# University of Alberta

Control of *Listeria monocytogenes* and Heat-Resistant *Escherichia coli* on Vacuum-Packaged Beef

by

Mandi Rai Socholotuik

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*To my grandparents, Harry and Nellie, for your sacrifices, your strength, your love, and for teaching me the value of hard work.*

**Abstract**

Novel methods to control *Listeria monocytogenes* and *Escherichia coli* on vacuum-packaged raw beef were investigated. Bacteriocin-negative *Lactobacillus sakei* FUA3058 and bacteriocin-positive *Carnobacterium maltaromaticum* UAL307 were tested as biopreservatives, alone or in combination with antimicrobial treatments. RT-qPCR was used to quantify bacteriocin gene expression by *C. maltaromaticum* UAL307 *in vitro* and on vacuum-packaged beef at 4°C and 10°C. Bacteriocin gene expression was highest at 10°C *in vitro*, and gene expression was higher *in vitro* than on meat. Carnocyclin A was the bacteriocin most consistently detected on meat. Phenolic acids were tested as outer membrane permeants to sensitize *E. coli* to bacteriocins produced by gram-positive bacteria. Syringic acid acted synergistically with *C. maltaromaticum* UAL307 cell-free culture supernatant to inhibit growth of *E. coli in vitro*. Green and red fluorescent proteins were evaluated as tools for monitoring bacterial viability and showed potential for use as intracellular pH probes for bacterial cells on meat.

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## List of Abbreviations

| | |
|---|---|
| ABC | - ATP-binding cassette |
| APT | - All Purpose Tween |
| ATP | - adenosine triphosphate |
| AU | - arbitrary activity unit |
| AW | - after wash |
| bp | - base pair |
| *C.* | - *Carnobacterium* |
| cDNA | - copy deoxyribonucleic acid |
| CFS | - cell-free culture supernatant |
| CFU | - colony forming units |
| $C_T$ | - cycle threshold value |
| DM | - drive mechanism |
| DNA | - deoxyribonucleic acid |
| DNase | - deoxyribonuclease |
| dNTP | - deoxyribonucleotide triphosphate |
| E | - polymerase chain reaction efficiency |
| *E.* | - *Escherichia* |
| EDTA | - ethylenediaminetetraacetic acid |
| eGFP | - enhanced green fluorescent protein |
| g | - gram |
| GFP | - green fluorescent protein |
| h | - hour |
| kDa | - kilodalton |

| | |
|---|---|
| kV | - kilovolts |
| L | - litre |
| *L.* | - *Listeria* |
| LAB | - lactic acid bacteria |
| LB | - Luria-Bertani |
| *Lb.* | - *Lactobacillus* |
| Man-PTS | - mannose phosphotransferase |
| MIC | - minimum inhibitory concentration |
| min | - minute |
| mM | - millimoles per litre |
| mRNA | - messenger ribonucleic acid |
| nm | - nanometre |
| OD | - optical density |
| PBS | - phosphate buffered saline |
| PCR | - polymerase chain reaction |
| qPCR | - quantitative polymerase chain reaction |
| RFP | - red fluorescent protein |
| RFU | - relative fluorescence units |
| RNase | - ribonuclease |
| rpm | - revolutions per minute |
| rRNA | - ribosomal ribonucleic acid |
| RT | - reverse transcription |
| TE | - tris ethylenediaminetetraacetic acid |
| UV | - ultraviolet |
| v | - volume |

| | |
|---|---|
| VRBA | - violet red bile agar |
| w | - weight |
| μF | - microfarad |
| Ω | - ohm |

# *INTRODUCTION*

## *1.1. Food safety*

There are an estimated 11 to 13 million cases of foodborne illnesses each year in Canada (Public Health Agency of Canada, 2009). As the international food supply relies more heavily on mass production and mass distribution, large recalls and outbreaks are becoming more widespread (Public Health Agency of Canada, 2009). In addition to the changing structure of our food supply, surveillance, detection and information sharing methods have greatly improved. An example of this is the Foodborne Diseases Active Surveillance Network (FoodNet) in the United States, a collaborative project joining the expertise and surveillance of many government departments (CDC, 2009) that was designed to gain more accurate estimates on the incidence of foodborne disease and to enhance food safety and better protect public health. The most recent surveillance data available for the United States indicates that there were 1034 food-related outbreaks in 2008, including 23,152 cases of foodborne illness and 22 deaths (CDC, 2011). Of these outbreaks, 36 were attributed to shiga toxin-producing *Escherichia coli*, and beef was identified as the food vehicle in 31 outbreaks.

## *1.2. Antimicrobial interventions on meat*

Growth of microorganisms on or in meat products can be controlled by curing, cooking, sterilization and refrigeration. Controlling the gaseous atmosphere inside packaging and correctly choosing and designing packaging

material will select for growth of certain organisms. For example, packages of fresh meat that contain little or no oxygen will select for gram-positive lactic acid bacteria rather than gram-negative spoilage bacteria such as *Pseudomonas* spp.. Growth of pathogens and spoilage organisms can be halted by reducing the water activity of the meat (by drying or salting) or by reducing the pH through the addition of acid-producing starter cultures or addition of lactic or acetic acids.

Antimicrobial intervention steps are commonly used in beef slaughter and meat processing facilities (FSIS, 2002). The United States Food Safety and Inspection Service recommends that larger processors use innovative interventions on carcasses to reduce *E. coli* O157:H7 and *Salmonella* contamination (FSIS, 2002). Antimicrobial interventions proven to be effective and recommended for use on beef carcasses include spray washing, steam vacuuming, steam pasteurization, trisodium phosphate sprays, chlorinated water washing, and acid washing (Cutter and Siragusa, 1994; Dorsa et al., 1997a,b; Nutsch et al., 1997; Phebus et al., 1997; Castillo et al., 1998; Dormedy et al., 2000; Bosilevac et al., 2006; Harris et al., 2006). Efficacy of antimicrobial treatments is often increased when two or more are used together as hurdles but high pressure spraying has the potential to embed pathogens into the outer layers of tissue, which could result in protection of pathogens from subsequent antimicrobial interventions (Dawson, 2003). Many of the interventions are performed at high temperatures, increasing the likelihood of strains acquiring heat resistance. Heat resistant strains of *E. coli* have been isolated from beef and beef processing facilities (Ahmed et al., 1995; Murphy et al., 2004). *E. coli* DM18.3

and AW1.7 were isolated from a beef processing facility (Aslam et al., 2004) and both are heat resistant with $D_{60}$-values of 15 and 71 min, respectively (Dlusskaya et al., 2011). Heat-sensitive *E. coli* GGG10 was isolated from a beef processing facility prior to the implementation of current intervention methods (Dlusskaya et al., 2011). It remains to be determined whether additional antimicrobial hurdles can increase the efficacy of steam and lactic acid treatments to destroy heat-resistant *E. coli* on beef.

### *1.3. 'Natural' preservatives*

In addition to the bacterial control methods mentioned above, there are a multitude of preservatives legally permitted for use in meat products in Canada (Department of Justice, Canada, 2011). One example is the use of nitrates and nitrites in ready-to-eat meats. Although effective, many of the approved preservatives may not be appealing to consumers because of perceived undesirable health effects associated with such products. Thus, there is a demand for more 'natural', preservative-free foods with 'clean labels', which presents challenges to processors who must find novel approved 'natural' additives. One class of compounds gaining considerable attention in the literature and popular media are phenolic compounds such as phenolic acids.

Phenolic acids are secondary plant metabolites found in fruits, vegetables, and other plant materials with antimicrobial activity against various bacteria (Tunçel and Nergiz, 1993; Olasupo et al., 2003; Merkl et al., 2010; Sánchez-Maldonado et al., 2011). Phenolics are a natural component of plants. Thus, they

are good candidates for use as natural food preservatives. Syringic acid and ferulic acid are phenolic acids with antimicrobial activity against *E. coli*; however, lactic acid bacteria are resistant (Sánchez-Maldonado et al., 2011).

### *1.4. Lactic acid bacteria*

#### *1.4.1. Introduction to the lactic acid bacteria*

Pathogen control and preservation of meat can also be achieved through the use of lactic acid bacteria (LAB) as competitive or protective cultures to inhibit growth of undesirable species. The LAB are a diverse group of gram-positive genera which convert sugars to lactic acid as the primary metabolite (Axelsson, 2004). LAB are catalase-negative, non-spore-forming, bacilli, cocci or coccobacilli with a G+C content of 55 mol % or less (Stiles and Holzapfel, 1997). LAB genera that have been isolated from food include *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Stiles and Holzapfel, 1997; Axelsson, 2004). Homofermentative LAB produce primarily lactic acid from glucose, whereas heterofermentative LAB convert hexoses to lactic acid, carbon dioxide, acetic acid, or ethanol (Schillinger and Holzapfel, 1995; Axelsson, 2004). Many LAB species are psychrotrophic, growing at 2 to 4°C, potentially making them useful as protective cultures applied to refrigerated meat.

### *1.4.2. Protective cultures*

Protective cultures prevent growth of other bacteria by outcompeting them for nutrients, through activity of their metabolites, or by producing bacteriocins (Bredholt et al., 1999; Nilsson et al., 1999; Alves et al., 2006). Protective cultures are present in fermented meats and as part of the natural microflora of fresh meat; in fact, many bacterial strains possessing protective properties were isolated from meat products, including species of *Lactobacillus* and *Carnobacterium*. Bacteria used as protective cultures in this study, *C. maltaromaticum* UAL307 and *Lb. sakei* FUA3058, were isolated from meat. Protective cultures may be added to raw, non-fermented, or heated foods as a weapon to destroy or prevent growth of pathogenic and spoilage organisms to ensure safety and a durable shelf life. In some cases, bacteriocins are responsible for antimicrobial activity of LAB, but in other cases, rapidly growing bacteriocin-negative strains of LAB effectively inhibit unwanted organisms in refrigerated foods.

### *1.4.3. Bacteriocin-negative protective cultures*

Bacteriocin-negative strains of *Lactobacillus sakei* inhibited *Listeria monocytogenes* in vacuum-packaged or modified atmosphere-packaged meat products (Bredholt et al., 1999; Nilsson et al., 1999; Bredholt et al., 2001; Alves et al., 2006; Vermeiren et al., 2006; Kaban et al., 2010). *Lb. sakei* grows at refrigeration temperatures and utilizes nutrients to produce lactic acid to inhibit growth of *L. monocytogenes.* The food system and desirable characteristics of the product must be considered when choosing an appropriate protective culture. A

protective culture must grow quickly and inhibit growth of undesirable organisms, but it must not fundamentally alter the sensory quality of the food (Holzapfel et al., 1995). Some strains of *Lb. sakei* produce hydrogen sulphide from cysteine in vacuum-packaged meat at refrigeration temperature, causing undesirable organoleptic changes (Hanna et al., 1983; Lee and Simard, 1984; Egan et al., 1989; Whitfeld, 1998). Heterofermentative LAB produce acids, alcohols, carbon dioxide, hydrogen peroxide and other compounds that may have a detrimental effect on the quality characteristics of foods, especially raw meat (Helander et al., 1997). Therefore, strains to be used as protective cultures must be chosen carefully. Some strains can develop resistance to the acids and other antibacterial metabolites produced by non-bacteriocinogenic cultures. It is more difficult for bacteria to develop resistance to bacteriocins (Helander et al., 1997; Cotter et al., 2005a, b; Gálvez et al., 2008), thus bacteriocinogenic strains might be a better choice for use as protective cultures.

