted exponential phase cultures of a wild type strain of *E. coli* were found to elongate for several days at temperatures $\leq$ 7°C before their viability declined (Jones et al., 2002; 2003). Cell mass, as indicated by optical absorbance of cultures, increased because of the formation of filaments at those temperatures. If temperatures fluctuated periodically above 7°C, as can occur during daily defrosting of refrigeration equipment, increases in cell mass at $\geq$ 2 to 7°C could apparently continue indefinitely (Jones et al., 2004). The extent to which filaments are formed and viability is lost at constant low temperatures varies between strains of *E. coli* (Gill et al., 2007). *Salmonella* too have been reported to form filaments at temperatures $\leq$ 8°C, to extents that vary between strains (Mattick et al., 2003a; Phillips et al., 1998). Reports on filamentation of *Salmonella* at low water activity (Mattick et al., 2000), *E. coli* O157:H7 and *Salmonella* at temperatures above the optimum for growth (Mattick et al., 2003b), *Listeria monocytogenes* at pH 5 and 10 % NaCl (Bereksi et al., 2002) and at pH > 9 (Giotis et al., 2007) as well as of *E. coli* at high hydrostatic pressure (Tamura et al., 1996) suggest that many types of stress can induce filamentation in food-borne pathogens.

Growth of mesophilic organisms with formation of filaments at refrigeration and somewhat higher temperatures gives rise to uncertainty about the rates of growth at those temperatures and the minimum temperatures at which growth occurs. As filamentous cells may divide into multiple daughter cells, filament formation may result in actual loads of mesophilic pathogens present in foods being underestimated from counts of colonies

recovered on agar plates. Good understanding of the behaviours of filamenting cells is therefore required if risks from mesophilic pathogens such as *E. coli* O157:H7 and *Salmonella* in chilled foods are to be properly assessed.

Studies with cultures of *E. coli* incubated at refrigeration temperatures have shown that cultures will ultimately begin to lose viability. However, it is not evident from the available information whether or not loss of viability involves cells of all sizes or only cells that have not formed filaments. Also, although it has been shown that filamenting cells can divide into multiple daughter cells at temperatures little above the minimum for filament formation (Jones et al., 2002), the behaviour of filamenting cells exposed abruptly to higher temperatures is not known. Some knowledge of both those matters seems to be required for broad understanding of possible consequences of filament formation by mesophilic pathogens. Therefore the loss of viability of cells in cultures of three strains of *E. coli* growing at 6°C, and the behaviours of cells from those cultures on transfer to 37°C were investigated. The three strains were selected for examination on the basis of the differences in their abilities to form filaments at 6°C that were identified in a previous study (Gill et al., 2007).

3.3 Materials and Methods

3.3.1 Bacterial strains and culture conditions

The strains of *E. coli* used in this study were 8WT, a meat plant isolate (Gill et al., 2001), ATCC 11775 and ATCC 23739. Organisms were stored at - 80ºC, grown on plates of Brain Heart Infusion agar (Oxoid, Mississauga, Ontario, Canada) at 37ºC for 18 h, and were maintained at 4°C with monthly transfer to new plates. *E. coli* cultures were obtained by

inoculating single colonies from plates into half-strength Brain Heart Infusion broth (BHIB, Accumedia, Lansing, MI, USA) that was incubated at 25°C for 16 to 18 h. A 0.1 ml portion of each culture was transferred to a separate 250 ml flask containing 100 ml of half-strength BHIB. Inoculated flasks were placed in a water bath (Brinkmann Lauda RC20, Brinkmann Instruments, Westbury, NY, USA) maintained at 15 $\pm$ 0.2°C and incubated with stirring until the absorbance at 600 nm (A_{600}) was between 0.6 and 0.7, to obtain exponential phase cells.

3.3.2 Treatment at 6°C

Three 1 L flasks containing 540 ml of half-strength BHIB were equilibrated at 15°C. Then, each flask was inoculated with approximately 60 ml of an exponential phase culture of *E. coli* to obtain cultures of each strain with A_{600} values between 0.06 and 0.07. The newly inoculated flasks were incubated at 6 ± 0.1°C for 10 d in a shaking water bath (Thermo Electron Precision, Winchester, VA, USA).

Samples were collected from each flask 1 h after incubation commenced and then daily, for determination of A_{600} values and viable counts, incubation at 37ºC and microscopy. Changes in A_{600} values were determined for duplicate 1 ml samples using a spectrophotometer (visible spectrophotometer, Novaspec Plus, Cambridge, UK). For viable counts, a 1 ml sample was decimally diluted from 10^{-1} to 10^{-7} in refrigerated (4ºC) 0.1% (w/v) peptone water (Difco, Becton Dickinson, Sparks, MD, USA). Duplicate 10 ml portions of 10^{-3}, 10^{-5} and 10^{-7} dilutions were each filtered through a hydrophobic grid membrane filter (HGMF, Neogen, Lansing, MI, USA) and each filter was placed on a plate of lactose monensin glucuronate agar (LMG; Accumedia) which allows resuscitation of injured cells. Another 10 ml sample from each of these same dilutions was filtered through an HGMF that

was placed on a plate of LMG agar supplemented with bile salts (Sigma Aldrich, Mississauga, Ontario, Canada) at 1.5 g/l (LMGB), on which growth of injured cells is inhibited. Plates were incubated at 37°C for 18-24 h and HGMFs containing > 10 < 1000 blue colonies were counted. A most probable number was calculated (MPN) using the formula: MPN = [N x $\log_e$ (N/ (N-X))], where N = the total numbers of squares on a filter and X = the number of positive squares.

The identities of the broth cultures were tested on day 10 by isolating a single colony from each culture on plate count agar (Oxoid) and comparing the biochemical profiles with those of stock cultures maintained at 4°C, using the API 20E test kit (bioMerieux Inc., Montreal, Quebec, Canada).

3.3.3 Treatment at 37°C

Duplicate 1 ml samples from cultures incubated at 6°C were added to 9 ml BHIB equilibrated to 6°C. The tubes containing the diluted cultures were incubated at 37°C for 2 h. Two 1 ml portions from each tube were serially diluted in peptone water to 10^{-9}. All of the 10^{-5}, 10^{-7} and 10^{-9} dilutions from each series were each filtered through an HGMF and duplicate filters were placed on plates of LMG or LMGB, to obtain MPN values for viable organisms as before.

3.3.4 LIVE/DEAD staining and microscopy

The LIVE/DEAD *BacLight* bacterial viability kit (Molecular Probes; Eugene, OR, USA) was used to compare the viability of cells with different morphologies. The kit contains two dyes, STYO 9 and propidium iodide. STYO 9 penetrates intact and damaged

cell membranes to stain cells fluorescent green. Propidium iodide penetrates only cells with damaged membranes to stain them fluorescent red. Due to its higher affinity for DNA, propidium iodide displaces SYTO 9 from dead cells so they fluoresce red (Berney et al., 2007).

A 10 ml portion collected daily from each culture incubated at 6°C was centrifuged at 4°C for 10 min at 10,000 x *g*. The supernatant was discarded and the pellet was re-suspended in 10 ml of refrigerated (4 °C) 0.85% (w/v) NaCl. The cells were again pelleted, and then re-suspended in 10 ml 0.85% NaCl at 4 ºC. A 1:1 mixture of the LIVE/DEAD dyes was prepared according to the manufacturer's instruction. A 1 ml portion of each bacterial suspension was treated with 3 µl of the dye mixture and incubated in the dark for 10 to 15 min at 4°C. A 10 µl sample of the stained bacterial suspension was placed on an agar-coated (Noble agar, Difco) microscope slide and covered with a glass cover slip. The edges of the cover slip were sealed with clear nail polish and the slide was observed using an AxioImager M1 photomicroscope (Carl ZeissMicroimaging GmBH, Gottingen, Germany) under differential interference contrast (DIC), epi-fluorescent GFP (green), and D*s*Red optics (channels) at 1,000 x magnification. One hundred images were taken of different fields of view using an AxioCam HRM camera (Carl Zeiss, München, Germany). AxioVision Release 4.7 multi-dimensional acquisition module software (Carl Zeiss) compiled the three channel images (DIC, GFP and D*s*Red) into a single image.

3.3.5 Cell length measurement

Photomicrographs were viewed using AxioVision Release 4.7 software (Carl Zeiss). For cell length measurements, DIC photomicrographs were obtained by deflecting the GFP

and D*s*Red channels from view. A set of 100 images of cells were randomly selected from the photomicrographs of each slide and the lengths of the images were measured.

3.3.6 Data analysis

Mean values for changes in A_{600} values, number of viable and uninjured cells (log CFU/ml) estimated from the numbers of colonies recovered on LMG and LMGB agars, respectively, and changes in numbers of viable and injured cells after transfer from 6°C to 37°C and holding for 2 h were separated by one-way analysis of variance (ANOVA). Tukey's test was used to assess differences among treatments. Differences between means were considered significant when $p \leq 0.05$.

Mean cell lengths were calculated from the measurements of cell images. Cells were grouped as regular (≤4 μm), elongated (> 4 ≤ 10 μm), or filamentous (> 10 μm). The images of 50 cells from each group were randomly selected and categorized as being of live or dead cells based on whether the cells were stained green or fluorescent red, respectively. When the total number of a cell length group was < 50 in 100 photomicrographs of a culture preparation, that particular group was not used for viability assessment. This limit was arbitrarily chosen to avoid generation of population proportions distorted by low numbers of observations. The percent of presumptive live cells in each size group was calculated.

3.4 Results

When incubation at 6°C commenced, the A_{600} values of all three cultures were about 0.07. A_{600} values for the culture of strain 8WT increased to 0.36 by day 6, then declined to 0.25 by day 10 (Fig. 3.1a). A_{600} values for strain 11775 increased to 0.20 by day 5, then

remained at or little above that level until day 10. A_{600} values for the culture of strain 23739 increased progressively to 0.52 on day 10 (Appendix I).

For strain 8WT, the numbers of cfu in the culture as determined from counts on LMG were in the range 7.5 to 7.8 log cfu/ml until day 6 (Fig. 3.1b). The numbers then declined, to 6.9 log cfu/ml on day 10. The numbers in the cultures of strains 11775 and 23739 determined from counts on LMG were in the ranges 7.5 to 7.8 log cfu/ml and 7.4 to 7.8 log cfu/ml, respectively, during incubation at 6°C for 10 days. The numbers in cultures determined from counts on LMGB were consistently less than the numbers determined from counts on LMG. However the differences were small, with the means ± standard deviations for differences between numbers in cultures determined from counts on LMG or LMGB being 0.09 ± 0.05, 0.08 ± 0.04 and 0.06 ± 0.04 log units for the cultures of strains 8WT, 11775 and 23739, respectively.

The mean length of cells in the culture of strain 8WT declined during the first day of incubation at 6°C, but then progressively increased to reach 6.5 µm (Fig. 3.2). The mean length of cells in the culture of strain 11775 increased slightly during the first day of incubation at 6°C, then changed little until day 4, when the mean length had increased. Subsequently the mean length increased from 3.7 to 5.2 µm on day 10. The mean length of cells in the culture of strain 23739 generally increased throughout the period of incubation at 6°C and reached 13.8 µm on day 10.

When incubation at 6°C commenced, cells $\leq$ 4 µm long predominated in the cultures of all three strains. In the culture of strain 8WT, the fraction of cells $\leq$ 4 µm long increased during the first day of incubation at 6°C, while the fraction of cells of length > 4 $\leq$ 10 µm

declined from about 30% to about 10% of the total cells (Fig. 3.3). Subsequently, the fraction of the shorter cells progressively declined, while the fraction of the longer cells increased until day 7. Cells > 10 μm long (filaments) were present on day 5; and their fraction of the total cells increased progressively to about 20% on day 10.

For strain 11775, the fraction of cells ≤ 4 μm long declined during day 1 and between days 3 and 7, but changed little at other times (Fig. 3.4). The fraction of cells > 4 ≤ 10 μm increased when the fraction of the shorter cells declined. Filaments were present on and after day 4, but they were ≤ 4% of the total cells at all times.

For strain 23739, the fraction of cells ≤ 4 μm declined to < 10% by day 5 (Fig. 3.5). On day 1, cells > 4 ≤ 10 μm long were the major fraction and cells > 10 μm long were a substantial fraction of the total. The fraction of cells > 4 ≤ 10 μm increased until day 5 and then declined. The fraction of cells > 10 μm long increased progressively from day 5 to form the major fraction of cells on days 9 and 10.

When incubation at 6°C commenced, > 95% of the cells of both ≤ 4 μm and >4 ≤ 10 μm lengths were viable, as determined by LIVE/DEAD staining (Fig. 3.6). In the culture of strain 8WT, the fraction of cells ≤ 4 μm long that were viable declined to fractions that fluctuated between about 40 and 70% on days 4 to 10. The fraction of cells >4 ≤ 10 μm long that were viable remained about 90% until day 3 and then declined to ≤ 70%. The fraction of cells of lengths > 10 μm that were viable was about 70% at day 8 and 9, but only about 30% on day 10. In the culture of strain 11775, the fractions of cells ≤ 4 μm or > 4 ≤ 10 μm that were viable were ≥ 90% on day 5 and before, and ≥ 75% on day 6 and after. In the culture of strain 23739, on days 1 to 6, the fraction of cells in each of the two or three length groups

present in the culture that were viable were mostly > 80%. On day 7 and after, the fractions of cells > 4 ≤ 10 µm or > 10 µm that were viable were < 80 > 60%.

With DIC optics, cells > 4 ≤ 10 µm and or > 10 µm with indented walls or incomplete septa at irregular distances along their length, or without septa were observed in preparations of all three cultures (Fig. 3.7a). Cells with damaged cell walls that fluoresced red when viewed under D*s*Red optics were observed in preparations of all three cultures at later incubation times (Fig.3.7b). Cells > 4 ≤ 10 µm and or > 10 µm with multiple intense green or red fluorescent regions (indicating nucleoids) in a less intense green or red background were observed under epi-fluorescent GFP or D*s*Red optics with all three strains after LIVE/DEAD staining (Fig. 3.8).

When samples from the newly prepared cultures that were to be incubated at 6°C were incubated at 37°C for 2 h, the increases in colony counts on LMG were 0.98, 0.92 and 1.27 log units for strains 8WT, 11775 and 23739, respectively (Table 3.1). For samples from the culture of strain 8WT that were incubated at 37°C after 1, 2 or 3 days of incubation 6°C, the mean increase in counts ± the standard deviation (SD) was 0.52 ± 0.17 log unit. However, after incubation at 6°C for longer times, the counts on LMG were generally less after than before incubation 37°C, the mean change in counts ± SD being -0.38 ± 0.34 log unit. With samples from strains 11775 and 23739 the numbers were higher after incubation at 37°C at all times, the means ± SDs for the increases being 0.73 ± 0.26 and 1.26 ± 0.17, respectively. There was no consistent trend in the fractions of injured cells in samples incubated at 37ºC (Table 3.1). After incubation at 37°C for 2 h, the cells in all samples were mostly < 4 µm long.

3.5 Discussion

The strains used in this study were selected on the basis of the previously observed differences in change of A_{600} values of cultures incubated at 6°C, and the maximum lengths attained by cells in those cultures (Gill et al., 2007). Those characteristics were the same for each of the cultures in this and the previous study.

In the previous study, changes in mean cell length were deduced from forward angle light scattering (FALS) data obtained by flow cytometry. Cell lengths were directly measured only for those samples indicated by FALS values as containing cells of the minimum or maximum mean length for a culture (Gill et al., 2007). The FALS data suggested that substantial fractions of the cells that grew by elongation ultimately divided to decrease the mean length of cells in cultures. The direct measurement of cell lengths in this study showed that this deduction from FALS data was erroneous. With all three strains the fractions of cells $\leq$ 4 µm long in cultures incubated at 6°C progressively declined. In addition, with all three strains the fractions of cells > 4 $\leq$ 10 µm long peaked after $\geq$ 5 d. These changes in cell length were most pronounced with strain ATCC 23739, but were evident with the other strains also. The presence of multiple nucleoid regions in cells of > 4 $\leq$ 10 µm or > 10 µm long were evident from LIVE/DEAD staining. The similar presence of multiple nucleoids in filamentous cells of *Bacillus cereus*, *Salmonella* and *L. monocytogenes* cultures grown in media containing $\geq$ 5 % NaCl (den Besten et al., 2009; Hazeleger et al., 2006) and in filamentous cells of *Salmonella* in cultures incubated at 8ºC (Mattick et al., 2003a) suggests that DNA replication and segregation are not affected in cells elongating in response to different stresses. Both the presence of multiple nucleoids and incomplete septa

in elongating and filamentous cells suggest that one or more proteins with a function or functions essential for septation or cell separation may be suppressed, or it or their enzyme activities may be inhibited by refrigeration temperatures and other stress. Division of cells that elongate at low temperatures would then seem to be suppressed indefinitely while temperatures that induce elongation are maintained.

Cells are obviously viable, i.e. capable of maintaining differential membrane permeability and increase in cell mass while they are elongating (Jones et al., 2002; Gill et al., 2007), but information on whether or not viability declined at different rates in cells that do or do not elongate at 6°C was lacking. The microscopic examination of LIVE/DEAD stained preparations indicated that generally, at any time, the fractions of dead cells among cells of different size ranges were similar. Moreover, from the differences in numbers of bacteria recovered on resuscitating or non-resuscitating media, it appears that injured cells remained approximately constant fractions of the viable cells in the cultures. It thus appears that the viability of cells of all sizes declined at similar rates.

Abrupt movement to an optimal temperature of *E. coli* growing at a low temperature is reported to induce a lag of between 40 and 50 min (Swinnen et al., 2005). At 37°C, the rate of growth of *E. coli* when not restricted by medium composition is about 2.5 generations/h (Bronikowski et al., 2001; Fujikawa et al., 2004). Thus, the observed increases of about 1 log unit in the numbers of strains 8WT and ATCC 11775 in samples collected from cultures on day 0 and incubated at 37°C for 2 h could be expected. The greater increase in the numbers of strain ATCC 23739 might indicate that the lag induced in this strain by the temperature shift was shorter than in the other strains, because a markedly faster maximum

rate of growth of ATCC 23739 seems unlikely.

The increases in cell numbers in samples of ATCC 11775 and ATCC 23739 cultures that were taken after longer exposure to 6ºC and incubated at 37°C were generally similar to the increases on day zero. As the viable cells in cultures of both those strains elongated considerably during incubation at 6°C, the limited increases in numbers after incubation at 37°C could occur only if elongated and filamentous cells did not divide, or large fractions of the cell populations were inactivated by the relatively rapid shift to 37°C. After 2 h at 37ºC, samples of all strains contained few elongated or filamentous cells. Thus, viable cells of those lengths initially present in the sample evidently divided. Moreover, the small increases at early times, and decreases at later times in the numbers of strain 8WT in samples incubated at 37°C shows that the temperature shift inactivated cells of this strain. It then seems that cells of the other strains were similarly inactivated, but to lesser extents, after division of many elongated or filamentous cells. Thus, the findings suggest that risks from consumption of chilled (4ºC) foods containing pathogens that have formed filaments at refrigeration temperature may be no greater, and possibly less than risks from the same numbers of cells of normal size.

However, in a previous study with *E. coli* strain 8WT, moving the filamenting organisms from 6 to 12°C resulted in numbers of viable cells rapidly increasing as filaments divided (Jones et al., 2002). It then seems likely that moderate increases of temperature would not result in the extensive inactivation of *E. coli* grown at refrigeration temperatures that apparently occur with abrupt movement to 37°C. Accelerated division on warming to 37ºC of filamentous *Salmonella* cells formed at 8ºC was observed by Mattick et al. (2003a).

Although the extent to which the daughter cells were viable was not reported, many or all may have been viable if the filaments were, as seems likely, warmed relatively slowly by placement of inoculated, agar-coated slides on a heated microscope stage. Division of filamentous cells in foods allowed to warm slowly from refrigeration temperatures before consumption might then pose greater risks than enumeration of viable counts in the refrigerated food would suggest. Further investigation of that aspect of filamenting cell behaviour seems to be required.

Table 3.1- Mean changes in numbers of viable cells and the per cent of injured cells in samples obtained daily from cultures of three strains of *Escherichia coli* incubated at 6ºC for 10 d, when samples were incubated at 37ºC for 2h.

Culture age	*E. coli* 8WT		*E. coli* ATCC 23739		*E. coli* ATCC 11775	
(days)	Change in numbers (log cfu/ml)	Injured cells (%)	Change in numbers (log cfu/ml)	Injured cells (%)	Change in numbers (log cfu/ml)	Injured cells (%)
0	0.98 ± 0.05^{a}	16.5	1.27 ± 0.02^{ac}	3.2	0.92 ± 0.02^{ac}	17.1
1	0.40 ± 0.09^{bc}	8.8	1.33 ± 0.02^{c}	8.0	0.54 ± 0.34^{bc}	19.1
2	0.64 ± 0.17^{abc}	4.6	1.11 ± 0.07^{ab}	8.4	1.11 ± 0.15^{a}	11.7
3	0.77 ± 0.05^{ab}	9.5	1.38 ± 0.06^{cd}	26.2	0.86 ± 0.01^{abc}	15.8
4	$- 0.71 \pm 0.27^{d}$	13.3	1.32 ± 0.05^{cd}	9.0	0.67 ± 0.00^{bc}	14.9
5	$- 0.62 \pm 0.14^{de}$	5.0	1.03 ± 0.16^{b}	10.1	0.85 ± 0.04^{abc}	8.1
6	$- 0.28 \pm 0.23^{df}$	41.5	1.00 ± 0.02^{b}	8.6	0.65 ± 0.14^{bcd}	4.7
7	0.28 ± 0.16^{c}	42.6	1.23 ± 0.06^{ac}	9.2	0.45 ± 0.14^{b}	9.8
8	$- 0.13 \pm 0.09^{f}$	11.6	1.54 ± 0.09^{d}	13.5	1.00 ± 0.12^{ad}	7.6
9	$- 0.10 \pm 0.21^{f}$	13.2	1.20 ± 0.04^{abc}	0.0	0.66 ± 0.15^{bcd}	8.8
10	$- 0.38 \pm 0.16^{def}$	10.8	1.41 ± 0.08^{c}	8.1	0.68 ± 0.11^{bcd}	8.5

Different superscript letters in each column denote significant difference ($P < 0.05$).

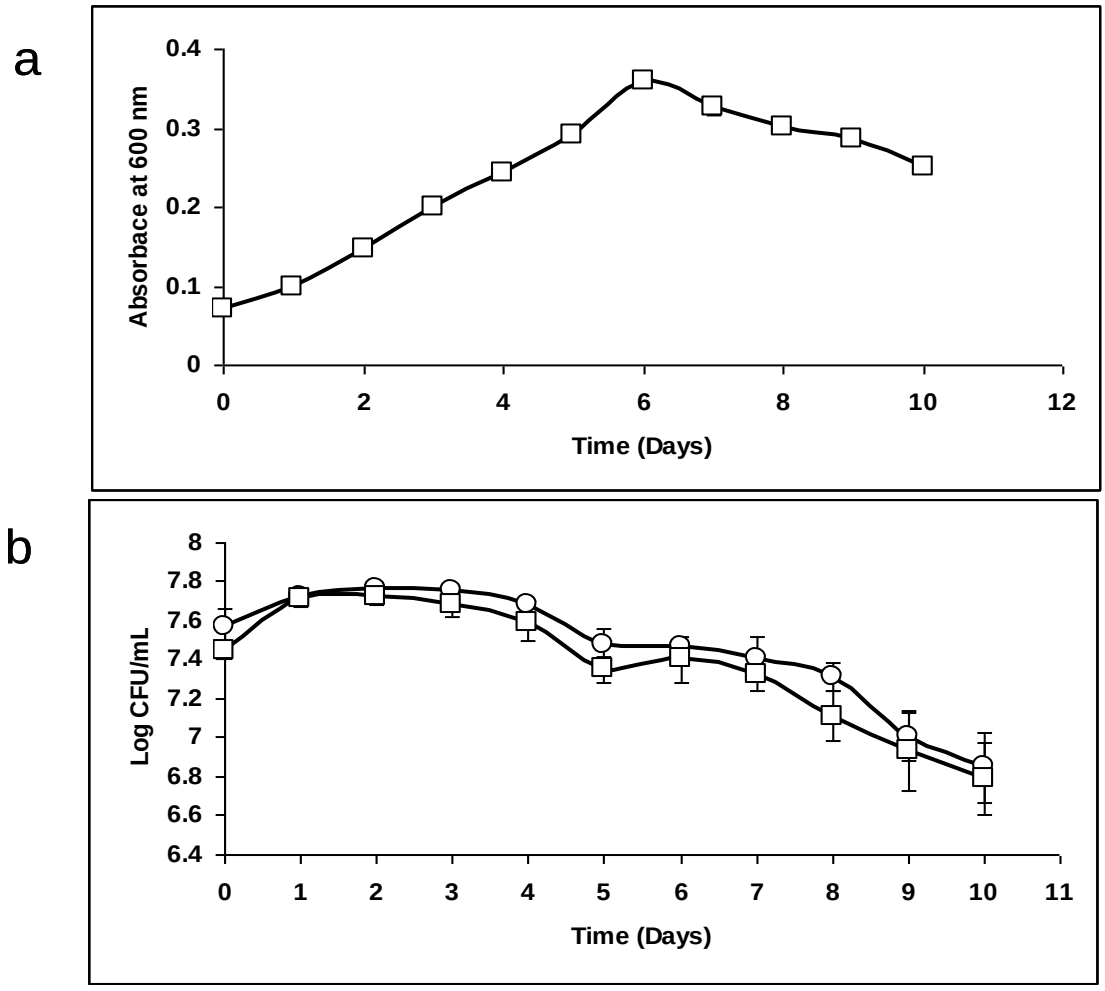

Fig. 3.1- Changes in A_{600} values (a) of a culture of *Escherichia coli* 8WT at 6ºC, and the mean number of cfu recovered from the culture (b) on agars that do (○) or do not (□) permit resuscitation of injured cells.

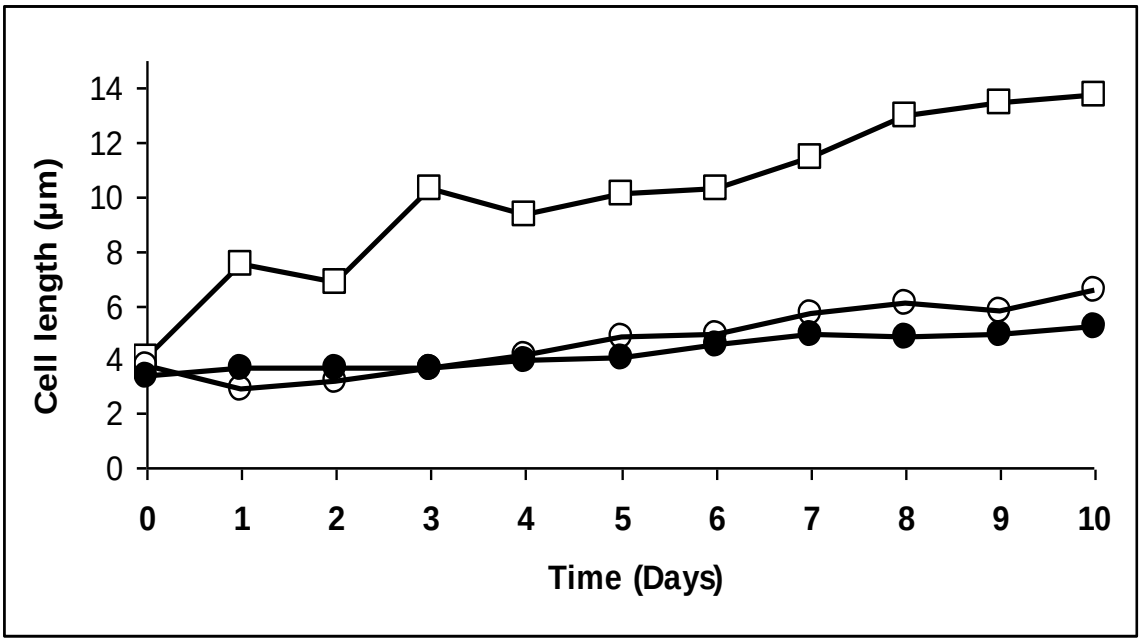

Fig. 3.2- Changes in the mean lengths of cells in cultures of *Escherichia coli* 8WT (○), *E. coli* ATCC 11775 (●) and *E. coli* ATCC 23739 (□) incubated at 6°C.

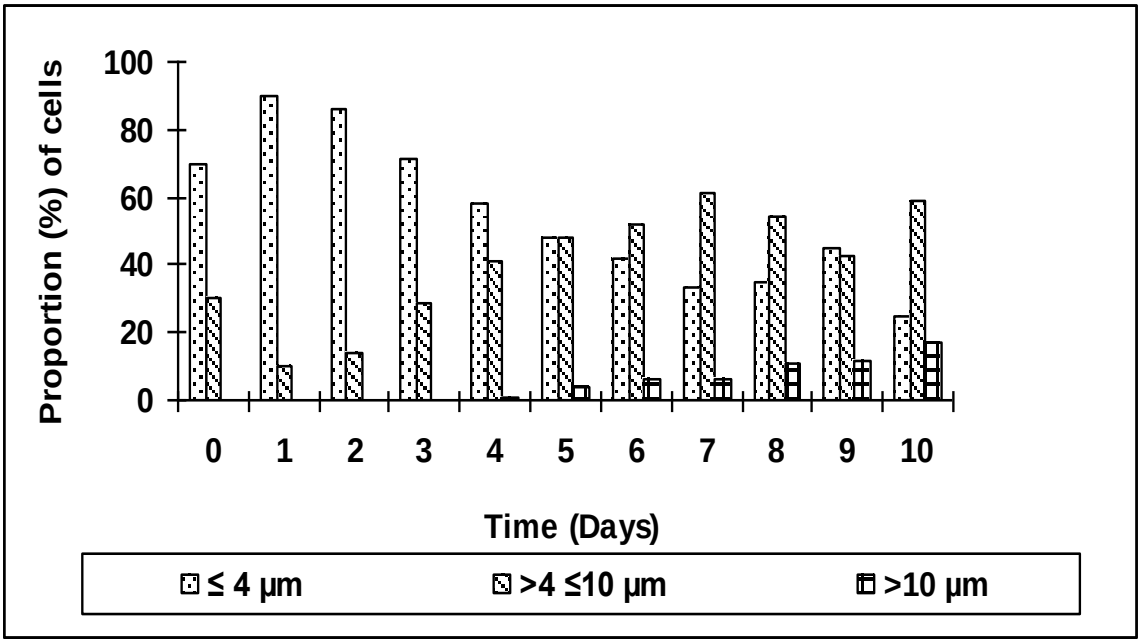

Fig. 3.3- Changes in the distribution of cell sizes in a culture of *Escherichia coli* 8WT incubated at 6°C for 10 d.

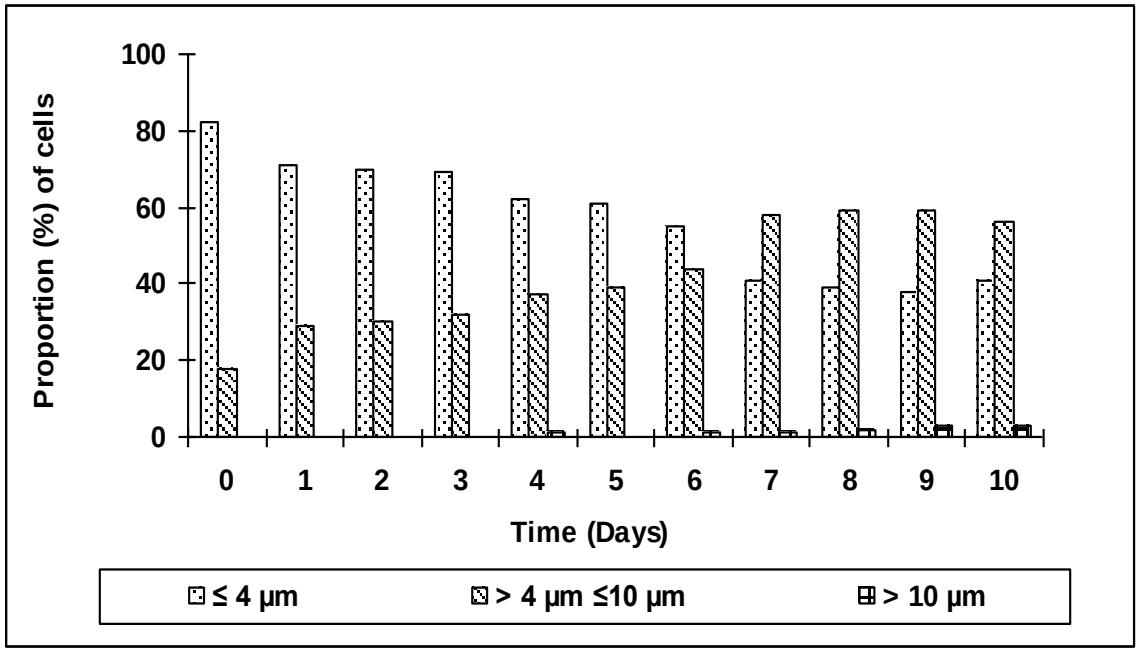

Fig. 3.4- Changes in the cells size distribution in a culture of *Escherichia coli* ATCC 11775 incubated at 6°C for 10 d.

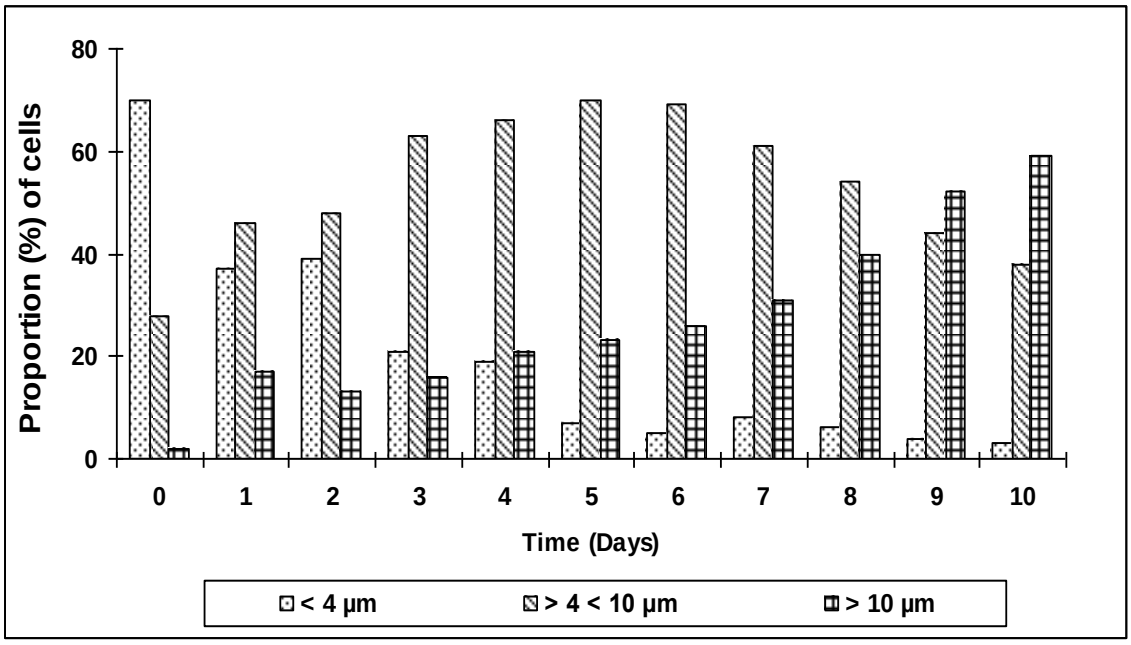

Fig. 3.5- Changes in the cell size distribution in a culture of *Escherichia coli* ATCC 23739 incubated at 6 °C for 10 d.