### *1.4.4. Bacteriocinogenic protective cultures*

Bacteriocins are small antibacterial peptides produced by bacteria. Bacteriocins produced by LAB can be grouped into two primary classes, lantibiotics and nonlantibiotics (Deegan et al., 2006). Lantibiotics (class I bacteriocins) are small, heat stable, post-translationally modified bacteriocins with the unusual amino acids lanthionine or β-methyllanthionine (Cotter et al., 2005a,b; Deegan et al., 2006; Bierbaum and Sahl, 2009). Nisin is the most extensively studied class I bacteriocin, and it has been approved for use in foods

in many countries. Class II bacteriocins are small (<10 kDa), heat-stable peptides that are not post-translationally modified (Deegan et al., 2006). Class II bacteriocins can be further divided into class IIa and class IIb bacteriocins. Class IIa bacteriocins are cationic peptides that contain 37 – 58 amino acid residues and have strong listericidal activity (Drider et al., 2006; Nissen-Meyer et al., 2011). Class IIa bacteriocins are characterized by a hydrophobic N-terminus that contains a highly conserved consensus sequence (YGNGVXC), as well as a disulfide bond between cysteine residues on the N-terminal (Drider et al., 2006; Leisner et al., 2007; Martin-Visscher et al., 2008). At least four genes must be expressed for class IIa bacteriocins to be produced: a pre-bacteriocin structural gene which is cleaved prior to export, an immunity gene encoding an immunity protein to protect itself from its cognate bacteriocin, an ABC transporter (or ATP-binding cassette) gene for secretion of the mature bacteriocin, and an accessory protein with an unknown function (Drider et al., 2006). Class IIb bacteriocins are two-component bacteriocins that require both components for high antimicrobial activity, with the genes encoding both componentss present on the same operon (Nissen-Meyer et al., 2011). Class III bacteriocins are large (> 10 kDa) heat-labile bacteriocins. These bacteriocins are either bacteriolytic enzymes (bacteriolysins) that kill bacterial cells by cell lysis, or non-lytic antimicrobial proteins (Heng et al., 2007). Class IV bacteriocins are circular bacteriocins. These peptides are post-translationally modified to covalently bond the N- and C-terminal ends to form a circular backbone (Heng and Tagg, 2006; Heng et al., 2007; Maqueda et al., 2008). Circularization of the peptide results in increased resistance to proteolyic

degradation and high temperatures compared to linear bacteriocins (Maqueda et al., 2008). Class IV bacteriocins have a broad activity spectrum and are stable across wide pH and temperature ranges (Maqueda et al., 2008), making these bacteriocins good candidates for use in foods.

Bacteriocins produced by LAB have varying activity spectra; some are active against a wide range of gram-positive organisms and others are active only against very closely-related bacterial strains. The bacteriocin-producing strain is immune to its bacteriocin(s); this innate protection is a result of immunity proteins expressed by the producing strain. Bacteriocins target the cell membrane of sensitive cells by binding to proteinaceous receptor molecules or phospholipid head groups of the cytoplasmic membrane, and inserting themselves into the membrane. The resulting pore that forms results in depletion of the proton motive force, depletion of intracellular ATP, and loss of low molecular-weight solutes (Abee et al., 1995; Montville et al., 1995; Moll et al., 1999; Drider et al., 2006). The mannose phosphotransferase system (Man-PTS), specifically the IIC and IID subunits, has been demonstrated to be the target site for many class IIa bacteriocins (Kjos et al., 2009, 2011). Binding of the bacteriocin to the membrane may be affected by the composition and charge of the membrane, and the presence, availability, and structure of the receptor molecule (Drider et al., 2006).

### *1.4.5. Bacteriocins as biopreservatives on meat*

Bacteriocins have been applied to foods with varying degrees of success. Bacteriocins are positively charged amphiphilic molecules; thus, they bind to

negatively charged, hydrophobic components such as protein and fat (Goff et al., 1996; Murray and Richard, 1997; Aasen et al., 2003; Hartmann et al., 2011), leaving less free, bacteriocin in foods. This may be problematic depending on the composition of the food and the particular bacteriocin structure. If the food contains high levels of proteolytic enzymes and the bacteriocin is sensitive to proteolytic degradation, bacteriocins will rapidly be destroyed. However, studies have shown that if the bacteriocin adsorbs onto the surface of a protein-rich substrate such as meat, it can retain activity while bound as long as the bacteriocin is not degraded by proteolytic enzymes (Goff et al., 1996; Aasen et al., 2003). Bacteriocins produced by LAB destroy closely-related organisms on ready to eat or cooked meat (Bredholdt et al., 1999; Uhart et al., 2004; Alves et al., 2006; Hartmann et al., 2011), while the bacteriocins remain unbound to the denatured protein on the meat surface. Cooked chicken breast meat does not bind pediocin, a class IIa bacteriocin; however, if pediocin is applied to raw chicken breast prior to cooking, it remains bound to the surface, and also retains antilisterial activity following cooking (Goff et al., 1996). Structure of the product may also affect bacteriocin activity. In ground pork and homogenized raw chicken and smoked salmon, bacteriocins were degraded rapidly (Murray and Richard, 1997; Aasen et al., 2003). Grinding or homogenization destroys cellular compartmentalization, which would allow release and dispersion of proteolytic enzymes. Thus, enzymes that do not have access to bacteriocins on intact meat can readily destroy the peptides in non-intact meat.

Some strains of bacteria produce more than one bacteriocin (Quadri et al., 1994; Revol-Junelles et al., 1996; Papathanasopoulos et al., 1998; Gursky et al., 2006; Martin-Visscher et al., 2008; Sawa et al., 2010; Aguilar-Galvez et al., 2011) enabling them to destroy competing organisms with a lower chance of acquisition of resistance, especially if the bacteriocins are from different classes. *Carnobacterium maltaromaticum* UAL307 is a psychrotrophic strain of LAB originally isolated from fresh pork (Martin-Visscher et al., 2008). This strain produces two class IIa bacteriocins, carnobacteriocin BM1 and piscicolin 126, and a circular bacteriocin, carnocyclin A. *C. maltaromaticum* UAL307 shows exceptionally high anti-listerial activity and demonstrated activity against a wide variety of gram-positive organisms including other bacteriocin producers (Martin-Visscher et al., 2008). It is likely that the antimicrobial potency is primarily due to carnocyclin A due to the physical properties of this particular bacteriocin (Martin-Visscher et al., 2008). Purified carnocyclin A has a broad spectrum of activity against gram-positive organisms, retains activity over a wide pH range (2 – 12 at 4°C), and is relatively heat resistant; heating at 121°C for 15 min did not affect activity, but heating at 100°C for 60 min reduced activity 32-fold (Martin-Visscher et al., 2008). It is cationic, has a high content of hydrophobic amino acids, and is susceptible to some proteases such as trypsin, pepsin, protease VII, α-chymotrypsin and thermolysin, but not to papain, chymopapain, endoproteinase Glu-C, or endoproteinase Asp-N (Martin-Visscher et al., 2008); therefore, retained activity in food would depend on the food components and enzymes present.

### *1.4.6. Carnobacterium maltaromaticum* UAL307 as a biopreservative

It remains unknown which of the 3 bacteriocins produced by *C. maltaromaticum* UAL307 is responsible for antibacterial activity. However, comparison with other strains of *C. maltaromaticum* may provide insight into antimicrobial activity of bacteriocins produced by this strain. *C. maltaromaticum* UAL26 was isolated from vacuum-packaged ground beef (Burns, 1987). It produces two of three bacteriocins produced by *C. maltaromaticum* UAL307, piscicolin 126 and carnobacteriocin BM1, and it grows at refrigeration temperatures (Gursky et al., 2006). *C. maltaromaticum* UAL8C2 is a plasmidless mutant of *C. maltaromaticum* LV17B (Quadri et al., 1997). It lacks the genes required for expression of the two bacteriocins that the parent strain produces, as they are plasmid mediated, but it contains the genetic determinants for carnobacteriocin BM1 and the immunity protein as they are located on the chromosome. *C. maltaromaticum* UAL307 is not active against gram-negative bacteria (Martin-Visscher et al., 2008), but outer membrane disrupting agents have made gram-negative strains susceptible to its bacteriocins in a model system (Martin-Visscher et al., 2011). It is unknown if *C. maltaromaticum* UAL307 can produce bacteriocins and show antimicrobial activity on raw meat. It also remains to be seen if the efficacy of current intervention methods used by the beef industry can be improved by the application of *C. maltaromaticum* UAL307 or its bacteriocins, or if it can act synergistically with phenolic acids to kill *E. coli* on beef. By detecting and quantifying bacteriocin gene expression, bacteriocin activity can be measured *in situ*, but this has not yet been performed.

### *1.5. Destabilizing the outer membrane of gram-negative bacteria*

Physical processes have been used to sensitize gram-negative bacteria to bacteriocins. High pressure processing, pulsed electric fields, high temperatures, low temperatures and freezing and thawing have been used with varying efficacy (Kalchayanand et al., 1992; Hauben et al., 1996; Kalchayanand et al., 1998; Boziaris and Adams, 2000, 2001). High pressure and pulsed electric field processing are not appropriate for fresh meat. Freezing and thawing also have detrimental effects on the quality of fresh meat and the process is more time consuming than heat treatment. Heat treatments are commonly used in meat processing, so the addition of bacteriocins to the meat before or after heating is possible, but depending on the processing facility, sterile addition of a bacteriocinogenic culture after treatment may not be possible.

The complex outer membrane of gram-negative bacteria acts as a barrier to gram-positive bacteriocins (Vaara, 1992; Gänzle et al., 1999). However, reports indicate that when bacteriocins produced by gram-positive organisms are used with agents or processes that disrupt the gram-negative outer membranes, these organisms can be destroyed (Kalchayanand et al., 1992; Scannell et al., 1997; Gänzle et al., 1999; Branen and Davidson, 2004; Martin-Visscher et al., 2011). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that destabilizes the gram-negative outer membrane by sequestering $Mg^{2+}$ and $Ca^{2+}$ that bind the lipopolysaccharides to the cell wall (Vaara, 1992). It releases the lipopolysaccharide layer, allowing hydrophobic bacteriocins to access the cytoplasmic membrane (Vaara, 1992; Branen and Davidson, 2004). Organic acids

disrupt gram-negative outer membranes, particularly undissociated organic acids, which are 10 – 600 times more effective than in dissociated form (Helander et al., 1997). Undissociated species are more prevalent at lower pH and they are able to cross bacterial membranes and acidify the cytoplasm resulting in cell death (Scannell et al., 1997). Few studies have combined lactate and bacteriocins other than nisin to destroy *E. coli* on meat. A novel way to assess the stability of gram-negative cell walls, the viability of acid-treated cells, and the internal pH of cells is to transform bacterial cells to express fluorescent proteins.

## *1.6. Fluorescent proteins*

### *1.6.1. Measuring cellular processes with fluorescent proteins*

Fluorescent proteins are useful tools for measuring cellular processes in living cells and for measuring responses of living cells to their immediate environment. Fluorescence can be measured non-invasively by fluorescence colony counting, fluorescence microscopy, flow cytometry or fluorescence spectroscopy. Cells expressing fluorescent proteins have been widely used to track cellular changes in yeast, bacteria, plants, fungi, insects, nematodes and mammals (Chalfie et al., 1994; Baird et al., 2000).

### *1.6.2. Characteristics of green fluorescent protein*

Wild-type green fluorescent protein (GFP) from the bioluminescent jelly fish *Aequorea victoria* is a 27-kDa protein that emits green light when excited by ultraviolet light (Vialette et al., 2004). GFP is chemically inert, non-toxic and may

be targeted to specific intracellular locations without leakage or migration (Kneen et al., 1998). Fluorescence is emitted from a chromophore in the centre of a distorted α-helix running through the middle of a β-barrel in the folded structure of GFP (Ormo et al., 1996; Yang et al., 1996). No cofactors or substrates other than molecular $O_2$ are required for fluorescence, and the reaction has only three steps: initial protein folding, cyclization and dehydration of the three adjacent amino acids (Ser 65-Tyr 66-Gly 67), and oxidation of the cyclised chromophore (Prasher et al., 1992; Cody et al., 1993; Reid and Flynn, 1997) to produce visible fluorescence. Problems associated with wild-type GFP include low brightness, low photostability and long time to obtain complete maturation of the protein. Improvements have been made to wild-type GFP through mutagenesis.

### *1.6.3. Green fluorescent protein as an intracellular pH probe*

Mutations of wild-type GFP have resulted in variants with qualities more desirable for use to study living cells such as enhanced brightness, greater photostability and lower toxicity (Jakobs et al., 2000; Shaner et al., 2005). GFP fluorescence intensity of GFP is affected by pH, with lower intensity at lower intracellular pH (Kneen et al., 1998; Ehrmann et al., 2001; Olsen et al., 2002; Kilimann et al., 2005). The pKa of GFP can be altered by mutagenesis. Wild-type GFP has a pKa of ~4.5 (Tsien, 1998; Ehrmann et al., 2001), mutants have pKa values ranging from 5.05 – 5.98 (Patterson et al., 1997; Kneen et al., 1998), and enhanced GFP (eGFP) has a pKa of 5.5 -6 (Patterson et al., 1997), similar to the pH of fresh meat. Enhanced GFP is more useful for measuring intracellular pH

than wild-type GFP because it is ~6 times brighter, does not display temperature dependent fluorescence, and the mature chromophore is oxidized at a much faster rate, allowing for more rapid fluorescence expression (Patterson et al., 1997; Ehrmann et al., 2001). Thus, eGFP appears to be a good candidate for use in studies to determine the intracellular pH of cells exposed to acidic environments, and its ease of detection makes it well-suited for use in food. Cabello et al. (2005) used GFP-labelled *Vibrio parahaemolyticus* to trace the organism in oysters, while GFP-labeled *Salmonella* has been used to measure the growth, inactivation and localization on jalapeño peppers, parsley plants, and alfalfa sprouts (Liao et al., 2010; Lapidot and Yaron, 2009; Gandhi et al., 2001). GFP-transformed *E. coli* has been used on meat to determine survival and growth (Ajjarapu and Shelef, 1999; Bloemberg et al., 1997; Cabrera Diaz, 2007; Pierce, 2009), but has not yet been tested as an intracellular pH probe on acid treated meat. Red fluorescent protein (DsRed-Express) is similar to GFP, but has not been studied as extensively on food.