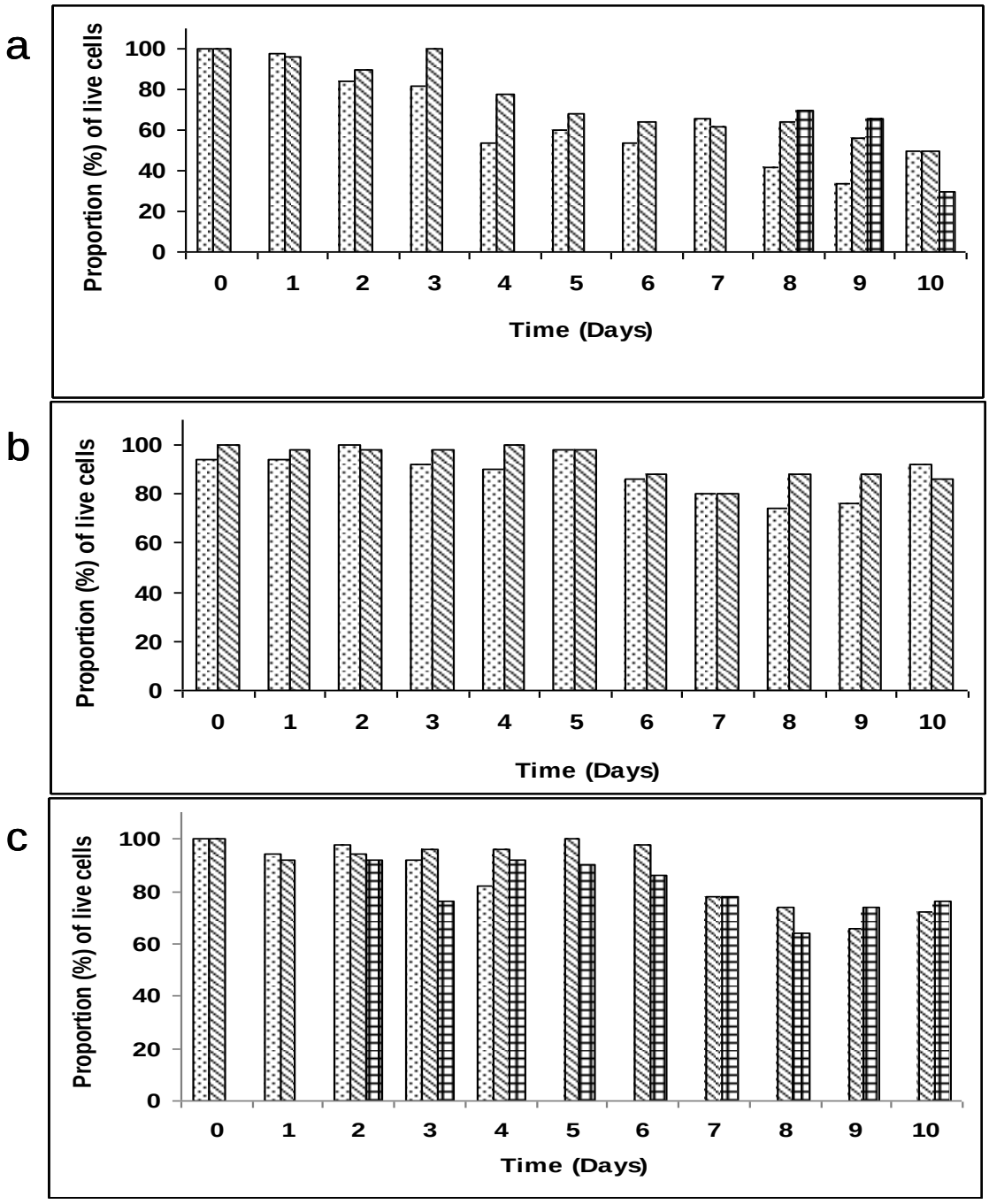

Fig. 3.6- Changes in the fractions of live cells with lengths ≤4 µm (⬚), >4≤10 µm (▨) or >10 µm (▦) in cultures of *Escherichia coli* 8WT (a), ATCC 11775 (b) and ATCC 23739 (c) incubated at 6°C for 10 d.

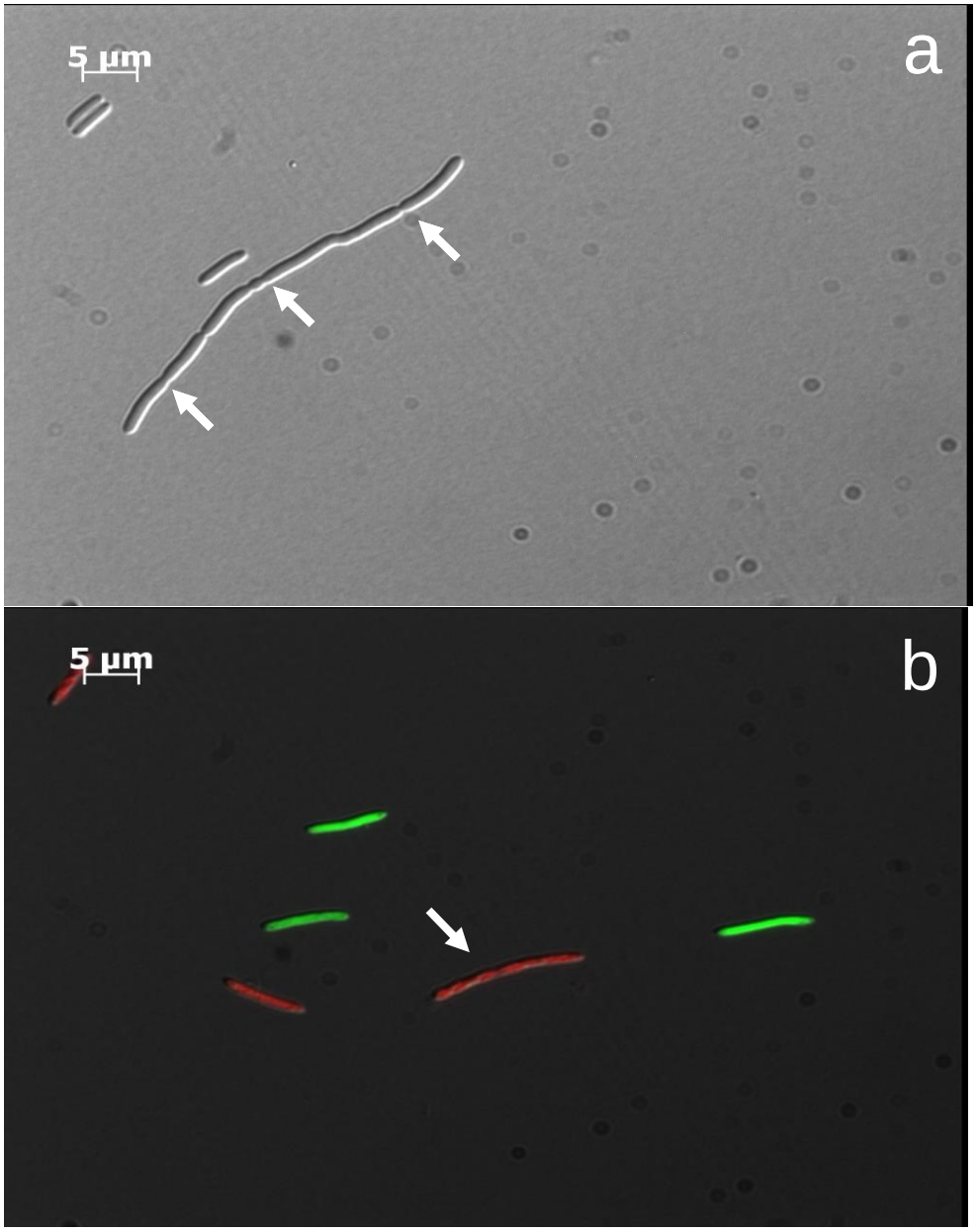

Fig. 3.7- Photomicrographs showing morphological characteristics of *Escherichia coli* ATCC 23739 incubated at 6ºC for (a) 6 d and (b) 7 d. Arrows indicate (a) a cell with multiple septa and (b) a red fluorescent, i.e. dead cell, with damaged cell wall.

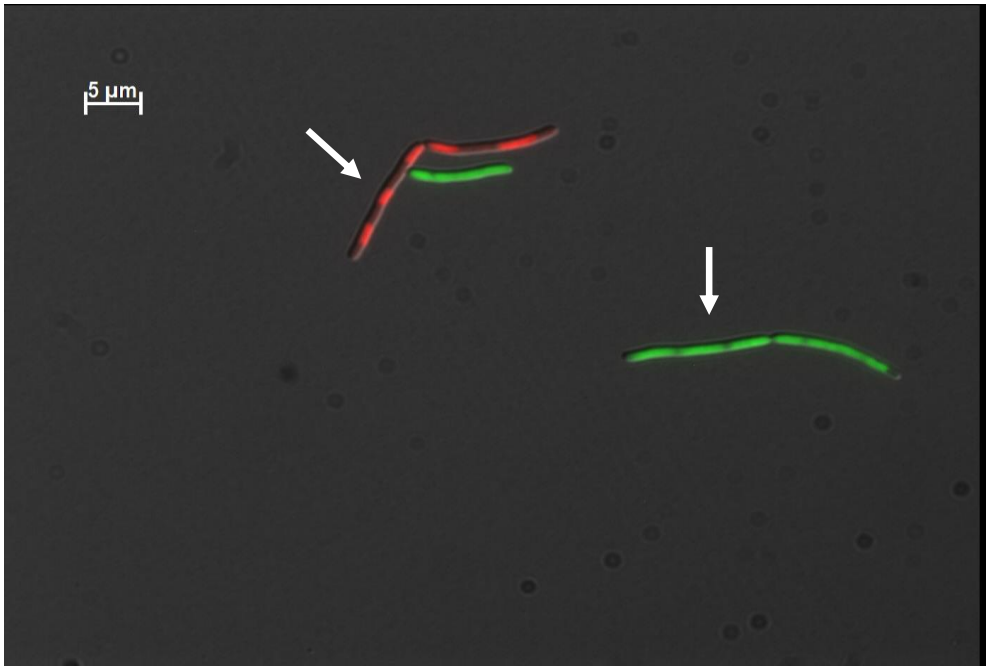

Fig. 3.8- Photomicrograph of filamentous cells of *Escherichia coli* ATCC 23739 which show multiple nucleoid regions (arrows) visualized by staining with propidium iodide and SYTO 9.

Chapter 4

Morphological and viability changes in *Escherichia coli* and *E. coli* O157:H7 cells upon rapid shift from 6ºC to 37°C

4.1 Abstract

Cells in log phase cultures of *Escherichia coli* ATCC 23739 and *E. coli* O157:H7 02:0627 incubated at 6°C for 8 days grew by elongation and the formation of filaments. When suspensions of cells from the cultures were incubated at 37°C for 4 h, there was little or no change in mean cell lengths during the first hour of incubation; but subsequently the fractions of elongated ($> 4 \leq 10$ µm) or filamentous (> 10 µm) cells declined with most cells being of normal size (≤ 4 µm) after 3 h. LIVE/DEAD BacLight staining indicated that > 94% of cells had intact cell membranes after all times at 37°C, but CTC vitality staining indicated that many cells lacked respiratory activity after 3 h at 37°C. Direct observation of cells on slides incubated at 37°C, from cultures incubated at 6°C for 5 d, showed that few or no cells of normal size divided. Elongated cells of both strains and filamentous cells of *E. coli* ATCC 23739 divided to multiple daughter cells; but filamentous cells of *E. coli* O157:H7 lysed. The results indicate that abrupt shifts of log phase *E. coli* from refrigeration to warm temperatures leads to inactivation of some cells and division of others to multiple daughter cells, and suggest that the extents of these opposing responses may vary widely among strains.

4.2 Introduction

Although the minimum temperature for sustained growth of *Escherichia coli* is about 7°C (Herbert and Sutherland, 2000), log phase, cold-adapted cells of the organism may continue growth by elongation at 6°C but not at 2°C (Jones et al., 2002). At temperatures below and somewhat above 7°C, growth proceeds with the elongation of cells and the formation of filaments. The extent to which filament formation occurs and the temperature range within which filaments are formed vary considerably among strains of *E. coli* (Gill et al., 2007).

In a recent study of the growth of three strains of *E. coli* during incubation at 6°C for up to 10 d, it was found that elongated and filamentous cells did not divide during incubation at that temperature; but that cells of all sizes lost viability as incubation was prolonged (Visvalingam et al., 2012). When cultures were shifted to 37°C and examined after 2 h at that temperature, the numbers of viable organisms had generally increased and most cells were of normal size. However, the increases in viable numbers varied with the time of incubation at 6°C; and after incubation of one strain at 6°C for > 3 d the numbers of viable cells were reduced by incubation at 37°C for 2 h. However, it was not clear whether these changes in viable numbers were attributable to elongated, filamentous cells or cells of normal size, and how the duration of exposure at 6ºC influenced lag time and growth rate upon transfer to 37ºC.

Some understanding of these responses would seem to be necessary for proper estimation of risks from small numbers of cells of mesophilic enteric pathogens like verotoxigenic (VTEC) *E. coli* O157:H7 that may have formed filaments in refrigerated foods.

A single cell regardless of its size develops into a single colony during standard plate counting. If filamentous cells divide simultaneously into multiple daughter cells, it may lead to a more rapid increase in numbers of pathogenic bacteria than anticipated when contaminated food is shifted to higher temperatures. In addition, the threat from pathogens with a low infective dose may be underestimated in refrigerated foods stored with uncertain temperature control. Therefore, changes in the sizes, numbers, and viabilities of *E. coli* cells at 37°C after incubation at 6°C for up to 8 d were examined. The organisms used in the study were a strain of *E. coli* that had previously been found to undergo extensive filamentation at 6°C (Gill at al., 2007; Visvalingam et al., 2012) and a human VTEC isolate that had not been studied with respect to its formation of filaments. The VTEC strain was included because it has been reported that some VTEC strains formed a larger proportion of filamentous cells than commensal *E. coli* strains at 8ºC (Mattick et al., 2003b), and because VTEC, which have a low infective dose, continue to be a major food safety concern (Pennington, 2010).

4.3 Materials and Methods

4.3.1 Preparation and sampling of *E. coli* cultures incubated at 6°C

The organisms used in the study were *E. coli* ATCC 23739, and *E. coli* O157:H7, strain 02:0627 from the National Microbiological Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada, which was kindly provided by Dr. R. Ahmed. The organisms were stored at -80°C and maintained on plates of Brain Heart Infusion Agar (BHIA; Oxoid, Mississauga, ON, Canada) stored at 4°C with monthly transfer to new plates. Three cultures of each organism were prepared for incubation at 6°C.

Active cultures of each strain were obtained by streaking on plates of BHIA and incubation at 37ºC for 18 h. Cold-adapted log phase cultures of each strain were prepared in Brain Heart Infusion Broth (BHIB; Accumedia, Lansing, MI, USA) as described previously (Visvalingam et al., 2012). Briefly, BHIB was inoculated from a single colony on agar, incubated at 25ºC for 16 to 18 h and used to inoculate fresh BHIB which was incubated with stirring at 15ºC for 24 to 28 h to get A_{600} values between 0.6 and 0.7. A 40 ml sample of the 15°C culture was transferred to 360 ml of BHIB equilibrated to 15°C, in a 1 L flask. The flask was placed in a shaking water bath operating at 6 ± 0.1°C and 100 rpm. The level of water in the bath was maintained 2.5 to 3 cm above the culture level in the flask. After incubation for 1 h and 4, 6 and 8 d, the A_{600} value of the culture was determined using a spectrophotometer and a 40 ml sample was collected for determination of the effects of a shift in temperature to 37°C.

4.3.2 Preparation and testing of suspensions incubated at 37°C

Cells in each sample from a 6°C culture were pelleted by centrifugation at 10,000 x *g* for 10 min at 4°C. The pellet was resuspended in 40 ml BHIB at 6°C. The suspension was transferred to a 100 ml conical flask and placed in a water bath for incubation with shaking at 37 $\pm$ 0.1°C for 4 h. Samples were collected from the suspension immediately and then at 30 min intervals for enumeration of viable numbers, and at the beginning of incubation and 60 min intervals for microscopic examination.

Each 1 ml sample collected for enumeration of viable cells was diluted ten-fold in 0.1% (wt/vol) peptone water (Difco; Becton Dickinson, Sparks, MD, USA), and the diluted suspension was placed on duplicate plates of BHIA using a spiral plater (Autoplate; Spiral

Biotech, Norwood, MA, USA). The plates were incubated at 37 °C for 18 h, and then colonies on appropriate sectors of the plates were counted. Numbers of viable cells in the suspension were calculated from those counts.

Each 5 ml sample collected for microscopy was centrifuged at 10,000 x *g* for 10 min at 4°C. The resultant pellet was resuspended in 5 ml of 0.85% (wt/vol) NaCl (Fisher Scientific, Edmonton, AB, Canada). A 1 ml portion of the suspension was mixed with 3 µl of a mixture of equal volumes of the stains SYTO 9 and propiduim iodide from a LIVE/DEAD BacLight^{TM} Bacterial Viability Kit (Kit L-7012; Molecular Probes, Eugene, OR, USA) and incubated in the dark at 25°C for 10 min. A 10 µl portion of the stained suspension was placed on a slide coated with Noble agar (Difco) and covered with a coverslip. The edges of the coverslip were sealed with clear nail varnish. The slide was observed using an AxioImager M1 photomicroscope (Carl ZeissMicroimaging, Gottingen, Germany), using differential interference contrast (DIC), epi-fluorescent GFP (green) and DsRed optical channels, at x 1000 magnification. An image was recorded of each of 10 different fields of view, using an AxioCam HRM camera (Carl Zeiss, München, Germany). AxioVision multi-dimensional acquisition module software, release 4.7 (Carl Zeiss) was used to compile the images of each field from the three channels into a single image. All cells in the 10 fields were counted. Cells that appeared green fluorescent were counted as live. Cells that appeared red were reported as dead.

An 850 µl portion of the cell suspension was mixed with 50 ml of 10 mM glucose (Sigma-Aldrich, Mississauga, ON, Canada) and then with 100 µl of a 50 mM solution of 5-cyano-2,3-ditolyltetrazolium chloride (CTC) prepared from CTC provided as part of a

BacLight™ RedoxSensor™ CTC Vitality Kit (Molecular Probes). After incubation in the dark at 25°C for 15 min, the cells were fixed by the addition of 100 µl of 37% (wt/vol) formaldehyde. The fixed cells were counterstained by the addition of 10 µl of a solution of 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), at a concentration of 5 mg/ml, provided as part of the CTC Vitality Kit. A microscope slide was prepared, and photomicrographs were obtained as before using DIC, epi-fluorescent D*s*Red and DAPI channels. Cells were counted as before, with cells that contained a red precipitate of CTC-formazan being counted as having been viable, and cells stained blue by DAPI being counted as having been dead.

The lengths of cells in each sample were determined by measuring the DIC images of 50 cells selected at random from the photomicrographs obtained for the sample, using the AxioVision software. Measurements made using the software were accurate to within 0.01 µm. Cells were grouped as being of normal length ($\leq$ 4 µm), elongated ($>$ 4 $\leq$ 10 µm) or filamentous ($>$ 10 µm).

4.3.3 Response of individual 5 day, 6°C -adapted cells to 37°C exposure

After incubation of cultures at 6°C for 5 d, a 10 µl sample from each culture was examined to identify the behaviours of individual cells. Each sample was deposited on a microscope slide coated with BHIA. The sample was covered with a coverslip, and the sides of the coverslip were sealed with nail varnish. Each slide was placed in an aerobic incubator at 37°C (XL S1; Carl Zeiss) fitted to an inverted microscope (Axiovert 200M; Carl Zeiss). Using a 100 x oil objective lens and DIC optics, repeated images of each selected field were obtained at 15 min intervals up to 4 h using an AxioCam MR3 camera with AxioVision 4.7.

software.

4.3.4 Analysis of data

Rates of increase in cell numbers during incubation of samples at 37°C were estimated from regression lines fitted to the viable counts recovered from each of three suspensions of the same organism after each time of incubation at 37°C. The significance of differences between mean values was determined by one-way analysis of variance, with $P \leq 0.05$. The significance of differences between treatment means was determined by application of a Tukey's test, with $P \leq 0.05$. Regression lines were fitted using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Statistical tests were performed using GraphPad Instat 3 (GraphPad Software, La Jolla, CA, USA).

4.4 Results

At 6ºC, A_{600} values of both strains increased similarly from 0.07 ± 0.01 (mean ± standard deviation) at day 0 to 0.2 ± 0.03 at day 4 and reached 0.4 ± 0.03 at day 8. The numbers of colony forming units (cfu) recovered from the *E. coli* ATCC 23739 cultures increased from 7.2 $\pm$ 0.1 log cfu/ml at day 0 to a maximum of 7.7 $\pm$ 0.04 log cfu/ml at day 6 and remained stable until day 8. The numbers in *E. coli* O157:H7 02:0627 cultures increased to the same extent, but within only 4 d and then decreased to 7.4 $\pm$ 0.06 log cfu ml^{-1} by day 8.

Following transfer of cultures held from zero to 8 d at 6°C to 37°C, no increases in numbers of cfu ml^{-1} were evident for 30 to 60 min of incubation at 37°C. However, after 60 min at 37ºC in all samples, the numbers of cfu ml^{-1} increased at rates that generally declined between 2 and 3 h at 37°C; with little or no further increase after incubation for 3.5 h. For

each strain, the initial rates of increase varied by $\leq$ 0.5 log cfu h^{-1} in samples obtained from cultures incubated at 6°C for different periods (Table 4.1). The rates of increase at 37°C in samples from cultures of *E. coli* ATCC 23739 were greater than the rates of increase in samples from cultures of *E. coli* O157:H7 02:0627 obtained after 4 and 8 d of incubation at 6°C. However, the numbers in all samples after incubation at 37°C for 4 h were generally similar.

With both strains of *E. coli* the mean lengths of cells in cultures incubated at 6°C on day zero were < 4 µm. The fractions of cells that were elongated (> 4 $\leq$ 10 µm) had substantially increased in all cultures by day 4 (Fig. 4.1). By day 8, filamentous cells (> 10 µ) predominated in cultures of *E. coli* ATCC 23739. At that time, filamentous cells in cultures of *E. coli* O157:H7 02:0627 were only small fractions of the cell populations, and elongated cells predominated. The mean lengths of cells in cultures of *E. coli* ATCC 23739 and *E. coli* O157:H7 02:0627 on day 8 were 12.0 µm and 6.4 µm, respectively.

When samples were transferred to 37°C, there was generally little or no change in mean cell lengths during the first hour of incubation, except with *E. coli* ATCC 23739 that had been incubated at 6ºC for 8 d (Fig. 4.1). In samples from the latter culture there were substantial decreases in the fractions of filamentous cells at 1 h. In all samples from cultures incubated at 6°C for $\geq$ 4 d, there were large reductions in the fractions of elongated or elongated and filamentous cells between 1 and 2 h of incubation at 37°C. After incubation for 3 h, cells of normal size ($\leq$ 4 µm) greatly predominated in all samples.

With samples from cultures of both strains, LIVE/DEAD BacLight staining indicated that $\geq$ 94% of cells were viable, irrespective of the time for which cultures had been

incubated at 6°C, or the time for which samples were incubated at 37°C. CTC vitality staining similarly indicated that ≥ 95% of cells of *E. coli* ATCC 23739 were viable during the first 2 h that samples were incubated at 37°C, irrespective of the time for which cultures had been incubated at 6 °C (Fig. 4.2). However, after 3 and 4 h of incubation at 37°C of samples from cultures incubated for ≥ 4 d at 6°C, CTC vitality staining indicated that ≤ 32% were viable. After 3 h of incubation at 37°C, ≤ 50% of cells of *E. coli* O157:H7 02:0627 in samples from cultures incubated at 6°C for 8 d were indicated to be viable by CTC vitality staining. After 4 h of incubation at 37°C, ≤ 60% of cells from cultures of *E. coli* O157:H7 02:0627 sampled at the earliest time, and < 14% of cells from cultures incubated at 6°C for longer times were indicated to be viable by CTC vitality staining. Extracellular CTC-formazan crystals were prevalent in CTC-stained preparations of samples of either strain that had been incubated at 37°C ≥ 2 h.

When slides of cultures incubated at 6°C for 5 d were incubated at 37°C, only 1 of the 5 cells of *E. coli* ATCC 23739 ≤ 4 µm long that were continuously (15 min intervals) observed for 1.5 h divided to give daughter cells, but none of the cells lysed (Table 4.2). All 19 cells > 4 µm long that were observed during that time divided to form daughter cells. Cells of *E. coli* O157:H7 02:0627 observed for 4 h that were ≤ 4 µm long also did not divide; but most lysed after about 1 h, as did 2 cells ≥ 10 µm long. In contrast most cells > 4 ≤ 10 µm long divided to form daughter cells. Division of cells was first apparent after 30 to 60 min in preparations of *E. coli* ATCC 23739, and between 45 and 120 min in preparations of *E. coli* O157:H7 02:0627. Divisions of daughter cells also occurred over ranges of times. Smaller mother and daughter cells increased in length before division, with some daughter

cells becoming > 10 µm long before division into multiple cells (Fig. 4.3).

4.5 Discussion

The formation of elongated cells and filaments by *E. coli* ATCC 23739 during incubation of log phase cultures at 6°C was as expected from previous study of the organism (Gill et al., 2007; Visvalingam et al., 2012). Although the less pronounced formation of elongated cells and filaments by *E. coli* O157:H7 02:0627 was comparable with the behaviour of other strains of *E. coli* incubated at 6°C in previous studies (Gill et al., 2007; Visvalingam et al., 2012), results contrasted with the report by Mattick et al. (2003b) where it was found that most *E. coli* O157 strains produced a greater proportion of filamentous (> 8µm) cells than commensal *E. coli* strains at 8ºC. However, few elongated and no filamentous cells were formed by yet another strain of *E. coli* O157:H7 examined by Gill et al. (2007). The findings for *E. coli* O157:H7 02:0627 suggest that, as with *E. coli* generally, the responses of log phase cells to incubation at refrigeration temperatures will vary considerably among strains.

When *E. coli* growing at temperatures < 20°C were abruptly shifted to 37°C, there was a lag of about 45 min before growth at the higher temperature proceeded (Swinnen et al., 2005). Therefore, a lag before the numbers of either strain of *E. coli* increased after being shifted from 6°C to 37°C was to be expected; however, the length of the lag before growth initiation at 37°C was not influenced by the time of prior exposure to 6°C. Interestingly, Phillips et al. (1998) found that with two strains of *Salmonella* Enteritidis PT 4, the lag at 37°C was different between strains (4 to 6 h) and was longer in the strain that produced longer filaments at 4°C, even though a proportion of filaments were injured. Since in that

study some cells reached 150 µm in length over 70 d at 4°C, and since elongated cells also returned to normal length upon 37°C exposure as noted in the present study, this difference in lag should not be surprising. Although there is uncertainty when making inter-generic comparisons, the return to normal length observed with these two genera highlight the importance of considering the potential for restoration of normal morphology/physiology by bacterial pathogens upon removal of low temperature stress.

Although the findings with the two staining systems for discrimination of viable and non-viable cells were similar for cells incubated at 37°C for $\leq$ 2 h, the findings diverged markedly with cells incubated at 37°C for longer times. The LIVE/DEAD BacLight stains allow discrimination between cells with intact cytoplasmic membranes (green) and those that have damaged membranes that allow entry of propidium iodide (red) which otherwise would be excluded (Berney et al., 2007). The CTC vitality stains allow discrimination between cells in which assimilated CTC is reduced to an insoluble, red fluorescent form by respiratory chain activities, and cells which lack respiratory activity (Rodriguez et al., 1992). The results obtained with the two sets of stains indicate that after 4 h of incubation at 37°C the cell populations of samples would consist predominantly of visibly intact but moribund cells. The direct observation of *E. coli* O157:H7 02:0627 cells during incubation at 37°C showed this was not the case, because after 4 h, most moribund cells had lysed while the great majority of cells were actively dividing. It therefore appears that in this study, CTC vitality staining did not allow reliable identification of viable cells. This may have been due to much CTC being reduced extracellularly by components of lysed cells. That possibility will require further investigation.

With both strains of *E. coli* there was little or no growth at 37°C of cells of usual size, i.e. ≤ 4 µm, from cultures that had been incubated at 6°C for 5 d. Inactivation of log phase cells that do not elongate during incubation at refrigeration temperatures may then be common among cells of *E. coli*. Both cells ≤ 4 µm long and filamentous cells of *E. coli* O157:H7 02:0627 lysed during incubation at 37°C. This suggests that the filamentous as well as the non-elongated cells had stopped growing, with lysis being a consequence of the failure to grow. As it was not possible to follow individual cells of *E. coli* ATCC 23739 at times of incubation at 37°C > 1.5 h because of confluent growth on the slides, it was not established whether cells of *E. coli* ATCC 23739 that failed to grow also lysed beyond 1.5 h at 37°C. However, the irregular division at 37°C of daughter cells of both strains of *E. coli* that had been incubated at 6°C indicated that the physiological effects of incubation at refrigeration temperatures are not wholly resolved within a single round of replication. It should be noted that because of the nature of microscopic observation, only a limited number of cells could be followed with this technique, making generalizations subject to some extent of uncertainty.

Abrupt shifts of *E. coli* from refrigeration to warm temperatures can evidently result in both division of elongated or filamentous cells to multiple daughter cells and inactivation of cells. The extents to which these opposing responses occur apparently vary widely between strains. In at least some strains, inactivation becomes increasingly prevalent with time of exposure to refrigeration temperatures (Gill et al., 2007). The findings with *E. coli* ATCC 23739 indicate that with some strains, abrupt shift to 37°C may result in increases in their numbers at rates above those of usual growth at that temperature. With other strains an

abrupt shift to 37°C may result in substantial reductions in numbers (Visvalingam et al., 2012). Risks assessed on the basis of numbers of VTEC recovered from refrigerated foods that are consumed without warming may then be exaggerated, or sometimes underestimated, because of the inability to take account of changes in numbers possible on warming to body temperature.

The situation might, however, be different with slow warming of contaminated, refrigerated foods to ambient temperatures before they are consumed. Previous work suggests that in such circumstances there may be little inactivation of the *E. coli* that were viable (Jones et al., 2002), whether growing or not at refrigeration temperatures. Risks assessed from numbers of VTEC in slowly warmed refrigerated foods might then be consistently underestimated. Further work will be required to clarify that matter.

Table 4.1- Maximum mean rates of increase of the numbers of cfu ml^{-1}, the initial numbers and numbers attained after incubation at 37°C for 4 h of cells from each of three cultures of *Escherichia coli* ATCC 23739 or *E. coli* O157:H7 02:0627 incubated at 6°C.

Cultures, time at 6°C (d)	*E. coli* ATCC 23739			*E. coli* O157:H7		
	Rate of increase[abc] (log cfu/h)	Numbers (log cfu/ml)		Rate of increase (log cfu/h)	Numbers (log cfu/ml)	
		Initial	Final		Initial	Final
0	0.68 ± 0.03^{BCa}	7.2	8.9	0.64 ± 0.02^{Aa}	7.3	9.1
4	0.75 ± 0.03^{ABa}	7.5	8.7	0.50 ± 0.03^{Bb}	7.7	8.8
6	0.60 ± 0.10^{Ca}	7.7	9.0	0.50 ± 0.03^{Ba}	7.6	9.0
8	0.77 ± 0.04^{ABa}	7.7	8.6	0.64 ± 0.02^{Ab}	7.4	8.8

[a]Mean rate of increase during the second hour of incubation at 37°C. [b]Different superscript letters ($^{A-C}$) in columns denote a significant difference ($P< 0.05$). [c]Growth rates of each organism in rows denoted by different superscript letters ($^{a-b}$) indicates a significant difference ($P< 0.05$).

Table 4.2- The numbers and sizes of cells of *Escherichia coli* ATCC 23739 and *E. coli* O157:H7 from cultures incubated at 6°C for 5 d and observed on microscope slides when slides were initially incubated at 37°C, the numbers of those cells that lysed and the numbers of daughter cells formed after incubation at 37°C for 1.5 and 4.0 h, respectively.

Organism	Initial cells		Lysed cells	Daughter cells	
	Size (µm)	Number		After 1.5 h	After 4.0 h
E. coli ATCC 23739	≤4	5	0	4	-[a]
	> 4 ≤ 10	13	0	59	-
	>10	6	0	79	-
E. coli O157:H7	≤ 4	8	6	-	0
	>4 ≤ 10	8	1	-	400
	>10	2	2	-	0

[a]Number of daughter cells not determined.