### *1.6.4. Characteristics of red fluorescent protein*

Wild-type red fluorescent protein (DsRed) from the Indo Pacific coral *Discosoma* sp. is a 28-kDa protein that emits red light when excited by ultraviolet light (Baird et al., 2000). The protein contains an internalized chromophore surrounded by an α-helix enclosed in an eleven-stranded β-barrel (Wall et al., 2000). DsRed has topology similar to wild-type GFP (Wall et al., 2000), and no cofactors or substrates other than molecular $O_2$ are required for fluorescence.

Similar to GFP, visible fluorescence is obtained by folding of the protein followed by autocatalytic cyclization of three adjacent amino acids (Gln 66-Tyr 67-Gly 68) (Wall et al., 2000) and oxidation of the cyclised chromophore (Gross et al., 2000).

### *1.6.5. Red fluorescent protein as an intracellular pH probe*

The pKa values of red fluorescent proteins range from 3.8 for TagRFP (Evrogen, 2011) to 5.0 (Shaner et al., 2005). These pKa values are lower than the pH of meat (~5.4 - 5.5), which may make RFP useful for determining the intracellular pH of acid-treated bacteria on meat. From pH 5.0 – 9.0, DsRed exhibits stable fluorescence (Gross et al., 2000; Heikal et al., 2000; Vrzheshch et al., 2000). At lower pH values, findings in the literature are inconclusive. Two groups found that fluorescence intensity of DsRed is reduced at pH ~4.0 (Baird et al., 2000; Vrzheshch et al., 2000), but another group found fluorescence to be unaffected by pH values as low as 3.9 (Heikal et al., 2000). DsRed-Express is a mutant of DsRed with faster maturation, lower toxicity and higher solubility than the wild type (Bevis and Glick, 2002). The plasmid encoding DsRed-Express, pDsRed-Ex, has been successfully transformed into yeast, bacteria and mammalian cells (Baird et al., 2000). It is unknown if cells transformed to express DsRed-Express would be effective as intracellular pH probes for use on acid treated meat.

### *1.7. RT-qPCR to quantify bacteriocin gene expression*

Examination of gene expression allows detection and differentiation of the production of multiple bacteriocins. Gene expression is measured using reverse transcriptase (RT) PCR. To quantify gene expression, reverse transcriptase quantitative real-time PCR (RT-qPCR) is necessary. Quantification can be absolute or relative. Absolute quantification compares the cycle threshold ($C_T$) values obtained from unknown samples to those on a standard curve generated using known amounts of the target gene. The cycle threshold value corresponds to the number of amplification cycles run before fluorescence intensity of the amplified product is significantly higher than the background fluorescence (Fleige and Pfaffl, 2006). Relative quantification quantifies the target gene in relation to an endogenous reference control (Pfaffl, 2006). It is easier to use relative quantification to examine gene expression in food as quantification of target RNA relative to a reference gene accounts for losses of RNA during handling.

Four studies have been performed recently to examine LAB gene expression in cheese. Taïbi et al. (2011) used RT-qPCR to quantify gene expression of four strains of *Lactococcus lactis* at different steps of cheddar cheese manufacture. Falentin et al. (2010) used RT-qPCR to assess the metabolic activity and stress level of *Propionibacterium freudenreichii* and *Lactobacillus casei* cultures at twelve stages during manufacture and ripening of Emmental cheese. Ribosomal, stress response, and house-keeping genes were quantified using the absolute quantification method. In 2008, Trmčić et al. used qPCR to detect LAB bacteriocin genes in raw milk cheese and in consortia of strains

isolated from cheese; however, because DNA rather than RNA was isolated, and secondary experiments to determine activity of bacteriocins against sensitive indicator organisms were not performed, they could not conclude whether the bacteriocins were active. This same group used RT-qPCR to quantify expression of nisin genes produced by *Lactococcus lactis* M78 in cheese-like medium (Trmčić et al., 2011). The relative quantification method was used to quantify eleven genes, and 16S and 23S rRNA were used as reference genes with no difference in expression between them, deeming either appropriate for use as the reference gene. RT-qPCR was used to quantify adherence-related genes produced by *E. coli* O157:H7 at 37°C and 48°C in ground beef with two reference genes: 16S rRNA and glyceraldehyde-3-phosphate dehydrogenase (Slanec and Schmidt, 2011). Hüfner and coworkers (2008) used RT-qPCR to analyse the transcriptional response of *Lactobacillus reuteri* during sourdough fermentation. The group measured six genes and found significant changes in gene expression when the strain grew in sourdough compared to chemically defined medium. Beltramo et al. (2006) used RT-qPCR to quantify the gene transcript level of *Oenococcus oeni* in wine-like medium to analyze its stress response. Torriani et al. (2008) used RT-qPCR to quantify tyrosine decarboxylase (*tdc*) gene expression in pure cultures and meat and meat products. They examined the influence of pH, temperature and salt concentration on gene expression. Tyrosine decarboxylase was detected at very low levels in some raw meat samples and at higher levels in fermented pork. In a model meat system, the expression of *tdc* was higher during exponential growth than during stationary phase and expression was higher in the presence of

glucose or salt. Rantsiou et al. (2008) used RT-qPCR to quantify *Listeria monocytogenes* in meat, milk, soft cheese, fermented sausage, cured ham and ready-to-eat salad. In all of the studies mentioned above, RNA was successfully isolated from the foods, but many of the authors indicated that the isolation method had to be optimized and that the food matrix made isolation more difficult than from a pure culture. Bacteriocin gene expression by *C. maltaromaticum* UAL307 has not yet been explored and it is unknown which bacteriocin(s) are produced on meat at refrigeration temperatures.

### *1.8. Hypothesis and research objectives*

This research aimed to test the hypothesis that pathogens could be controlled *in vitro* and *in situ* on raw beef using novel biopreservatives and natural antimicrobial agents. To achieve this overall aim, this research evaluated the role of bacteriocin production by LAB on inhibition of gram-positive and gram-negative pathogens on meat. Bacteriocin-negative and –positive strains of lactic acid bacteria were examined for use as protective cultures on fresh beef. *Lb. sakei* FUA3058 was tested against a 5-strain cocktail of *L. monocytogenes* on vacuum-packaged beef stored at abusive refrigeration temperature to determine if growth of the pathogen could be inhibited. *Lb. sakei* FUA3058 and *C. maltaromaticum* UAL307 were tested as antimicrobial hurdles with steam and lactic acid to inhibit growth of heat-resistant *E. coli* on vacuum-packaged beef stored at refrigeration temperature. Phenolic acids were tested as potential outer membrane disrupting agents on heat-resistant *E. coli* with and without bacteriocins and their minimum

inhibitory concentrations were determined. Bacteriocin gene expression in *C. maltaromaticum* was quantified in liquid growth medium and on meat to determine how many of the three bacteriocins produced by *C. maltaromaticum* UAL307 are expressed and in what proportion at 4 and 10°C in both systems. Fluorescent proteins were tested as intracellular pH probes in model systems.

## *2. MATERIALS AND METHODS*

### *2.1. Culture maintenance and propagation*

Bacterial strains used in this study (Table 1) were obtained from the Food Microbiology laboratory culture collection at the University of Alberta. All materials and solutions were sterilized prior to use either by autoclaving (121°C for 30 min) or filter sterilization (Fisherbrand, 13 mm, 0.22 μm; Fisher Scientific Canada, Ottawa, ON). Strains of *Carnobacterium maltaromaticum*, *Carnobacterium divergens* and *Lactobacillus sakei* grown to stationary phase were stored at -80°C in APT broth (All-Purpose Tween; Difco, Becton Dickinson; Sparks, MD) containing 27% (v/v) glycerol. In preparation for each experiment, frozen stock cultures were streaked onto APT medium containing 1.5% (w/v) agar and incubated aerobically at 22°C for 48 h. Single colonies were transferred to APT broth and incubated at 22°C for 48 h, followed by a 1% (v/v) subculture in APT broth and incubation at 22°C for 24 h (stationary culture). For strains of *L. monocytogenes*, the same procedures were followed as for *C. maltaromaticum* except all incubation steps were at 30°C for 16-18 h. For *E. coli* AW1.7, DM18.3 and GGG10, similar procedures were followed as for *C. maltaromaticum* with the following changes: all incubation steps were at 37°C for 16-18 h in LB broth (Luria-Bertani broth, Miller; Difco, Becton Dickinson) and broth cultures were agitated at 200 rpm in a shaking incubator. For *E. coli* strains expressing fluorescent proteins, LB broth or LB medium containing 1.5% (w/v) agar was supplemented with ampicillin to a final concentration of 100 ppm after the medium had cooled to 50°C (LB-amp100). The ampicillin stock solution (50 g

$L^{-1}$) was prepared using distilled water and was filter sterilized (0.22 μm) and stored at -20°C. LB medium with ampicillin was stored at 4°C for a maximum of 14 d prior to use. To attain maximum aeration of broth cultures during propagation of *E. coli* FUA1039 and DM18.3 eGFP, cultures were shaken at 200 rpm in sterile Erlenmeyer flasks with foam stoppers at 37°C.

Table 1: Organisms used and their relevant characteristics.

| Bacterial Strain | Characteristics | Source or reference |
|---|---|---|
| *Carnobacterium maltaromaticum* UAL307 | Meat isolate<br>Produces carnocyclin A, piscicolin 126 and carnocyclin BM1 | (Martin-Visscher, 2008) |
| *Carnobacterium maltaromaticum* UAL26 | Meat isolate<br>Produces piscicolin 126 and carnobacteriocin BM1 | (Burns, 1987; Gursky et al., 2006) |
| *Carnobacterium maltaromaticum* UAL8C2 | Derivative of *C. maltaromaticum* LV17<br>Non-bacteriocinogenic<br>Plasmids removed | (Ahn and Stiles, 1990) |
| *Carnobacterium divergens* LV13 | Meat isolate<br>Carnobacteriocin-sensitive indicator | (Shaw and Harding, 1984) |
| *Lactobacillus sakei* FUA3058 | Meat isolate | (Vyas and Gänzle, unpublished) |
| *Listeria monocytogenes* FS13 | Meat isolate | (Bohaychuk et al., 2006) |
| *Listeria monocytogenes* FS14 | Meat isolate | (Bohaychuk et al., 2006) |
| *Listeria monocytogenes* FS19 | Meat isolate | (Bohaychuk et al., 2006) |
| *Listeria monocytogenes* FS30 | Meat isolate | (Bohaychuk et al., 2006) |
| *Listeria monocytogenes* ATCC 7644 | Outbreak strain | (Webb and Barber, 1937) |
| *Escherichia coli* AW1.7 | Heat resistant ($D_{60}$ = 71 min)<br>Meat plant isolate | (Aslam et al., 2004)<br>(Dlusskaya et al., 2011) |
| *Escherichia coli* DM18.3 | Heat resistant ($D_{60}$ = 15 min)<br>Meat plant isolate | (Aslam et al., 2004)<br>(Dlusskaya et al., 2011) |
| *Escherichia coli* FUA1039 | Derivative of *E. coli* AW1.7 (pDs-REx) | (Pierce, 2009) |
| *Escherichia coli* GGG10 | Heat sensitive strain isolated from a beef processing facility | Greer, G.G. AAFC Lacombe<br>(Dlusskaya et al., 2011) |
| *Escherichia coli* DM18.3 eGFP | Derivative of *E. coli* DM18.3<br>pEGFP: 3,4 kb, $amp^r$, pUC ori, lac | (Pierce, 2009)<br>(Ehrmann et al., 2001) |

## *2.2. Inhibition* of the *growth* of Listeria monocytogenes *by* Lactobacillus sakei *on meat*

### *2.2.1. Meat sample preparation (30 cm$^2$ surface area)*

Vacuum-packaged lean beef striploins were obtained from a federally inspected meat processing facility and stored at -18°C until use. The primal cut was thawed at 0°C for 18 h and cut against the direction of the muscle tissue into 3 mm thick slices with a slicer (Berkel, Russel Food Equipment, Edmonton, AB) that had been cleaned and sanitized (Matrixx™; Ecolab Food and Beverage, Mississauga, ON) prior to use. Meat slices were aseptically transferred to a sterile plastic cutting board and cut into 30 cm$^2$ samples using a sterilized knife with a sterilized piece of stainless steel as a template. Rectangular meat samples were placed 3 to 5 millimetres apart on sterilized aluminum foil in a single layer using sterilized forceps. Aluminum foil was folded to exclude air and packages were stored at -20°C for up to 2 weeks.