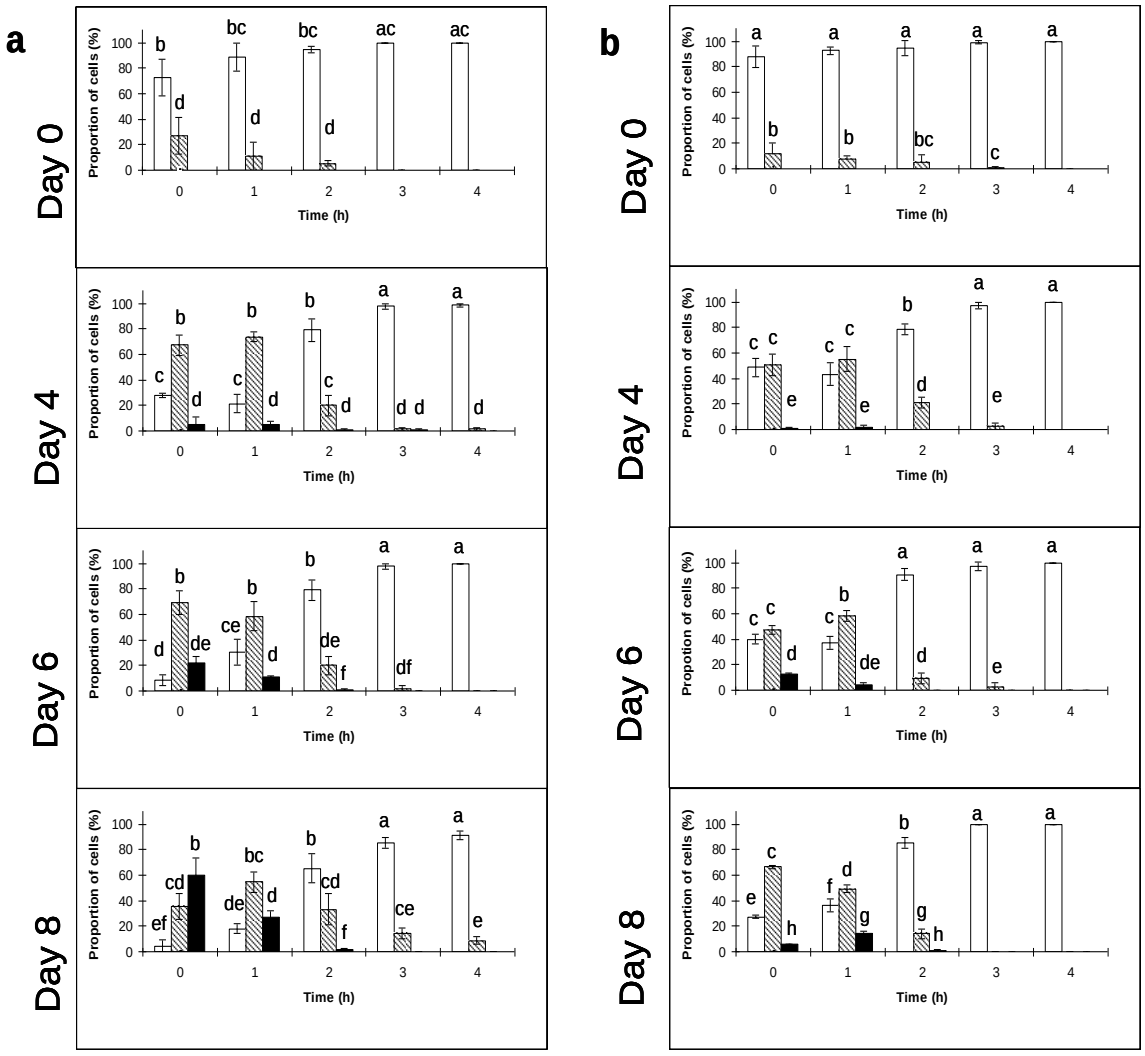

Fig. 4.1- Changes in the fractions of cells ≤4 µm (□), >4 ≤10 µm (▨) or >10 µm (■) long in suspensions of cells from cultures of a) *Escherichia coli* ATCC 23739 or b) *E. coli* O157:H7 02:0627 incubated at 6°C for 8 d, when suspensions were incubated at 37°C for 4 h.

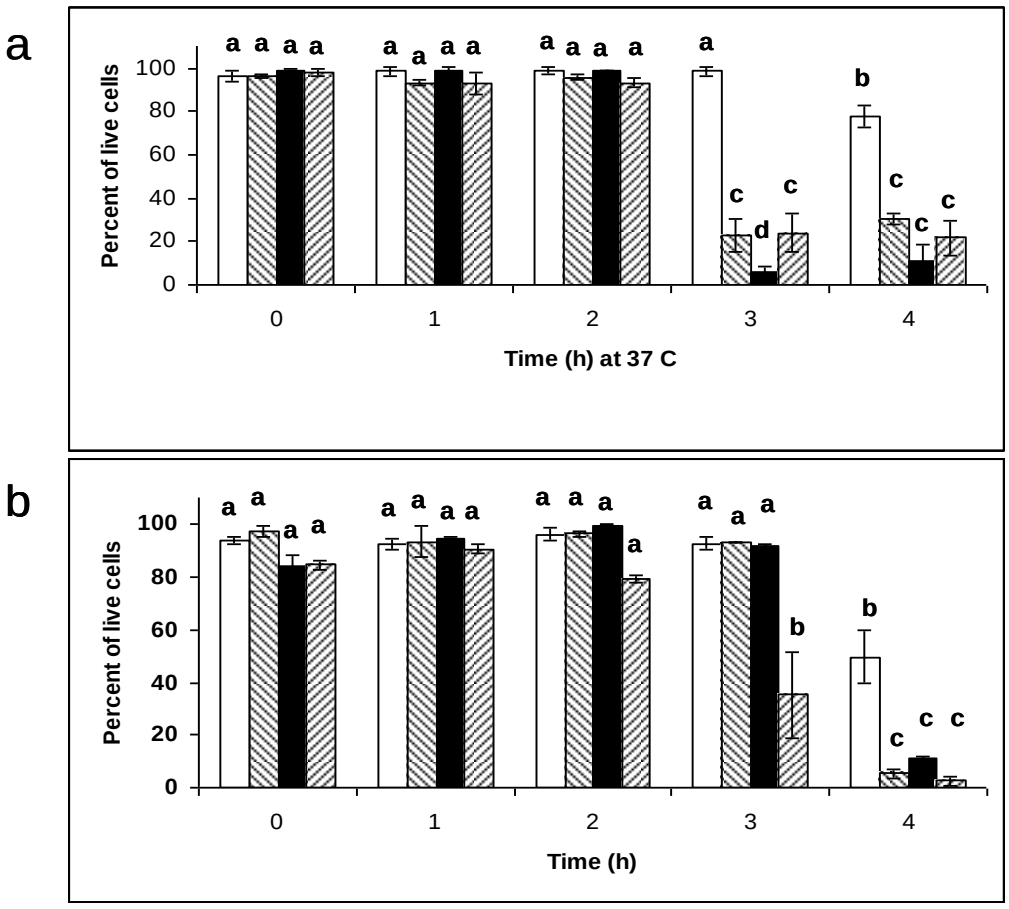

Fig. 4.2- Changes in the percent of viable cells of a) *Escherichia coli* ATCC 23739 and b) *E. coli* O157:H7, by CTC vitality stain, in suspensions of cells from cultures incubated at 6°C for 0 (□), 4 (▨), 6 (■) or 8 (▨) d, when suspensions were incubated at 37°C for 4 h.

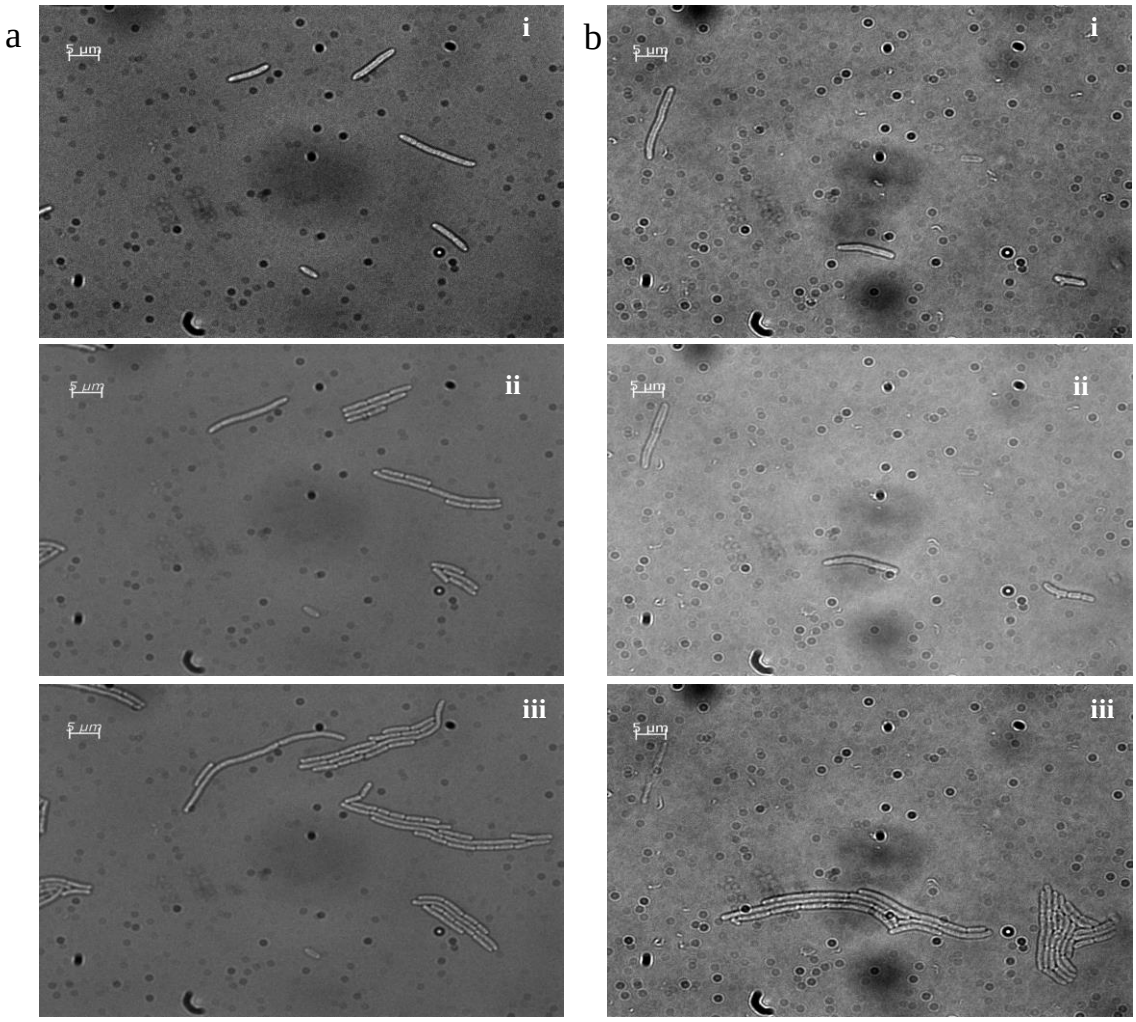

Fig. 4.3- Photomicrographs of agar-coated sides inoculated with a) *Escherichia coli* ATCC 23739 previously held at 6°C for 5 d, when slides were transferred to 37°C for 0 (i), 1 (ii) or 1.5 h (iii) and b) *E. coli* O157:H7 02:0627 previously held at 6°C for 5 d, when slides were transferred to 37°C for 0 (i), 2 (ii) or 4 h (iii). Photomicrographs in a) or b) are of the same organism in the same field of view at different times.

Chapter 5

Adherence of cold-adapted *Escherichia coli* O157:H7 strain 02:0627 to stainless steel and glass surfaces

5.1 Abstract

The effects of previous cold-induced cell elongation on adherence of *Escherichia coli* O157:H7 02:0627 to glass slides and stainless steel surfaces was evaluated at 4ºC for ≤ 48 h. Planktonic *E. coli* O157:H7 02:0627 with and without cold adaptation were prepared at 15 and 37ºC, respectively, and planktonic *E. coli* O157:H7 02:0627 containing elongated (>4≤10 μm) and filamentous (>10 μm) cells were prepared at 6ºC. Despite morphological differences in planktonic *E. coli* O157:H7 02:0627 preparations, all three cell types attached to a greater extent to glass than to the stainless steel surfaces. *E. coli* O157:H7 02:0627 cells adapted to growth at 15ºC attached better to both glass and stainless steel surfaces (3.2 and 2.6 log cfu/cm^2, respectively) than cells of the other treatments ≥ 24 h. Cells adapted at 6ºC attached to glass slides and stainless steel coupons at levels of 3.0 and 1.8 log cfu/cm^2, respectively, while *E. coli* O157:H7 02:0627 cells grown at 37ºC attached to these surfaces at levels of 2.0 and 1.7 log cfu/cm^2, respectively. No further attachment of cells from any of the treatments was noted between 24 and 48 h at 4°C. These results suggest that *E. coli* O157:H7 02:0627 cells adapted at 6ºC to 15ºC have greater potential to attach to food contact surfaces than those grown at higher temperature. The enhanced biofilm-forming ability of 6ºC or 15°C-adapted, elongated and filamentous *E. coli* O157:H7 02:0627 cells did not appear to be related to the greater adherence of longer cells.

5.2 Introduction

Strains of *Escherichia coli* O157:H7 are organisms of significant public health concern in most developed countries because of serious illness often caused when it contaminates food. The majority of illness outbreaks caused by this organism have involved meat, meat products, fruits and vegetables, particularly those that are ready-to-eat. Contamination of food can occur in processing environments via cross-contamination from raw material or from contaminated food contact surfaces (Pennington, 2010). After entry into processing facilities, *E. coli* O157:H7 can form biofilms on food contact surfaces which are resistant to regular cleaning/sanitizing and can become a persistent source of contamination for otherwise unadulterated food products (Pennington, 2010; Sofos and Geornaras, 2010; Van Houdt and Michiels, 2010).

The safe distribution of perishable raw foods for processing is dependent upon the effective operation of the cold supply chain (Montanari, 2008). However, if *E. coli* O157:H7 is present on contaminated food materials, the organism may become adapted to cold exposure during processing, distribution or storage (Vidovic et al., 2011). Studies conducted with mesophilic enteric organisms like *E. coli* at constant cool (about 7ºC) and fluctuating refrigeration temperatures that are similar to those experienced during daily defrosting of refrigeration equipment (Jones et al., 2004) and with *Salmonella* at a constant 8ºC (Mattick et al., 2003b), revealed that such conditions can induce cell elongation or filamentation. Similar behaviour was also observed with *E. coli* O157:H7 at various refrigeration temperatures (Gill et al., 2007). Subsequently, *E. coli* O157:H7 may also be able to multiply at faster than expected rates when temperatures exceed 7ºC (Sumner and Krist, 2002; Wang, 2004; Huang,

2010). Cold adaptation can change the extent and types of proteins expressed and change the cytoplasmic membrane composition of *E. coli* O157:H7 (Russell, 2002; Jones et al., 2006; Vidovic et al., 2011). These changes can alter the surface hydrophobicity, the surface charge of bacteria and affect their ability to attach and form biofilms on food contact surfaces (Hood and Zottola, 1995). Furthermore, altered morphology during extended exposure to cool temperatures may also affect the ability of *E. coli* O157:H7 to attach to food contact surfaces. However, these possibilities have not been considered in recent studies of *E. coli* O157:H7 biofilm formation on food contact surfaces where planktonic cells grown at 20 to 37ºC were used in tests (Hood and Zottola, 1997; Pawar et al., 2005; Silagyi et al., 2009; Skandamis et al., 2009; Dourou et al., 2011). Therefore, the objective of this study was to evaluate the effects of cold adaptation and cold-induced cell elongation upon the adherence of *E. coli* O157:H7 to food contact surfaces.

5.3 Materials and Methods

5.3.1 Bacterial strains and inoculum preparation

The *E.coli* O157:H7 strain 02:0627 used was provided by R. Ahmed, National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada). Stock cultures were preserved at - 80ºC and working cultures were maintained on Brain Heart Infusion Agar plates at 4ºC (BHIA; Oxoid, Mississauga, ON, Canada) with monthly transfer.

To obtain planktonic *E. coli* O157:H7 02:0627 without cold adaptation, a single colony from a streak plate was added to half-strength Brain Heart Infusion broth (BHIB; Accumedia, Lansing, MI, USA) and incubated at 37ºC overnight (16 to 18 h). Then 100 µl of

the overnight culture was used to inoculate 9.9 ml half-strength BHIB and incubated at 37ºC to obtain exponential phase cells with absorbance at 600 nm (A_{600}) between 0.6 and 0.7. A 1 ml sample of exponential phase culture was added to 199 ml half-strength BHIB to obtain bacterial numbers of about 10^6 cfu/ml.

To obtain 15 ºC-adapted planktonic cells, *E. coli* O157:H7 02:0627 was grown at 15ºC as previously described (Visvalingam et al., 2012) to get exponential phase cells with A_{600} between 0.6 and 0.7 and diluted in half-strength BHIB that was previously equilibrated at 15ºC to obtain bacterial numbers of about 10^6 cfu/ml.

To obtain 6 ºC-adapted planktonic cells, *E. coli* O157:H7 02:0627 was grown at 15ºC to A_{600} values between 0.6 and 0.7 and used to inoculate 500 ml flasks containing 180 ml half-strength BHIB that had been previously equilibrated at 15 ºC, yielding cultures with an A_{600} value between 0.06 to 0.07 (about 1.5 x 10^7 cfu/ml). The newly inoculated flasks were incubated at 6 ± 0.1°C for 8 d in a shaking water bath (Thermo Electron Precision, Winchester, VA, US) rotating at 100 rpm and generated cultures containing elongated and filamentous cells. A 20 ml sample collected after 8 d of incubation at 6°C was added to 180 ml half-strength BHIB that had been equilibrated at 6ºC to obtain bacterial numbers of about 10^6 cfu/ml.

5.3.2 Stainless steel coupon and glass slide preparation

Glass slides (7.6 cm x 2.5 cm, Fisher Scientific, Edmonton, AB, Canada) and stainless steel coupons (7.5 cm x 2.0 cm x 0.2 cm, grade 304, no. 4 finish; JC Stainless

Equipment Ltd, Winnipeg, MB, Canada) were cleaned using detergent, distilled water and 70 % ethanol as previously described (Dourou et al., 2011) and air-dried. Air-dried glass slides and stainless steel coupons were autoclaved at 121ºC for 15 min in a dry cycle.

5.3.3 Testing adherence

Sterile glass slides or stainless steel coupons were placed individually in separate sterile disposable Petri dishes (100 mm diameter, Fisher). Diluted samples (8 ml, about 10^6 cfu/ml) from *E. coli* O157:H7 02:0627 cultures grown at 37 ºC and samples from cold-adapted cultures at 6°C or 15°C were each added to 8 glass slides and 6 stainless steel coupons and incubated at 4ºC for ≤ 48 h. Test tubes containing 8 ml identical planktonic cultures were also incubated at 4ºC for ≤ 48 h to determine changes in the number of organisms and cell morphology in the liquid media. Three glass slides and stainless steel coupons were removed at 24 or 48 h, individually placed in a new Petri dish, and washed twice with 10 ml 4 ºC, 0.1% peptone water to remove unattached cells (Silagyi et al., 2009). The adherent cells were removed from glass slides or stainless steel coupons by swabbing 100 times with a sterile cotton swab (Fisher). The swab was transferred to a tube containing 10 ml 4ºC, 0.1 % peptone and vortex-mixed at 3000 rpm for 2 min to suspend the cells. To determine the number of organisms recovered from biofilms and in liquid media, cell suspensions were plated in duplicate on BHIA using a spiral plater (Spiral Biotech, Norwood, MA, US). Cell suspensions were decimally diluted in 0.1 % peptone before spiral plating, if needed. Plates were incubated at 37ºC for 18 to 24 h and colonies were counted.

5.3.4 Microscopy

One ml samples collected from test tubes immediately before and 24 or 48 h after incubation at 4ºC were fixed with formaldehyde (3.7%, w/v final concentration) and centrifuged for 10 min at 10,000 x*g*. The cell pellet was re-suspended in 100 µl 0.85 % (w/v) NaCl (Fisher) to obtain about 10^7 cells/ml. Microscope slides were prepared and 20 photomicrographs were taken of different fields of view with a 100x, 1.3 NA oil objective lens and an Axio Cam HRM camera (Carl ZeissMicroimaging GmBH, Gottingen, Germany) using differential interference contrast (DIC) optics. Cell length measurements were made and results were grouped into normal (≤ 4 µm), elongated (> 4 ≤ 10) or filamentous (> 10 µm) types as previously described (Visvalingam et al., 2012).

After 24 h were allowed for cell adherence, a glass slide was removed and washed with 0.1 % peptone as described above and fixed with 70 % ethanol for 2 min. One side of the slide was cleaned to remove adherent cells using sterile cotton wool. Then 10 µl 0.85% NaCl was placed on the uncleaned surface of the slide and covered with a glass coverslip. At least 200 different fields of view of each duplicate slide preparation were observed as described above.

5.3.5. Statistical analysis

Results presented are means ± standard deviations (SD) of triplicate samples. One-way analysis of variance (ANOVA) was used to determine differences in adherence by different planktonic culture types, and in changes in the number of organism and cell size distribution in BHIB at 4ºC. Tukey's test was used to assess differences between means and

statistical significance was identified when $p \leq 0.05$.

5.4 Results and Discussion

This study was designed to observe the differences in adherent properties of *E. coli* O157:H7 02:0627 grown at 37ºC compared to those of 15ºC or 6ºC-adapted cultures when exposed to 4ºC. Glass (slides) was chosen because of its use in refrigerated display cabinets and to facilitate light microscopic examination of adherent cells. Stainless steel coupons were chosen because of the use of stainless steel in food processing and storage environments. Both glass slides and stainless steel are hydrophilic materials (Chavant et al., 2002; Cerca et al., 2005).

When planktonic *E. coli* O157:H7 02:0627 cultures prepared without cold adaptation were incubated at 4ºC, a 0.20 log cfu/ml reduction in their numbers occurred at 24 h and viability remained stable during the next 24 h. The number of viable planktonic *E. coli* O157:H7 02:0627 cells adapted at 15ºC remained stable for 48 h at 4ºC, while a 0.64 log cfu/ml reduction was noted when planktonic *E. coli* O157:H7 02:0627 previously adapted at 6 ºC were incubated at 4ºC for $\geq$ 24 h (Fig 5.1).

The mean length distribution of planktonic *E. coli* O157:H7 02:0627 cells is presented in Table 5.1 and photomicrographs of different planktonic cells are presented in Figure 5.2a. Initially at 4ºC, the mean lengths of planktonic cells prepared at 37ºC, 15ºC and 6ºC were 2.7, 3.5 and 6.1 µm, respectively, and about 98%, 80% and 30 % of cells present were of normal size, respectively. The rest of the cells in these cultures were initially either elongated or filamentous at 4°C exposure. Mean cell lengths and cell size distribution of

organisms in the three treatments did not change during 4ºC incubation for 48 h (Table 5.1).

Incubation at 4ºC was chosen because it had been proven to keep viable numbers and morphology of 15 ºC-adapted exponential phase cells of a commensal *E. coli* strain stable for several days (Jones et al., 2004), and also was done to reflect the recommended temperature for holding perishable foods. Results obtained showed that not only were viable numbers and morphology of 15ºC-adapted planktonic *E. coli* O157:H7 02:0627 cells stable at 4ºC for ≤ 48 h, but also 6 and 37 ºC-adapted planktonic cells were stable as well (Fig 5.1, Table 5.1).

Quantitative microscopic determination of the size distribution of cells adherent to glass slides was unsuccessful because of the low number of fields of view (≤ 4/200) at 24 h which contained visible bacteria (Fig. 5.2). More elongated cells from 6°C-adapted cultures were observed to attach to the glass but this may have been related to the higher (almost 2-fold greater) content of elongated cells in this treatment (Table 5.1). Although microscopic observation was discontinued at 24 h, it is unlikely (but unknown) whether elongated *E. coli* O157:H7 02:0627 cells change their length upon physical attachment to surfaces (Annous et al., 2001).

It has been reported that in a nutrient rich medium, planktonic *E. coli* O157:H7 02:0627 prepared at 37ºC attached to the extent of about 1.8 log cfu/cm^2 to stainless steel surfaces after 72 h at 4 ºC (Dourou et al., 2011). Thus, the attachment of 1.7 log cfu/cm^2 to stainless steel after 24 h at 4ºC by planktonic *E. coli* O157:H7 02:0627 prepared at 37 ºC could be expected (Fig. 5.3a).

The adherence of 15ºC-adapted planktonic *E. coli* O157:H7 02:0627 was significantly

higher on both glass slides and stainless steel coupons than was observed with cultures prepared at 37°C or cold-adapted at 6ºC (Fig. 5.3a,b). *Listeria monocytogenes* grown to exponential phase at 8ºC were found to have more hydrophilic surface properties and tended to attach better to hydrophilic surfaces (Chavant et al., 2002). In addition to surface hydrophilicity, flagellar motility can play an important role in bacterial adherence to food contact surfaces under static conditions (Van Houdt and Michiels, 2010). Flagellin (FliC), the major protein component of flagella, was found to play an important role in biofilm formation, and its synthesis was found to be increased when *E. coli* O157:H7 was moved from 37 to 15°C (Carey et al., 2009). It is possible that the increased adherence observed here by 15ºC-adapted planktonic *E. coli* O157:H7 02:0627 to stainless steel and glass surfaces might have been due to increased hydrophilic interactions and motility. Even though *E. coli* O157:H7 02:0627 cold-adapted at 6ºC and non-cold-adapted (37ºC) cells attached to glass or stainless steel surfaces with similar frequency (Fig 5.3a,b), adherent cells of 6ºC-adapted planktonic cultures may have greater potential to multiply at a faster rate (by dividing into > 2 daughter cells simultaneously) than adherent cells of 37ºC-adapted planktonic cultures upon exposure to higher temperatures (Mattick et al., 2003a). Since the safety of the food supply is dependent upon proper cold-chain operation and since initial attachment is the key for successful biofilm development (Hood and Zottola, 1995), it is unsettling that 6 or 15ºC-adapted *E. coli* O157:H7 02:0627 may have enhanced ability to establish biofilms on food processing equipment and/or chilled processed foods.

5.5 Conclusion

Results of this study revealed that 15ºC-adapted *E. coli* O157:H7 02:0627 had

increased ability to attach to food contact surfaces at 4°C than cells from 6 or 37°C. Elongated and filamentous *E. coli* O157:H7 02:0627 cells formed at 6°C retained their ability to attach to food contact surfaces and their attachment was similar to planktonic *E. coli* O157:H7 02:0627 prepared at 37°C. Since the mean length of 15ºC-adapted cells was roughly half that of 6ºC-adapted cells, and since 15ºC-adapted cells attached significantly better to glass and stainless steel, these results suggest that entanglement of elongated and filamentous cells is an unlikely factor in enhancing attachment of these cells during biofilm formation.

Table 5.1- Influence of 48 h exposure at 4ºC upon the morphology of planktonic *Escherichia coli* O157:H7 02:0627 cells having different initial cell lengths and prior cold (6 or 15ºC) adaptation.

Planktonic culture type	Hours at 4ºC	Morphological characteristic			
		Mean cell length (µm)	Cell length distribution[1] (%)		
			≤ 4 µm	> 4 ≤ 10 µm	> 10 µm
Not cold-adapted (37°C)	0	2.7	98.3 ± 1.5[a]	1.7 ± 1.5[a]	0.00[a]
	24	2.8	98.3 ± 0.6[a]	1.7 ± 0.6[a]	0.00[a]
	48	2.8	97.3 ± 0.6[a]	2.7 ± 0.6[a]	0.00[a]
Cold-adapted (15ºC)	0	3.5	84.0 ± 6.0[b]	15.6 ± 6.5[b]	0.33 ± 0.6[a]
	24	3.9	77.0 ± 6.0[b]	22.3 ± 5.8[b]	0.7 ± 0.6[a]
	48	ND[2]	ND	ND	ND
Cold-adapted (6ºC)	0	6.1	29.3 ± 3.1[c]	64.0 ± 6.24[c]	6.7 ± 3.8[b]
	24	6.3	31.7 ± 5.5[c]	59.0 ± 4.4[c]	9.3 ± 2.1[b]
	48	6.3	31.3 ± 5.5[c]	60.3 ± 5.1[c]	8.3 ± 1.2[b]

[1]Different letters in each column denote significant difference ($P \leq 0.05$). [2]ND – Not determined, because of sample loss during centrifugation and re-suspension.

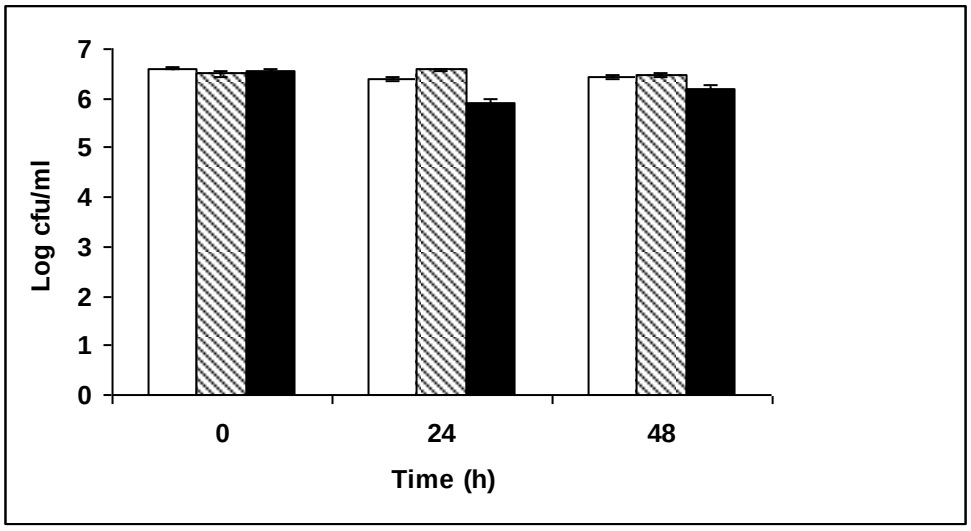

Fig. 5.1- Changes in the number of planktonic *E. coli* O157:H7 02:0627 (log cfu/ml) in BHIB held at 4°C for ≤ 48 h after inoculation of cells prepared at 37ºC without cold adaptation (□), with prior adaptation at 15 ºC (▨) and in cultures exposed at 6°C for 8 d before transfer to 4ºC, (■).

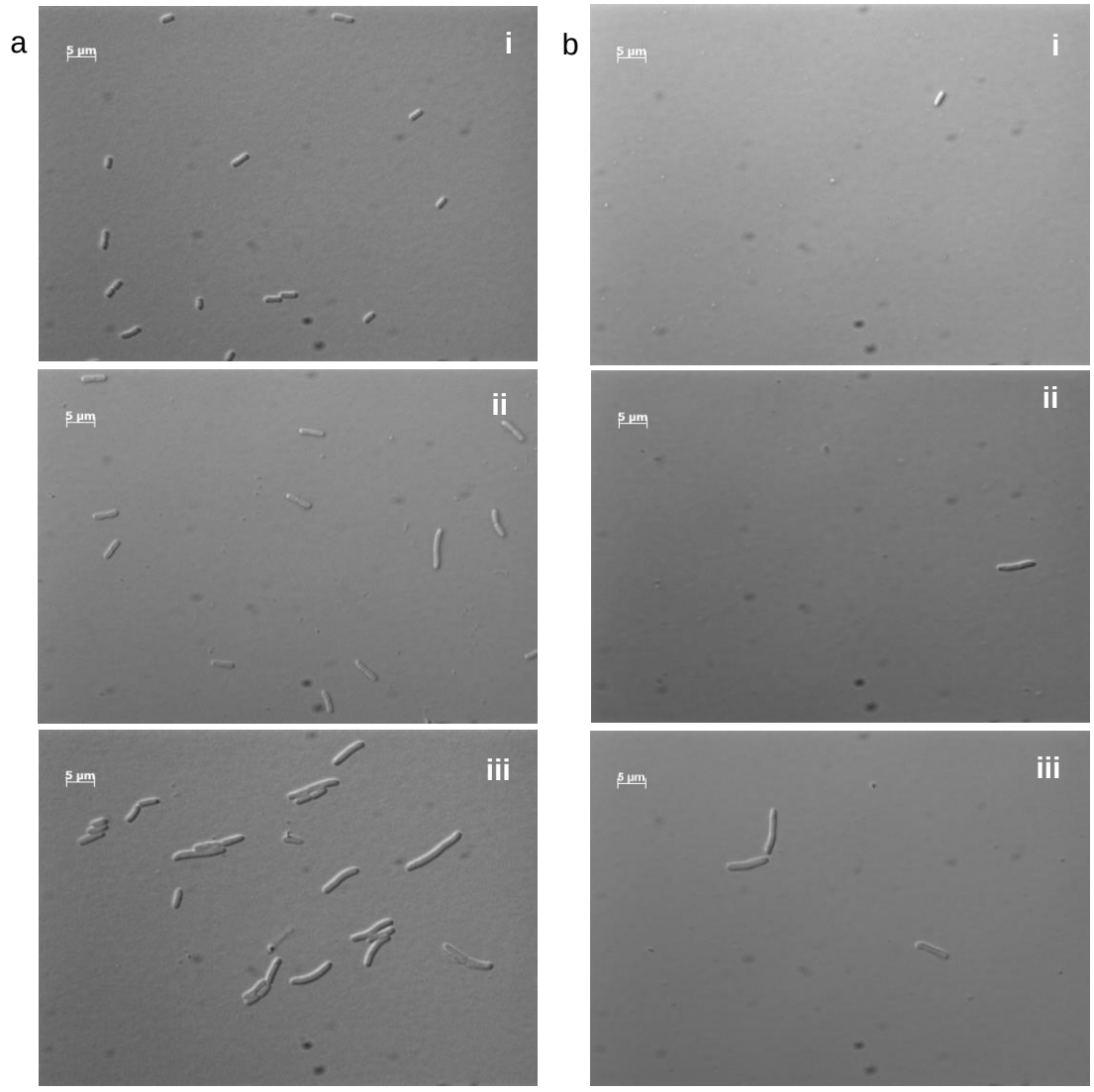

Fig. 5.2- Photomicrographs showing a) planktonic *E. coli* O157:H7 02:0627 cells prepared (i) at 37°C without cold adaptation, (ii) with prior adaptation at 15ºC and (iii) after adapted to 6°C for 8 d, and b) glass attachment observed after 24 h at 4 ºC by cultures prepared (i) at 37°C without cold adaptation, (ii) with prior adaptation at 15ºC and (iii) after adapted to 6°C for 8 d.

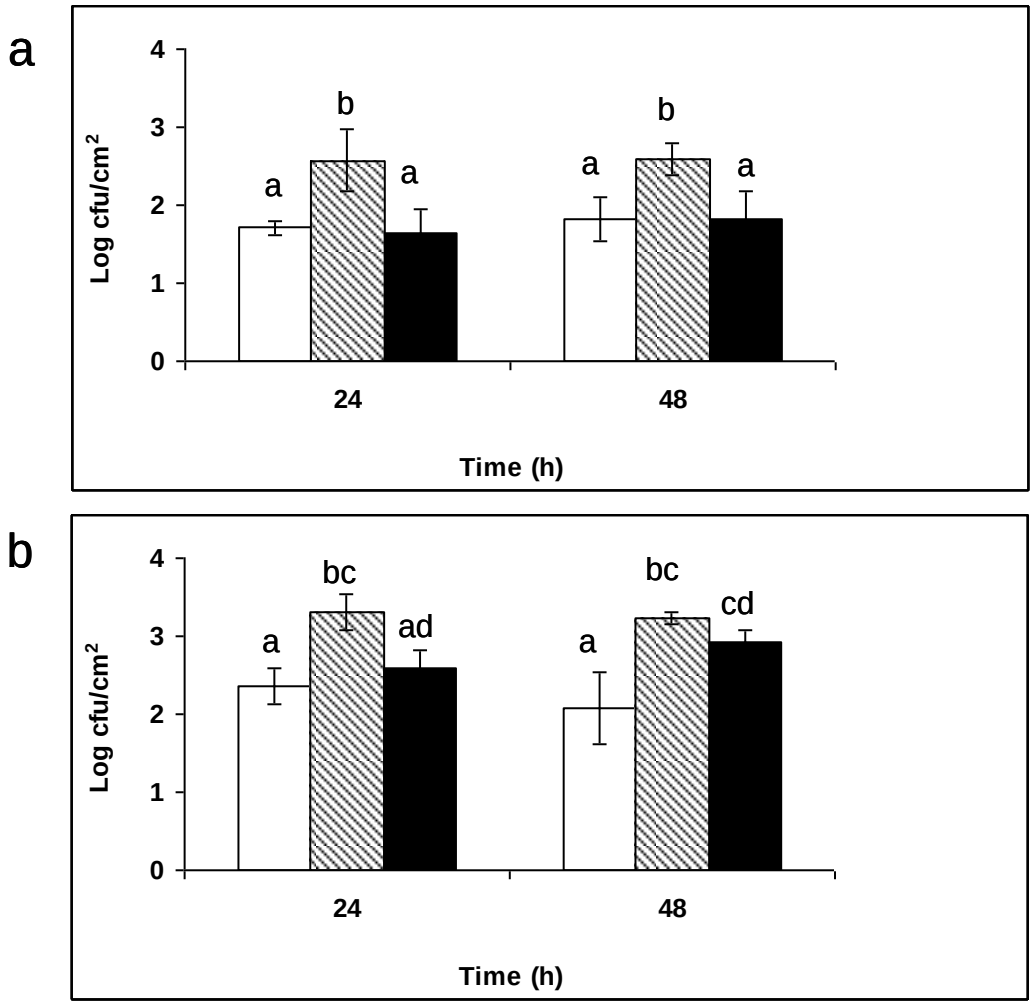

Fig. 5.3- The number of *E. coli* O157:H7 02:0627 (log cfu/cm^2) cells attached to a) stainless steel coupons and b) glass slides, after being submerged in planktonic *E. coli* O157:H7 02:0627 prepared at 37ºC without cold adaptation (□), with prior adaptation at 15ºC (▨) and in cultures exposed at 6ºC for 8 d containing elongated and filamentous cells before treatment at 4ºC for ≤ 48 h (■).

Chapter 6

Temperature-dependent effect of sub-lethal levels of cinnamaldehyde on viability and morphology of *Escherichia coli*

6.1 Abstract

Effects of sub-lethal levels of cinnamaldehyde on the viability and morphology of *Escherichia coli* O157:H7 and *E. coli* 8WT were investigated at 6ºC and 37ºC. The minimum inhibitory concentration of cinnamaldehyde against *E. coli* O157:H7 and *E. coli* 8WT was 400 mg/l. At 37ºC and ≤ 300 mg/l cinnamaldehyde delayed multiplication of both strains, causing ≤ 5 h and ≤ 13 h lag, respectively. Delayed multiplication at ≤ 300 mg/l was partly due to cell elongation and injury as determined by LIVE/DEAD viability, CTC vitality and bis-(1, 3-dibutylbarbituric acid) trimethine oxonol staining. The greatest extent of cell elongation (87 %) and greatest mean length (6.4 µm) occurred with *E. coli* O157:H7 at 2 h exposure to 200 mg/l cinnamaldehyde. After initial delays in multiplication, both *E. coli* O157:H7 and *E. coli* 8WT returned to exponential growth and normal morphology before reaching the stationary phase. In contrast at 6ºC, cinnamaldehyde at ≥ 100 mg/l prevented cell elongation which occurred in untreated control cells. Treatment with 200 or 300 mg/l cinnamaldehyde at 6ºC was lethal to both *E. coli* strains. At 300 mg/l cinnamaldehyde caused a ≥ 5 log cfu/ml reduction at ≤ 3 d and completely inactivated both of these organisms, causing ≥ 7 log cfu/ml reduction at 7 d. Sub-lethal levels of cinnamaldehyde at 37ºC delayed multiplication of *E. coli* cells by causing transient cell elongation, but at 6ºC ≥ 200 mg/l cinnamaldehyde was lethal to *E. coli*. Inhibition of cold-induced cell elongation and the enhanced lethal effect of cinnamaldehyde at 6ºC against *E. coli* O157:H7 suggest

cinnamaldehyde may be useful for control of this pathogen at refrigeration temperatures.

6.2 Introduction

Escherichia coli O157:H7 is one of the major foodborne pathogens in developed countries, causing serious infections that contribute significantly to the economic burden (Scharff, 2010) as well as the overall morbidity and mortality of foodborne illnesses (Henson et al., 2008; Buzby and Roberts, 2009). *E. coli* O157:H7 illness outbreaks have been associated with water as well as foods including meat and processed meat products, milk, fruits, juices, vegetables and ready-to-eat foods with extended shelf life (Pennington, 2010; Taban and Halkman, 2011).

Increasing consumer demand for foods that are treated with natural preservatives or by mild (gentle) processing methods instead of chemicals has stimulated effort to find and use alternative preservation methods. While minimal processing methods like modified atmosphere packaging and refrigeration can extend the shelf life of perishable foods, they cannot control the growth of psychrotrophic pathogens such as *Listeria monocytogenes* and *Yersinia enterocolitica* (Smid and Gorris, 2007). Even mesophilic enteric organisms like *E. coli* O157:H7 may be able to multiply when refrigerators operate at minima of 7ºC (Sumner and Krist, 2002) or produce elongated cells when held at > 4ºC (Gill et al., 2007). Since elongated cells formed at 6ºC can simultaneously yield multiple daughter cells upon exposure to growth permitting temperatures (Jones et al., 2002; Mattick et al., 2003a), *E. coli* O157:H7 filamentation under these conditions may represent an uncharacterized risk.

Spice essential oils can be potently antimicrobial and have the potential to be used as

natural alternatives to chemical preservatives for improving the safety and shelf life of perishable foods (Smid and Gorris, 2007). A wide range of studies have shown that many essential oils and their components are active against both Gram-positive and -negative foodborne pathogens. The antimicrobial action of essential oils is often determined by their components, most especially eugenol, cinnamaldehyde, thymol, and carvacrol (Burt, 2004; Holley and Patel, 2005).

Understanding how essential oils and their components act on microorganisms is key for their effective application in food preservation. Cinnamon oil has been reported to damage the cytoplasmic membrane and decrease the intracellular pH of *E. coli* O157:H7 (Oussalah et al., 2006). Its major constituent, cinnamaldehyde has also been reported to cause membrane damage, decrease the cellular ATP level, and inhibit membrane ATPase (Gill and Holley, 2004, 2006ab). But in these studies, lethal concentrations of cinnamon oil or cinnamaldehyde were used and treatment duration was short (< 30 min). Brief exposure to sub-lethal levels of cinnamaldehyde has not been reported to cause membrane damage or intracellular ATP depletion in *E. coli* O157:H7 (Helander et al., 1998). Although Kwon et al. (2003) reported that cinnamaldehyde treatment inhibited cell separation and caused cell elongation in *Bacillus cereus* at 37ºC; there is no information on viability of these elongated cells. As many foods are reported to contain cinnamaldehyde, often at concentrations that are lower than the levels lethal to *E. coli* O157:H7 (Helander et al., 1998; Friedman et al., 2000; Pei et al., 2009), it is of interest to understand how exposure to sub-lethal levels of cinnamaldehyde for longer durations would affect growth and morphology of *E. coli* O157:H7. It is understood that exposure to 6ºC can cause cell elongation in *E. coli* O157:H7; however, how

cinnamaldehyde alone or when combined with low temperature (6ºC) affects the viability or elongation of *E. coli* O157:H7 cells has not been characterized. Therefore, the objective of this study was to examine changes in growth and morphology of *E. coli* O157:H7 cells and cells of a commensal *E. coli* strain during exposure to sub-lethal concentrations of cinnamaldehyde at 6ºC for ≤ 7 d and at 37ºC for ≤ 24 h. The commensal *E. coli* strain used has been widely studied for its ability to form elongated cells at refrigeration temperatures (Jones et al., 2002, 2006; Gill et al., 2007; Visvalingam et al., 2012).

6.3 Materials and Methods

6.3.1 Bacterial strains and inoculum preparation

E. coli O157:H7 02:0627 and commensal *E. coli* 8WT used in this study were kindly provided by R. Ahmed, National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada) and C.O Gill, Agriculture Agri-Food Canada, Lacombe Research Centre (Lacombe, AB, Canada), respectively. Cultures were maintained on Brain Heart Infusion Agar plates (BHIA; Oxoid, Mississauga, ON, Canada) at 4 ºC with monthly transfer to new plates.

For cinnamaldehyde treatment at 37ºC, stirred *E. coli* cultures were obtained by inoculating a single colony from a streak plate in Brain Heart Infusion Broth (BHIB; Accumedia, Lansing, MI, US) with incubation at 37ºC for 16 to 18 h. Then 1 ml of an overnight culture was used to inoculate 99 ml BHIB and incubated at 37ºC to obtain exponential phase cells with absorbance at 600 nm (A_{600}) between 0.6 and 0.7.

For cinnamaldehyde treatment at 6ºC, stirred overnight *E. coli* cultures were obtained

by transferring a single colony from a plate to 50 ml BHIB followed by incubation at 25ºC. A 0.1 ml sample of overnight culture was transferred to 250 ml flasks containing 100 ml BHIB. Inoculated flasks were placed in a water bath (Brinkmann Lauda RC20, Brinkmann Instruments, Westbury, NY, US) maintained at 15ºC and incubated with stirring (magnetic bar) to obtain exponential phase cells with A_{600} between 0.6 and 0.7. Twenty ml of this culture was used to inoculate 200 ml BHIB pre-equilibrated at 6ºC (Visvalingam et al., 2012).

6.3.2 Cinnamaldehyde stock solution preparation

Cinnamaldehyde was obtained from SAFC Supply Solutions (St Louis, MO, US). Filter-sterilized (0.2 µm syringe filter unit, Fisher Scientific, Edmonton, AB, Canada) cinnamaldehyde was dissolved in BHIB containing 4% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) to yield a 6400 mg/l cinnamaldehyde stock solution, which was prepared immediately prior to each use.

6.3.3. Minimal inhibitory concentration determination

The broth macrodilution assay described by Wilson et al. (2005) was used with some modifications to determine the minimal inhibitory concentration (MIC) of cinnamaldehyde. Two-fold serial dilutions of cinnamaldehyde in BHIB were prepared from the stock solution at concentrations ranging from 3200 to 100 mg/l and 5 ml was added to 10 ml screw-capped glass tubes. Exponential phase culture prepared at 37ºC was diluted in BHIB to yield 2.5 x 10^6 cfu/ml. Five ml inoculum was added to tubes to achieve a total volume of 10 ml. A set of tubes containing similar concentrations of cinnamaldehyde in BHIB without inoculum

(negative control), and a tube containing only inoculum (positive control) were included in each experiment. The contents of the tubes were vortex-mixed and incubated at 37ºC for 24 h with shaking at 150 rpm (Titer Plate Shaker; Barnstead International, Dubuque, IA, US). The lowest concentration of cinnamaldehyde showing no visible growth or turbidity after 24 h at 37ºC was considered the MIC.

6.3.4 Cinnamaldehyde treatment at 6ºC or 37ºC

The effect of sub-lethal concentrations of cinnamaldehyde on exponential phase cells of *E. coli* at 37 ºC was examined. Appropriate amounts of the cinnamaldehyde stock solution were added to 250 ml screw-capped Erlenmeyer flasks to achieve final concentrations of 100, 200 and 300 mg/l after the total volume was adjusted to 200 ml by addition of inoculum and BHIB. Twenty ml of exponential phase culture prepared at 37 ºC was added to each flask to yield 2.5 x 10^7 cfu/ml. Cultures without cinnamaldehyde were maintained as controls. Flask contents were mixed and incubated at 37 ºC for ≤ 24 h with shaking at 100 rpm (Titer Plate Shaker).

Similar volumes of cold-adapted (15ºC) exponential phase cultures were used to inoculate flasks containing BHIB tempered at 6ºC with similar concentrations of cinnamaldehyde as used in the 37ºC treatments, but were incubated at 6 ºC for ≤ 7 d in a shaking water bath operating at 100 rpm (Thermo Electron Precision, Winchester, VA, US).

At 37ºC samples were collected immediately after cinnamaldehyde addition, hourly until the bacterial population reached its maximum threshold number (N_{max}), and then at 12 and/or 24 h for A_{600}, viable number estimation and microscopy. At 6ºC samples were

collected immediately after cinnamaldehyde addition, 6 h and daily for A_{600}, viable number estimation and microscopy. Changes in A_{600} values were monitored in 1 ml samples using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK). For viable number determination, a 1 ml sample was decimally diluted using 0.1 % (w/v) peptone (Difco, Becton Dickinson, Sparks, MD, US) and plated on duplicate BHIA plates using Spiral Plater (Autoplate 4000; Spiral Biotech, Norwood, MA, US). Plates were incubated at 37ºC for 18 to 24 h and colonies were counted.

To examine the presence of surviving cells in the 300 mg/l cinnamaldehyde treatment, a 30 ml sample collected from each flask on day 7 was centrifuged at 4ºC for 10 min at 10,000 x*g*, resuspended in 30 ml BHIB and incubated at 37ºC for 24 h. Then samples were plated on BHIA without dilution and incubated at 37ºC for 18 to 24 h.

6.3.5 Microscopy

Five ml samples collected from 6ºC or 37 ºC treatments were centrifuged at 4ºC for 10 min at 10,000 x*g*. Cell pellets were resuspended in 5 ml 0.85 % (w/v) NaCl (Fisher). The effect of cinnamaldehyde on cytoplasmic membrane integrity was evaluated with the LIVE/DEAD Bac*Light* viability kit (Invitrogen Canada, Burlington, ON, Canada) containing the green cytoplasmic membrane permeable dye SYTO 9 and the red impermeable dye propidium iodide, which enable the detection of cells with an intact cytoplasmic membrane (Boulos et al., 1999). One ml of bacterial suspension was stained with a 1:1 mixture of SYTO 9 and propidium iodide, and 10 photomicrographs were taken of different fields of view using an AxioImager M1 photomicroscope equipped with an AxioCam HRM camera (Carl ZeissMicroimaging GmBH, Gottingen, Germany) as previously described

(Visvalingam et al., 2012). The fraction of cells with an intact cytoplasmic membrane (fluorescent green) was calculated.

The numbers of actively respiring cells were estimated using the Bac*Light* RedoxSensor CTC vitality kit (Invitrogen). Electron transport activity during bacterial respiration reduces oxidized 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) and forms a red fluorescent precipitate in the cytoplasm. The DNA-binding fluorochrome 4', 6-diamino-2-phenylindole (DAPI), which gives blue fluorescence to cells, was used as a counter stain (Rodriguez et al., 1992). An 850 µl sample of cell suspension was mixed with 50 µl of 10 mmol/l glucose (Sigma-Aldrich) in a microcentrifuge tube. Then the cell suspension was stained at 25ºC with CTC, counterstained with DAPI as described by the manufacturer and photomicrographs were taken as described above using the DIC, epi-fluorescent DsRed and DAPI channels of the microscope. Cells that gave red fluorescence were counted and considered to represent actively respiring cells.

The effect of cinnamaldehyde on cytoplasmic membrane potential was evaluated with the membrane potential sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Invitrogen). The dye selectively penetrates cells with depolarized plasma membranes, binds to lipid-containing cell components, and makes cells fluoresce green (Suller and Lloyd, 1999). A 1 ml cell suspension was stained with 1µl, 1 mg/ml $DiBAC_4(3)$ and photomicrographs were taken as described above using the DIC, and epi-fluorescent GFP channels. Cells with no fluorescence were considered to have a polarized cytoplasmic membrane.

Microscopic examination was discontinued when the cell population of each

treatment reached their N_{max} at 37ºC and when numbers decreased to $\leq 10^5$ cfu/ml at 6ºC. In addition, after 24 h at 37ºC, cells were observed again to check for changes in morphology during the stationary phase.

6.3.6 Measurement of cell size

Digital photomicrographs were viewed using AxioVision Release 4.7 software (Carl Zeiss, München, Germany). For cell measurements, DIC optics were obtained by deflecting epi-fluorescent channels from view. A set of 100 cells were randomly selected from images and their lengths and widths were measured using the same software. Cells that measured > 4 µm were considered to be elongated.

6.3.7 Statistical analysis

All experiments were conducted in triplicate and results tabulated are the means ± standard deviation (SD). Treatment means were compared by one-way analysis of variance (ANOVA). Tukey's test was used to assess differences among treatments and statistical difference between treatment means was concluded when $P \leq 0.05$.

6.4 Results

6.4.1 Effect of cinnamaldehyde at 37ºC

Since the MIC value of cinnamaldehyde for both *E. coli* O157:H7 and *E. coli* 8WT was 400 mg/l, the effects of lower concentrations on growth were examined. When *E. coli* O157:H7 and *E. coli* 8WT were treated with ≤ 300 mg/l of cinnamaldehyde at 37ºC, the length of the lag before growth occurred was increased as cinnamaldehyde concentrations

increased (Fig. 6.1). Maximum growth delays of 5 h with *E. coli* O157:H7 and 13 h with *E. coli* 8WT were observed at 300 mg/l. Following growth delays, both *E. coli* O157:H7 and *E. coli* 8WT achieved similar rates of exponential growth. The duration and growth rates during the exponential phase were ≥ 4 h and ≤ 1.7 generations h^{-1} for controls and 100 mg/l cinnamaldehyde, and were ≤ 3 h and ≥ 1.8 generations h^{-1} with ≥ 200 mg/l cinnamaldehyde. Once the numbers of these organisms reached N_{max}, they remained stable for 24 h in all treatments.

Initially, A_{600} values of all treatments were ≤ 0.09 with *E. coli* O157:H7 and *E. coli* 8WT. A_{600} values of both organisms increased at a rate of 0.31 unit h^{-1} for ≥ 4 h without any delay for controls and 100 mg/l cinnamaldehyde, but at 200 and 300 mg/l cinnamaldehyde the A_{600} of these organisms slowly increased to about 0.2 to 0.3 before entering exponential growth, where they increased at about 0.34 unit h^{-1} for ≤ 3 h. At the end of the exponential phase, the A_{600} values of both organisms were about 1.2 units in all treatments.

When incubation at 37ºC commenced, the mean length of both *E. coli* O157:H7 and *E. coli* 8WT cells was ≤ 3.2 µm (Fig. 6.2a, b), and the fraction of elongated cells was ≤ 8 % (Fig. 6.3a, b) in all treatments. The mean lengths of untreated cells remained ≤ 3.5 µm for ≤ 24 h. The fraction of elongated cells of both organisms increased for 1 to 2 h at 100 and 200 mg/l cinnamaldehyde and reached maximum values of 87 % and 62 % at 2 h with *E. coli* O157:H7 and *E. coli* 8WT, respectively. At 300 mg/l cinnamaldehyde, the peak values of mean lengths and fractions of elongated cells occurred at 5 h with *E. coli* O157:H7 (Fig. 6.2a, 6.3a) and at 13 h with *E. coli* 8WT (Fig. 6.2b, 6.3b).The greatest mean length of *E. coli* O157:H7 was 6.4 µm and was observed after 2 h at 200 mg/l, while the longest cells of *E.*

coli 8WT which averaged 5.8 µm were observed after 13 h at 300 mg/l cinnamaldehyde. The maximum mean length and fraction of elongated cells observed with *E. coli* O157:H7 at ≤ 300 mg/l cinnamaldehyde were generally greater than values observed with *E. coli* 8WT in similar treatments. After reaching peak values, the mean length and fraction of elongated cells gradually decreased to ≤ 3 µm and ≤ 1 %, respectively, during exponential growth and reached normal morphology which was retained after 24 h at ≤ 300 mg/l cinnamaldehyde.

Initially, the mean width/diameters of *E. coli* O157:H7 and *E. coli* 8WT cells were 1.2 µm and remained at ≤1.2 µm throughout the treatment at ≤ 200 mg/l cinnamaldehyde. However, at 300 mg/l cinnamaldehyde, the mean width of *E. coli* O157:H7 and *E. coli* 8WT cells increased to 1.5 µm at 5 and 13 h, respectively, before returning to ≤1.2 µm at the end of exponential growth.

When incubation with ≤ 300 mg/l cinnamaldehyde at 37 ºC began, ≥ 94 % of cells of both *E. coli* O157:H7 and *E. coli* 8WT had intact cytoplasmic membranes, active respiration and were polarized. With longer treatment at 100 and 200 mg/l cinnamaldehyde no changes in these characteristics were noted. Following 300 mg/l cinnamaldehyde treatment for 3 h, the fraction of *E. coli* O157:H7 and *E. coli* 8WT cells with intact cytoplasmic membranes decreased to about 72 and 40 %, respectively (Appendix II, III). Examination of cells for intact cytoplasmic membranes was discontinued after 4 h because a large proportion of cells of both strains remained uncoloured by the LIVE/DEAD stain (Fig. 6.4a). However, uniform green staining of cells was observed after 24 h at 300 mg/l with both organisms (Fig. 6.4b).

The fraction of *E. coli* O157:H7 cells with both active respiration (Table 6.1) and polarized membranes (Appendix IV) decreased to a minimum of 71 % at 3 h of treatment

with 300 mg/l cinnamaldehyde, and this fraction later increased to ≥ 97 % at ≥ 6 h. With *E. coli* 8WT, the fraction of cells with active respiration (Table 6.2) and polarized membranes (Appendix V) reached a minimum of about 23 and 50 %, respectively, at 7 h treatment with 300 mg/l cinnamaldehyde and later this fraction gradually increased to 98 % at 16 h.

6.4.2 Effect of cinnamaldehyde at 6ºC

The number of viable organisms remained relatively stable for 7 d with control and 100 mg/l cinnamaldehyde-treated *E. coli* O157:H7 and *E. coli* 8WT (Table 6.3). Treatment with 200 mg/l cinnamaldehyde caused 3 log cfu/ml reductions in the numbers of both strains at 7 d. When both organisms were treated with 300 mg/l cinnamaldehyde, a ≥ 5 log cfu/ml reduction occurred at 3 d, which was followed by complete inactivation at 7 d (confirmed by 24 h enrichment in BHIB).

Initially, A_{600} values of control and cinnamaldehyde-treated *E. coli* O157:H7 and *E. coli* 8WT were ≤ 0.08. In the absence of cinnamaldehyde, A_{600} values of *E. coli* O157:H7 and *E. coli* 8WT progressively increased to 0.4 and 0.3, respectively on day 7, while A_{600} values remained ≤ 0.08 for 7 d in the presence of 100 to 300 mg/l cinnamaldehyde.

When incubation at 6°C began, the mean length and the fraction of elongated *E. coli* O157:H7 and *E. coli* 8WT cells were ≤ 4.5 µm and ≤ 40 % for both strains. In the absence of cinnamaldehyde, the mean length and fraction of elongated *E. coli* O157:H7 cells progressively increased to 7.5 µm and 77 %, respectively, at day 7 (Appendix VI). With *E. coli* 8WT, these values decreased for ≤ 1 d and then progressively increased for ≥ 6 d to reach 5.3 µm (Fig 6.5). In contrast, in the presence of cinnamaldehyde the mean length and

extent of cell elongation with both strains remained ≤ 4 μm and ≤ 40 % for ≤ 7 d. The mean widths of cinnamaldehyde-treated and control cells were unchanged at ≤ 1.1 μm for ≤ 7 d.

Also at the start of 6ºC incubation, the fractions of *E. coli* O157:H7 and *E. coli* 8WT cells with intact cytoplasmic membranes were ≥ 99 % in all treatments, except at 300 mg/l cinnamaldehyde with *E. coli* 8WT, which was 83 % (Table 6.4). With the control, the fraction of *E. coli* O157:H7 cells with intact cytoplasmic membranes remained ≥ 94 % for 7 d while with *E. coli* 8WT it decreased to 75 % at 7 d. Substantially greater reductions in the fraction of *E. coli* 8WT than *E. coli* O157:H7 cells with intact cytoplasmic membranes were observed after ≥ 2 d at 100 mg/l, ≥ 1 d at 200 mg/l or ≥ 6 h at 300 mg/l cinnamaldehyde.

The observed changes in the fractions of *E. coli* O157:H7 and *E. coli* 8WT cells with active respiration and a polarized cytoplasmic membrane were very similar to those observed with intact cytoplasmic membranes at ≤ 300 mg/l cinnamaldehyde for ≤ 7 d (data not presented).

6.5 Discussion

Antibacterial potency of essential oils and their components is conventionally expressed as the MIC (Burt and Reinders, 2003; Helander et al., 1998; Hammer et al., 1999). Since these compounds are hydrophobic to varying degrees, a wide variety of emulsifying or stabilizing agents have been used to enable MIC determination (Burt, 2004). While their use can influence experimental outcomes (Burt 2004; Blaszyk and Holley, 1998) it was unlikely that DMSO used here affected the results. It was not inhibitory when used alone at the test concentrations and the MIC values obtained were consistent with those reported by Helander

et al. (1998) and Pei et al. (2009) who used different solubilizing agents.

Exponential phase cultures were used in this study to avoid a delay (lag) in growth upon transfer to new media (White, 2007), and to observe how sub-lethal concentrations of cinnamaldehyde affected growth of *E. coli* unrestrained by substrate limitation at 37ºC. Growth was delayed by cinnamaldehyde in a concentration-dependent manner with both *E. coli* strains at 37ºC. However, cell mass as measured by A_{600}, and cell length increased during this delay. At 300 mg/l cinnamaldehyde and 37ºC cells of both strains become wider. Increases in cell mass as a result of cell elongation without substantial changes in the number of *E. coli* have been well documented at chill temperatures (Jones et al., 2002; Gill et al., 2007; Visvalingam et al., 2012), and similar increases were also observed with A_{600} and cell length/ number of elongated cells in the absence of cinnamaldehyde at 6ºC. The width of cells of both organisms was not affected by incubation at 6ºC alone or when combined with cinnamaldehyde.

Previous studies on cold-induced cell elongation of *E. coli* (Gill et al., 2007) and *Salmonella* (Mattick et al., 2003a) showed that the extent of cell elongation varied greatly among bacterial strains. Then, differences in cell elongation observed between *E. coli* O157:H7 and *E. coli* 8WT in the presence of cinnamaldehyde at 37ºC and in the absence of cinnamaldehyde at 6ºC could be expected.

Fluorescent dyes that are used to detect cytoplasmic membrane injury and respiration may also be used for rapid detection of bacterial inactivation (Rodriguez et al., 1992; Suller and Lloyd, 1999; Boulos et al., 1999). Divergent results from fluorescent staining and viable numbers at 37ºC with 300 mg/l cinnamaldehyde treated *E. coli* O157:H7 at the first 5 h were

likely due to sub-lethal injury, and this appeared to be reversed later in some cells. On the other hand, substantial reductions in the number of cells with active respiration, showing intact and polarized cytoplasmic membranes as well as the number of viable *E. coli* 8WT between 10 to 12 h indicate that part of the *E. coli* 8WT population was inactivated.

Overall, at 37ºC the observed delays in multiplication of *E. coli* O157:H7 and *E. coli* 8WT at 300 mg/l were partly due to sub-lethal injury and cell elongation. The longer growth delay, partial inactivation, and greater cell injury observed with *E. coli* 8 WT at 300 mg/l indicate that *E. coli* 8WT was more sensitive to the inhibitory effects of cinnamaldehyde at 37ºC. Skandamis et al. (2001) found that when oregano essential oil was used at 0.03 % v/v (close to the minimum bactericidal concentration of 0.4 % v/v), against *E. coli* O157:H7 at 37ºC, inhibitory effects on growth dynamics were similar to those seen in the present study. It was concluded that oregano caused injury (membrane roughness increased and there was loss of granular cytoplasmic substance), however changes in bacterial length were not reported.

Lack of green fluorescence of 300 mg/l cinnamaldehyde-treated *E. coli* cells after LIVE/DEAD staining and the absence of red fluorescence (propidium iodide) in many of these cells suggests that the normal uptake of SYTO 9 by viable cells was prevented by cinnamaldehyde at 37ºC without affecting membrane integrity. However, it is possible that 300 mg/l cinnamaldehyde treatment altered the composition or structure of either or both membranes of *E. coli* and reduced SYTO 9 penetration at 37ºC (Di Pasqua et al., 2006; Berney et al., 2007). Similarities in the changes caused by both $DiBAC_4(3)$ (which can only penetrate cells with a depolarized plasma membrane) and CTC suggest that structural alteration in either or both membranes caused by cinnamaldehyde at 300 mg/l did not affect

the ability of these dyes to enter cells.

Eventually, both *E. coli* strains overcame the inhibitory effects of cinnamaldehyde at 37ºC and began exponential growth. Significant reductions in the fractions of elongated cells within the first 2 h of exponential growth and return to normal length (≤ 4 μm) suggest elongated cells either lysed or divided into normal size cells (Mattick et al., 2003a; Visvalingam et al., 2012).

At 6ºC, cinnamaldehyde treatment was lethal to both *E. coli* O157:H7 and *E. coli* 8WT, and lethal effects were concentration dependent. Further, cold-induced cell elongation which occurred in the absence of cinnamaldehyde (Gill et al., 2007; Visvalingam et al., 2012) was prevented at 6ºC by 100 to 300 mg/l cinnamaldehyde. The maintenance of cellular activities and cell elongation at 6ºC have been reported to be dependent upon continuous energy generation (Jones et al., 2006). The observed lethal effects at 6ºC and inhibition of cold-induced cell elongation by cinnamaldehyde may have been due to retardation of energy generation by inhibition of glucose uptake or its utilization and reduced ATP synthesis (Gill and Holley, 2004, 2006b), as well as through interference with maintenance of plasma membrane integrity or respiratory activity.

Results obtained indicate that at 37ºC the temporary growth delay caused by ≤ 300 mg/l cinnamaldehyde was due to damage of the cytoplasmic membrane, inhibition of respiratory activity and the formation of elongated cells which were reversible. Commensal *E. coli* 8WT was more sensitive to the inhibitory effects of cinnamaldehyde than *E. coli* O157:H7. At 6ºC, levels of cinnamaldehyde ≥ 200 mg/l were lethal to both *E. coli* O157:H7 and *E. coli* 8WT. Cinnamaldehyde at ≤ 300 mg/l did not cause cell elongation at 6ºC, in fact,

even 100 mg/l prevented cell elongation caused by 6ºC exposure. The observations at 6ºC suggest that cinnamaldehyde may be used as an alternative to control *E. coli* O157:H7 in perishable refrigerated foods.

Table 6.1- The fractions of actively respiring of *E. coli* O157:H7 cells observed by CTC vitality staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 8 h at 37ºC.

Time (h)	Percent of actively respiring cells*†			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	97.0 ± 2.2^{A}	98.7 ± 0.5^{A}	96.1 ± 1.7^{A}	94.9 ± 1.2^{AC}
1	97.1 ± 5.0^{A}	96.5 ± 0.6^{A}	96.7 ± 1.5^{A}	81.7 ± 6.2^{BC}
2	99.3 ± 0.6^{A}	99.8 ± 0.3^{A}	95.4 ± 1.6^{A}	74.2 ± 9.1^{B}
3	99.5 ± 0.8^{A}	99.3 ± 0.6^{A}	96.0 ± 0.7^{A}	70.7 ± 5.6^{B}
4	99.3 ± 0.6^{A}	99.4 ± 0.5^{A}	92.8 ± 2.4^{A}	75.1 ± 1.7^{B}
5	98.6 ± 1.8^{A}	96.8 ± 1.6^{A}	99.6 ± 0.7^{A}	76.9 ± 6.3^{B}
6	95.7± 0.7^{A}	99.6 ± 0.5^{A}	98.6 ± 0.6^{A}	97.1± 2.2^{A}
7	ND	ND	ND	97.3 ± 1.3^{A}
8	ND	ND	ND	88.9 ± 2.3^{AC}

*ND – Not determined. †Different superscript letters ($^{A-C}$) in each column denote a significant difference ($P < 0.05$).

Table 6.2- The fractions of actively respiring *E. coli* 8WT cells observed by CTC vitality staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 24 h at 37ºC.

Time (h)	Percent of actively respiring cells*†			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	99.4 ± 0.8^{A}	98.1 ± 1.8^{A}	92.6 ± 1.2^{A}	97.9 ± 0.5^{A}
1	99.6 ± 0.9^{A}	93.8 ± 2.2^{A}	98.3 ± 0.5^{A}	52.9 ± 4.9^{C}
2	100 ± 0^{A}	97.7 ± 0.7^{A}	98.6 ± 0.5^{A}	85.5 ± 4.9^{AD}
3	99.7 ± 0.6^{A}	99.0 ± 1.2^{A}	96.5 ± 2.5^{A}	63.6 ± 11.4^{CF}
4	99.7 ± 0.4^{A}	98.6 ± 0.6^{A}	96.0 ± 1.4^{A}	65.8 ± 6.7^{CF}
5	99.6 ± 0.7^{A}	98.6 ± 0.6^{A}	99.3 ± 0.6^{A}	62.7 ± 6.6^{CF}
6	99.6 ± 0.6^{A}	99.6 ± 0.1^{A}	98.4 ± 0.4^{A}	63.6 ± 3.9^{C}
7	ND	ND	ND	23.0 ± 5.0^{E}
8	ND	ND	ND	58.8 ± 11.8^{C}
9	ND	ND	ND	62.1 ± 6.8^{C}
10	ND	ND	ND	61.7 ± 10.1^{C}
11	ND	ND	ND	71.0 ± 4.8^{CDF}
12	ND	ND	ND	75.4 ± 8.3^{BDF}
13	ND	ND	ND	77.0 ± 1.5^{BDF}
14	ND	ND	ND	83.7 ± 7.9^{A}
15	ND	ND	ND	92.0 ± 3.2^{A}
16	ND	ND	ND	98.0 ± 2.0^{A}
18	ND	ND	ND	96.8 ± 1.7^{A}
19	ND	ND	ND	98.0 ± 0.3^{A}

*ND – Not determined. †Different superscript letters ($^{A-F}$) in each column denote a significant difference ($P < 0.05$).

Table 6.3- Changes in the number of viable *E. coli* O157:H7 and *E. coli* 8WT cells after treatment with sub-lethal levels of cinnamaldehyde at 6ºC for ≤ 7 d.