### *2.2.2. Inoculation and sampling*

Stationary cultures of *L. monocytogenes* and *Lb. sakei* were washed twice in 0.85% saline (w/v) (centrifugation at 7000 x *g*, 10 min) and suspended in 0.1% (w/v) Bacto™ Peptone (Difco, Becton Dickinson) buffered with 0.15 mM NaCl. Equal volumes of washed cells of *L. monocytogenes* strains FS13, FS14, FS19, FS30 and ATCC 7644 were mixed and diluted in 0.1 % buffered peptone to obtain a 5.1 log CFU mL$^{-1}$ cocktail. Meat samples were held at room temperature for 10 min prior to inoculation. Each bag contained one 30 cm$^2$ meat sample

inoculated with five strains of *L. monocytogenes* (FS13, FS 14, FS19, FS30 and ATCC 7644; 3.1 log CFU $cm^{-2}$ meat), *Lb. sakei* FUA3058 (3.1 log CFU $cm^{-2}$ meat) and/or saline as follows: (1) *Listeria* with 300 µL saline; (2) *Listeria* + *Lb. sakei* FUA3058; (3) *Lb. sakei* with 300 µL saline. Meat samples were aseptically placed into a vacuum-packaging bag (oxygen transmission rate of 52 $cm^3$ $m^{-2}$; Allied Pak; Toronto, ON) using sterile forceps and were inoculated as described above. Each inoculum was allowed to adsorb onto the surface of the meat for 15 min, and packages were sealed using a commercial vacuum-packaging machine (Model C200, Multivac Canada; Woodbridge, ON). All treatments were prepared in duplicate, and samples were incubated at 10°C for 1, 7, 14, 21 or 28 days.

For sampling, duplicate packages per treatment were opened aseptically using sterile scissors. Sterile Tris EDTA (TE) buffer (10 mM Tris, 1mM EDTA, pH 8.0) (25 mL) was added to each bag, and samples were homogenized for 2 min (Colworth 400 stomacher, Seward, and Co., London, EN). Homogenates were serially diluted in sterile 0.1% buffered peptone water and cell counts were determined by plating onto the surface of APT medium and PALCAM medium containing PALCAM selective supplement (Oxoid; Fisher Scientific Canada). Plates were incubated aerobically at 22°C for 72 h (APT) and 30°C for 24 h (PALCAM). Mean log CFU $cm^{-2}$ meat was calculated from duplicate samples per treatment for two replicate experiments.

## *2.3. Inhibition* of the *growth* of Escherichia coli *on vacuum-packaged beef*

### *2.3.1. Aseptic preparation of meat samples (4.9 $cm^2$ surface area)*

Vacuum-packaged lean beef (outside round) was obtained from a federally inspected meat processing facility and stored at -18°C. The primal cut was thawed at 2°C for 24 h and sliced against the direction of the muscle tissue into steaks ~7 cm thick using a chef's knife. Steaks were tightly wrapped in a 0.9 MIL low density polyethylene bag (17.8 x 7.6 x 50.8 cm) (Pur™ Value; Unisource Canada Inc., Edmonton, AB) sealed with an elastic band and two layers of aluminum foil (Fisherbrand®, Fisher Scientific Canada) and stored at -18°C for up to 2 weeks.

Prior to coring, steaks were partially thawed at 4°C for 5 to 6 h. Steaks were immersed in ethanol and the ethanol was ignited to sterilize the surface. A sterilized circular metal corer (2.54 cm diameter) was hammered into steaks parallel to the direction of the muscle fibres. Cores were sliced into meat tokens 2 to 3 mm in depth with a surface area of 4.9 $cm^2$ using a sterilized fillet knife. Tokens were placed 3 to 5 millimetres apart on autoclaved aluminum foil in a single layer using sterilized forceps. Aluminum foil was folded to exclude air and packages were stored at -20°C for up to 2 weeks.

### *2.3.2. Inoculation, antimicrobial treatments, and sampling*

To investigate whether psychrotrophic lactic acid bacteria can act as an additional hurdle against heat-resistant *E. coli* on meat, lean beef was inoculated with *E. coli*, subjected to steam and lactic acid treatment, and inoculated with bacteriocin-producing or non-producing strains of lactic acid bacteria. Stationary

cultures of *E. coli* AW1.7, *E. coli* DM18.3, *Lb. sakei* FUA3058 and *C. maltaromaticum* UAL307 were washed twice in 0.85 % (w/v) saline (centrifugation at 7000 x *g*, 10 min) and resuspended in 0.1 % buffered peptone. Washed cells were serially diluted in 0.1 % buffered peptone, and viable counts were determined by spread plating on the surface of APT agar. Plates were incubated aerobically at 22°C for 72 h.

To determine the initial inoculum level of each bacterial strain, cultures were diluted and aliquots were spread onto LB or APT agar, for *E. coli* and lactic acid bacteria, respectively. A cocktail of *E. coli* was made by mixing equal volumes of washed cells of *E. coli* AW1.7 and DM18.3. Meat tokens (4.9 $cm^2$) were tempered at 22°C for 10 min, placed aseptically into separate sterile disposable petri dishes (60 x 15 mm; Fisher Scientific Canada), and inoculated with 100 µL of the *E. coli* cocktail. Control samples were inoculated with 100 µL saline. Following inoculation, meat samples were incubated for 15 min at room temperature in closed petri dishes, after which samples were subjected to steam treatment using a bench-top steaming apparatus (Pierce, 2009) (Figure 1). The steaming apparatus consisted of a round-bottom flask containing distilled water fitted with a custom-made glass curved nozzle covered with aluminum foil for insulation. The flask was supported by a stand with a metal ring placed directly above a Bunsen burner to heat the flask. Inoculated meat samples in petri dishes were placed 2 cm below the outlet of the steam nozzle, and samples were exposed to saturated steam for 15 sec. The surface temperature of the samples was measured by threading a temperature probe into the top surface of the meat

sample, and measuring the temperature using a Barnant T/C type K thermocouple (model 600-0000; Barnant Company, Barrington, IL). The temperature of the steam at the nozzle ranged from 99.6°C to 100.2°C, while the surface temperature of the meat sample rose above 81°C in 2 sec, reaching 98 to 99°C after 15 sec of steam treatment. Immediately after steam treatment, samples were removed from the steam, and were sprayed with ~2.5 mL of 4.5 % (v/v) lactic acid using a plastic spray bottle. Samples were held in closed petri dishes at 22°C for 5 min before they were aseptically transferred to vacuum packaging bags (oxygen transmission rate of 52 $cm^3$ $m^{-2}$; Allied Pak; Toronto, ON) using sterile forceps. Each vacuum-packaging bag contained one 4.9 $cm^2$ meat sample. Meat tokens were inoculated with 100 µL of washed *Lb. sakei* FUA3058 or *C. maltaromaticum* UAL307 and held for 15 min at 22°C. The following controls were used in this experiment: (1) Steam and lactic acid treatment only: 100 µL saline, steam and lactic acid treatment, 100 µL saline; (2) 100 µL saline, no steam and lactic acid treatment, 100 µL saline; (3) 100 µL saline, no steam and lactic acid treatment, 100 µL *Lb. sakei*; (4) 100 µL saline, no steam and lactic acid treatment, 100 µL *C. maltaromaticum*. After the inoculum absorbed into the meat samples, packages were vacuum-packaged using a commercial vacuum-packaging machine (Model C200, Multivac Canada; Woodbridge, ON). Samples were incubated at 4°C for 4, 7, 14, 21 or 28 days. Initial counts were also determined 1 to 2 h after packaging.

For sampling, two packages per treatment were opened aseptically using sterile scissors. To facilitate bacterial enumeration, 0.1 % buffered peptone (90 mL) was added to each package before homogenizing in a stomacher for 2 min. Serial dilutions were prepared in 0.1% buffered peptone, and viable counts were determined in triplicate for each dilution using a spot-plating technique (20 µL) on one plate. For enumeration, Violet Red Bile Agar (VRBA; Difco), LB, and APT were used for uninjured *E. coli*, total *E. coli*, and lactic acid bacteria, respectively. LB and VRBA plates were incubated at 37°C for 16 to 20 h, while APT plates were incubated at 22°C for 48 to 72 h. Mean log CFU $cm^{-2}$ meat was calculated for duplicate experiments.

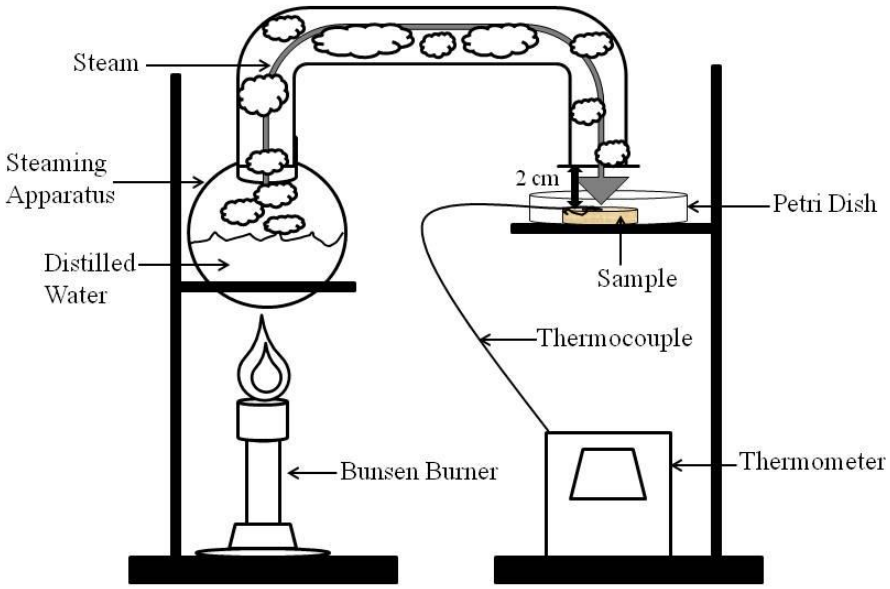


Figure 1: Bench top steam apparatus (Adapted from Pierce, 2009).

### *2.4. Bacteriocin gene expression in broth cultures and on meat*

#### *2.4.1. RNA stabilization from broth cultures*

To determine the relative expression ratio for bacteriocin genes of strains of *C. maltaromaticum* on meat at refrigeration temperatures compared to 16S rRNA genes, cultures were grown at 4°C and 10°C in liquid medium and on meat. Strains of *C. maltaromaticum* grown in APT broth at 4°C were used as reference conditions to quantify gene expression. *C. maltaromaticum* UAL26, UAL8C2 and UAL307 frozen stock cultures were streaked onto APT agar, and single colonies from each strain were inoculated into APT broth and incubated at 15°C for 48 h until late exponential phase was reached. Fresh broth (10 mL) was inoculated with 100 µL of a 48 h culture and incubated at either 4°C or 10°C until late exponential phase was reached ($OD_{600}$ = 0.6 to 0.7; 7 to 8 d at 4°C and 4 to 5 d at 10°C) (GeneQuant *pro* UV/Vis Spectrophotometer; Biochrom Ltd., Cambridge, UK). Optical density measurements were taken daily starting at 3 d and growth curves were constructed. A second subculture was made [1 % (v/v)] into fresh broth, and cultures were incubated at 4 or 10°C until an $OD_{600}$ of 0.53 to 0.63 was reached. Two aliquots of culture (500 µL) were mixed with 1 millilitre of RNAprotect Bacteria Reagent (Qiagen; Mississauga, ON), mixed on a vortex for 5 s, incubated at 22°C for 5 min, and centrifuged (20,800 x *g*, 5 min) to harvest cells. The supernatant was discarded and tubes were inverted onto a paper towel to remove residual liquid. Cell pellets were frozen at -80°C for up to 8 weeks until RNA isolation was performed.