Time (Days)	Number of cells (log cfu/ml)*†							
	E. coli O157:H7				*E. coli* 8WT			
	Cinnamaldehyde concentration (mg/l)				Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300	Control	100	200	300
0	7.2 ± 0.1^{Ca}	7.2 ± 0.0^{Aa}	7.3 ± 0.0^{Aa}	7.3 ± 0.0^{Aa}	7.3 ± 0.1^{Bab}	7.4 ± 0.0^{Aa}	7.2 ± 0.1^{Ab}	7.3 ± 0.0^{Aab}
0.25	7.4 ± 0.1^{Ba}	7.2 ± 0.0^{Ab}	7.3 ± 0.0^{Aab}	7.0 ± 0.1^{Ac}	7.6 ± 0.0^{Aa}	7.3 ± 0.1^{Ab}	7.1 ± 0.1^{Ab}	6.4 ± 0.2^{Bd}
1	7.5 ± 0.0^{ABa}	7.2 ± 0.0^{Ab}	7.4 ± 0.0^{Aa}	6.7 ± 0.1^{Bc}	7.6 ± 0.0^{Aa}	7.0 ± 0.2^{Bb}	6.7 ± 0.1^{Bc}	2.8 ± 0.5^{Cd}
2	7.5 ± 0.0^{ABa}	7.1 ± 0.1^{ABb}	6.5 ± 0.1^{Bc}	2.5 ± 0.1^{Cd}	7.5 ± 0.0^{Aa}	6.7 ± 0.1^{Cb}	6.1 ± 0.1^{Bc}	3.1 ± 0.6^{Cd}
3	7.6 ± 0.1^{Aa}	7.0 ± 0.1^{BCb}	5.9 ± 0.1^{Cc}	1.7 ± 0.2^{Dd}	7.6 ± 0.1^{Aa}	6.5 ± 0.1^{Db}	5.7 ± 0.3^{Cc}	1.8 ± 0.6^{Dd}
4	7.5 ± 0.1^{ABa}	7.0 ± 0.0^{BCb}	5.3 ± 0.0^{Dc}	1.1 ± 0.2^{Ed}	7.4 ± 0.0^{Ba}	6.4 ± 0.1^{DFb}	5.3 ± 0.2^{Dc}	1.7 ± 0.0^{Dd}
5	7.5 ± 0.1^{ABa}	6.9 ± 0.1^{Cb}	4.6 ± 0.1^{Ec}	0.4 ± 0.4^{Fd}	7.3 ± 0.0^{Ba}	6.3 ± 0.1^{Fb}	5.0 ± 0.1^{Ec}	$< 0.4^{Ed}$
6	7.4 ± 0.1^{Ba}	6.8 ± 0.1^{Cb}	4.2 ± 0.1^{Fc}	$< 0.4^{Gd}$	7.1 ± 0.1^{Cb}	6.0 ± 0.1^{Gb}	4.7 ± 0.2^{Fc}	$< 0.4^{Ed}$
7	7.2 ± 0.2^{Ca}	6.6 ± 0.1^{Db}	3.2 ± 0.1^{Gc}	0.0 ± 0.0^{Gd}	6.8 ± 0.0^{Cb}	5.7 ± 0.1^{Hb}	4.4 ± 0.1^{Gc}	0.0 ± 0.0^{Ed}

*Different superscript letters ($^{A-G}$) in each column denote a significant difference ($P < 0.05$). †Different superscript letters ($^{a-d}$) in each row of each organism denote a significant difference ($P < 0.05$).

Table 6.4- The fractions of *E. coli* O157:H7 and *E. coli* 8WT cells with intact cytoplamic membranes observed by LIVE/DEAD staining after exposure to sub-lethal levels of cinnamaldehyde at 6ºC for ≤ 7 d.

Time (Days)	Percent of cells with intact cytoplasmic membrane*†							
	E. coli O157:H7				*E. coli* 8WT			
	Cinnamaldehyde concentration (mg/l)				Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300	Control	100	200	300
0	99.0 ± 1.0^{A}	99.0 ± 1.0^{A}	99.0 ± 1.0^{A}	100 ± 0.0^{A}	99.8 ± 0.3^{A}	99.9 ± 0.2^{A}	100 ± 0.0^{A}	82.5 ± 2.8^{A}
0.25	98.9 ± 1.0^{A}	94.3 ± 2.5^{A}	95.2 ± 6.7^{A}	99.5 ± 0.5^{A}	99.6 ± 0.4^{A}	85.5 ± 4.0^{A}	40.9 ± 8.9^{B}	15.3 ± 4.1^{B}
1	98.2 ± 0.7^{A}	97.3 ± 1.2^{A}	89.2 ± 5.2^{A}	66.3 ± 9.2^{B}	95.0 ± 2.3^{A}	44.1 ± 5.3^{B}	28.5 ± 10.3^{B}	ND
2	97.5 ± 1.6^{AB}	95.7 ± 1.8^{A}	76.1 ± 4.5^{A}	ND	94.3 ± 2.8^{A}	26.1 ± 3.1^{B}	35.9 ± 15.4^{B}	ND
3	97.8 ± 1.2^{AB}	84.1 ± 4.1^{B}	46.9 ± 15.1^{B}	ND	86.6 ± 0.4^{B}	36.5 ± 16.7^{B}	22.8 ± 12.0^{BC}	ND
4	97.9 ± 0.6A	87.9 ± 0.5^{B}	50.4 ± 10.0^{B}	ND	84.3 ± 1.5^{BD}	34.6 ± 22.1^{BC}	ND	ND
5	97.4 ± 1.2^{AB}	84.5 ± 2.2^{B}	ND	ND	77.7 ± 3.0^{CD}	20.5 ± 5.5^{BC}	3.8 ± 1.4^{C}	ND
6	94.0 ± 2.6^{B}	86.5 ± 2.6^{B}	ND	ND	74.5 ± 4.3^{C}	7.3 ± 1.9^{C}	ND	ND
7	96.6 ± 1.0^{AB}	84.1 ± 1.8^{B}	ND	ND	75.1 ± 1.9^{C}	ND	ND	ND

*ND – Not determined. †Different superscript letters ($^{A-F}$) in each column denote a significant difference (P< 0.05).

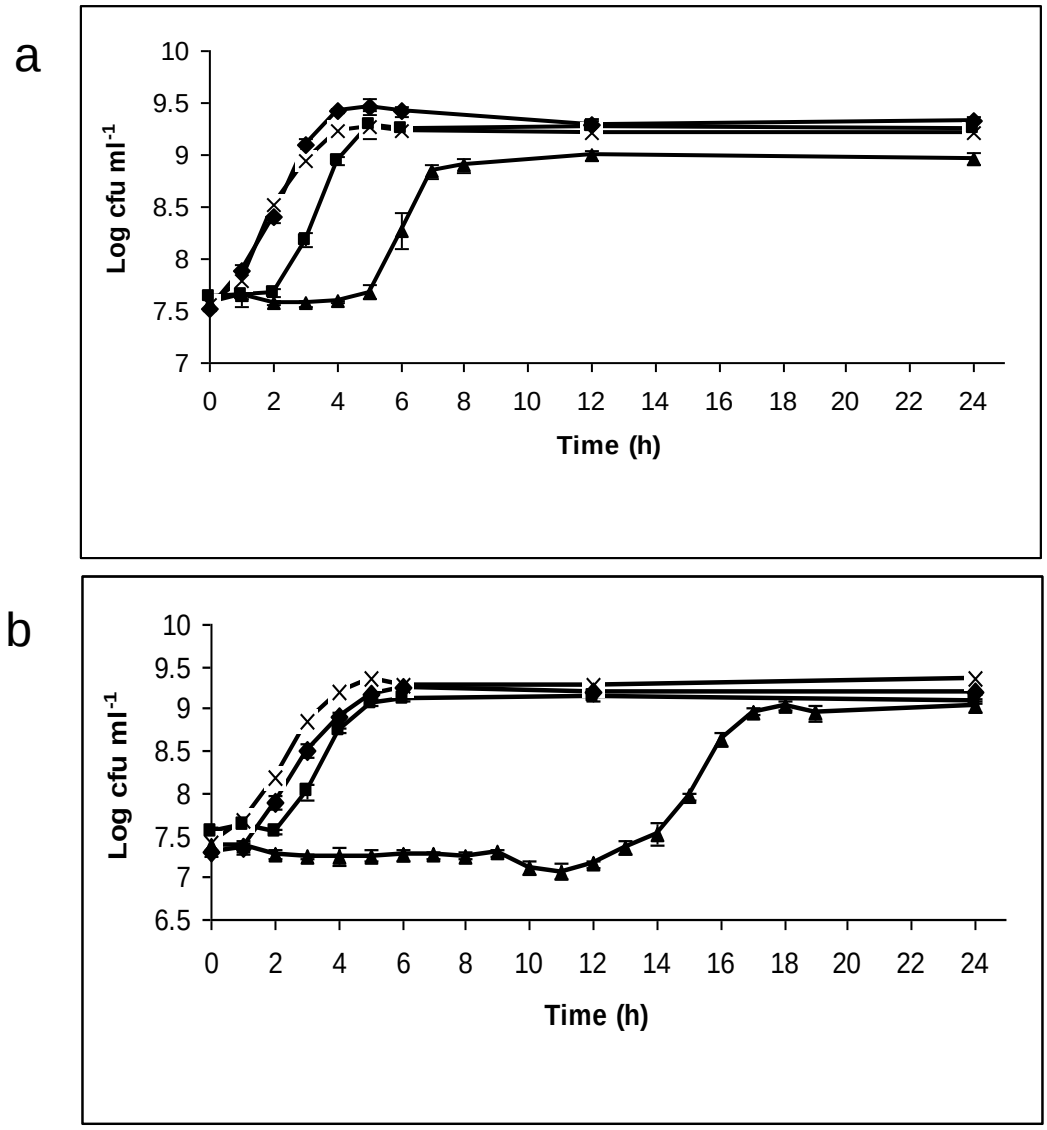

Fig. 6.1- Changes in the numbers (log cfu/ml) of a) *E. coli* O157:H7 and b) *E. coli* 8 WT in the absence (×) or presence of 100 mg/l (♦), 200 mg/l (■) or 300 mg/l (▲) cinnamaldehyde at 37ºC.

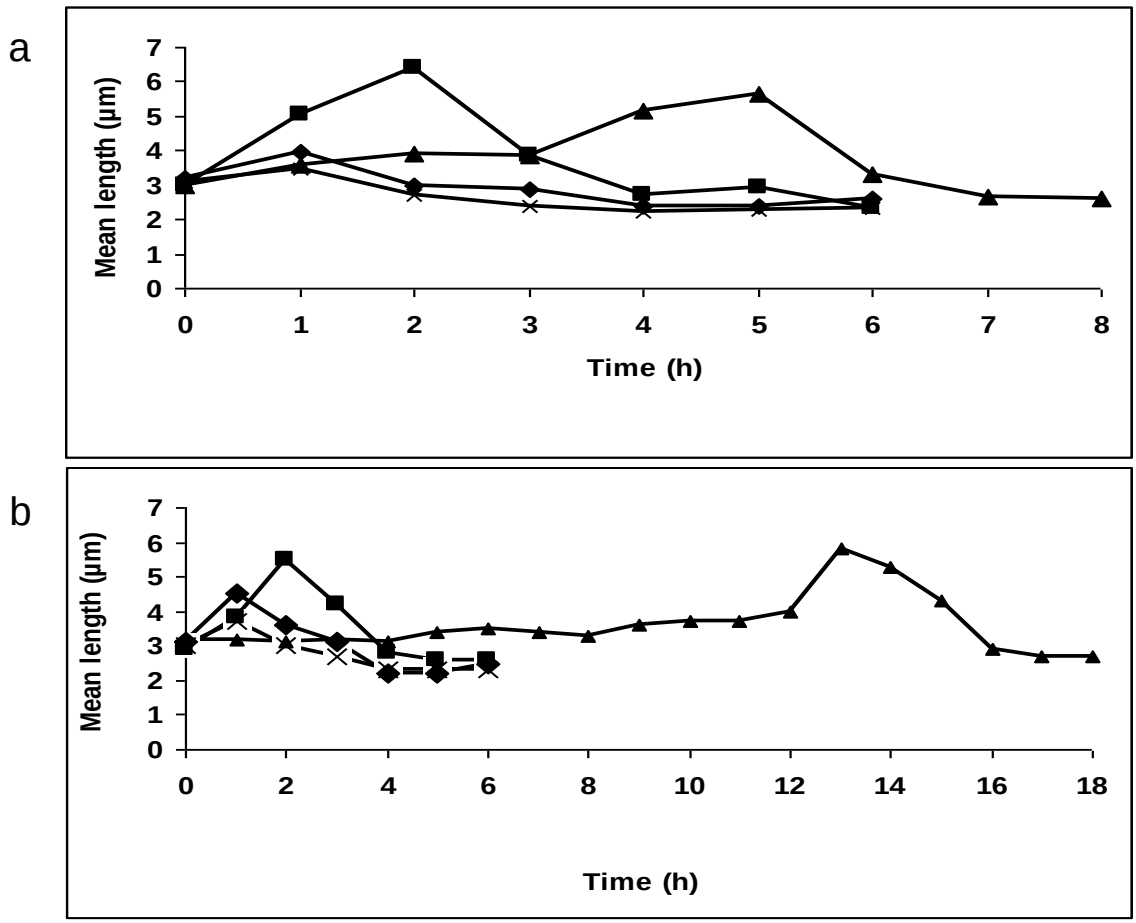

Fig. 6.2- Changes in the mean length of a) *E. coli* O157:H7 and b) *E. coli* 8WT in the absence (×) or presence of 100 mg/l (♦), 200 mg/l (■) or 300 mg/l (▲) cinnamaldehyde at 37ºC.

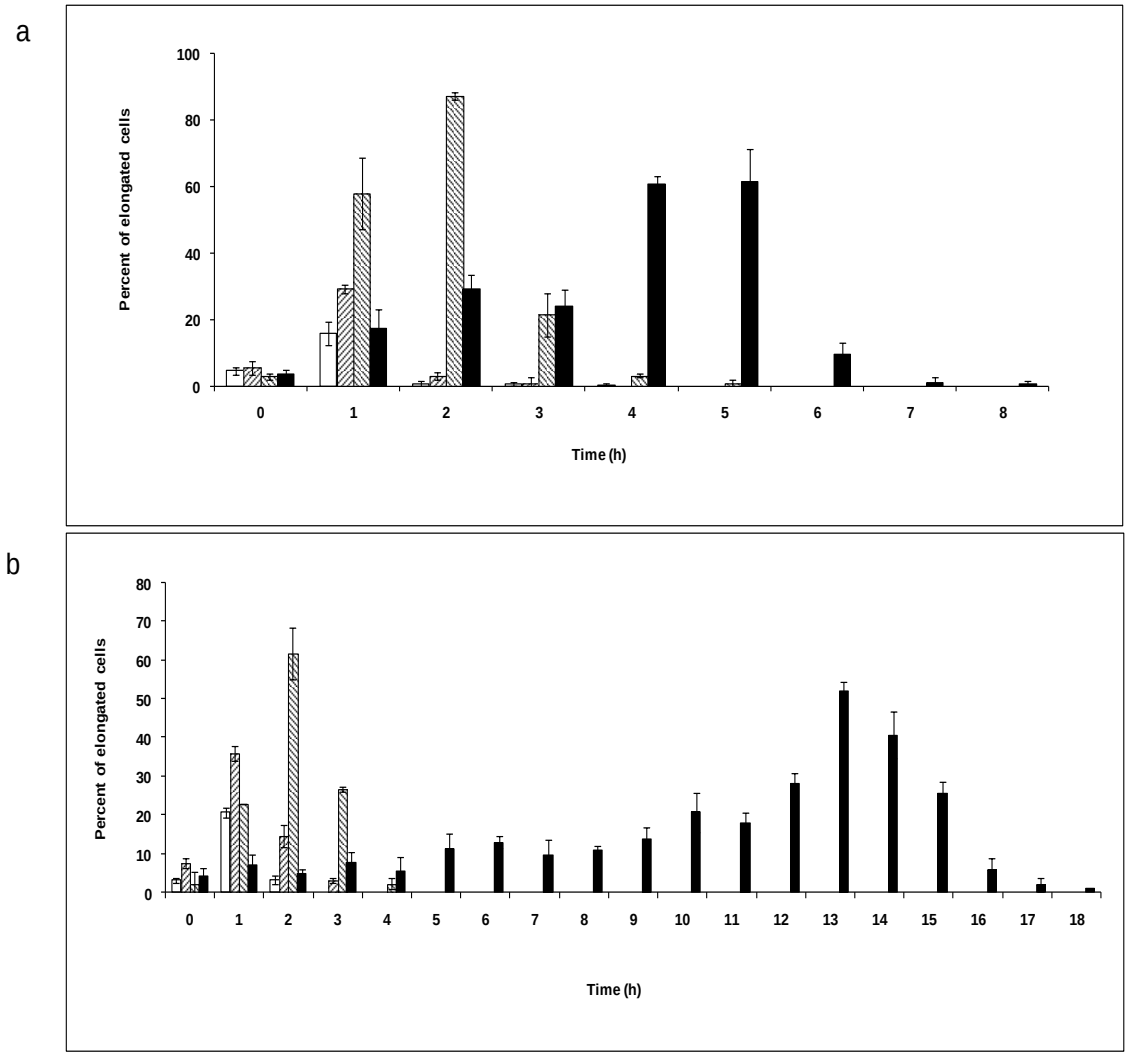

Fig. 6.3- The fractions of elongated cells observed with a) *E. coli* O157:H7 and b) *E. coli* 8 WT in the absence (□) or presence of 100 mg/l (▨), 200 mg/l (▨) or 300 mg/l (■) cinnamaldehyde at 37ºC.

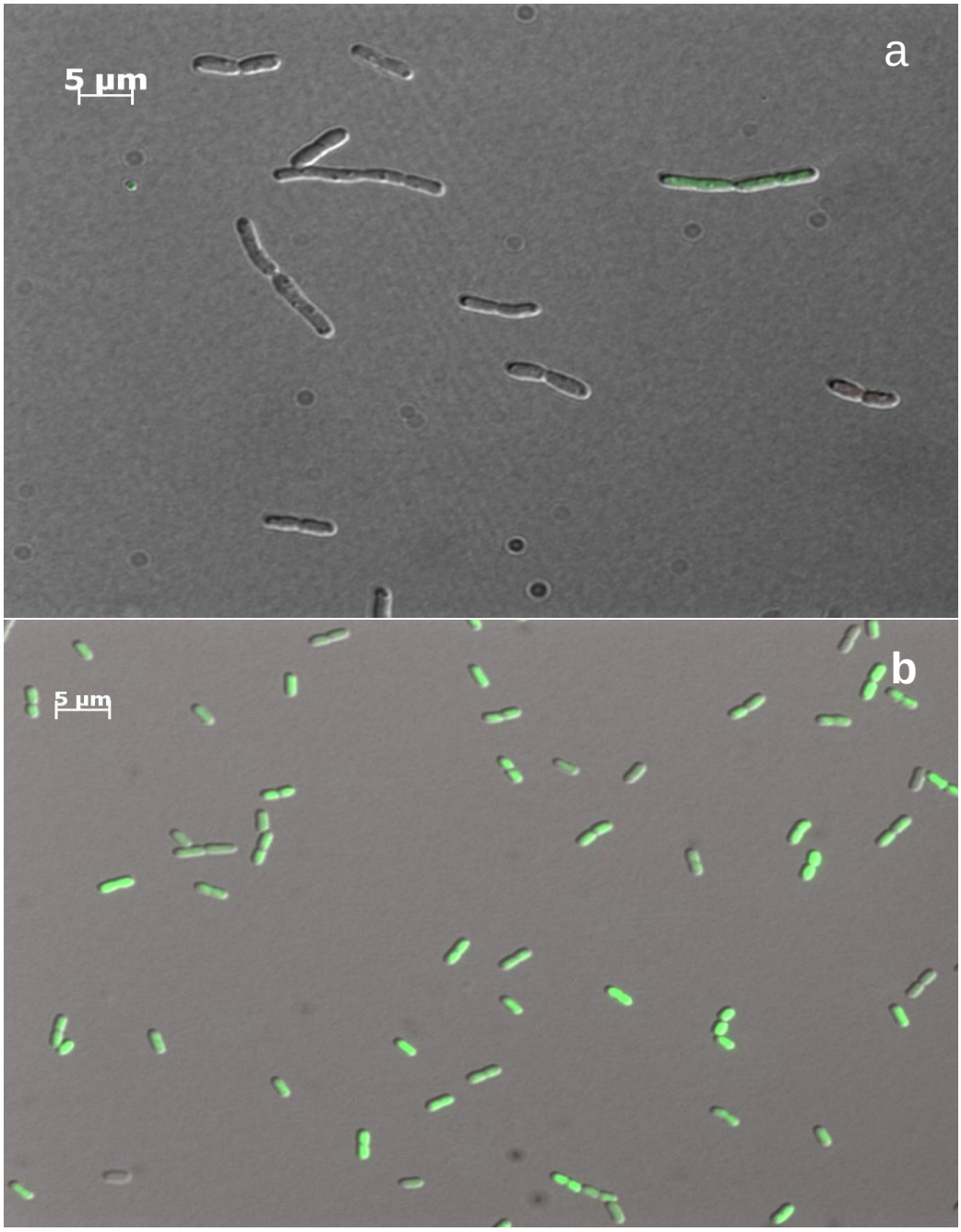

Fig. 6.4- Photomicrographs of *E. coli* O157:H7 treated with 300 mg/l cinnamaldehyde at 37ºC for (a) 5 h; showing a large proportion of elongated and non elongated cells being unstained by the LIVE/DEAD stain and (b) 24 h; showing more uniform green LIVE/DEAD staining.

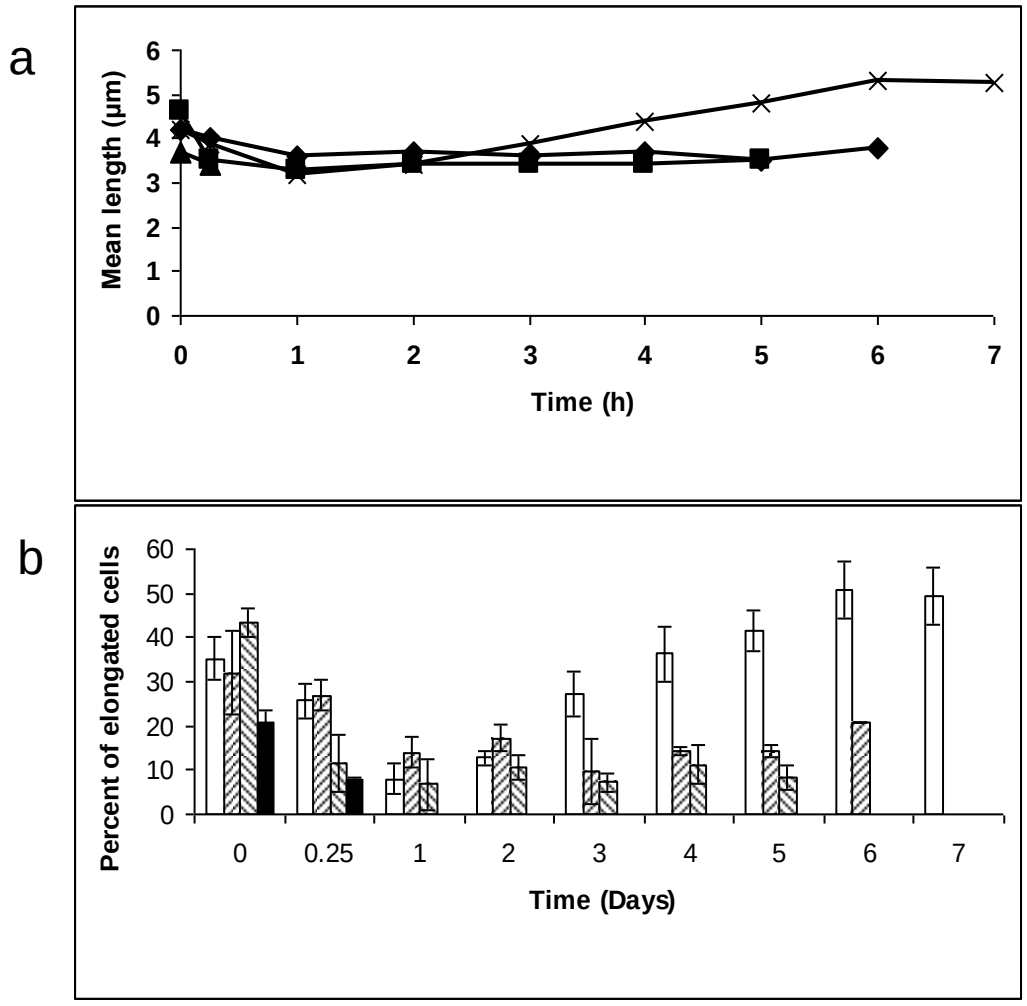

Fig. 6.5- Changes in the mean length of a) *E. coli* 8WT in the absence (×) or presence of 100 mg/l (♦), 200 mg/l (■) or 300 mg/l (▲) cinnamaldehyde at 6ºC and b) the fractions of elongated cells observed with *E. coli* 8 WT in the absence (☐) or presence of 100 mg/l (▨), 200 mg/l (▧) or 300 mg/l (■) cinnamaldehyde at 6ºC.

Chapter 7

Examination of the genome-wide transcriptional response of *Escherichia coli* O157:H7 to cinnamaldehyde exposure

7.1 Abstract

Cinnamaldehyde is a natural antimicrobial and has been found to be effective against many foodborne pathogens including *Escherichia coli* O157:H7. Although its antimicrobial effects have been well investigated, limited information is available on its effects at the molecular level. Sub-lethal treatment at 200 mg/l cinnamaldehyde inhibited growth of *E. coli* O157:H7 at 37^{o}C and for $\leq$ 2 h caused cell elongation, but from 2 to 4 h growth resumed and cells reverted to normal length. To understand this transient behaviour, genome-wide transcriptional analysis of *E. coli* O157:H7 was performed at 2 and 4 h exposure to cinnamaldehyde in conjunction with reverse phase-high performance liquid chromatography (RP-HPLC) analysis of cinnamaldehyde and other cinnamic compounds. Drastically different gene expression profiles were obtained at 2 and 4 h. RP-HPLC analysis showed that cinnamaldehyde was structurally stable for at least 2 h. At 2 h exposure, cinnamaldehyde induced overexpression of many oxidative stress-related genes, reduced DNA replication, and synthesis of protein, O-antigen and fimbriae. At 4 h, many cinnamaldehyde-induced repressive effects on *E. coli* O157:H7 gene expressions were reversed and cells became more motile and grew at a slightly faster rate. Data indicated that by 4 h, *E. coli* O157:H7 was able to convert cinnamaldehyde into the less toxic cinnamic alcohol using dehydrogenase/reductase enzymes (YqhD and DkgA). This is the first study to characterize the ability of *E. coli* O157:H7 to convert cinnamaldehyde into cinnamic alcohol, which in

turn, showed that the antimicrobial activity of cinnamaldehyde is mainly attributable to its carbonyl aldehyde group.

7.2 Introduction

Safety and shelf life of perishable foods can be improved by altering pH, reducing water activity through dehydration or by adding solutes, by low temperature or modified atmosphere storage or by a combination of these techniques (Rahman, 2007). However, studies showed that marginal conditions created by these alterations like low pH (4.3) or low water activity (0.95) with *Escherichia coli* O157, commensal *E. coli* and *Salmonella* (Mattick et al., 2003b), low temperature with *E. coli* O157 and commensal *E. coli* (6 to 8 °C) (Mattick et al., 2003b; Visvalingam et al., 2012) and *Salmonella* (4 to 8 °C) (Mattick et al., 2003b), 5 % NaCl with *Bacillus cereus* (den Besten et al., 2009), 100 % CO_2 and 4 °C with *Listeria monocytogenes* (Nilsson et al., 2000), or pH 5 and 10% NaCl with *L. monocytogenes* (Bereksi et al., 2002) as well as high hydrostatic pressure with *E. coli* (Kawarai et al., 2004), caused cell elongation often without substantial changes in viable numbers. The elongation of cells under many of these conditions seemed to continue indefinitely while the conditions that caused elongation were imposed (Phillips et al., 1998; Mattick et al., 2000; Visvalingam et al., 2012). Removal of restrictions and exposure of elongated cells to favourable conditions resulted in rapid division of elongated cells into multiple daughter cells (Mattick et al., 2003a; Jones et al., 2002).

Use of natural antimicrobials as alternatives to traditional preservation techniques like heat treatment, dehydration and chemical preservatives has gained popularity in recent years as consumers increasingly prefer foods processed with milder preservation techniques, which

have enhanced natural appeal and perceived nutritional quality (Smid and Gorris, 2007). Plant essential oils and their components like eugenol, cinnamaldehyde, thymol, and carvacrol have been reported to be effective against many foodborne pathogens including *E. coli* O157:H7 (Holley and Patel, 2005), which continues to be one of the major foodborne pathogens in many developed countries (Pennington, 2010). *E. coli* O157:H7 infections not only contribute significantly to the economic burden (Scharff, 2010), but also contribute to the overall morbidity and mortality of foodborne illnesses (Buzby and Roberts, 2009; Henson et al., 2008). Cinnamaldehyde has been shown to improve the safety of many perishable foods by inactivating *E. coli* O157:H7 and other foodborne pathogens (Juneja and Friedman, 2008; Obaidat and Frank, 2009; Amalaradjou et al., 2010; Baskaran et al., 2010; Ayari et al., 2012; Yossa et al., 2012).

In a recent study, it has also been found that with *E. coli* O157:H7, cinnamaldehyde at concentrations between 100 and 300 mg/l can also cause cell elongation without substantial changes in viability during exposure at 37 °C for ≤ 5 h. Elongation was more extensive at 2 h exposure to 200 mg/l cinnamaldehyde. Unlike exposure to other marginal conditions, cinnamaldehyde-induced cell elongation did not last indefinitely and cells resumed multiplication after 2 h of treatment and returned to normal morphology by 4 h (Visvalingam and Holley, 2012). This transient cell elongation could have been the result of one or more of the following: (i) *E. coli* O157:H7 became adapted or acclimated to cinnamaldehyde by modifying gene expression, which may occur under other marginal conditions (House et al., 2009; Vidovic et al., 2011); (ii) reduction in the concentration of cinnamaldehyde occurred through chemical instability or by its volatilization from media during constant agitation

and/or (iii) the conversion of cinnamaldehyde to cinnamic alcohol occurred by the action of alcohol dehydrogenases/reductases, as can occur in human skin cells during its detoxification (Smith et al., 2000). It has been shown that *E. coli* has multiple alcohol dehydrogenases (Atsumi et al., 2010), which could convert cinnamaldehyde into cinnamic alcohol. Therefore, the objectives of the study were to examine how transcriptional changes differed in *E. coli* O157:H7 when growth was inhibited at 2 h, and at 4 h when growth at 200 mg/l cinnamaldehyde was measurable. Changes in cinnamaldehyde concentration and its possible metabolite, cinnamic alcohol, were also monitored in the cultures during this period. Furthermore, even though ample literature describes the antimicrobial potency of cinnamaldehyde and other plant essential oil components, comparatively less information describes their influence on transcriptomic changes in *E. coli* O157:H7. The study was undertaken to provide better understanding of the antimicrobial action of cinnamaldehyde at the molecular level.

7.3 Materials and Methods

7.3.1 Bacterial strain and growth conditions

E. coli O157:H7 02:0627 and a non-motile strain 02:1840 were used in this study. Strain 02:0627 was chosen for the examination of cinnamaldehyde-induced transcriptomic changes because it has been found to undergo extensive cell elongation in response to cinnamaldehyde treatment (Visvalingam and Holley, 2012). The non-motile strain was only used as a negative control for motility assays. Both *E. coli* O157:H7 strains were provided by R. Ahmed, National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada). *E. coli* cultures were stored at – 80 ºC and maintained on Brain Heart Infusion

agar plates (BHIA, Oxoid, Mississauga, ON, Canada) at 4 ºC with monthly transfer to new plates. Active *E. coli* cultures were obtained by transferring a single colony from a plate to 10 ml Brain Heart Infusion Broth (BHIB; Accumedia, Lansing, MI, US) with incubation at 37 ºC for 16 to 18 h. A flask containing 99 ml BHIB was inoculated with 1 ml overnight culture and incubated at 37 ºC to get exponential phase cultures with absorbance at 600 nm (A_{600}) between 0.6 to 0.7.

7.3.2 Cinnamaldehyde treatment

Cinnamaldehyde was obtained from SAFC Supply Solutions (St Louis, MO, US). Filter-sterilized (0.2 µm syringe filter unit; Fisher Scientific, Edmonton, AB, Canada) cinnamaldehyde was dissolved in BHIB containing 4% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, Oakville, ON, Canada) to yield a 6400 mg/l cinnamaldehyde stock solution, which was prepared immediately prior to each use.

Appropriate amounts of the cinnamaldehyde stock solution were added to each of two 250 ml screw-capped Erlenmeyer flasks to achieve a final concentration of 200 mg/l after the total volume was adjusted to 150 ml by addition of inoculum and BHIB. Approximately 15 ml of exponential phase culture was added to each flask to yield an initial bacterial population near 2.5×10^7 (A_{600} value between 0.06 and 0.07). Two control culture flasks containing a similar bacterial concentration without cinnamaldehyde were also prepared. The contents of the flasks were mixed well and incubated at 37 ºC for ≤ 4 h with shaking at 100 rpm.

7.3.3 Viable number and microscopy measurements

Samples were collected immediately after cinnamaldehyde addition, and then hourly for viable number estimation and at 2 and 4 h for microscopy. For viable numbers, a 1 ml sample was decimally diluted using 0.1 % (w/v) peptone (Difco, Becton Dickinson, Sparks, MD, US) and plated on duplicate BHIA plates using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA, US). Plates were incubated at 37 ºC for 18 to 24 h and colonies were counted.

Five ml samples collected from each culture for microscopy were centrifuged at 4 ºC for 10 min at 10,000 x*g*. The pellets were re-suspended in 5 ml 0.85 % (w/v) NaCl (Fisher). A 10 µl sample of bacterial suspension was placed on an agar-coated (Noble agar, Difco) microscope slide and covered with a coverslip. The edges of the coverslip were sealed with clear nail polish and the slide was observed using an AxioImager M1 photomicroscope under differential interference contrast (DIC) optics at x1000 magnification (Carl ZeissMicroimaging GmBH, Gottingen, Germany). Ten images were taken of different fields of view using an AxioCam HRM camera (Carl Zeiss). Photomicrographs were viewed using AxioVision Release 4.7 software (Carl Zeiss). A set of 100 cells were randomly selected from photomicrographs and their lengths were measured using the same software. Cells that measured > 4 µm were considered to be elongated.

7.3.4 RNA extraction

Duplicate control and 200 mg/l cinnamaldehyde-treated cultures of *E. coli* O157:H7 were obtained as described above and incubated at 37 ºC with shaking at 100 rpm for 4 h. At 2 and

4 h, three 600 µl samples were collected from each culture, with the exception of the 200 mg/l treatment at 2 h where three 1 ml samples were collected, and transferred to sterile DNA/RNA-free 2 ml-sized microcentrifuge tubes. These volumes were chosen based on preliminary experiments to yield ≥ 10 µg of total RNA from each control or treatment culture. Samples were centrifuged at 13000 x*g* for 5 min at 4 ºC. After discarding supernatants, 1 ml TRIzol® reagent (Invitrogen Canada, Burlington, ON, Canada) was added, vortex-mixed and incubated at 65 ºC for 10 min. Total RNA was isolated using chloroform phase separation, isopropanol precipitation, 75 % ethanol washing and air drying as described by the TRIzol® manufacturer. The RNA pellet was dissolved using 100 µl RNase-free water, digested with DNase I and cleaned-up using RNeasy mini columns (Qiagen Sciences, Germantown, MD, US) according to the manufacturer's protocol. RNA was re-suspended in 40 µl nuclease-free water (Applied Biosystems, Foster City, CA, US) and its quality and quantity were measured using a spectrophotometer (Beckman Coulter, Du® 800; Fullerton, CA, US).

7.3.5 cDNA synthesis, labelling and hybridization

For cDNA synthesis, labelling and hybridization were performed according to the Affymetrix protocol (Affymetrix, Santa Clara, CA, US). The RNA/primer hybridization mix was prepared by combining 10 µg RNA, 10µl 75 ng/µl random primers (Invitrogen), and 2 µl poly-A RNA controls (Affymetrix), adjusting total volume to 30 µl by addition of nuclease-free water (Applied Biosystems) followed by incubation at 70 ºC for 10 min, and cooling at 25 ºC for 10 min. The cDNA synthesis reaction buffer containing SuperScript II was prepared as described by the manufacturer (Affymetrix) and mixed with the RNA/primer

hybridization mix. Then samples were incubated at 25 ºC for 10 min, 37 ºC for 60 min and 42 ºC for 60 min. RNA was removed by adding 20 µl of 1 N NaOH and incubating at 65 ºC for 30 min. The resulting mixture was neutralized with 20 µl of 1 N HCl. The neutralized cDNA synthesis product was purified using MiniElute PCR purification columns (Qiagen) and eluted in EB buffer (Qiagen). The purified cDNA product was quantified by measuring absorbance at 260 nm. A mixture of 3 to 7 µg of cDNA and 0.6 U/µg DNase I (Invitogen) was prepared in DNase I buffer (Invitrogen) and incubated at 37 ºC for 10 min for fragmentation. The reaction mixture was heated at 98 ºC for 10 min to inactivate the DNase I.

For terminal labelling, fragmented cDNAs were mixed with GeneChip® DNA labelling reagent at 7.5 mM (Affymetrix), reaction buffer and 60 U of terminal deoxynucleotidyl transferase (Promega, Madison, WI, US), and incubated at 37 ^{o}C for 60 min. The reaction was terminated by adding 2 µl of 0.5 M EDTA. Hybridization of the cDNAs was conducted at Genome Québec Innovation Centre (McGill University, Montréal, QC, Canada) using Affymetrix Genechip® *E. coli* Genome 2.0 Array (Affymetrix) as described by the manufacturer.

7.3.6 Microarray data analysis

Raw microarray data was imported into FlexArray 1.6.1 software and statistical tests were performed (Blazejczyk et al., 2007). Data normalization, background correction and expression value calculation were done using the robust multi-array average algorithm (RMA) (Irizarry et al., 2003a). The EB (Wright and Simon) algorithm was employed to enhance the robustness of the data. Because the RMA algorithm reduced the false positive

rate, increased sensitivity and compressed fold change (FC), an FC treatment minus control value ≥ 1.5 or ≤ -1.5 $\log_2$ with a p value ≤ 0.05 was considered as a cut-off point to determine differentially expressed genes (Irizarry et al., 2003b). The differentially expressed genes were classified into functional groups using the Database for Annotations, Visualizations and Integrated Discovery (DAVID) version 6.7 (Huang et al., 2009ab).

7.3.7 Microarray data access

Data from microarray analyses were deposited at the National Center for Biotechnology Information Gene Expression Omnibus database http://www.ncbi.nlm.nih.gov.geo with the accession number GSE40693.

7.3.8 Motility assay

Soft BHIA (BHIB + 0.25 % agar, Fisher) plates with 100, 200 and 300 mg/l cinnamaldehyde, and without cinnamaldehyde were prepared and immediately vacuum-sealed in aroma impermeable packages (ESE 1275 R; WINPAK, Winnipeg, MB, Canada) containing a polyethylene terephthalate layer (PET) (Johansson and Leufven, 1994). The vacuum-sealed plates were stored at 4 °C overnight for solidification. Exponential phase cultures of the motile and non-motile *E. coli* O157:H7 strains were prepared as described above and diluted to near10^7 cfu/ml. Soft BHIA plates were removed from vacuum packages and a 1 µl sample of diluted culture (about 10^4 cfu/plate) was placed in the middle of each agar plate by stabbing incompletely through with a micropipette tip to avoid swarming on the bottom surface (Lane et al., 2005). Four plates from each concentration were again vacuum-sealed and incubated at 37 °C for 12 h and duplicates were held without vacuum packaging.

The diameter of each motility halo was recorded. Since vacuum packaging did not affect the halo, the experiment was repeated without a vacuum. Following measurements, the contents of each plate were transferred to a stomacher bag and decimally diluted with 0.1 % peptone. Diluted samples were plated as described above, incubated at 37 ºC for 18 to 24 h and colonies were counted.

7.3.9 Sample preparation for high performance liquid chromatography

Duplicate *E. coli* O157:H7 02:0627 cultures with (treatment), and without (positive control) 200 mg/l cinnamaldehyde plus uninoculated BHIB with 200 mg/l cinnamaldehyde (negative control) were prepared as described above and incubated at 37 °C with shaking at 100 rpm. Ten ml samples collected from treatment and positive controls at 0, 2 and 4 h were centrifuged at 10000 x*g* for 10 min at 4 ºC and the pellet and supernatant were separated. The cell pellets were re-suspended in methanol (Fisher) and sonicated for 15 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, US). The suspension was filtered through 0.2 µm nylon syringe filter units (Fisher) and directly used for reverse phase high performance liquid chromatography (RP-HPLC) analysis. The supernatant was mixed with 10 ml ethyl acetate (Sigma-Aldrich) to avoid interference by materials present in BHIB with RP-HPLC detection. The mixture was vortex-mixed at maximum speed for 3 min. After 3 min rest, the clear top ethyl acetate phase was collected, and the procedure was repeated twice. Ethyl acetate extracts prepared from negative controls were not centrifuged. The pooled ethyl acetate extracts generated from each 10 ml sample were evaporated using a rotary evaporator (IKA, RV10 digital, Santa Clara, CA, US) and the residue was dissolved in 20 ml methanol. The solution was further diluted in methanol, if needed, filtered through a 0.2 µm nylon

syringe filter unit (Fisher) and directly used for RP-HPLC analyses. The extracts prepared from duplicate supernatants and corresponding cell pellets were analysed separately.

7.3.10 Preparation of standard solutions

Standard solutions of cinnamaldehyde and cinnamic alcohol (Sigma) ranging from 0.5 to 50 µg/ml were prepared in methanol immediately before each use. The solutions were filtered through 0.2 µm nylon syringe filter units before injection.

7.3.11 RP-HPLC analysis

The analysis was performed using a Waters HPLC system consisting of a model 486 detector operated at 280 nm and a 600E system controller (Waters Corporation, Milford, MA, US). The data were processed using Waters LC-module 1 millennium software version 32. A reverse phase column, Gemini C18 (150 x 4.6 mm, 5 µm), was connected by a security guard column (Gemini-NX C18, 4 x 3.0 mm, Phenomenex, Torrance, CA, US) to the instrument. The mobile phases used for separation were: A; 1 % acetic acid (Sigma-Aldrich) in methanol, and B; 1 % acetic acid in water. A gradient flow of mobile phases (0-10 min at 15 % A, 85 % B; 10-11 min at 50 % A, 50% B; 11-18 min at 65 % A, 35% B; and 18-30 min at 15 % A, 85% B) at a rate of 1 ml/min was used for separation. The retention times and standard curves for cinnamaldehyde and cinnamic alcohol were obtained by injecting 0.5 to 50 µg/ml of 10 µl standard solution of each compound. The linearity of the curves and the respective correlation coefficients were calculated from the peak area at each standard concentration. Ten µl extract from treatment, positive, or negative controls were injected. The methanol extracts obtained from cells were injected at same level, separately, and final

concentrations of cinnamaldehyde or cinnamic alcohol were obtained by combining values of the cell extract and the extract from corresponding supernatants.

7.3.12 Determination of the minimal inhibitory concentration of cinnamic alcohol

The broth macrodilution assay described previously was used to determine the minimal inhibitory concentration (MIC) of cinnamic alcohol (Visvalingam and Holley, 2012). Exponential phase culture prepared at 37 ºC was diluted in BHIB to yield 2.5 x 10^6 cfu/ml, and added to screw-capped glass tubes containing two-fold serial dilutions of cinnamic alcohol in BHIB to achieve final concentrations of 100 to 3200 mg/l. A set of tubes containing similar concentrations of cinnamic alcohol in BHIB without inoculum, and a tube containing only inoculum were included in each experiment. The contents of the tubes were vortex-mixed and incubated at 37 ºC for 24 h with shaking at 150 rpm. The lowest concentration of cinnamic alcohol showing no visible growth or turbidity after 24 h at 37 ºC was considered the MIC.

7.3.13 Data analysis

Growth curves were plotted and regression lines were fitted using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, US). Statistical differences between growth rate, and the fraction of elongated cells were examined using the student t-test. Cell motility and RP-HPLC data were compared by one-way analysis of variance (ANOVA). Tukey's test was used to assess differences among treatments and statistical difference between treatment means was concluded when $p \leq 0.05$.

7.4 Results

7.4.1 Growth and morphology

Treatment with 200 mg/l cinnamaldehyde caused a 2 h delay in the growth of *E. coli* O157:H7, and once growth began cells grew at a rate of 1.9 generations/h. The growth of untreated cells was 1.5 generations/h, and there was no delay prior to growth (Fig. 7.1). When treatment began, the fraction of elongated cells and mean length of both treated and untreated cells were 4.7 % and 3.09 μm, respectively. The mean length of untreated cells remained < 3.09 μm for ≤ 4 h, whereas the mean length of treated cells increased to 6.04 μm at 2 h with 72.5 % cells being elongated. By 4 h their mean length returned to 2.83 μm with only 0.5 % of the cells being elongated.

7.4.2 General changes in transcriptomic response

Whole genome transcriptomic profiling of *E. coli* O157:H7 at 2 h and 4 h exposure to 200 mg/l cinnamaldehyde was performed using the commercially available Affymetrix GeneChip 2.0 Array. This array contained 10208 oligonucleotide probe sets corresponding to 20336 genes of 4 *E. coli* genomes (strains K-12 MG1655, O157:H7 EDL033, O157:H7 Sakai, and uropathogenic CFT073). Genes that showed a high degree of similarity among the *E. coli* strains were represented by a single probe and were the equivalent ortholog in all 4 strains (Affymetrix, 2005). A total of 8662 probe sets (84.9 %) were detected in hybridized chips and used for data analysis.

A total of 393 genes were differentially expressed after treatment with cinnamaldehyde for 2 h, of which 185 were upregulated. While after 4 h of cinnamaldehyde

treatment, a total of 843 genes were differentially expressed, of which 633 were upregulated. One hundred genes were found to be differentially expressed at both 2 and 4 h; however, only 64 genes with well or putatively identified functions are presented in Table 7.1. Hypothetical genes that were differentially expressed either at 2 h or 4 h are not identified, but a complete list of these genes was deposited in the National Center for Biotechnology Information Gene Expression Omnibus database.

7.4.3 Cell shape and cell division cycle

The gene encoding cell filamentation protein (Fic) was the only gene related to cell shape/cell division cycle overexpressed (1.7-fold) after 2 h of treatment and was not differentially expressed at 4 h. However, at 4 h of treatment, more than 20 genes which included those related to cell division (*ftsBHW*), peptidoglycan synthesis (eg: *dacA*, *pbpC*, *mrcA*, *mrdA*, murG) and chromosome segregation (*mukEF*, *xerC*) were overexpressed, while genes encoding cell division inhibitors *dicB*, *dicF* (ncRNA, non-coding small RNA), and transcriptional regulator *dicC*, which controls the transcription of *dicB* were downregulated (Appendix VII).

7.4.4 Energy derivation and oxidation reduction

More than 61 genes related to energy derivation and oxidation/reduction reactions were differentially expressed at 2 h of treatment while 49 genes belonging to this group were differentially expressed at 4 h. Many genes involved in aerobic respiration and electron transport were upregulated (Table 7.1, Appendix VIII) while many genes involved anaerobic respiration and all three hydrogenases were downregulated at 2 h (Table 7.1, Appendix IX).

Of genes upregulated at 2 h, the highest expression (6.1-fold) was observed with the gene encoding alcohol dehydrogenase (*yqhD*); which was followed by a 5.0-fold increase in the gene encoding 2, 5-diketo-D-gluconate reductase A (*dkgA*). At 4 h no differential expression was observed with *yqhD*, *dkgA* and many aerobic respiratory genes. The expression of many previously suppressed genes associated with anaerobic respiration and hydrogenase 2 were increased by 3-to 6-fold at 4 h. The upregulated genes involved in electron transport at 2 h remained upregulated at 4 h of treatment. Additionally, genes encoding F0F1 ATP synthase (*atpABDEFGH*), NADH: ubiquinone oxidoreductase (*nuoEFGHIJKLM*), trimethylamine N-oxide (TMAO) reductase I (*torCD*) and the electron transport complex (*rsxACDE*) were also overexpressed at 4 h of treatment (Appendix X).

The genes *ansB* and *speF* involved in anaerobiosis of *E. coli* were downregulated at 2 h and subsequently induced by ≥ 4-fold at 4 h, while another gene, *narU*, which is involved in nitrate metabolism during anaerobiosis and nutrient starvation (Clegg et al., 2006), was upregulated at both 2 and 4 h.

7.4.5 DNA metabolism

Treatment with cinnamaldehyde for 2 h caused a significant reduction in the expression of some genes responsible for DNA replication. But at 4 h of treatment, more than 8 genes for DNA replication were upregulated, including many of those that were downregulated at 2 h.

7.4.6 Translation/protein synthesis and amino acid metabolism

Cinnamaldehyde treatment for 2 h had a substantial negative effect on expression of

many translation/ protein synthesis- related genes, including those involved in RNA synthesis, 50S and 30S ribosomal protein synthesis, and transcription elongation (Table 7.1, Appendix IX). An exception was *rpsV*, a gene responsible for stationary phase induced 30S ribosomal subunit S22 synthesis, which was overexpressed by 2.5-fold at 2 h of treatment (Appendix VIII). The overall negative effect was reversed at 4 h and the expression of many translation/ protein synthesis genes increased by 1.8- to ≥ 5-fold (Table 7.1, Appendix X). Interestingly, genes (*higAB*, *rdlB* and *sokC*) that control translation of toxin/anti-toxin systems were downregulated (Appendix XI).

Genes involved in synthesis of various amino acids such as the aromatic types (*shiA*), plus threonine (*latE*) and arginine (*argG*) were induced at 2 h. The expression of the gene encoding D-cysteine desulfhydrase (*dcyD*), which enables cells to use D-cysteine as a source of sulphur and protects cells from D-cysteine-mediated growth inhibition (Soutourina et al., 2001), was also upregulated at 2 h, while several genes involved in cysteine synthesis were downregulated.

7.4.7 Stress response

Various stress response genes were differentially expressed at 2 and 4 h of treatment. The genes *sodA, katE, gshA, gshB, sufA,* and *yhcN* that are known to be involved in various oxidative stress responses in *E. coli* were upregulated at 2 h (Hopkin et al., 1992; Outten et al., 2004; Wang et al., 2009; Lee et al., 2010b), but many of these genes were not differentially expressed at 4 h (Table 7.1). Some osmotically inducible genes (*osmB, osmY, otsB,* and *treF*) were also upregulated at 2 h, but the many of these genes were either not differentially expressed or were downregulated (*osmB, osmC* and *osmE*) at 4 h. The RNA

polymerase sigma factor RpoS which is responsible for generalized, starvation or stationary phase stress response was upregulated only at 2 h. The gene encoding ncRNA *rprA* that has been reported to positively regulate *rpoS* translation (McCullen et al., 2010) and *rydB* which has been reported to negatively regulate *rpoS* expression (Wassarman, et al. 2001), were also overexpressed at 2 h and repressed at 4 h. Similar differential expressions of the genes for DNA starvation/stationary phase protection (*dps*) and for phosphate starvation (*psiE*) were found.

With the exception of the gene for chaperone protein (*hchA*), none of the heat-shock proteins were differentially expressed at 2 h, and many of these including *hchA* were repressed at 4 h (Appendix XI). In contrast, the genes encoding the cold-shock proteins *cspH* and *cspG* were downregulated by ≥ 2.4-fold at 2 h and were upregulated by ≥ 6-fold at 4 h. Interestingly, the genes for stationary phase/starvation inducible cold-shock protein (*cspD*), and universal stress proteins (*uspB, uspD and uspF*) were downregulated at 4 h (Appendix XI). The periplasmic repressor protein CpxP, which combats extracytoplasmic protein-mediated toxicities by inducing the expression of serine endopeptidase (DegP) (Danese and Silhavy, 1998), and the genes (*cpxP* and *degP*) encoding both of these proteins were induced at 4 h.

The expression of genes involved in glutamate-dependent acid resistance (*gadE*, *gadAB*, and *gadC*) and acid resistant proteins (*hdeAB and hdeD*) were repressed by ≥ 3.5-fold at 2 h and were not differentially expressed at 4 h. In contrast, genes for acid-inducible protein InaA and acid-shock-inducible periplasmic protein Asr were upregulated at 2 h by 3.9- and 2.6-fold, respectively. Subsequently, at 4 h the expression of *asr* was decreased by

5.3-fold. The lysine- and arginine-dependent acid-resistant genes were only upregulated at 4 h.

7.4.8 Antibiotic resistance

The genes of the multiple antibiotic resistance (*mar*) operon, *marRAB*, the genes for the predicted multidrug transporter (*mdlAB*), and the gene for the acriflavin resistant protein A (*acrA*) were significantly induced by cinnamaldehyde treatment for 2 h, while genes for the multidrug efflux system *mdtEF* were repressed. At 4 h *marRB* was no longer differentially expressed but *marA* expression was slightly repressed (by 1.5-fold). Furthermore, repressed expression of *mdtEF* was reversed and *acrA* continued to be overexpressed at 4 h. Some other antibiotic resistance genes (e.g.: *acrB, acrD, ampC, emrAD* and *mdtIJ*) that were not differentially expressed at 2 h were overexpressed at 4 h (Appendix X).

7.4.9 Membrane/membrane transport

Maltose (*malEFG*, and *lamB*), mannose (*manXYZ*), ferric iron (*afuB*), and hemin (*hmuV*) transport genes, outer membrane porin protein C (*ompC*) and outer membrane protein (*ompF*) genes were downregulated at 2 h (Appendix IX), and no differential expression of these genes was observed at 4 h. The genes encoding proteins for O-antigen synthesis, fimbriae, and Mg^{2+} transport were suppressed at 2 h, but the expression of some of these genes was increased by ≥ 3.5-fold at 4 h. Genes for amino acid transport (*cydD, yecS*), ferrous iron permease (*efeU*), outer membrane channel protein (*tolC*) and vitamin B_{12} transport (*btuF*) were overexpressed at both 2 and 4 h.

Many genes associated with lipid-A biosynthesis, oligopeptide transport, and

lactose/glucose efflux were only upregulated at 4 h, while genes for thiosulfate transporter (*cysP*), a membrane protein induced after carbon starvation (*slp*), the arginine exporter (*yggA*), and many inner membrane proteins were downregulated at 4 h. Many genes associated with long chain fatty acid biosynthesis (ECs4332, *accABC, fabB, fabH, fabD,* and *fabG*) were also upregulated only at 4 h.

7.4.10 Transcriptional regulation

Many transcriptional regulators were differentially expressed at 2 or 4 h. The gene *yqhC* encoding the putative ARAC-type regulatory protein and the gene, *fnr*, encoding the fumarate/nitrate reduction transcriptional regulator were upregulated by 3.8- and 2.0-fold at 2 h. The Na^+/H^+ antiporter regulator (*nhaR*) involved in Na+ adaptation and regulation of biofilm genes *pgaAB* (Goller et al., 2006) was downregulated at 2 h. Induction of genes encoding the global DNA binding transcriptional regulator, Fis, the transcriptional activator of the maltose regulon, MalT, and the biofilm gene transcriptional regulator, McbR, were observed at 4 h. However, repression of genes encoding the transcriptional activator in response to Zn^{2+} (*zntR*), the nitrogen regulatory gene (*glnK*), and a putative transcriptional regulator (*yafC*) was observed at 4 h. The expression of *grlR*, a negative regulator of *E. coli* O157:H7 locus of enterocyte effacement (LEE) genes, was increased from 1.5-fold at 2 h to 2.2-fold at 4 h.

7.4.11 Motility and chemotaxis

None of the motility and chemotaxis-related genes was differentially expressed at 2 h, but > 20 of these genes were upregulated at 4 h. The largest expression of all genes was

observed with genes encoding flagellar motor proteins (*motAB*, ≥ 6.7-fold) (Appendix X).

7.4.12 Biofilm-related genes and others

The biofilm adhesin polysaccharide PGA export genes *pgaAB* were downregulated at 2 h while another gene for biofilm cell surface signalling protein, BhsA was upregulated at 2 h. Three other biofilm-related genes were downregulated at 4 h.

The genes encoding isoprenoid biosynthesis (*idi*, *ispA*), delta-amino levulinic acid dehydratase (*hemB*) and a predicted enzyme were upregulated only at 2 h.

7.4.13 Motility assay

The soft BHIA plate motility assay was performed to examine whether elevated expression of motility/chemotaxis genes observed at 4 h of cinnamaldehyde treatment resulted in increased motility of *E. coli* O157:H7. The diameters of the *E. coli* O157:H7 motility halo in control, 100, 200 and 300 mg/l cinnamaldehyde-containing soft agar plates were 4.2, 4.5, 5.4, and 0.6 cm, respectively. The motility halo diameter of cells treated with 200 mg/l was significantly ($p \leq 0.05$) higher than the control (Fig. 7.2). Growth was observed only at the inoculation site of the non-motile *E. coli* O157:H7 control. The numbers of *E. coli* O157:H7 in control and 200 mg/l cinnamaldehyde-containing soft agar plates were 8.8 and 9.2 log cfu/cm^2, respectively.

7.4.14 RP-HPLC analysis

The changes in concentrations of cinnamaldehyde and its possible metabolite cinnamic alcohol in BHIB with *E. coli* O157:H7 (treatment) and without bacterial

inoculation, but with cinnamaldehyde (negative control) were monitored by RP-HPLC analysis (Fig. 7.3). The concentration of cinnamaldehyde detected immediately before incubation at 37 °C (0 h) was 154.5 ± 10.2 mg/l with the negative control and treatment (Table 7.2). This indicates that only about 80 % of the cinnamaldehyde added (200 mg/l) was extracted using ethyl acetate. At 2 h the cinnamaldehyde concentration decreased by 20 mg/l in both the negative control and treatment and remained stable in the negative control at 4 h. However, with *E. coli* O157:H7, the cinnamaldehyde concentration decreased to ≤ 10 mg/l at 4 h and the cinnamic alcohol concentration increased to about 120 mg/l. Cinnamic alcohol was not detected in the positive control (*E. coli* O157:H7 alone) for ≤ 4 h.

7.4.15 MIC value of cinnamic alcohol

Since cinnamic alcohol was detected at 4 h in treated samples, its antimicrobial potency (MIC value) against *E. coli* O157:H7 was determined and found to be 1600 mg/l.

7.5 Discussion

Since exposure of *E. coli* O157:H7 to 200 mg/l cinnamaldehyde for 2 and 4 h yielded two distinctive phenotypes (at 2 h cells were elongated with a mean length of ≥ 6 µm and at 4 h cells were of normal, ≤ 3.0 µm, length), gene expression analysis of the whole *E. coli* O157:H7 genome was conducted at these time-points. The changes in growth and morphology of *E. coli* O157:H7 during its exposure to 200 mg/l were as expected from a previous study of this organism (Visvalingam and Holley, 2012). Cinnamaldehyde has been reported to bind with FtsZ via its carbonyl group and inhibited FtsZ polymerization and its guanosine-5'-triphosphatase (GTPase) activity (Domadia et al., 2007). Since FtsZ

polymerization is dependent on GTP hydrolysis (Mohammadi et al., 2009), it is possible that inhibition of GTPase activity led to inhibition of septum development and subsequently caused elongation of *E. coli* O157:H7 cells. Although upregulation of the gene for cell filamentation protein Fic was observed at 2 h, it is considered unlikely that the elevated expression of this gene simply caused cell elongation. This is probable because it has been reported that Fic-induced cell elongation occurred only in the presence of cyclic AMP at 43 °C (Utsumi et al., 1982). The observed overexpression of *fic* may be the result of an elevated level of RpoS, which has been reported to be the only known regulator of this gene (Utsumi et al., 1993), and it was coincident with the upregulation of the gene encoding RpoS at 2 h.

E. coli is known to produce catalase (KatE) and superoxide dismutase (SodA) to remove hydrogen peroxide, superoxide molecules, or other reactive oxygen species and reduce oxidative stress (Wang et al., 2009). Oxidizing agents like hydrogen peroxide and chlorine have been reported to induce genes responsible for glutathione synthesis in *E. coli* (Wang et al., 2009; Lee et al., 2010b) and enhance its protective response to oxidative stress (Carmel-Harel and Storz 2000). Furthermore, the reactive oxygen species produced under high oxidative stress can interact with Fe-S clusters, which serve as cofactors in enzymatic proteins, causing their decomposition. To overcome this detrimental effect, *E. coli* may upregulate the genes for Fe-S cluster assembly (Wang et al., 2009). In addition to genes for catalase (*katE*), superoxide dismutase (*sodA*), glutathione synthetase (*gshA*, *gshB*) and Fe-S cluster assembly scaffold protein (*sufA*), genes for biofilm cell signaling protein (*bhsA*) and periplasmic protein (*yhcN*) which have been known to be upregulated under oxidative stress (Pomposiello et al., 2001; Lee et al., 2010b; Schaefer et al., 2010) were also upregulated at 2

h.

The protein MarR negatively regulates the transcription of the *marRAB* operon under normal growth conditions. However, inactivation of MarR by antibiotics, oxidizing agents and phenolic compounds leads to overexpression of the *marRAB* operon (Alekshun and Levy, 1999). Upregulation of *marRAB* at 2 h exposure indicates, as with other oxidizing agents such as paraquat, sodium salicylate (Pomposiello et al., 2001), chlorine (Wang et al., 2009), and reuterin (3-hydroxypropionaldehyde), an antimicrobial compound produced by *Lactobacillus reuteri* (Schaefer et al., 2010), that cinnamaldehyde may also have inhibited the repressive effect of MarR. Coincidently, > 15 genes that have been reported to be under the direct or indirect control of MarA (Barbosa and Levy, 2000; Ruiz et al., 2008) were also differentially expressed. Of particular importance were repression of *ompF* and activation of *acrA/tolC* expression, which are involved in MarA-mediated antibiotic resistance, and repression of acid resistance genes *hdeAB, hdeD, gadE, gadAB, gadC,* and *gadE*. It is worth noting that genes related to the antibiotic efflux pump *acrAB*/*tolC* were actively transcribed at 4 h, and when this effect is considered with the upregulation of other antibiotic resistance genes, *acrD, ampC, emrAD, mdtEF* and *mdtIJ* at 4 h, enhanced antibiotic resistance following cinnamaldehyde challenge appears probable.

Dps, a non-specific DNA-binding protein, not only protects DNA from oxidative damage by binding with it (Martinez and Kolter, 1997), but Dps also protects cells by sequestering iron during oxidative (H_2O_2) stress (Ceci et al., 2004). In combination with the overexpression of *dps* at 2 h, the observed downregulation of DNA replication-related genes suggest that cinnamaldehyde may negatively affect DNA synthesis in *E. coli* O157:H7.

Similar to other studies that examined oxidative stress (Pomposiello et al., 2001; Wang et al., 2009; Lee et al., 2010b), overexpression of genes related to osmotic stress and heat shock at 2 h indicate that different stress response networks may either be interconnected or this may represent a cross-protective response to cinnamaldehyde-induced oxidative stress (Gunasekera et al., 2008; Lee et al., 2010b). However, it was apparent that cold-shock response gene involvement differed, since these genes were simultaneously downregulated at 2 h. No differential expression of cold-shock genes was observed in other studies that examined oxidative stress response in *E. coli* (Pomposiello et al., 2001; Wang et al., 2009; Lee et al., 2010b).

Overall, these observations indicate that cinnamaldehyde induced oxidative stress response in *E. coli* O157:H7. Many enzymes involved in oxidative stress response have been reported to reduce the intracellular NADPH level in *E. coli*, which not only lowers the reducing power of the cells but also causes growth arrest by limiting its biosynthetic capability (Cabiscol et al., 2000; Pomposiello et al., 2001; Miller et al., 2009; Krapp et al., 2011). This may explain the observed repression of biosynthetic genes such as genes for protein synthesis (as indicated by ribosomal and RNA synthesis) and O-antigen synthesis, which have been reported to be important for attachment to plants and for bovine colonization (Sheng et al., 2008; Boyer et al., 2011), as well as fimbriae synthesis. Furthermore, increased levels of tricarboxylic acid cycle enzymes including *fumC* and *acnA* that are resistant to oxidative stress (superoxide) (Storz and Zheng, 2000) suggests that overexpression of these enzymes may aid *E. coli* in combating effects of cinnamaldehyde-induced oxidative stress by increasing the reducing power of *E. coli* cells via reduction of

NAD^+ (Pomposiello et al., 2001). These observations also suggest that detrimental effects of cinnamaldehyde may not be limited to the cell membrane (Holley and Patel, 2005) and may involve the cytoplasm as well.

Although there was a small but significant reduction in cinnamaldehyde concentration at 2 h relatable to extraction efficacy, no new metabolite(s) were detected by RP-HPLC, confirming that cinnamaldehyde was stable up to this point in tests. Thus, transcriptomic changes observed at 2 h were the result of cinnamaldehyde exposure. However, in the presence of *E. coli* O157:H7 at 4 h, the cinnamaldehyde concentration decreased to ≤ 10 mg/l, while cinnamic alcohol increased to ≥ 120 mg/l. Since the MIC value of cinnamic alcohol was 4 times higher than that of cinnamaldehyde (400 mg/l) against this *E. coli* strain (Visvalingam and Holley, 2012), it is evident that the antimicrobial activity of cinnamaldehyde is mainly due to its carbonyl-carrying aldehyde group. *E. coli* has been reported to overexpress the alcohol dehydrogenase YqhD in the presence of toxic aldehydes as a way to afford protection from them (Perez et al., 2008; Lee et al., 2010a; Turner et al., 2011). Another aldehyde reductase, 2,5-diketo-D-gluconate reductase A (DkgA) has also been reported to be overexpressed in the presence of aldehydes (Jeudy et al., 2006; Turner et al., 2011). Both of these enzymes have broader substrate specificity (Jeudy et al., 2006; Perez et al., 2008; Atsumi et al., 2010; Lee et al., 2010a) and the genes encoding these enzymes (*yqhD* and *dkgA*) were positively regulated by YqhC in the presence of aldehydes including cinnamaldehyde (Turner et al., 2011). The observed elevated expression of *yqhC*, *yqhD*, and *dkgA* at 2 h and subsequent conversion of cinnamaldehyde to cinnamic alcohol by *E. coli* O157:H7 during tests strongly suggests these enzymes may have played a role in this

reaction.

The conversion of cinnamaldehyde to cinnamic alcohol resulted in the repression of many genes related to stress response and overexpression of genes related to protein synthesis, O-antigen, fimbriae and lipid-A synthesis at 4 h. Reduction of the cinnamaldehyde concentration through its dissimilation by *E. coli* O157:H7 was likely responsible for its reduced/ neutralized inhibition of cell division, which at the molecular level can involve the cell division protein FtsZ (Domadia et al., 2007). Many genes, including those for motility/chemotaxis, cell division cycle/cell shape, DNA synthesis, fatty acid synthesis, F0F1 ATP synthase as well as the global transcriptional regulator, Fis, that positively controls energy metabolism, protein synthesis and motility during exponential growth (Bradley et al., 2007), were also upregulated. It has also been reported that *E. coli* uses different respiratory enzymes in a hierarchal order and that the enzymes NouA-N, HybABC and GlpABC preferentially contribute to the generation of proton motive force by proton pumping during high metabolic fluxes or growth rates (Unden and Bongaerts, 1997). The upregulation of these genes observed at 4 h is consistent with the elevated growth rate found between 2 and 4 h of cinnamaldehyde exposure and the increased motility of *E. coli* O157:H7 on soft agar plates containing 200 mg/l cinnamaldehyde.

7.6 Conclusion

The whole genome transcriptional profiling of *E. coli* O157:H7 at 2 and 4 h exposure to cinnamaldehyde revealed substantially different gene expression at each of these intervals. RP-HPLC analysis showed that cinnamaldehyde was stable in the absence of bacteria for 4 h, and in their presence for at least 2 h. The transcriptional profile at 2 h showed that

cinnamaldehyde induced oxidative stress as indicated by overexpression of many oxidative stress-related genes, reduced DNA replication and the synthesis of protein, as well as O-antigens and fimbriae by down regulation of the respective functional genes. *E. coli* O157:H7 was able to detoxify cinnamaldehyde by its conversion to cinnamic alcohol, probably through use of the dehydrogenasesYqhD and DkgA. This is the first study to characterize the transformation of cinnamaldehyde to cinnamic alcohol by *E. coli* O157:H7 and this result suggested that the antimicrobial activity of cinnamaldehyde was mainly attributable to its carbonyl aldehyde group. Evidence was obtained at the molecular level suggesting that cinnamaldehyde challenge may increase resistance to antibiotic treatment. Because of cinnamaldehyde degradation by 4 h, many repressive effects on *E. coli* O157:H7 gene expression were reversed, whereupon cells became more motile and grew at a slightly faster rate.