### *2.4.2. Meat sample inoculation and sampling*

To prepare washed cells for inoculation onto meat, cells from 24 h (stationary) cultures of *C. maltaromaticum* UAL26, UAL307 or UAL8C2 were recovered by centrifugation at 7000 x *g* for 10 min, washed twice in saline, and serially diluted in 0.1 % buffered peptone. Viable counts were determined by plating on APT agar in triplicate.

Aseptically prepared meat tokens (4.9 $cm^2$) were tempered at room temperature for 10 min and were aseptically placed into a vacuum-packaging bag (oxygen transmission rate of 52 $cm^3$ $m^{-2}$; Allied Pak; Toronto, ON) using sterile forceps. Each bag contained three meat tokens, each inoculated with 3.2 log CFU $cm^{-2}$ of washed cells from one *C. maltaromaticum* strain (*C. maltaromaticum* UAL26, UAL307 or UAL8C2) (100 µL of a 4.9 log CFU $mL^{-1}$ culture per token). The inoculum was allowed to absorb into the surface of the meat for 15 min, and the package was vacuum-packaged using a commercial vacuum-packaging machine (Model C200, Multivac Canada). Samples were incubated at 4°C and 10°C for 1, 4, 7, 14, 21, 28 or 35 days.

To determine bacterial counts on the meat samples and to prepare cell extracts for RNA isolation, duplicate packages per treatment were aseptically opened using sterile scissors. Sterile TE buffer (25 mL) was added to the bag and the samples were homogenized for 60 s using a stomacher. From each bag, two aliquots of homogenate (500 µL) were mixed with 1 mL of RNAprotect Bacteria Reagent, 20 µL of homogenate was plated directly onto APT agar in triplicate, and one mL was serially diluted in 0.1 % buffered peptone and plated onto APT

agar. Plates were incubated at 22°C for 72 h and log CFU $cm^{-2}$ was calculated. Tubes containing homogenate and RNAprotect were mixed by vortexing for 5 sec, incubated at 22°C for 5 min, and centrifuged at 20,800 x *g* for 5 min to harvest the cells. The supernatant was discarded and tubes were inverted on a paper towel to remove residual liquid. Cell pellets were frozen at -80°C.

### *2.4.3. RNA isolation from broth cultures and meat sample homogenates*

A modified acidified phenol chloroform RNA isolation method (Torriani et al., 2008) did not provide DNA-free RNA, so RNA isolation was performed using the Qiagen RNeasy Mini kit (Qiagen) with a modified protocol for Gram-positive bacteria. Frozen cell pellets were thawed at 22°C for 5 min and centrifuged for 5 min to harvest cells. All centrifugation steps were done at 8000 x *g*. Residual supernatant was removed using a pipette. To facilitate cell wall disruption, cell pellets were re-suspended in TE buffer containing 1.5 % (w/v) lysozyme, mixed by vortexing for 10 s and incubated at 37°C for 30 min followed by a second vortexing step. Ribonucleases were destroyed by addition of 525 µL buffer RLT (25 to 50 % guanidine thiocyanate) containing 1% (v/v) 2-mercaptoethanol, followed by 10 s of mixing by vortex. To precipitate RNA, 375 µL of ethanol was added, samples were mixed with a pipette, and RNA was collected by centrifugation for 30 s on the column provided in the kit. Precipitated RNA was washed with 700 µL buffer RW1 that contained over 50% guanidinium chloride to denature any remaining RNases, washed twice with 500 µL of buffer

RPE [80% (v/v) ethanol] , with each washing step followed by a 2 min centrifugation. A final 2 min centrifugation was performed to ensure complete removal of ethanol from the column prior to eluting the RNA with nuclease-free water (40 µL). Concentration of total nucleic acids was determined using a spectrophotometer at 260 nm **(**NanoDrop™ Spectrophotometer; Thermo Fisher Scientific; Nepean, ON).

### *2.4.4. DNase treatment and reverse transcription*

Purified RNA was treated with RNase-free DNase I (Ambion, Applied Biosystems; Streetsville, ON) to remove contaminating DNA prior to reverse transcription. Briefly, 10 µL of RNA (≤ 40 ng $\mu L^{-1}$) was added to 1.25 µL 10X DNase I buffer and 1.25 µL DNase I in 0.2 mL thin wall PCR tubes, and incubated at 37°C for 40 min. DNase I was inactivated at 75°C for 10 min in a thermocycler after adding EDTA to a final concentration of 5 mM.

First-strand cDNA synthesis and reverse transcription were performed according to the manufacturer's directions for SuperScript™ III Reverse Transcriptase (Invitrogen Canada; Burlington, ON). For first-strand cDNA synthesis, each reaction contained 250 ng random primers (Promega Corporation; Madison, WI), 2 µL DNase treated RNA, and 1 µL 10 mM dNTP mix (Invitrogen) diluted to a final volume of 13 µL using sterile RNase-free water. The mixture was heated at 65°C for 7 min and cooled to 4°C in a thermocycler. Tube contents were collected by centrifugation for 5 s and reverse transcription reagents including RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) were

added as per manufacturer's directions. Copy DNA was synthesized using the following program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. Samples were stored at -20°C until qPCR was performed.

### *2.4.5. Quantitative PCR to quantify bacteriocin gene expression*

Primers used in this study are listed in Table 2. Primers for *pisT* (GenBank accession #: AY812745) and *cclA* (GenBank accession #: EU624394) were designed using Primer Quest software (www.idtdna.com/scitools/applications/primerquest/). The specificity of each primer was checked *in silico* using the NCBI nucleotide BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi). Secondary structure and dimer formation were analyzed using OligoAnalyzer 3.1 software (www.idtdna.com/analyzer/applications/oligoanalyzer/). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Bacteriocin gene expression was measured using Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) and the QuantiFast SYBR Green PCR Kit (Qiagen).

Each 25 µL reaction contained 12.5 µL SYBR Green Master Mix, 10.5 µL sterile, nuclease-free water, 1 µL primer mix (5 µM each, forward and reverse primers in sterile nuclease-free water), and 1 µL cDNA. Negative controls contained 1 µL sterile nuclease-free water in place of template. For each cDNA template, a corresponding sample of DNase-treated RNA diluted ten-fold was run as a negative control to ensure that there was no DNA present in the RNA samples. Chromosomal DNA isolated from *C. maltaromaticum* UAL26, UAL307 and UAL8C2 were used as controls. Products were amplified using primers

specific for *cbnBM1, pisT* and 16S rRNA (Table 2) with the following program: 95°C for 10 min, followed by 40 cycles of 94°C for 20 sec, 55°C for 15 sec and 72°C for 30 sec, followed by a melt curve analysis to confirm primer specificity. For *cclA*-specific primers the same program was used, but with an annealing temperature of 62°C rather than 55°C. Primer specificity was confirmed based on the absence of secondary amplification products in melt curves. Samples were collected from two independent experiments and two duplicate samples were analyzed on each sampling day.

PCR efficiencies were calculated in duplicate for each primer pair. Briefly, *C. maltaromaticum* UAL307 chromosomal DNA was diluted two-fold, 14 times, in nuclease-free water, and qPCR was run in duplicate on the 15-step dilution series. Reaction volumes, primer concentration and temperature programs for qPCR reactions were as previously described. Negative controls contained nuclease-free water in the place of chromosomal DNA. Primer efficiency (E) was calculated by averaging the difference in $C_T$ values between successive two-fold dilutions, and by taking the reciprocal of the average to the power of two.

Table 2: Primers used for RT-qPCR with RNA isolated from strains of *C. maltaromaticum*.

| Gene | Primer name | Primer sequence (5' – 3') | Amplicon (bp) | Reference |
|---|---|---|---|---|
| *cbnBM1* | BM1F1 | ATATGAATTCATGAAAAGCGTTAAAGA | 200 | Gursky et al., 2006 |
| *cbnBM1*[a] | BM1R1 | ATATTCTAGATTAATGTCCCATTCCTG | | Gursky et al., 2006 |
| *pisT* | pisT-F | AGCAAGGTTGCCTTAGTTGGTGTT | 146 | This study |
| *pisT*[a] | pisT-R | TCGGTCGCGTAATTGATGAAAAGGA | | This study |
| *cclA* | cclA-F | GCATATGGTATCGCACAAGGTACAGC | 125 | This study |
| *cclA*[a] | cclA-R | GCTGTGAAGACACCTGATAAACCG | | This study |
| 16S rRNA | RNA3 | TAGCGGTGAAATGCGT | 279 | Gursky et al., 2006 |
| 16S rRNA[a] | RNA4 | TCGAATTAAACCACATGCTC | | Gursky et al., 2006 |

### *2.4.6. Calculation to determine relative gene expression*

Results from two experiments (two biological replicates with duplicate samples for each replicate) were analyzed using the Comparative $C_T$ method (Pfaffl, 2001) to calculate the relative expression ratio of bacteriocin genes compared to the reference gene, 16S rRNA. Reference conditions were the same strains of *C. maltaromaticum* grown to an $OD_{600}$ of 0.53 to 0.63 in APT broth at 4°C.

The equation used to determine relative fold change in gene expression follows (Pfaffl, 2001):

$$\text{Relative expression ratio} = \frac{(E_{target})^{\Delta Ct \text{ target (control – sample)}}}{(E_{reference})^{\Delta Ct \text{ reference (control – sample)}}}$$

Where: $E_{target}$ = Calculated PCR efficiency of target gene transcript

$E_{reference}$ = Calculated PCR efficiency of reference gene transcript

$\Delta C_{T\ target}$ = ($C_T$ control - $C_T$ sample) for target gene transcript

$\Delta C_{T\ reference}$ = ($C_T$ control - $C_T$ sample) for reference gene transcript

Control = broth cultures at 4°C

Sample = broth cultures at 10°C, meat samples at 4°C or

meat samples at 10°C

### *2.5. Synergistic activity of bacteriocins and lactic acid or phenolic acids*

To determine whether phenolic acids can permeabilize the outer membrane of *E. coli*, minimum inhibitory concentrations (MIC) were determined using bacteriocin containing preparations [*C. maltaromaticum* UAL307 cell-free culture supernatant (CFS), Micocin X™ or Chrisin™] and phenolic acids. Lactate and EDTA were used as controls as they are known to disrupt the outer membrane of gram-negative bacteria.

### *2.5.1. Chemicals and bacteriocin-containing preparations*

A solution of EDTA (200 mM) was prepared by dissolving disodium EDTA dihydrate (Fisher Scientific) in distilled water and filter sterilizing the solution (0.22 μm).

Lactate buffers (600 mM, pH 3.0, 3.5 and 4.0; 800 mM and 400 mM, pH 3.5) were prepared using sodium DL-lactate syrup, 60% (w/w) (Sigma-Aldrich; St. Louis, MO) and DL-lactic acid, 85% (w/w) (Sigma Aldrich). Amounts of each component required for a given pH were calculated using the Henderson-Hasselbach equation and final pH was adjusted using 10 N NaOH prior to filter sterilization (0.45 μm). Syringic acid (Sigma-Aldrich) and ferulic acid (Sigma-Aldrich) were dissolved in 1:1 ethanol:water to a final concentration of 20 g $L^{-1}$ prior to filter sterilization (0.22 μm).

Chrisin™ (Chr. Hansen, DK), which contains 2.5 % (w/w) nisin, was dissolved in 0.02 N HCl to make a concentrated stock solution (250 mg $L^{-1}$ nisin) and stored at -20°C until use. For use in MIC experiments, nisin was diluted to 2.5 mg $L^{-1}$ and 25 mg $L^{-1}$ in LB broth or in syringic acid (20 g $L^{-1}$), ferulic acid (20 g $L^{-1}$) or lactate buffer (600 mM, pH 3.5).

Micocin X™ (Griffith Laboratories; Toronto, ON) is a commercially available spray dried cell-free culture supernatant produced by *C. maltaromaticum* UAL307. A concentrated solution [5 % (w/v)] was prepared by dissolving Micocin X™ in sterile LB or APT broth. Aliquots (10 mL) were aseptically transferred into 15 mL polypropylene centrifuge tubes, heated in a

circulating water bath at 80°C for 20 min, and were stored at -20°C. Micocin X™ solutions were turbid after heating.