Table 7.1- *E. coli* O157:H7 genes that were differentially expressed at 2 and 4 h of cinnamaldehyde exposure

Gene	Function	Fold change (log_2)	
		2 h	4 h
Energy derivation and oxidation reduction			
yqhD	alcohol dehydrogenase, NAD(P)-dependent	6.1	(0.31)
dkgA	2,5-diketo-D-gluconate reductase A	5.0	(-0.08)
fumB	anaerobic class I fumarate hydratase (fumarase B)	-4.8	2.1
glpABC[1]	anaerobic sn-glycerol-3-phosphate dehydrogenase	-2.1/-2.8/-6.4	1.5/2.8/4.4
hybOABDEFG[1]	hydrogenase 2	-2.4/-3.0/-2.4/-3.4/-2.1/-2.1/-3.3	1.5/2.3/2.9/2.2/1.9/1.6/1.6
frdD	fumarate reductase subunit D (anaerobic)	-3.4	1.5
dcuA	anaerobic C4-dicarboxylate transporter	-2.1	1.9
gapC	glyceraldehyde 3-phosphate dehydrogenase C	2.0	2.6
torA	trimethylamine-N-oxide reductase 1	1.5	5.5
cyoA	cytochrome o ubiquinol oxidase subunit II	3.4	2.2
rsxB	electron transport complex protein RnfB	1.6	4.1
nemA	N-ethylmaleimide reductase	5.0	3.8
DNA metabolism			
dnaB	replicative DNA helicase	-1.8	3.2
fimB	tyrosine recombinase/inversion of on/off regulator of fimA	-1.6	2.3
iraD	DNA replication/recombination	-2.8	2.1
Translation/ protein synthesis			
dusB	tRNA-dihydrouridine synthase B	-2.7	3.1
rnt	ribonuclease T	2.4	1.8

Table 7.1- continued

Gene	Function	Fold change (log_2)	
		2 h	4 h
rimP	ribosome maturation factor for 30S subunits	-1.6	1.8
rplC	50S ribosomal protein L3	-2.6	2.6
rimJ	ribosomal-protein-S5-alanine N-acetyltransferase	-1.5	-1.7
Stress response			
cspHG[1]	cold shock protein	-4.2/-2.4	6.2/5.9
asr	acid shock-inducible periplasmic protein	2.6	-5.3
hchA	chaperone protein HchA	2.4	-1.9
osmB	osmotically inducible lipoprotein	1.6	-3.4
dps	stationary phase protection protein Dps	3.4	-2.4
psiE	phosphate-starvation-inducible protein PsiE	2.1	-2.6
rydB	ncRNA	5.3	-2.3
rprA	ncRNA	1.8	-1.5
gloA	glyoxalase I, Ni-dependent	1.9	1.8
yhcN	conserved protein; periplasmic protein	5.1	2.0
Antibiotic resistance			
marA	DNA-binding transcriptional dual activator of multiple antibiotic resistance	3.8	-1.5
acrA[2]	acriflavin resistance protein A	2.9	1.6
mdtEF[1]	multidrug efflux system protein	-2.8/-3.3	1.6/2.8
Membrane/membrane transport			
wzx	O antigen flippase	-2.0	1.6
wzzE	Enterobacterial Common Antigen (ECA) polysaccharide chain length modulation protein	-1.9	1.6
gfcE	predicted lipoprotein required for formation of the O-antigen capsule	-1.9	3.7
ECs2113	Type-1 fimbrial protein, A chain precursor	-2.2	2.4
ECs2112	putative fimbrial chaperone protein	-3.2	2.1
ECs4328	putative acyl carrier protein	-2.0	3.7
ECs4327	putative phospholipid biosynthesis acyltransferase	-1.6	3.9

Table 7.1- continued

Gene	Function	Fold change (log_2)	
		2 h	4 h
yhiD	putative Mg(2+) transport ATPase	-4.2	2.1
pgaB	biofilm adhesin polysaccharide PGA export lipoprotein	-2.3	1.8
yedE	putative inner membrane protein	-2.6	2.1
ynjD	predicted transporter subunit: ATP-binding component of ABC superfamily	-2.3	2.5
nupC	permease of transport system for 3 nucleosides	-1.9	2.6
ynjE	putative thiosulfate sulfur transferase	-2.5	2.4
gfcB	predicted outer membrane lipoprotein	-2.1	2.7
ydhC	inner membrane transport protein YdhC	1.6	-2.2
yhjX	Inner membrane protein/MFS transporter	3.9	-2.3
pncB	nicotinate phosphoribosyltransferase	1.6	1.9
efeU	ferrous iron permease	2.8	-3.1
cydD	cysteine/glutathione ABC transporter membrane/ATP-binding component	1.5	2.1
ydhJ	predicted membrane fusion protein (MFP) of YdhJK efflux pump	1.5	3.7
btuF	vitamin B12 transporter subunit: periplasmic-binding component of ABC superfamily	1.6	1.7
tolC[2]	outer membrane channel protein	1.8	1.5
yejE	putative transport system permease protein	1.6	2.4
yecS	amino-acid ABC transporter permease protein	1.6	1.6
Anaerobiosis			
narU	nitrite extrusion protein 2	1.9	1.6
ansB	L-asparaginase II	-3.6	2.2
speF	ornithine decarboxylase	-2.2	2.1
Transcription			
grlR	negative regulator GrlR	-1.5	2.2

[1] Changes in expression for each gene of the complex or the same group are listed sequentially in respective order. [2] Genes that are directly or indirectly controlled by MarA (Barbosa and Levy, 2000; Ruiz et al., 2008).

Table 7.2- Conversion of cinnamaldehyde to cinnamic alcohol by *Escherichia coli* O157:H7 in BHIB at 37°C

Treatment	Concentration (mg/l)					
	Cinnamaldehyde			Cinnamic alcohol		
	0 h	2 h	4 h	0 h	2 h	4 h
BHIB + cinnamaldehyde, uninoculated (negative control)	156.4 ± 2.7^{Aa}	125.9 ± 1.0^{Ab}	115.9 ± 4.7^{Ab}	ND[1]	ND	ND
BHIB + *E. coli* O157:H7 + cinnamaldehyde (Treatment)	151.1 ± 16.1^{Aa}	120.6 ± 3.1^{Ab}	7.1 ± 0.1^{Bc}	ND	ND	114 ± 8.3
BHIB + *E. coli* O157:H7 (positive control)	ND	ND	ND	ND	ND	ND

[1]ND- Not detected; Different superscript letters ($^{A-B}$) in each column indicate a significant difference ($p < 0.05$). Different superscript letters ($^{a-c}$) in each row denote a significant difference ($p < 0.05$)

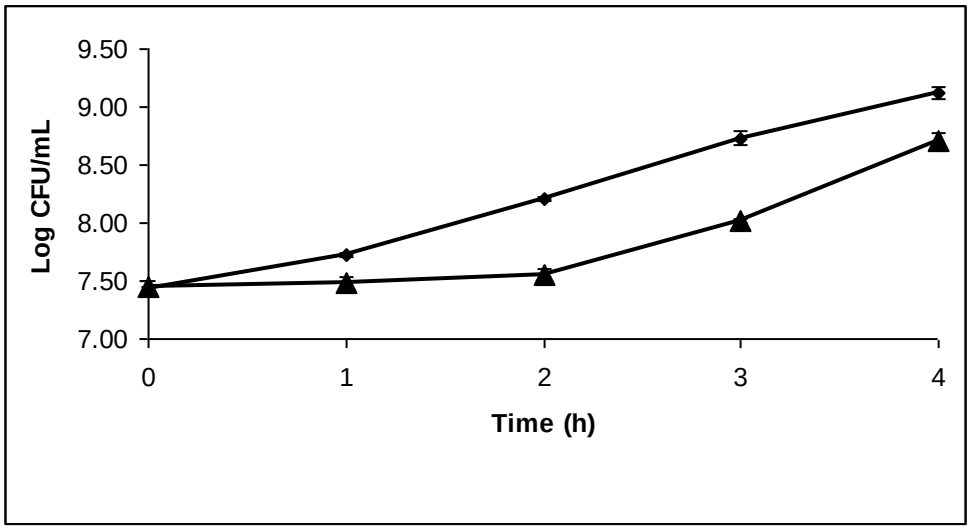

Fig. 7.1- Changes in the numbers (log cfu/ml) of *E. coli* O157:H7 in the absence (♦) or presence of 200 mg/l (▲) cinnamaldehyde at 37ºC.

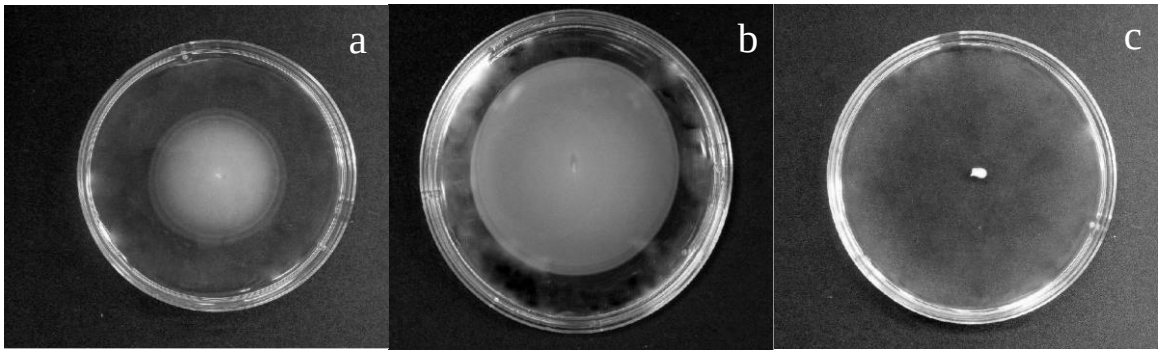

Fig. 7.2- Photographs of agar plates showing active motility of *E. coli* O157:H7 within the agar of a) control and (b) 200 mg/l cinnamaldehyde, soft-BHI agar plates, and (c) non-motile *E. coli* O157:NM 02:1840 control after 12 h incubation at 37ºC.

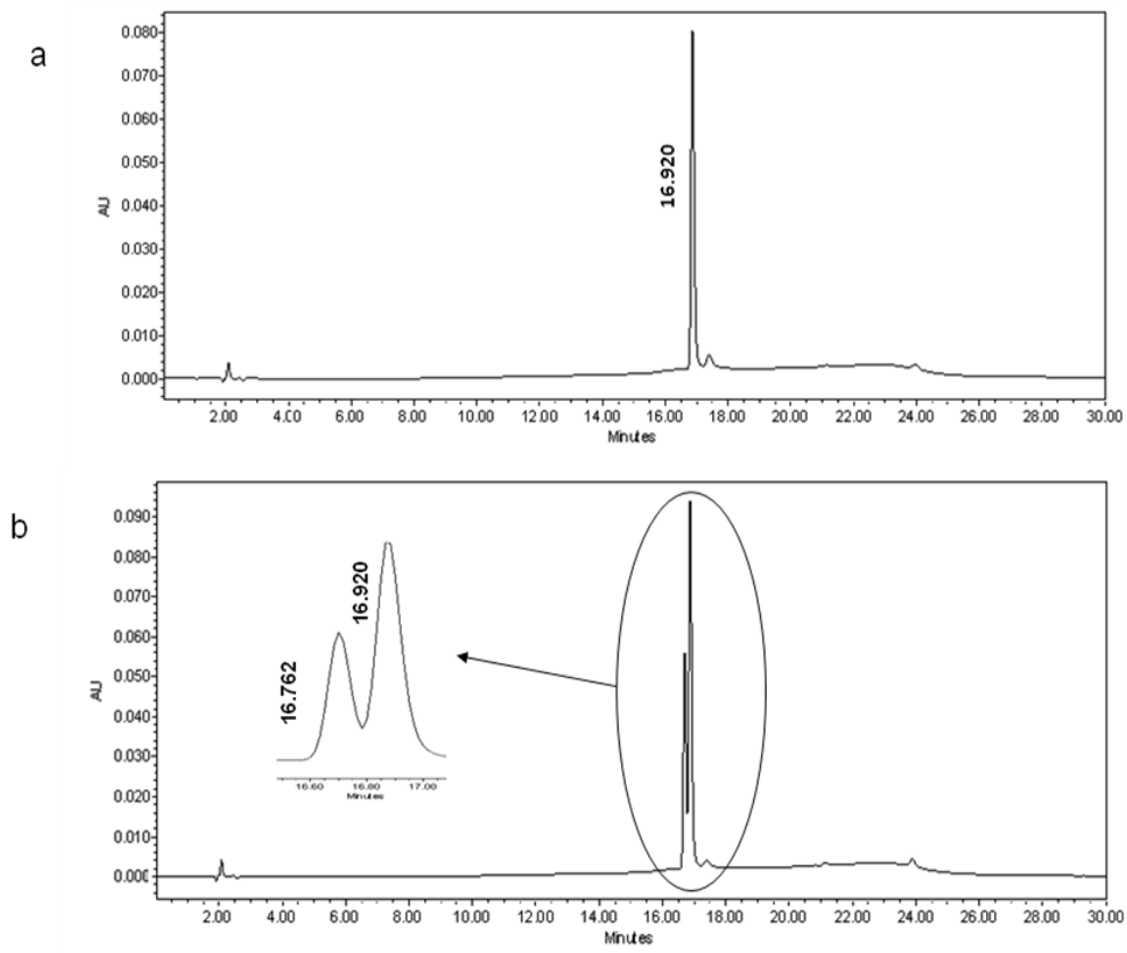

Fig. 7.3- RP-HPLC detection of a) cinnamaldehyde and b) A mixture of 5 mg/l cinnamaldehyde and 50 mg/l cinnamic alcohol standards at 280 nm using 1% acetic acid in water and 1 % acetic acid in methanol as the mobile phase. Cinnamaldehyde retention time 16.920 min; cinnamic alcohol retention time 16.762 min.

Chapter 8

General discussion

One of the important aspects of improving safety and shelf life of perishable foods is controlling microbial growth. While application of heat treatment or dehydration can enhance food safety, such techniques often affect the nutritional and sensory quality of food. Refrigeration is proven technology which not only extends the safety and shelf life of perishable foods but also reduces losses during processing, storage, transport and sale (Coulomb, 2008). Refrigerated storage is becoming more important than ever because of increased consumer demand for fresh, less processed food and for convenience foods requiring minimal preparation such as minimally processed and ready-to-eat refrigerated foods with extended shelf life (Cleland, 1996; Juneja, 2003). The food industry responded to this with improved operation of the industrial cold chain. In addition, increased access to affordable domestic refrigeration has resulted in the increased use of household refrigerators world-wide (Coulomb, 2008). The maximum recommended refrigeration temperature is often determined based on the minimum temperature for sustained growth of *E. coli* and related mesophilic pathogens, $\leq 7°C$ (Smith, 1985; Mackey et al., 1980; Sumner and Krist, 2002; Huang, 2010).

This increased utilization of refrigeration in combination with recent reports on formation of filaments during 7ºC storage by mesophilic enteric organisms (Jones et al., 2002; Mattick et al., 2003b; Gill et al., 2007) highlights the necessity of acquiring better understanding of how mesophilic pathogens like *E. coli* O157:H7 behave during refrigerated storage.

During the present work at 6°C, cells of all *E. coli* strains studied grew by elongation (>4 μm), with formation of filaments (>10 μm) to different extents, while viable numbers increased slightly for ≤ 3 d and these later decreased to varying extents, depending on the strain (Chapter 3; Gill et al., 2007). As the cultures of *E. coli* strains contained varying proportions of cells of different size (≤ 4; >4≤ 10 and > 10 μm), it was not clear whether the observed loss in viability involved cells of all sizes or just a particular size group. Because each cell, regardless of its size develops into a single colony on agar plates, the plate count technique can not discriminate the differences in viabilities of elongating and non-elongating cells. Furthermore, if the observed loss in viability is mainly attributable to elongated or filamentous cells, then the risk associated with these cells via their rapid multiplication could be minimal. Therefore, estimation of viability of different size cells was a key to properly assess the risk associated with the elongated cells. The viability of cells of different sizes was estimated using microscopy after LIVE/DEAD staining, and results showed that both elongating and non-elongating cells lost their viability to similar degrees (Chapter 3). This suggests that unlike cell division *fts* mutants, which elongate, do not divide and eventually lyse at restrictive temperatures (White, 2007), elongated or filamentous cells formed at 6°C were as equally viable as their non-elongating counter parts.

Despite the observations that the maximum mean length and extent of cell elongation observed at 6°C over ≤ 10 d varied greatly among *E. coli* strains (Chapter 3 and 4), cell elongation was evident with all strains. This was consistent with previous findings on cold-induced cell elongation of *E. coli* and *Salmonella* (Mattick et al., 2003a; Gill et al., 2007). However, the mechanism that induces/causes cell elongation at refrigeration temperature is

not well understood. Studies conducted with RpoS sigma factor mutant of *Salmonella* strains showed that RpoS was not responsible for cell elongation at refrigeration temperatures (Mattick et al., 2003a).

Jones et al. (2006) proposed that increased synthesis of proteins related to energy generation, especially proteins involved in the TCA cycle and electron transport, in cells incubated at 6°C for ≤ 8 d could be used as an indicator of limited energy generation. This exposure was suggested to lead to the development of a stringent response and conversion of GTP to guanosine pentaphosphate or tetraphosphate, (p)ppGpp. As GTP is essential for polymerization of the cell division protein FtsZ, the reduced level of GTP would inhibit FtsZ polymerization, subsequent septal ring assembly and cause cell elongation. The stringent response or elevated levels of (p)ppGpp has also been reported to inhibit DNA replication in *E. coli* (Srivatsan and Wang, 2008). Cell division is a complex process which involves a large number of proteins associated with septum development and peptidoglycan synthesis. This process occurs in a well coordinated and sequential order. The FtsZ protein initiates septal ring development by forming a ring before invagination and leads the constriction of the cell wall at the division site (White, 2007). Since both the presence of multiple nucleoids and incomplete septa were observed along the length of elongated or filamentous cells (Chapter 3) the induction of cell elongation upon refrigeration was unlikely due to the inhibition of FtsZ polymerization or inhibition of DNA synthesis. However, filament formation might result from reduced synthesis or inhibition of the activity of one or more of the protein(s) involved in the later stages of septum formation/cell separation (Cabeen and Jocobs-Wagner, 2005). Further studies that systematically examine the expression profiles of

cell division-related genes or proteins and their assembly by immunofluorescent or fluorescent microscopic examination (Lutkenhaus, 2002; Mannik et al., 2012) of *E. coli* cells at 6°C may provide more insight regarding cell elongation.

Removal of filamentous *Salmonella* cells from a condition that caused cell elongation to allow growth resulted in rapid division of filamentous cells and a rapid increase in viable numbers (Mattick et al., 2000; Mattick et al., 2003a). To examine whether similar observations can be made with *E. coli*, cultures incubated at 6°C for ≤10 d were shifted to 37°C for 2 h and viable numbers were estimated (Chapter 3). It was found that the increases in viable numbers after 2 h at 37°C by the cultures of *E. coli* ATCC 11775 and ATCC 23739 previouly incubated at 6°C for 1 to 10 d were generally similar to the increases by cells exposed to < 1 d to 6°C. While viable cells of both *E. coli* ATCC11775 and ATCC 23739 elongated considerably during incubation at 6°C ≤ 10 d, the limited increases in numbers observed after incubation at 37°C could be explained if elongated and filamentous cells did not divide; if the longer exposure to 6°C extended the lag time upon transfer to 37°C (Phillips et al., 1998) or if large fractions of the cells were inactivated by the sudden shift to 37 °C as observed with *E. coli* 8WT. The observation of fewer elongated or filamentous cells after 2 h at 37ºC in samples of all strains, regardless of the duration of prior incubation at 6°C, suggests that viable cells of those lengths initially present in the samples either divided or lysed.

To further understand the effect of rapid shift from 6 to 37°C on morphology and viability, *E. coli* cultures were examined for a longer period (≤ 4 h) at 37ºC after being

shifted from 6°C (Chapter 4). For this study, *E. coli* ATCC 23739 that underwent extensive filamentation at 6°C (Chapter 3) and a strain of *E. coli* O157:H7 that had not been studied with respect to filamentation was used. Even though *E. coli* O157:H7 grew by elongation at 6°C, the extent of cell elongation was lower than with *E. coli* ATCC 23739. Results also revealed that the duration of incubation at 6ºC had no effect on the lag time at 37°C and had little effect on growth rate at 37 ºC. Direct microscopic observation of cells from cultures incubated at 6°C for 5 d on slides incubated at 37°C for ≤ 4 h showed that abrupt shifts of *E. coli* from refrigeration to warm temperatures can result in both division of elongated cells to multiple daughter cells and inactivation of cells. The majority of cells that did not elongate at 6ºC failed to multiply upon abrupt shift to 37ºC (Chapter 4). Although previous studies speculated the possible division of elongated cells formed during refrigeration to multiple daughter cells under favourable growth conditions (Jones et al., 2002, 2003, 2004; Gill et al., 2007), this study provided the direct evidence that elongated or filamentous cells can indeed produce multiple daughter cells when exposed to favourable growth condition. Similarly, high hydrostatic pressure-induced filamentous *E. coli* cells were also reported to yield multiple daughter cells upon removal from high hydrostatic pressure (Kawarai et al., 2004).

Both in Canada and the USA, *E. coli* O157:H7 infections have been considered notifiable diseases and are subject to national reporting (Gill and Gill, 2010). The Canadian Food Inspection Agency states that no *E. coli* O157:H7 should be detected in a ground beef or raw beef samples and the number of quality indicator organisms like generic *E. coli* or coliform numbers should be < 100 cfu/g, when tested (Health Canada, 2012). The USDA has similar regulations for the US food processors (USDA, 2012). As per these regulations, meat

samples should be tested for the presence or absence of *E. coli* O157:H7 using recognized enrichment protocols (Health Canada, 2012). Testing for non-O157:H7 verotoxigenic *E. coli* (non-O157:H7 VTEC) is not necessary under these regulations. Thus, the presence of elongated or filamentous *E. coli* O157:H7 cells in these products may not necessarily lead to underestimation of this pathogen. However, the absence of *E. coli* O157:H7 in tested samples does not indicate that food is devoid of this organism or guarantee the absence of non-O157:H7 VTEC. A considerable proportion of beef carcasses have been reported to be contaminated with non-O157:H7 VTEC in Canada and the USA (Gill and Gill, 2010). Non-O157:H7 infections have also been reported to cause 30 to 50 % of VTEC illnesses and a variety of refrigerated foods have been linked to illness outbreaks caused by these VTEC serotypes (Doyle et al., 2006; Gill and Gill, 2010). The current practice of assessing safety risk based on the numbers of generic *E. coli* or coliforms (< 100 cfu/g) in refrigerated foods does not distinguish commensal *E. coli* and non-O157:H7 VTEC. As infectious does of VTEC have been considered to be ≤ 40 cells (Fitzpatrick, 1999; Doyle et al., 2006), the division of single filamentous cells of *E. coli* into multiple daughter cells at warmer temperatures suggests that the threat from mesophilic pathogens may be underestimated in refrigerated foods.

L. monocytogenes has also been reported to produce elongated or filamentous cells under various marginal conditions (Nilsson et al., 2000; Bereksi et al., 2002; Vail et al., 2012). The current Canadian policy on ready-to-eat foods notes that "*L. monocytogenes* can be reduced, but cannot always be eradicated from finished product or the plant environment, even though the industry should work towards elimination". The Canadian policy placed

ready-to-eat foods into three categories relative to *L. monocytogenes* risk. The first category includes foods which are linked to illness outbreaks such as soft cheese, liver pâté, hot dogs and jellied pork tongue. The second category specifies those foods supporting growth of *L. monocytogenes* that have a shelf-life of more than 10 days such as vacuum packaged meat, refrigerated sauces and modified atmosphere packaged sandwiches. The third category includes those foods supporting growth of *L. monocytogenes* with refrigerated shelf-lives of less than 10 days (e.g. fresh salad) and all ready-to-eat foods not supporting *L. monocytogenes* growth (dry salami, ice cream, and hard cheese). For foods in categories 1, 2 and 3, their recall is required if there is detection of any viable *L. monocytogenes* in a 50g sample, in a 25g sample, or ≥100 cfu/g sample in these food categories, respectively (Health Canada, 2004). If filamentous *L. monocytogenes* cells can divide into multiple daughter cells, like *E. coli* does when exposed to favourable growth conditions, the current limit of ≤ 100 cfu/g in ready-to-eat foods may potentially underestimate the risk and may have potential health implications.

Since evidence obtained with *Salmonella* showed that food materials can influence the extent of filamentation during refrigeration (Mattick et al., 2003a) and since these experiments on *E. coli* filamentation were carried out using laboratory media, future studies are needed to examine the effect of food materials on *E. coli* O157:H7 filamentation during refrigeration. Moreover, the significant variability observed in filamentation among *E. coli* strains at 6°C points outs the importance of strain selection for future studies.

It remains unclear how virulence properties of elongating *E. coli* O157:H7 cells during refrigeration change and how subsequent exposure of elongated cells to warmer

temperatures might influence virulence. Matter of fact, when filamentous cells are ingested, like all *E. coli* cells, they must survive exposure to gastric acid in the stomach before colonization of the intestinal epithelium (House et al., 2009). Understanding the virulence properties of cold-elongated *E. coli* O157:H7 cells and their sensitivity to low pH exposure are therefore necessary if risks of *E. coli* O157:H7 from refrigerated foods are to be properly assessed.

Chapter 3 and 4 documented cell elongation of cold-adapted *E. coli* and potential risks associated with filamentous cells. In addition, cold adaptation can alter the extent and types of proteins expressed, and the cytoplasmic membrane composition of *E. coli* O157:H7 (Russell, 2002; Jones et al., 2006; Carey et al., 2009; Vidovic et al., 2011). The work in Chapter 5 was carried out to gather information on how cold adaptation and cell elongation influence adherence of *E. coli* O157:H7 to food contact surfaces. Previous cold adaptation at 6 or 15°C was found to improve adherence of *E. coli* O157:H7 to food contact surfaces at 4°C, but cell elongation had little/no influence on adherence. Because the farm-to-folk continuum heavily depends on the cold chain, if *E. coli* O157:H7 is present on contaminated food materials, the organism may become adapted to cold and may have greater ability to adhere to food contact surfaces. Future work on biofilm development by *E. coli* O157:H7 would be more meaningful if conducted using refrigerated conditions rather than 20 to 37°C.

In addition to refrigeration, the use of natural antimicrobials has gained attention among researchers and the food industry in recent years because of consumer demand for fresh foods or foods preserved with natural compounds rather than synthetic chemicals. The components of plant essential oils like carvacrol, thymol and cinnamaldehyde, have been

found to be effective against many foodborne pathogens (Holley and Patel, 2005) and could be used as natural alternatives. Interestingly, prolonged exposure to cinnamaldehyde has also been reported to cause cell elongation in *B. cereus* (Know et al., 2003). However, few studies have examined the effect of cinnamaldehyde upon bacteria at sub-lethal levels. Considering the fact cinnamaldehyde is found in many foods at concentrations that are lower than the levels lethal to *E. coli* O157:H7 (Helander et al. 1998; Friedman et al. 2000; Pei et al. 2009), the experiments described in Chapter 6 were designed to investigate how sub-lethal levels of cinnamaldehyde affect its viability and morphology at 6 and 37ºC. The minimum inhibitory concentration was established at 400 mg/l for both *E. coli* O157:H7 and commensal *E. coli* 8WT. Thus concentrations ≤ 300 mg/l were used for this study. Treatment at 6ºC was chosen because cell elongation was common among the *E. coli* strains at this temperature and was used to evaluate whether cinnamaldehyde could enhance or reduce cold-induced cell elongation. Meanwhile, 37ºC was chosen for treatment to examine the effect of sub-lethal levels of cinnamaldehyde without the influence of other stresses.

In Chapter 6 it was shown that concentrations of cinnamaldehyde (200 to 300 mg/l) sub-lethal at 37^{o}C were lethal to both *E. coli* strains at 6°C, and 300 mg/l cinnamaldehyde caused ≥ 5 log cfu/ml reduction in 3 d. Complete inactivation of *E. coli* (≥ 7 log cfu/ml) was observed at day 7. Cell elongation was not observed with ≥ 100 mg/l cinnamaldehyde at 6°C. These observations suggest that cinnamaldehyde may be used as an effective natural antimicrobial to inhibit cold-induced cell elongation or to inactivate *E. coli* O157:H7 during refrigerated storage.

Contrary to the observations at 6ºC, sub-lethal levels of cinnamaldehyde caused cell

elongation and delayed cell multiplication for up to 13 h at 37ºC; depending on the concentration used and the *E. coli* strain (Chapter 6). The greatest proportion of elongated cells and the greatest mean length were observed with 200 mg/l cinnamaldehyde at 2 h. Following delays in cell multiplication, treated cells returned to active growth and normal size. Growth rates observed at 200 and 300 mg/l cinnamaldehyde were higher than the control. To understand the mechanism behind this transient behaviour, the transcriptional response of *E. coli* O157:H7 was analysed at 2 and 4 h exposure to cinnamaldehyde at 37°C. These times were selected because of the delay in multiplication (growth arrest) and greatest cell elongation ($\geq$ 6 μm) occurred at 2 h, and because of the reversal to normal ($\leq$ 3.0 μm) length and growth occurred at 4 h (Chapter 6 and 7). Changes in cinnamaldehyde and its possible metabolites were also monitored by RP-HPLC.

Cinnamaldehyde remained stable for at least 2 h and thus, changes in gene expression observed at this time would be the result of cinnamaldehyde exposure. Transcriptome data showed induction of an oxidative stress response at 2 h. Reduction of NADPH levels in *E. coli* O157:H7 via utilization by oxidative stress response enzymes may have lowered the reducing power of cells and arrested multiplication by limiting their biosynthetic capability (Cabiscol et al., 2000; Pomposiello et al., 2001; Miller et al., 2009; Krapp et al., 2011). Evidence of reduced expression of biosynthetic genes such as genes for protein, O-antigen, fimbriae and DNA synthesis at 2 h as well as delayed multiplication between 0 to 2 h further supports this possibility.

Activation of the MarA-mediated antibiotic resistance network, AcrAB/TolC, in *E. coli* has been shown to confer resistance to tetracycline, chloramphenicol, penicillin G,

erythromycin, novobiocin, ampicillin and rifampicin (Alekshun and Levy, 1999; Kieboom and De Bont, 2000). Deletion of *acrAB* genes in *E. coli* led to increased susceptibility to tetracycline, norfloxacin, chloramphenicol, ampicillin, puromycin, nalidixic acid and rifampicin (Alekshun and Levy, 1999). Although it is yet to be determined whether cinnamaldehyde challenge enhances antibiotic resistance in *E. coli* O157:H7 the evidence presented above and the over expression of MarA-mediated antibiotic resistance genes at 2 and 4 h, and other antibiotic resistance genes at 4 h (Chapter 7), strongly suggests that a sub-lethal cinnamaldehyde challenge may increase the antibiotic resistance of *E. coli* O157:H7 and cause pumping of cinnamaldehyde out of cells.

E. coli O157:H7 was able to convert cinnamaldehyde to the less toxic cinnamic alcohol probably by using alcohol dehydrogenase (YqhD) or aldehyde reductase (DkgA) by 4 h. Cinnamaldehyde can inhibit cell the division protein FtsZ by attachment through its carbonyl aldehyde group (Domadia et al., 2007) and might have contributed to cell elongation at $\leq$ 2 h of cinnamaldehyde exposure. It is believed that the dissimilation of cinnamaldehyde to cinnamic alcohol between 2 and 4 h reversed the inhibition of FtsZ, which allowed successful septum development and the return to normal length. This conversion also relieved *E. coli* O157:H7 from the repressive effects on genes involved in protein, O-antigen, fimbriae, lipid-A and DNA synthesis. After this conversion, *E. coli* O157:H7 grew at a faster rate and became highly motile with increased metabolic fluxes as indicated by over expression of many electron transport enzymes (e.g: F_0F_1 ATPase, NouA-N and HybABC).

Given the 4-fold higher MIC value of cinnamic alcohol (1600 mg/l) compared to

cinnamaldehyde, it can be concluded that the antimicrobial action of cinnamaldehyde is likely due to its carbonyl aldehyde group. Based on the transcriptomic data (Chapter 7) and results of Domadia et al., (2007), a model depicting the overall response of *E. coli* O157:H7 to cinnamaldehyde is presented in Fig 8.1. Activation of the MarA-mediated antibiotic resistance network is not included in this model because it would essentially be a reproduction of the model presented by Alekshun and Levy (1999), and because confirmatory studies are needed to confirm the effects of cinnamaldehyde exposure upon antibiotic resistance of *E. coli* O157:H7.

Even though this is the first study to describe the capability of *E. coli* O157:H7 to convert cinnamaldehyde to cinnamic alcohol, it has been well understood that *E. coli* is equipped with multiple enzymes that can dissimilate phenolic compounds and enable their use as energy sources (Diaz et al., 2001**)**. However it remains unclear if the observed conversion of cinnamaldehyde to cinnamic alcohol will lead to further degradation of this compound.

Overall this study demonstrated that cell elongation could be a common response of mesophilic enteric organisms like *E. coli* O157:H7 to cold exposure. Elongated cells formed at refrigeration temperatures can simultaneously form multiple progeny when exposed to warmer temperatures. Therefore, the current practice of bacterial enumeration/risk assessment using conventional plate count techniques immediately after removal of food from refrigeration, which does not take account cell elongation, may potentially underestimate the risk of *E. coli* O157:H7. Interestingly, application of cinnamaldehyde could prevent cell elongation and potentially eliminate *E. coli* O157:H7 during refrigeration.

The transcriptomic study revealed that the antimicrobial action of cinnamaldehyde was attributable to its aldehyde group and that the aldehyde group was responsible for cell elongation and activation of various stress response networks at 37°C. *E. coli* O157:H7 was able to neutralize the effect of sub-lethal levels cinnamaldehyde by its convertion to cinnamic alcohol at 37°C.

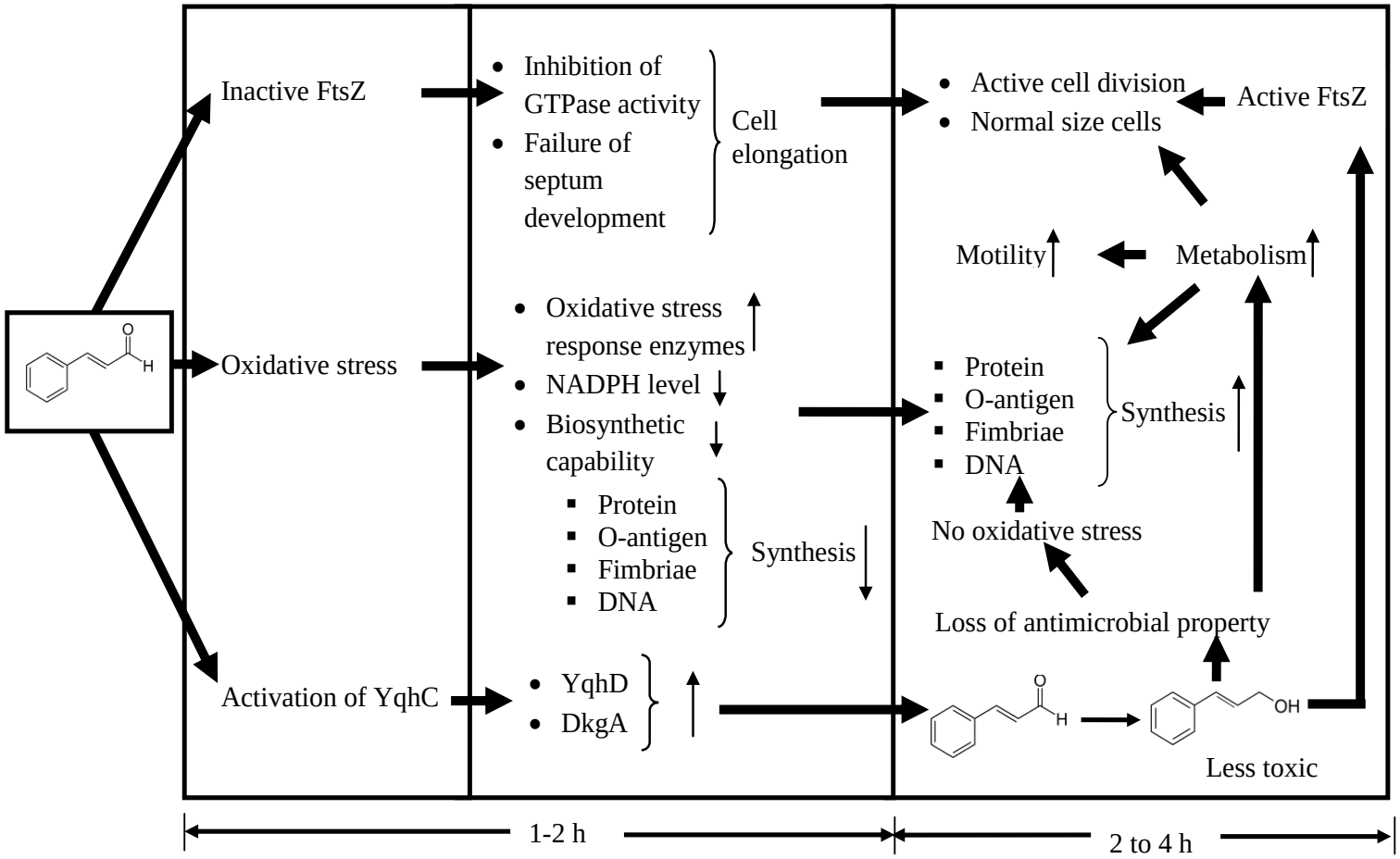

Fig. 8.1- Model showing response of *E. coli* O157:H7 to cinnamaldehyde exposure at 2 and 4 h. YqhD, Alcohol dehydrogenase; DkgA, 2,5-diketo-D-gluconate reductase A. (Visvalingam et al. 2013. Appl. Environ. Microbiol. 79: 942-950)

Chapter 9

Conclusions

Elongation of both commensal *E. coli* and *E. coli* O157:H7 at refrigeration (6°C) appears to be common although the extent to which elongated or filamentous cells formed varied greatly among strains. Elongated or filamentous cells better retained their viability upon transfer from 6 ºC to 37 ºC than expected and appeared to be the major contributors to the viability of resulting cultures. Since elongated or filamentous cells formed at 6 ºC are able to divide at warmer temperatures, filament formation by mesophilic enteric pathogens that have low infective dose at refrigeration temperatures (which are not detected by measuring viable numbers of cells present) may cause underestimation of risk if contaminated food temperature rises above 6ºC. Prior cold adaptation can enhance adherence of *E. coli* O157:H7 to food contact surfaces. However, cell size/ elongation has no influence on this enhanced adherence.

Cinnamaldehyde is a natural antimicrobial and its MIC value against *E. coli* strains tested here was 400 mg/l. interestingly, levels of cinnamaldehyde (≤ 300 mg/l) sub-lethal at 37^{o}C not only inhibited cell elongation under refrigeration (6°C) but were also lethal to *E. coli* at this temperature. Thus, cinnamaldehyde could be an effective agent to inhibit cell elongation and inactivate *E. coli* O157:H7.

In contrast, at 37ºC the temporary growth delay caused by ≤ 300 mg/l cinnamaldehyde was partly due the formation of elongated cells which was reversible. Treatment at 200 mg/l cinnamaldehyde inhibited growth of *E. coli* O157:H7 at 37ºC and for

≤ 2 h caused cell elongation, but from 2 to 4 h growth resumed and cells reverted to normal length. Analysis of gene expression by microarray and cinnamic compounds by RP-HPLC revealed that the antimicrobial action of cinnamaldehyde was attributable to its aldehyde group and that the aldehyde group was responsible for cell elongation, activation of various stress response networks and the growth delay at 37°C. However *E. coli* O157:H7 was able to neutralize the inhibitory effects of cinnamaldehyde by converting it to the less toxic cinnamic alcohol at 37°C.

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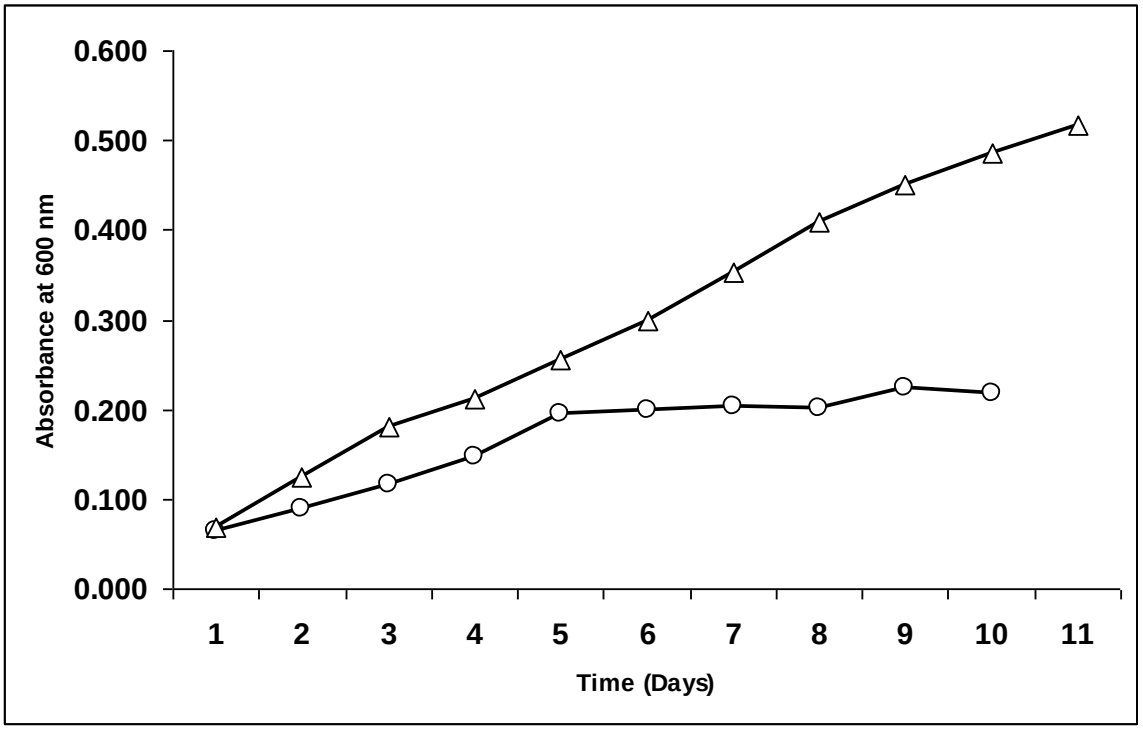


Appendix I– Changes in A_{600} values of cultures of *Escherichia coli* ATCC 11775 (o) and *E. coli* ATCC 23739 (Δ) incubated at 6°C.

Appendix II– The fractions of *E. coli* O157:H7 cells with intact cytoplasmic membrane observed by LIVE/DEAD staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 6 h at 37 ºC

Time (h)	Percent of cells with intact cytoplasmic membrane*			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	99.2 ± 0.9^{A}	98.1 ± 2.4^{A}	97.8 ± 0.7^{A}	98.3 ± 0.7^{A}
1	100 ± 0^{A}	100 ± 0^{A}	98.9 ± 1.2^{A}	93.2 ± 2.9^{A}
2	99.2 ± 0.5^{A}	99.4 ± 1.1^{A}	96.7 ± 2.9^{A}	86.9 ± 6.6^{A}
3	99.5 ± 0.2^{A}	99.7 ± 1.0^{A}	97.3 ± 1.7^{A}	71.9 ± 5.9^{B}
4	99.5 ± 0.2^{A}	100 ± 0^{A}	99.6 ± 0.4^{A}	ND
5	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}	ND
6	100 ± 0^{A}	100 ± 0^{A}	99.6 ± 0.4^{A}	ND

ND – Not determined.
*Different superscript letters ($^{A-F}$) in each column denote a significant difference (P< 0.05).
determined.

Appendix III– The fractions of *E. coli* 8WT cells with intact cytoplasmic membrane observed by LIVE/DEAD staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 24 h at 37 ºC

Time (h)	Percent of cells with intact cytoplasmic membrane*			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	98.9 ± 2.0^{A}	99.6 ± 0.6^{A}	94.3 ± 1.5^{A}	82.6 ± 1.6^{A}
1	99.8 ± 0.4^{A}	87.7 ± 1.4^{C}	97.5 ± 1.1^{AC}	40.5 ± 7.1^{B}
2	100 ± 0^{A}	99.8 ± 0.3^{A}	99.1 ± 1.6^{BC}	36.8 ± 1.2^{B}
3	99.7 ± 0.6^{A}	97.1 ± 1.3^{B}	98.7 ± 0.7^{BC}	42.8 ± 11.3^{B}
4	99.3 ± 0.5^{A}	99.2 ± 0.1^{A}	98.2 ± 1.1^{AC}	ND
5	98.2 ± 0.5^{A}	99.4 ± 0.7^{A}	99.5 ± 0.0^{BC}	ND
6	99.3 ± 0.9^{A}	99.5 ± 0.2^{A}	99.3 ± 0.7^{BC}	ND

ND – Not determined.
*Different superscript letters ($^{A-B}$) in each column denote a significant difference ($P < 0.05$).

Appendix IV– The fractions of *E. coli* O157:H7cells with polarized cytoplamic membrane observed by $DiBAC_4(3)$ staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 8 h at 37 ºC

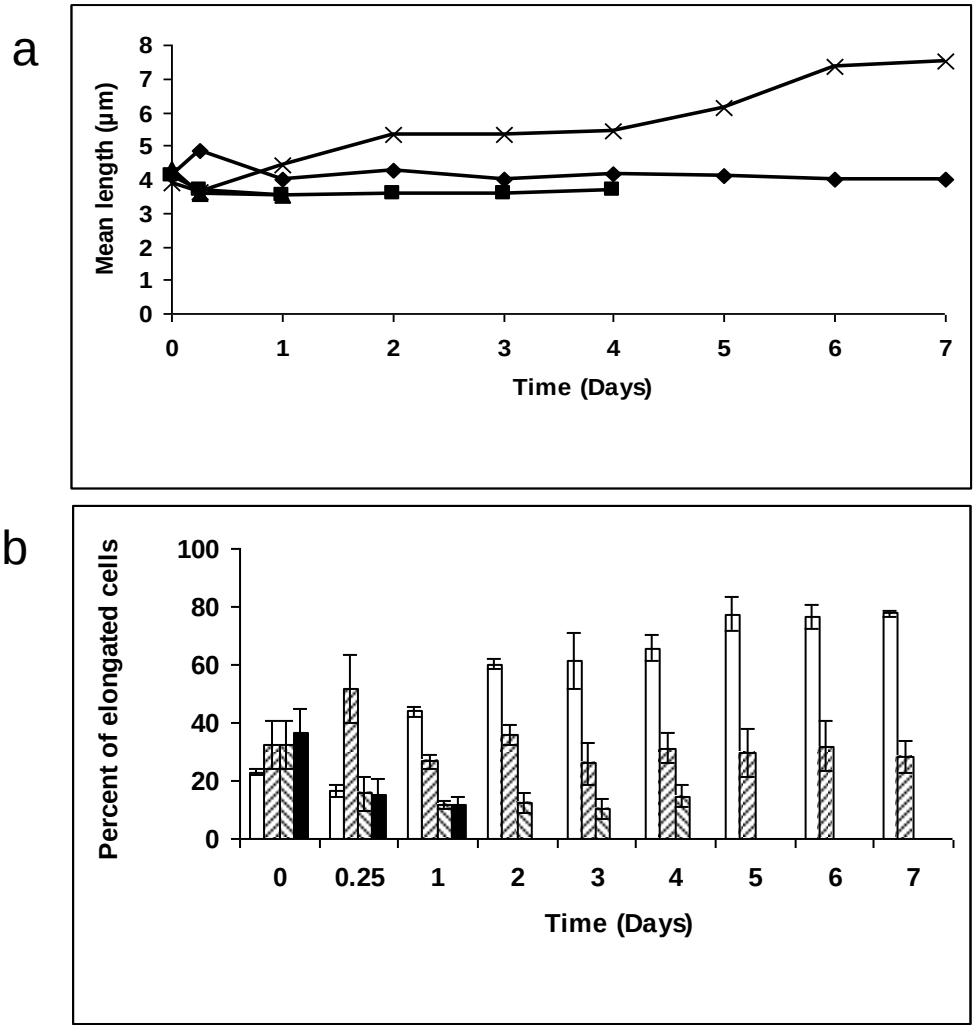
Time (h)	Percent of cells with polarized cytoplasmic membrane*			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	100 ± 0^{A}	100 ± 0^{A}	99.8 ± 0.4^{A}	97.8± 2.0^{A}
1	99.6 ± 0.6^{A}	100 ± 0^{A}	99.8 ± 0.4^{A}	96.1 ± 2.3^{A}
2	100 ± 0^{A}	99.7± 0.5^{A}	98.6 ± 0.8^{A}	91.9 ± 3.0^{A}
3	98.6± 0.2^{A}	100 ± 0^{A}	97.3 ± 2.0^{A}	91.4 ± 1.7^{A}
4	99.8 ± 0.3^{A}	100 ± 0^{A}	99.3 ± 0.2^{A}	89.7 ± 3.6^{A}
5	99.2 ± 1.2^{A}	100 ± 0^{A}	99.6 ± 0.2^{A}	73.8 ± 6.6^{B}
6	99.4± 0.3^{A}	100 ± 0^{A}	99.1 ± 1.0^{A}	97.1 ± 2.2^{A}
7	ND	ND	ND	98.0 ± 1.0^{A}

ND – Not determined.
*Different superscript letters ($^{A-B}$) in each column denote a significant difference (P< 0.05).

Appendix V– The fractions of *E. coli* 8WT cells with polarized cytoplasmic membrane observed by $DiBAC_4(3)$ staining after exposure to sub-lethal levels of cinnamaldehyde for ≤ 24 h at 37 ºC

Time (h)	Percent of cells with polarized cytoplasmic membrane*			
	Cinnamaldehyde concentration (mg/l)			
	Control	100	200	300
0	100 ± 0^{A}	99.5 ± 0.8^{A}	99.2 ± 0.9^{A}	88.3 ± 4.1^{A}
1	100 ± 0^{A}	96.9 ± 1.2^{A}	100 ± 0^{A}	50.7 ± 6.5^{CD}
2	100 ± 0^{A}	99.0 ± 0.5^{A}	100 ± 0^{A}	58.0 ± 8.6^{BC}
3	100 ± 0^{A}	99.7 ± 0.3^{A}	99.4 ± 0.5^{A}	68.5 ± 4.4^{B}
4	99.8 ± 0.3^{A}	99.8 ± 0.2^{A}	95.9 ± 1.8^{B}	54.1 ± 3.6^{CD}
5	99.7 ± 0.4^{A}	99.7 ± 0.1^{A}	100 ± 0^{A}	54.6 ± 5.1^{CD}
6	99.8 ± 0.2^{A}	99.4 ± 0.4^{A}	99.9 ± 0.2^{A}	52.2 ± 3.8^{CD}
7	ND	ND	ND	53.0 ± 5.2^{CD}
8	ND	ND	ND	46.0 ± 1.7^{D}
9	ND	ND	ND	52.7 ± 2.3^{CD}
10	ND	ND	ND	56.7 ± 1.6^{BCD}
11	ND	ND	ND	55.4 ± 3.4^{CD}
12	ND	ND	ND	58.6 ± 4.7^{BCD}
13	ND	ND	ND	90.9 ± 1.8^{A}
14	ND	ND	ND	96.5 ± 1.8^{A}
15	ND	ND	ND	97.5 ± 1.0^{A}
16	ND	ND	ND	99.7 ± 0.3^{A}
18	ND	ND	ND	98.7 ± 0.5^{A}
19	ND	ND	ND	99.0 ± 0.4^{A}

ND – Not determined.
*Different superscript letters ($^{A-D}$) in each column denote a significant difference ($P < 0.05$).



Appendix VI- Changes in the mean length of a) *E. coli* O157:H7 in the absence (×) or presence of 100 mg/l (♦), 200 mg/l (■) or 300 mg/l (▲) cinnamaldehyde at 6ºC and b) the fractions of elongated cells observed with *E. coli* O157:H7 in the absence (□) or presence of 100 mg/l (▨), 200 mg/l (▨) or 300 mg/l (■) cinnamaldehyde at 6ºC.

Appendix VII- Cell shape/cell cycle-related *E. coli* O157:H7 genes that were differentially expressed at 2 and 4 h exposure to cinnamaldehyde

Gene	Function	Fold change (log_2)	
		2 h	4 h
fic	cell filamentation protein Fic	1.7	NS
alr	alanine racemase 1	NS	2.8
ftsW	cell division protein	NS	2.8
ftsH	ATP-dependent metalloprotease	NS	1.9
ftsB	cell division protein	NS	1.5
dacA	penicillin-binding protein 5	NS	1.8
pbpC	penicillin-binding protein 1C	NS	1.6
mrcA	fused penicillin-binding protein 1a:murein transglycosylase	NS	2.4
mrdA	penicillin-binding protein 2	NS	2.4
mraY	phospho-N-acetylmuramoyl-pentapeptide-transferase	NS	2.5
mreBCD	rod shape-determining protein	NS	2.4/2.5/3.2
murG	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	NS	2.5
murC	UDP-N-acetylmuramate--L-alanine ligase	NS	1.8
murE	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	NS	2.7
murF	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	NS	2.3
ispU	undecaprenyl pyrophosphate synthase	NS	1.5
mukEF	chromosome condensin	NS	2.0/2.0
bcsQ	hypothetical protein /cell division protein	NS	2.6
mraY	phospho-N-acetylmuramoyl-pentapeptide transferase	NS	2.5
tig	peptidyl-prolyl cis/trans isomerase (trigger factor)	NS	2.4
xerC	site-specific tyrosine recombinase XerC	NS	1.9
yiiU	hypothetical protein	NS	-1.8
dicB	putative inhibitor of cell division encoded by cryptic prophage CP-933P/DicB	NS	-3.0
dicC	DNA-binding transcriptional regulator DicC	NS	-1.9
dicF	ncRNA	NS	-2.8

Appendix VIII- *E. coli* O157:H7 genes that were highly upregulated only at 2 h exposure to cinnamaldehyde[1]

Gene	Function	Fold change (log_2)
Energy derivation and oxidation reduction		
poxB	pyruvate dehydrogenase	3.5
cyoCD[2]	cytochrome o ubiquinol oxidase subunit III	3.1/1.8
nfnB[2]	dihydropteridine reductase, NAD(P)H-dependent, oxygen-insensitive	3.1
acnA[3]	aconitate hydratase 1	3.0
aceE[3]	pyruvate dehydrogenase, decarboxylase component E1, thiamin-binding	2.9
mdaA/nfsA[3]	nitroreductase A, NADPH-dependent, FMN-dependent	2.9
talA	transaldolase A	2.9
aceF[3]	dehydrogenase, dihydrolipoyltransacetylase component E2	2.8
gcd	glucose dehydrogenase	2.8
fumC[3]	fumarate hydratase (fumarase C),aerobic Class II	2.4
betA	choline dehydrogenase	2.2
betB	betaine aldehyde dehydrogenase	2.0
mltD	membrane-bound lytic murein transglycosylase D	2.0
gdhA	glutamate dehydrogenase	2.0
erpA	iron-sulfur cluster insertion protein	2.0
deoB	Phosphopentomutase	2.4
deoC	2-deoxyribose-5-phosphate aldolase, NAD(P)-linked	2.3
Translation/ protein synthesis		
rpsV	30S ribosomal subunit S22 / Stationary-phase-induced ribosome-associated protein	2.5
Stress response		
inaA[3]	conserved protein, acid-induced	3.9
sodA[3]	superoxide dismutase, Mn	3.5

Appendix VIII- continued

Gene	Function	Fold change (log_2)
katE	hydroperoxidase II	2.7
gshB[3]	glutathione synthetase	2.1
sufA	iron-sulfur cluster assembly scaffold protein	1.9
otsB	trehalose-6-phosphate phosphatase	3.3
treF	cytoplasmic trehalase	2.4
osmY	osmotically inducible periplasmic protein	2.3
gshA	glutamate--cysteine ligase	1.7
rpoS	RNA polymerase sigma factor RpoS (σ^{38})	1.6
Antibiotic resistance		
marR[3]	DNA-binding transcriptional repressor of multiple antibiotic resistance	4.2
marB[2]	Multiple antibiotic resistance protein	3.1
mdlAB[2]	fused predicted multidrug transporter	2.9/2.3
Membrane/membrane transport		
bhsA	biofilm, cell surface and signaling protein	4.6
chaC	cation transport protein	3.7
ECs2055	putative outer membrane receptor for iron transport	2.6
ompX[3]	outer membrane protein X	1.9
ylaC	inner membrane protein, DUF1449 family	2.2
nlpD	activator of AmiC murein hydrolase activity, lipoprotein (S-phase)	2.0
nhoA	N-hydroxyarylamine O-acetyltransferase	1.9
Amino acid synthesis/metabolism		
shiA	shikimate transporter	2.8
argG	argininosuccinnamaldehydeate synthase	1.9
ltaE	L-allo-threonine aldolase	2.5
dcyD	D-cysteine desulfhydrase	1.9
Transcriptional regulators		
yqhC	putative ARAC-type regulatory protein	3.8

Appendix VIII- continued

Gene	Function	Fold change (log_2)
fnr	fumarate/nitrate reduction transcriptional regulator	2.0
Other genes		
yhbW	predicted enzyme	3.8
hemB[3]	delta-aminolevulinic acid dehydratase	2.3
idi/ ECs3761	isopentenyl-diphosphate delta-isomerase (isoprenoid biosynthesis)	2.3
ispA	Geranyltranstransferase (isoprenoid biosynthesis)	1.9

[1] Genes with expression value ≥ 1.6 are presented.
[2]Changes in expression for each gene of the complex or the same group are listed sequentially in respective order.
[3]Genes that are directly or indirectly controlled by MarA (Barbosa and Levy, 2000; Ruiz et al., 2008).

Appendix IX- *E. coli* O157:H7 genes that were highly downregulated only at 2 h exposure to cinnamaldehyde[1]

Gene	Function	Fold change (log_2)
Energy derivation and oxidation reduction		
hycABCDEFGHI[2]	hydrogenase 3/ formate hydrogenlyase	-3.9/-2.3/-3.1/-5.0/-4.5/-3.5/-1.7/-2.0/-2.0
hyaABC[2]	hydrogenase-1	-4.0/-3.1/-1.9
hydN	electron transport protein HydN	-4.1
fdhF	formate dehydrogenase H	-3.4
frdABC[2]	fumarate reductase flavoprotein subunit (anaerobic)	-2.7/-3.2/-2.2
dmsABC[2]	anaerobic dimethyl sulfoxide reductase	-2.8/-3.0/-2.0
nuoA	NADH dehydrogenase subunit A	-2.1
nrfA	cytochrome c552 /nitrite reductase	-2.0
hypB	hydrogenase nickel incorporation protein HypB	-2.1
hypE	hypothetical protein /plays structural role in maturation of all 3 hydrogenases	-1.9
nrdG	anaerobic ribonucleotide reductase activating protein	-2.1
dcuB	anaerobic C4-dicarboxylate transporter	-3.5
DNA metabolism		
priB	primosomal replication protein N	-2.7
cnu	oriC-binding nucleoid-associated protein	-2.4
Translation/ protein synthesis		
greA	transcription elongation factor GreA	-1.8
tdcF	predicted L-PSP (mRNA) endoribonuclease	-2.1
rpmEG[2]	50S ribosomal protein L31/L33	-2.1/-2.9
rplOP[2]	50S ribosomal protein L15/16	-2.1/-2.2
rpsHRS[2]	30S ribosomal protein S8/S18/S19	-3.2/-2.2/-2.3
rpsH	30S ribosomal protein S8	-3.2
Stress response		
gadE[3]	transcriptional activator involved in acid resistance	-3.6

Appendix IX- continued

Gene	Function	Fold change (log_2)
gadAB[3]	glutamate decarboxylase alpha	-4.1/-4.8
gadC[3]	glutamate:gamma-aminobutyric acid antiporter/ acid sensitivity protein	-3.5
hdeAB[23]	acid-resistance protein	-4.8/-6.0
hdeD[3]	acid-resistance membrane protein	-4.8
Membrane/membrane transport		
malEFG[2]	maltose transporter	-4.0/-1.9/-2.5
wbdNO[2]	putative glycosyl transferase	-3.1/-2.0
manXYZ[2]	PTS system mannose-specific transporter	-2.3/-2.3/-2.7
lamB	maltose outer membrane porin (maltoporin)	-2.5
hmuV	hemin importer ATP-binding subunit	-1.9
afuB	putative permease component of transport system for ferric iron	-2.1
ydeQRS[3]	predicted fimbrial-like protein	-2.5/-3.8/-3.6
wzy	O antigen polymerase	-3.0
wbdR	acetyl transferase; O-antigen biosynthesis	-2.1
gfcE	lipoprotein required for formation of the O-antigen capsule	-1.9
ompF[3]	outer membrane protein F	-2.2
ompC	outer membrane porin protein C	-2.8
pgaA	biofilm adhesin polysaccharide PGA export/ PGA export lipoprotein with a polysaccharide deacetylase activity needed for export	-2.6
ycdT	diguanylate cyclase	-2.8
etk	cryptic autophosphorylating protein tyrosine kinase Etk	-2.1
Transcription		
nhaR	DNA-binding transcriptional activator	-2.0

Appendix IX- continued

Gene	Function	Fold change (log_2)
Amino acid synthesis/metabolism		
cysJ	sulfite reductase subunit alpha	-1.9
cysM	cysteine synthase B	-1.8
cysW	sulfate/thiosulfate transporter permease subunit	-2.7
cysD	sulfate adenylyltransferase subunit 2	-3.2
aspA	aspartate ammonia-lyase	-4.2

[1] Genes with expression value ≤ -1.9 are presented.
[2]Changes in expression for each gene of the complex or the same group are listed sequentially in respective order.
[3]Genes that are directly or indirectly controlled by MarA (Barbosa and Levy, 2000; Ruiz et al., 2008).

Appendix X- *E. coli* O157:H7 genes that were highly upregulated only at 4 h exposure to cinnamaldehyde[1]

Gene	Function	Fold change (log_2)
Motility and chemotaxis		
motB	flagellar motor protein	6.8
motA	flagellar motor protein	6.7
fliC	flagellin	5.6
fliD	flagellar capping protein	5.2
tap	methyl-accepting protein IV	5.6
cheA	chemotaxis protein	5.6
cheZ	chemotaxis regulator CheZ	4.5
cheW	purine-binding chemotaxis protein	4.5
cheY	chemotaxis regulator transmitting signal to flagellar motor component	4.0
tar	methyl-accepting chemotaxis protein II	4.8
fliS	flagellar protein potentiates polymerization	4.6
flgK	flagellar hook-filament junction protein 1	4.1
flgN	flagella synthesis protein	3.6
fliT	flagellar biosynthesis protein	3.4
flgM	anti-sigma factor for FliA	3.2
cheR	chemotaxis methyltransferase	3.1
flhC	transcriptional activator FlhC	2.2
tsr	methyl-accepting chemotaxis protein I	2.6
fliA	flagellar biosynthesis sigma factor (σ^{28})	2.6
flhD	transcriptional activator FlhD	2.6
flgM	anti-sigma factor for FliA (σ^{28})	3.2
yhjH	cyclic-di-GMP phosphodiesterase, FlhDC-regulated	4.2
Energy generation and oxidation reduction		
atpABDEFGH[2]	F0F1 ATP synthase	2.9/2.0/2.7/2.7/2.8/2.8/3.0

Appendix X- continued

Gene	Function	Fold change (log_2)
nuoEFGHIJKLM[2]	NADH:ubiquinone oxidoreductase	2.0/1.8/1.8/2.0/2.7/2.0/2.4/2.5/1.9
torC	trimethylamine N-oxide (TMAO) reductase I cytochrome c-type subunit	6.2
torD	chaperone involved in maturation of TorA subunit of trimethylamine N-oxide reductase system I	5.3
rsxACDE[2]	electron transport complex	4.1/3.8/1.8/1.8
DNA metabolism		
yjjV	predicted DNase	3.5
nrdA	ribonucleotide-diphosphate reductase subunit alpha	3.0
dnaE	DNA polymerase III subunit alpha	2.5
holA	DNA polymerase III subunit delta	2.1
ECs4325	putative O-methyltransferase	5.0
Translation/ Protein synthesis		
hemK	N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase	3.6
tgt	queuine tRNA-ribosyltransferase	3.1
rlmH	rRNA large subunit methyltransferase	2.7
rhlE	ATP-dependent RNA helicase RhlE	3.1
rpsJP[2]	30S ribosomal protein S10/S16	2.5/2.1
rplBDV[2]	50S ribosomal protein L2/L4/L22	2.4/2.3/2.1
rpmH	50S ribosomal protein L34	2.0
glyQS[2]	Glycyl-tRNA synthetase	1.5/2.0
pheST[2]	Phenylalanyl-tRNA synthetase	2.8/2.2
prfABC[2]	Peptide chain release factor 1/2/3	3.2/1.8/2.2
rnpA	protein C5 component of RNase P	3.4
Stress response		

Appendix X- continued

Gene	Function	Fold change (log_2)
cpxP	periplasmic repressor CpxP	4.3
cadA	lysine decarboxylase, acid-inducible	4.0
cadB	lysine/cadaverine antiporter	4.0
adiA	biodegradative arginine decarboxylase	3.7
adiC	arginine:agmatin antiporter	3.2
degP	serine endoprotease	3.1
gppA	guanosine pentaphosphate phosphohydrolase	3.0
Antibiotic resistance		
acrB	acriflavin resistance protein B	2.8
acrD	aminoglycoside/multidrug efflux system	2.6
ampC	beta-lactamase	2.2
emrAD[2]	multidrug resistance secretion protein	2.2/1.8
bcr	bicyclomycinnamaldehyde/multidrug efflux system	1.7
mdtIJ[2]	multidrug efflux system protein	2.7/4.6
Membrane/membrane transport		
spr	mutational suppressor of prc thermosensitivity, outer membrane lipoprotein	4.7
lpxT	Lipid A 1-diphosphate synthase/ lipid A 1-phosphate phosphotransferase	4.4
eptB	KDO phosphoethanolamine transferase, Ca^{2+}-inducible	4.3
waaL	lipid A-core:surface polymer ligase	4.2
ECs4324	lipoprotein	4.1
ECs4329	acyl carrier protein	3.9
ECs4332	putative (3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	3.6
ddg	lipid A biosynthesis palmitoleoyl acyltransferase	3.0
lgt	prolipoprotein diacylglyceryl transferase	3.0

Appendix X- continued

Gene	Function	Fold change (log_2)
oppCD[2]	oligopeptide transport system	3.3/2.9
acpT	holo-(acyl carrier protein) synthase 2	2.8
mipA	scaffolding protein for murein synthesizing machinery	3.1
prc	carboxy-terminal protease for penicillin-binding protein 3	3.3
setB	lactose/glucose efflux system	3.4
lpxB	lipid-A-disaccharide synthase	2.7
mlaF	ABC transporter maintaining OM lipid asymmetry, ATP-binding protein	2.7
accABC[2]	acetyl-CoA carboxylase carboxyltransferase	1.6/2.5/2.1
fabB	3-oxoacyl-(acyl carrier protein) synthase I	1.6
fabH	3-oxoacyl-(acyl carrier protein) synthase III	1.8
fabD	malonyl-CoA-[acyl-carrier-protein] transacylase	1.6
fabG	3-ketoacyl-(acyl-carrier-protein) reductase	1.8
aaeAB[2]	p-hydroxybenzoic acid efflux system	1.6/2.0
Transcription		
fis	global DNA-binding transcriptional dual regulator	2.9
malT	transcriptional regulator MalT	3.1
mcbR	colanic acid and biofilm gene transcriptional regulator, MqsR-controlled	3.3

[1] Genes with expression value ≥1.6 are presented.
[2]Changes in expression for each gene of the complex or the same group are listed sequentially in respective order.

Appendix XI- *E. coli* O157:H7 genes that were highly downregulated only at 4 h exposure to cinnamaldehyde[1]

Gene	Function	Fold change (log_2)
Stress response		
ibpAB[2]	Small heat shock protein	-2.9/-3.6
htpG	heat shock protein 90	-2.7
rmf	ribosome modulation factor (heat resistant)	-2.5
hspQ	heat shock protein HspQ	-2.0
clpB	protein disaggregation chaperone	-1.9
grpE	heat shock protein GrpE	-1.9
dnaK	chaperone DnaK/chaperone Hsp70	-1.9
grxA	glutaredoxin 1	-5.0
gadY	ncRNA (acid resistant)	-3.0
cspD	stationary phase/starvation inducible regulatory protein CspD	-2.8
uspBDF[2]	universal stress protein	-2.1/-2.2/-3.0
ldhA	fermentative D-lactate dehydrogenase, NAD-dependent	-2.2
ECs1120	putative copper/zinc-superoxide dismutase	-2.5
osmCE	osmotically inducible protein	-2.1/-1.5
Translation/protein synthesis		
higAB[2]	HigB-HigA toxin-antitoxin system	-2.3/-4.0
rdlB	ncRNA	-3.3
sokC	ncRNA	-2.6
Membrane/membrane transport		
macB	macrolide transporter ATP-binding permease protein	-3.3
cysP	thiosulfate transporter subunit	-2.9
slp	membrane protein induced after carbon starvation	-2.9
ygdI	lipoprotein ygdI precursor	-4.2
yggA	arginine exporter protein	-2.1
ybhL	inner membrane protein, UPF0005 family	-2.3
yohC	inner membrane protein	-2.2
ydhC	inner membrane transport protein YdhC	-2.2

Appendix XI- continued

Gene	Function	Fold change (log_2)
Transcription		
zntR	DNA-binding transcriptional activator in response to Zn(II)	-3.6
glnK	nitrogen regulatory protein P-II	-2.3
yafC	putative transcriptional regulator YafC	-2.1
Biofilm formation		
bdm	biofilm-dependent modulation	-2.3
bssR	biofilm formation regulatory protein BssR	-3.5
BssS	biofilm formation regulatory protein BssS	-1.8
Others		
ryjA	ncRNA	-3.1

[1] Genes with expression value ≤ -1.8 are presented.
[2] Changes in expression for each gene of the complex or the same group are listed sequentially in respective order.