### *2.5.2.* C. maltaromaticum UAL307 *Cell-free culture supernatant*

*C. maltaromaticum* UAL307 CFS was prepared using cultures grown at 15°C for 48 h in 20 mL APT broth. Two 20-mL cultures were transferred into one 50 mL centrifuge tube and centrifuged (5311 x *g*, 10 min at 10°C) to harvest the cells. Residual cells in the supernatant were inactivated by heating in a circulating water bath at 60°C for 40 min, and at 85°C for 10 min. Cell-free supernatant was stored at -80°C. The antibacterial activity of the *C. maltaromaticum* UAL307 CFS was determined by diluting the CFS in a two-fold series with APT broth, spotting 5 µL of each dilution onto APT agar, and overlayering with semi-solid APT agar [0.75 % (w/v) agar] seeded with 1 % (v/v) of a 24 h culture of the sensitive indicator organism *Carnobacterium divergens* LV13. Plates were dried for 30 min prior to aerobic incubation at 22°C, and were examined for zones of clearing after 24 h. Arbitrary activity units per mL (AU $mL^{-1}$) were calculated from the reciprocal of the final dilution showing inhibition (Ahn and Stiles, 1990).

### *2.5.3. MIC of Micocin X™ or nisin against Carnobacteria*

To determine the antimicrobial activity of Micocin X™ or nisin against *C. divergens* LV13, *C. maltaromaticum* UAL8C2, UAL26 and UAL307, MIC assays were performed in 96-well microtiter plates. Nisin stock solution was diluted in APT broth to a concentration of 25 mg $L^{-1}$ and was serially diluted two-fold in

APT broth. The concentration of nisin tested ranged from 0.0245 mg $L^{-1}$ to 12.5 mg $L^{-1}$, while concentrations of Micocin X™ ranged from 0.0049 % (w/v) to 2.5 % (w/v). For the assay, 100 µL of an overnight culture diluted to ~5 log CFU $mL^{-1}$ was added to wells in duplicate containing 100 µL of nisin or Micocin X™ in APT broth. Positive controls (100 µL diluted culture in 100 µL APT broth) and negative controls (nisin or Micocin X™ in APT broth) were included in duplicate on each microtiter plate for each bacterial strain tested. Plates were sealed with sterile adhesive film and incubated aerobically at 22°C for 24 h. Optical density was measured at 630 nm using a plate reader (Varioskan Ascent; Thermo Fisher Scientific). Optical density ≤ 0.1 indicated the inhibition of growth.

#### *2.5.4. MIC of C. maltaromaticum UAL307 CFS with phenolic acids to inhibit growth of* **E. coli**

To determine the antimicrobial activity of *C. maltaromaticum* UAL307 CFS and syringic acid or ferulic acid against *E. coli*, minimum inhibitory concentration (MIC) assays were performed using *E. coli* AW1.7, DM18.3 and GGG10. EDTA was used as a positive control with *C. maltaromaticum* UAL307 CFS. Assays were performed in 96-well microtiter plates using two-fold serial dilutions of CFS in APT broth. The range of concentrations of syringic and ferulic acid tested was 0.625 to 5 g $L^{-1}$ and the range of EDTA tested was 6.25 to 50 mM. Acids or EDTA were diluted 1:1 (v/v) across columns in the 96-well microtiter plates using sterile distilled water (50 µL acid or EDTA mixed with 50 µL water in each well; contents were mixed and 50 µL transferred to the next column for

dilution). Final concentrations of CFS ranged from 2.5 to 80 AU per well (undiluted full strength CFS activity was 1600 AU $mL^{-1}$), and APT medium was the negative control. Cell-free supernatant was diluted and added to wells of the microtiter plate after acids and EDTA had been diluted in the wells, with AU concentration decreasing down plate rows. For the assay, 100 µL of *E. coli* AW1.7, DM18.3 or GGG10 diluted to ~$10^6$ CFU $mL^{-1}$ was added to the appropriate well containing 50 µL CFS and 50 µL EDTA, syringic acid or ferulic acid, resulting in a final volume of 200 µL. Positive and negative controls were included on each plate for each strain of *E. coli* and antimicrobial agent. Plates were sealed with adhesive film and incubated with shaking (175 rpm) at 37°C for 24 h. The optical density was measured at 630 nm and values ≤ 0.1 indicated inhibition of growth.

To determine the antimicrobial activity of *C. maltaromaticum* UAL307 CFS and lactate buffers against *E. coli* AW1.7, DM18.3 and GGG10, MIC assays were performed as described above. Lactate buffers at pH 3.0, 3.5 and 4.0 were used at concentrations of 18.75 to 150 mM lactate, while CFS was used 0, 20, 40, and 80 AU. Positive and negative controls were included on each plate for each strain of *E. coli* and pH buffer. Plates were sealed with adhesive film and incubated aerobically with shaking (175 rpm) at 37°C for 24 h. The optical density was measured at 630 nm and values ≤ 0.1 indicated inhibition of growth.

### *2.5.5. MIC of Micocin X™ or nisin with phenolic acids against* E. coli

A cross-over dilution MIC assay was used to determine the antimicrobial activity of nisin and syringic acid (20 g $L^{-1}$) or ferulic acid (20 g $L^{-1}$) against *E. coli* AW1.7, DM18.3 and GGG10. Lactate buffer at pH 3.5 (600 mM) was used as a control. Separate 96-well microtiter plates were used for each acid with nisin diluted across columns and acids diluted down rows. Steps were followed in a set order as follows. Nisin stock solution (250 mg $L^{-1}$) was diluted to a working concentration of 2.5 mg $L^{-1}$ in LB broth, syringic acid, ferulic acid and lactate buffer, pH 3.5. Eleven 200-µL aliquots of each solution were dispensed into 1.5 mL tubes; ten were used to make a two-fold dilution series using nisin (2.5 mg $L^{-1}$) in LB broth. Lowest and highest concentrations in each dilution series were: 2.5 µg $L^{-1}$ and 1.25 mg $L^{-1}$ nisin. Luria Bertani broth (100 µL) was dispensed into the appropriate wells of a 96-well microtiter plate, and, using a multi-channel pipette, nisin in LB broth (100 µL) was diluted across columns to the same concentrations as in tubes. Aliquots (100 µL) of two-fold dilution series of nisin in syringic acid, ferulic acid or lactate buffer were dispensed into the appropriate well of the 96-well plate (row 1) and acids were serially diluted two-fold (100 µL) down the rows of the plate, stopping before the final row. For the assay, each well containing 100 µL nisin solution and acid was inoculated with 100 µL of a 20 h culture of *E. coli* AW1.7 diluted to ~5 log CFU $mL^{-1}$ in LB broth. Wells for negative controls contained LB broth (100 µL), nisin (2.5 mg $L^{-1}$ in LB broth, 50 µL) and acid at the highest concentration used (50 µL). Wells for positive controls contained LB broth (50 µL), nisin (2.5 mg $L^{-1}$ in LB broth, 50 µL) and *E .coli*

AW1.7 (20 h culture diluted to ~5 log CFU $mL^{-1}$ in LB broth, 100 μL) or LB (100 μL) and *E. coli* (20 h culture diluted to ~5 log CFU $mL^{-1}$ in LB broth, 100 μL). Concentration ranges of nisin, lactate, and ferulic and syringic acids tested were 1.2 μg $L^{-1}$ to 0.625 mg $L^{-1}$, 2.3 to 150 mM, and 0.08 g $L^{-1}$ to 5 g $L^{-1}$, respectively. Plates were sealed with adhesive film and incubated aerobically with shaking (175 rpm) at 37°C for 24 h. The optical density was measured at 630 nm and values ≤ 0.1 indicated growth inhibition.

To determine the antimicrobial activity of Micocin X™ against *E. coli* AW1.7 with syringic acid, or ferulic acid, cross-over dilution MIC assays were performed as described above for nisin. Positive and negative controls were included on each plate. Plates were sealed with adhesive film and incubated aerobically with shaking (175 rpm) at 37°C for 24 h. The optical density was measured at 630 nm and values ≤ 0.1 indicated growth inhibition.

### *2.6. Fluorescent proteins*

#### *2.6.1. Cloning fluorescent proteins into* **E. coli** *AW1.7 and DM18.3*

To determine whether bacteriocins penetrate the cell membrane after antimicrobial agents permeabilize outer membrane of *E. coli*, standard methods of electroporation (Sambrook and Russell, 2001) were used to transform electrocompetent *E. coli* AW1.7 and DM18.3 cells with plasmids encoding enhanced green fluorescent protein (eGFP) and DsRed-Express red fluorescent protein (pRFP) (Clontech; Mountain View, CA). Electrocompetent cells of *E. coli* AW1.7 and DM18.3 cells were prepared using a modified method of Cabreria-

Diaz (2007). All cultures were propagated with shaking (250 rpm) at 37°C. Briefly, 100 mL LB broth was inoculated with 1 mL of an 18 h culture of *E. coli* AW1.7 or DM18.3 and grown to an optical density (600 nm) of 0.6 – 0.7 (2 to 2.5 h). For the next steps, all materials (tubes, pipette tips, pipettes) were chilled at -20°C, and solutions were chilled in an ice water bath. Aliquots (40 mL) were cooled in an ice water bath for 10 min in chilled 50 mL centrifuge tubes and centrifuged (5000 x *g*, 10 min at 4°C) to harvest cells. Cell pellets were resuspended in 25 mL of 10 % glycerol (v/v) and centrifuged at 5000 x *g* for 10 min at 4°C three times. Washed cells were resuspended in 1.0 mL 10 % glycerol, and 100 µL aliquots were dispensed into sterile chilled 1.5 mL tubes using chilled pipette tips. Electrocompetent cells were flash-frozen by placing the 1.5 mL tubes in liquid nitrogen and were stored at -80°C until transformation.

Plasmids (pEGFP and pDs-Rex) were isolated from *E. coli* Top 10 eGFP and from *E. coli* FUA1039, respectively (Pierce, 2009), according to the manufacturer's instructions using the QIAprep Spin Miniprep Kit (Qiagen). Plasmids were stored at -20°C in 5 µL aliquots. Plasmids were cloned into *E. coli* AW1.7 and DM18.3 using a modified method of Pierce (2009). Plasmid DNA and electrocompetent cells were thawed on ice and electroporation was performed as follows: plasmid DNA (2 µL) and 100 µL electrocompetent cells were transferred into a chilled 0.2 cm gap cuvette (Gene Pulser, BIO-RAD Laboratories, Hercules, CA) and subjected to electroporation under the following conditions: resistance 200 Ω, capacitance 25 µF and voltage 2.5 kV. LB broth (1 mL) was added immediately after treatment, and transformed cultures were incubated at 37°C for

1 h without agitation. Each transformed sample was spread (100 mL and 20 μL in duplicate) on LB agar containing 100 ppm ampicillin (LB-amp100) and incubated at 37°C for 12 – 14 h. Colonies were screened for fluorescent protein expression (red or green colonies) and the colonies with the brightest fluorescence were streaked onto LB-amp100 solid agar and incubated at 37°C for 22 – 24 h. Liquid cultures were propagated by inoculating single colonies with the brightest fluorescence into LB-amp100 broth in Erlenmeyer flasks with foam stoppers, and incubating at 37°C and 200 rpm for 24 h. Cultures grown to stationary phase were stored at -80°C in LB-amp 100 containing 27 % (v/v) glycerol. Bacterial strains were designated as *E. coli* DM18.3 RFP, *E. coli* AW1.7 RFP, *E. coli* DM18.3 eGFP and *E. coli* AW1.7 eGFP.

### *2.6.2. Use of eGFP and RFP as pH probes: calibration curves*

To determine whether the transformed strains of *E. coli* could be used to assess intracellular pH of cells exposed to acid treatments, calibration curves were constructed using intact and permeabilized cells at pH values ranging from 4 to 7. Cultures were prepared using a brightly-fluorescing single colony of *E. coli* AW1.7 RFP, *E. coli* DM18.3 RFP, *E. coli* AW1.7 eGFP or *E. coli* DM18.3 eGFP grown on LB-amp100 agar and propagated in 50 mL of LB-amp100 broth in a 250 mL Erlenmeyer flask with a foam stopper (37°C, 24 h, 200 rpm). Stationary phase cultures were aseptically transferred to 50 mL centrifuge tubes, and cells were harvested by centrifugation (5000 x *g*, 4°C, 10 min), and washed in 50 mL phosphate buffered saline, pH 7.2 (NaCl, 137 mM; KCl, 2.7 mM; $Na_2HPO_4$, 10

mM; $KH_2PO_4$, monobasic, 2 mM; PBS). Cells were collected by centrifugation and resuspended in 10 mL of PBS to obtain 5X concentrated cells.

Citrate phosphate buffers (30 mM; pH 4, 4.5, 5, 5.5 and 7) were prepared for constructing calibration curves for RFP- and GFP-transformed *E. coli* cells. Two citrate phosphate buffers were prepared, one acidic and one basic, and were mixed to obtain the desired pH. One litre of the acidic buffer was prepared by mixing 5.76 g $C_6H_8O_7$ (Fisher Scientific) with 4.08 g $KH_2PO_4$ (Fisher Scientific) in distilled water and one litre of alkaline buffer was prepared by mixing 9.73 g $C_6H_5K_3O_7 \cdot H_2O$ (Sigma-Aldrich) with 4.25 g $Na_2HPO_4$ (EM Science; Gibbstown, NJ) in distilled water. Buffers were sterilized by autoclaving (121°C for 30 min).

To obtain 'permeabilized' cells of RFP- and eGFP-transformed *E. coli*, 200 µL of 5X concentrated cells was mixed with either 300 µL of ethanol to obtain a final concentration of 60 % ethanol (v/v) or 300 µL of 10 mg $L^{-1}$ polymyxin B in water in a 2 mL microcentrifuge tube. Samples were mixed by vortexing for 5 sec, and incubated at 37°C for 30 min. Cells were harvested by centrifugation (8600 x *g*, 5 min) and were washed in 500 µL 30 mM citrate phosphate buffer at pH 4, 5, or 7. After centrifugation (8600 x *g*, 5 min), cells were suspended in the same buffer (500 µL) and incubated at 22°C for 15 min. To obtain 'intact' cells, 200 µL of 5X concentrated cells was mixed with 300 µL phosphate buffered saline (pH 7.2) containing 10 mM glucose, mixed by vortexing for 5 sec, and incubated at 37°C for 30 min. Cells were harvested by centrifugation (8600 x *g*, 5 min) and washed in 500 µL 30 mM citrate phosphate buffer at pH 4, 5, or 7 containing 10 mM glucose. After centrifugation (8600 x *g*,

5 min), cells were suspended in 500 μL of the same buffer and incubated at 22°C for 15 min. 'Permeabilized' and 'intact' GFP-transformed cells at pH 4, 5.5, and 7 or RFP-transformed cells at pH 4, 4.5, or 7 (200 μL) were transferred to individual wells of a 96-well microtiter plate in duplicate and fluorescence was measured using a microtiter plate reader (Varioskan Flash; Thermo Fisher Scientific; Nepean, ON) with SkanIt® software, version 2.4.

For strains of *E. coli* transformed with eGFP, excitation spectra were obtained using an emission wavelength of 507 nm and the instrument was set to scan between 458 and 486 nm in steps of 4 nm. Emission spectra were obtained using an excitation wavelength of 488 nm and the instrument was set to scan between 507 and 535 nm in steps of 4 nm. Fluorometric measurements were taken with the excitation and emission wavelengths set to their experimentally determined optimum values of 488 and 507 nm, respectively.

For strains of *E. coli* transformed with RFP, excitation spectra were obtained using an emission wavelength set at 585 nm and the instrument was set to scan between 535 and 565 nm in steps of 5 nm. Emission spectra were obtained using an excitation wavelength set at 557 nm and the instrument was set to scan between 577 to 605 nm in steps of 4 nm. Fluorometric measurements were taken with the excitation and emission wavelengths set to their experimentally determined optimum values of 557 nm and 585 nm, respectively.

Files were exported into Microsoft Excel 2007 and values were converted into emission and excitation spectra. Due to large variations in fluorescence

intensity between samples, results originating from different single colonies were not combined.

## *3. RESULTS*

### *3.1. Activity of bacteriocin-negative* Lb. sakei *against* Listeria monocytogenes *on beef*

A challenge study was performed to determine whether bacteriocin-negative *Lb. sakei* FUA3058 could inhibit growth of *Listeria monocytogenes* on vacuum packaged beef. Viable counts determined on APT and PALCAM agars are shown in Figure 2. In packages that had not been inoculated with *Lb. sakei*, *L. monocytogenes* remained in exponential phase of growth until day 14, with counts increasing slowly to a maximum of 6.4 and 7.2 log CFU $cm^{-2}$ on PALCAM and APT, respectively, after 28 days of storage at 10°C. Conversely, when meat was inoculated with *Lb. sakei* alone, counts on APT reached the maximum population density by day 7 and remained stable through day 28. When meat was inoculated with both *L. monocytogenes* and *Lb. sakei*, *Listeria* counts on PALCAM dropped below the detection limit by day 14 and remained below detection through the duration of the study, while *Lb. sakei* grew rapidly to its maximum population density of ~7.5 log CFU $cm^{-2}$ by day 14.

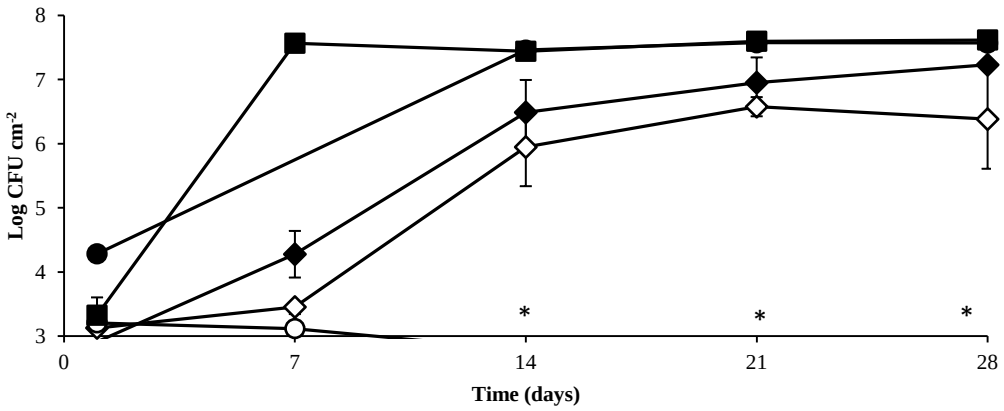


Figure 2: Mean log counts of *Lactobacillus sakei* FUA3058 and *Listeria monocytogenes* cocktail on APT medium (black symbols) and *L. monocytogenes* on PALCAM medium (white symbols) originating from inoculated vacuum packaged lean beef during 28 days of storage at 10°C. *L. monocytogenes* cocktail + saline inoculated meat (◆); *L. monocytogenes* cocktail + *Lb. sakei* FUA3058 inoculated on meat (●); *Lb. sakei* FUA3085 + saline inoculated meat (■). Experiment was replicated twice with duplicate samples within a replicate. Minimum y-axis value is the limit of detection (3 log CFU $cm^{-2}$); * = counts below limit of detection.

### *3.2. Activity of steam, lactic acid, and LAB against* E. coli *on beef*

To determine whether competitive LAB cultures act as hurdles along with intervention steps used by the beef industry, meat was inoculated with heat-resistant *E. coli*, treated with steam and lactic acid, and inoculated with either *Lb. sakei* FUA3058 or *C. maltaromaticum* UAL307 prior to incubation at 4°C. Steam and lactic acid treatment alone reduced the number of uninjured *E. coli* to the detection limit within 14 days (counts on VRBA; Figure 3A), but total *E. coli* (counts on LB) remained relatively constant for the duration of the experiment. When meat was inoculated with *Lb. sakei* after steam and lactic acid treatment, there was a 1.5 log CFU $cm^{-2}$ decrease in uninjured *E. coli* (Figure 3B), but total *E. coli* remained relatively constant and *Lb. sakei* counts dropped by approximately 1 log CFU $cm^{-2}$ by day 28. *C. maltaromaticum* appeared to be an

effective protective culture, with counts of uninjured *E. coli* and total *E. coli* below detection by 14 and 28 days, respectively (Figure 3C).

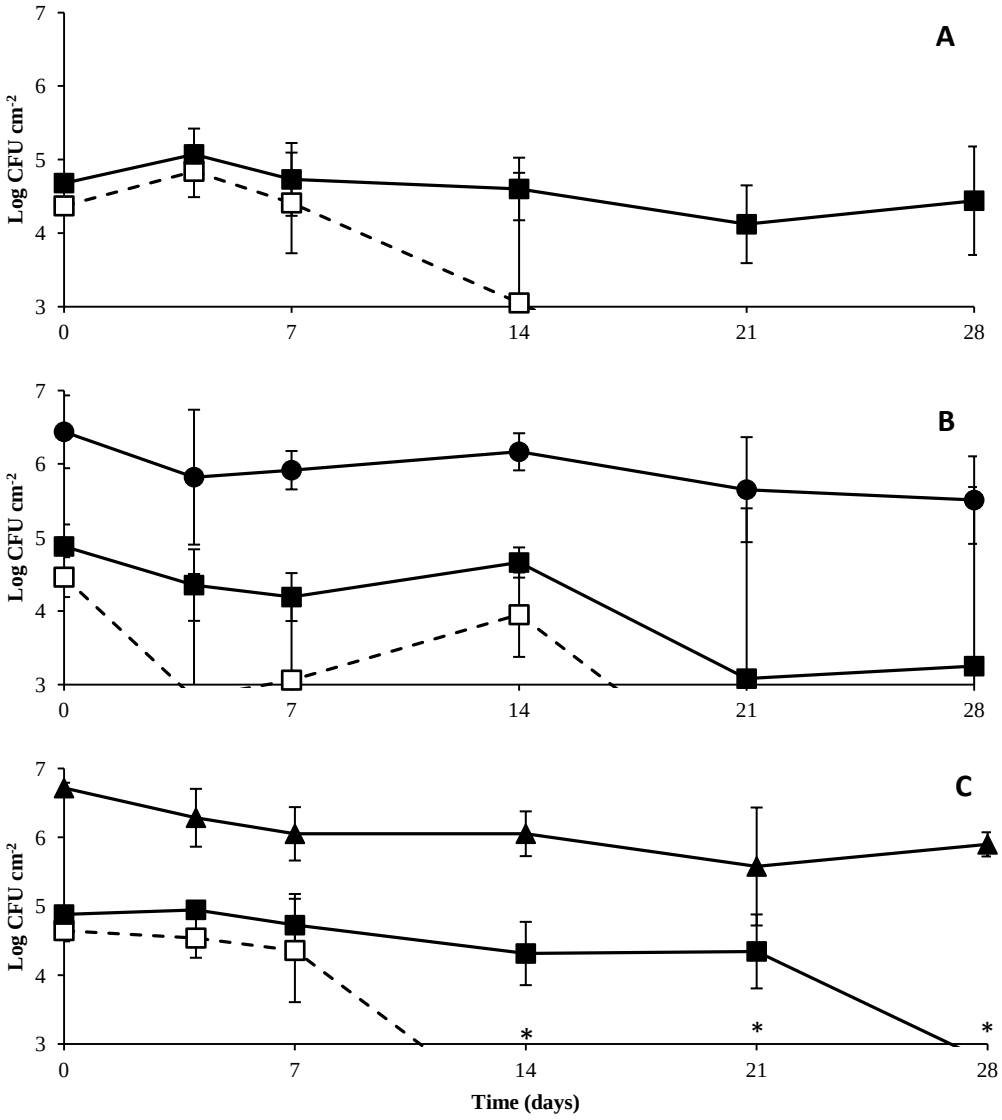


Figure 3: Mean log counts of a cocktail of *E. coli*, *Lb. sakei* FUA3058 and *C. maltaromaticum* UAL307 on vacuum-packaged beef incubated at 4°C for 28 days. Total *E. coli* were enumerated on LB agar (■), uninjured *E. coli* were enumerated on VRBA agar (□) and *Lb. sakei* (●) and *C. maltaromaticum* (▲) were enumerated on APT medium. Treatments: (A) *E. coli* cocktail subjected to steam and lactic acid treatments; (B) *E. coli* cocktail subjected to steam and lactic acid treatments and *Lb. sakei*; (C) *E. coli* cocktail subjected to steam and lactic acid treatments and *C. maltaromaticum.* Data points represent the mean of 3 replicates sampled in duplicate and error bars represent ± SD. Minimum y-axis value is the limit of detection (3 log CFU $cm^{-2}$); * = counts below limit of detection.

### *3.3.* C. maltaromaticum *growth and bacteriocin production in broth cultures*

*C. maltaromaticum* UAL307 produces three bacteriocins (Martin-Visscher et al., 2008), is strongly listeriocidal, and showed promise in inhibiting growth of *E. coli* on meat. To further evaluate bacteriocin production by *C. maltaromaticum* UAL307, a study was performed to quantify bacteriocin gene expression by this strain in liquid medium at 4°C and 10°C. Two other strains of *C. maltaromaticum* were used as controls: *C. maltaromaticum* UAL26, which produces two of the same bacteriocins as *C. maltaromaticum* UAL307 (piscicolin 126 and carnobacteriocin BM1), and *C. maltaromaticum* UAL8C2, a plasmidless non-bacteriocin producing strain used as a negative control. The results in broth cultures at 4°C were then used a reference conditions for quantification of bacteriocin gene expression by the same strains of *C. maltaromaticum* at 10°C and on meat.

To determine the approximate amount of time required for *C. maltaromaticum* UAL26, UAL307 and UAL8C2 to reach a given optical density at 4 and 10°C, optical density measurements were taken over time at 600 nm (Figure 4). All three strains grew more rapidly at 10°C than at 4°C. At 10°C, the desired optical density (0.53 – 0.63) was reached within 3 to 4 days, while 6 to 8 days of growth were required to reach the same optical density at 4°C.

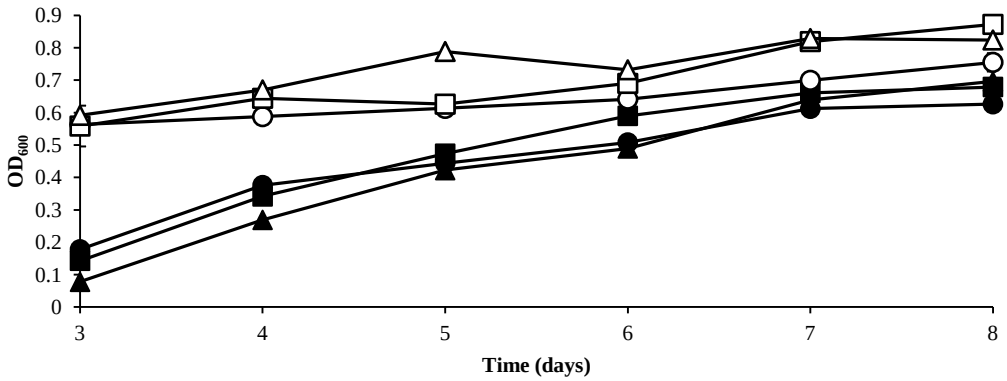


Figure 4: Optical density (600 nm) of cultures of *Carnobacterium maltaromaticum* UAL26 (❍), UAL307 (□) and UAL8C2 (△) growing in APT medium at 4°C (closed symbols) and 10°C (open symbols) for 8 days. Data points represent the mean of 2 replicates sampled in duplicate.

RT-qPCR was used with the comparative $C_T$ relative quantification method to determine whether incubation temperature (4°C or 10°C) affected bacteriocin gene expression in *C. maltaromaticum* UAL307.

To confirm specificity of primer pairs, melting curves were run after each RT-qPCR reaction. A single peak was observed at the expected melting temperature for all positive samples. Efficiency of each primer pair was determined with values of 1.84, 1.91, 2.0 and 1.84 for *cbnBM1*, *pisT*, *cclA* and 16S rRNA, respectively.

Results from two independent biological replicates show that *C. maltaromaticum* UAL307 expresses genes for all three bacteriocins at 4°C and 10°C and *C. maltaromaticum* UAL26 expresses genes for its two bacteriocins. The negative control, *C. maltaromaticum* UAL8C2, was negative for the three bacteriocin genes assayed, but 16S rRNA was amplified (data not shown). At both

incubation temperatures, *C. maltaromaticum* UAL307 expressed *cclA* at the same level with a ratio of 1.03, but *pisT* and *cbnBM1* were more highly expressed at 10°C, with ratios of 4.11 and 1.671, respectively. For *C. maltaromaticum* UAL26, both *pisT* and *cbnBM1* were more highly expressed at 10°C than at 4°C, with ratios of 1.54 and 1.891, respectively.

### *3.4.* C. maltaromaticum *growth and bacteriocin production on beef*

To examine the growth rate of and bacteriocin production by three strains of *C. maltaromaticum* on lean vacuum-packaged beef at 4°C and 10°C, ~3 log CFU $cm^{-2}$ of each strain was inoculated onto meat and viable counts were determined over 4 to 5 weeks of storage (Figure 5). At 4°C, there was a 4 day lag phase for all three strains, but at 10°C the lag phase was less than 24 h. At 4°C, *C. maltaromaticum* UAL26 and UAL307 reached 6.2 and 6.6 log CFU $cm^{-2}$, respectively, on day 14, and both remained at those levels until the end of storage. *C. maltaromaticum* UAL8C2 reached stationary phase by day 7 at 4°C, and stayed below 6 log CFU for the duration of storage. At 10°C, all strains reached the stationary phase after 7 days of storage and *C. maltaromaticum* UAL26 and UAL307 remained in stationary phase for the final 4 weeks. At day 14 of incubation at 10°C, *C. maltaromaticum* UAL8C2 dropped to 5.8 log CFU $cm^{-2}$, but reached 7.3 log CFU $cm^{-2}$ by the end of storage.

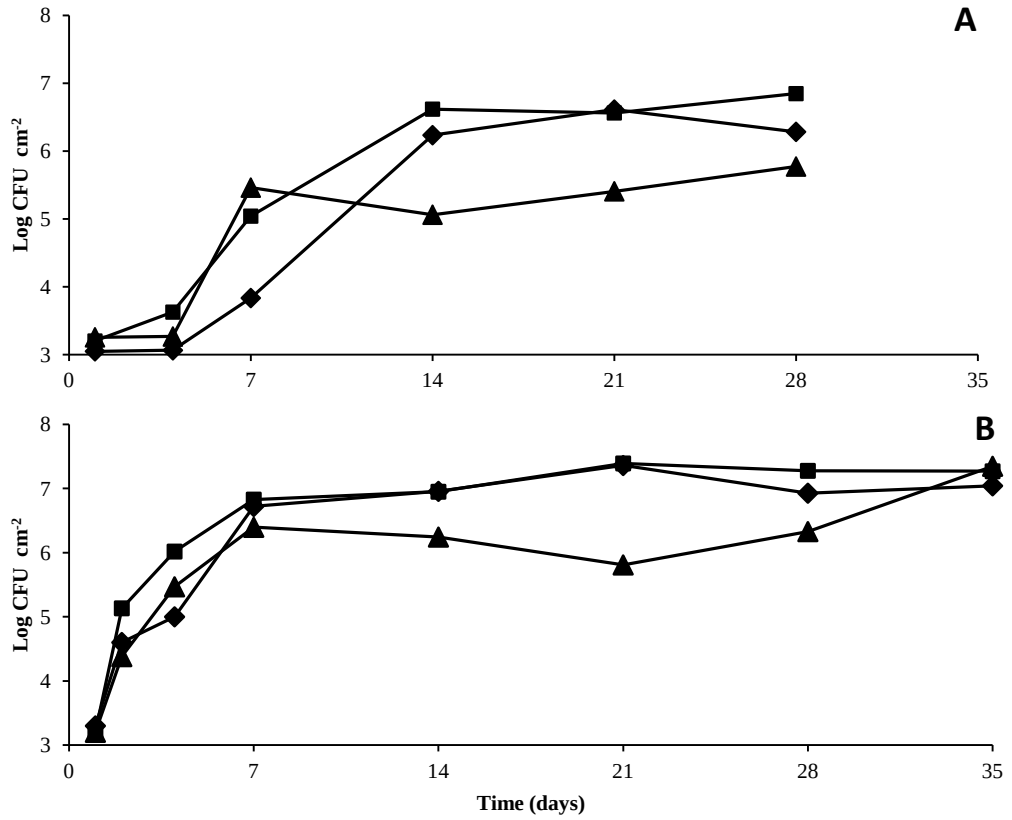


Figure 5: Mean log counts of *C. maltaromaticum* UAL26 (◆), UAL307 (■) and UAL8C2 (▲) enumerated on APT agarfrom inoculated vacuum-packaged lean beef during storage at (A) 4°C and (B) 10°C. Minimum y-axis value is the limit of detection (3 log CFU $cm^{-2}$) for n=2 replicates analyzed in duplicate.

Bacteriocin expression by *C. maltaromaticum* UAL307 on refrigerated raw vacuum packaged beef was analyzed using RT-qPCR. *C. maltaromaticum* UAL26 was used as a control for production of piscicolin 126 and carnobacteriocin BM1, while *C. maltaromaticum* UAL8C2 was used as a non-bacteriocin producing control. Chromosomal DNA from each strain acted as a positive or negative control depending on the bacteriocin gene targeted, and as a positive control for 16S rRNA primers.

Results from two independent replicates showed that *C. maltaromaticum* UAL307 was the only strain for which bacteriocin gene expression was detected on meat. The gene *cclA* was most consistently detected and most highly expressed on meat incubated at 4°C and 10°C (Tables 3 and 4) based on limit of detection and $C_T$ values (not shown). *PisT* was detected twice at 4°C and three times at 10°C, but expression was not consistent and $C_T$ values (not shown) were near the detection limit. *CbnBM1* was not detected in any of the meat samples, but it had previously been detected in liquid medium at 4°C and 10°C. Overall, detection of bacteriocin gene expression was lower on meat than in broth cultures.

Table 3: Relative expression ratios of bacteriocin genes *cclA*, *pisT* and *cbnBM1* obtained using the comparative $C_T$ method with 16S rRNA as the reference gene. RNA was isolated from vacuum-packaged meat samples previously inoculated with *C. maltaromaticum* strains UAL307, UAL26 and UAL8C2 stored for up to 35 days at 4°C. qPCR reactions were run on all samples using primers for 3 bacteriocin genes and the reference gene. *C. maltaromaticum* UAL8C2 was a negative control for all 3 bacteriocin genes and *C. maltaromaticum* UAL26 was a negative control for *cclA*. Reference conditions were broth cultures of the same strains grown to $OD_{600}$ of 0.53-0.63 at 4°C. Results are averages of duplicate samples for 2 replicate experiments.

| | *C. maltaromaticum* UAL307 | | | *C. maltaromaticum* UAL26 | |
|---|---|---|---|---|---|
| | Bacteriocin gene | | | Bacteriocin gene | |
| Time (d) | *cclA* | *pisT* | *cbnBM1* | *pisT* | *cbnBM1* |
| 1 | ND | ND | ND | ND | ND |
| 4 | ND | ND | ND | ND | ND |
| 7 | 0.0045 | 0.1356 | ND | ND | ND |
| 10 | ND | ND | ND | ND | ND |
| 14 | 0.0031 | ND | ND | ND | ND |
| 21 | 0.0096 | ND | ND | ND | ND |
| 28 | 0.0063 | ND | ND | ND | ND |
| 35 | 0.0221 | 0.1134 | ND | ND | ND |

ND = not detected

Table 4: Relative expression ratios of bacteriocin genes *cclA*, *pisT* and *cbnBM1* obtained using the comparative $C_T$ method with 16S rRNA as the reference gene. RNA was isolated from vacuum-packaged meat samples previously inoculated with *C. maltaromaticum* strains UAL307, UAL26 and UAL8C2 stored for up to 35 days at 10°C. qPCR reactions were run on all samples using primers for 3 bacteriocin genes and the reference gene. *C. maltaromaticum* UAL8C2 was a negative control for all 3 bacteriocin genes and *C. maltaromaticum* UAL26 was a negative control for *cclA*. Reference conditions were broth cultures of the same strains grown to $OD_{600}$ of 0.53-0.63 at 4°C. Results are averages of duplicate samples for 2 replicate experiments.

| | *C. maltaromaticum* UAL307 | | | *C. maltaromaticum* UAL26 | |
|---|---|---|---|---|---|
| | Bacteriocin gene | | | Bacteriocin gene | |
| Time (d) | *cclA* | *pisT* | *cbnBM1* | *pisT* | *cbnBM1* |
| 1 | ND | ND | ND | ND | ND |
| 4 | 0.0326 | 0.1311 | ND | ND | ND |
| 7 | 0.0076 | 0.1034 | ND | ND | ND |
| 10 | ND | ND | ND | ND | ND |
| 14 | 0.0185 | 0.1182 | ND | ND | ND |
| 21 | 0.0101 | ND | ND | ND | ND |
| 28 | 0.0169 | ND | ND | ND | ND |
| 35 | 0.04 | ND | ND | ND | ND |

ND = not detected

### *3.5. MIC of* C. maltaromaticum *UAL307 cell-free culture supernatant and outer membrane permeants to inhibit the growth of* E. coli

*C. maltaromaticum* UAL307 showed some synergism with steam and lactic acid against heat resistant strains of *E. coli* on meat. Thus, experiments were performed to determine the MIC of bacteriocin-containing *C. maltaromaticum* UAL307 cell-free culture supernatant (CFS) and outer membrane permeants required to inhibit growth of heat resistant strains of *E. coli*. EDTA and lactate were positive controls as they are known to disrupt the *E. coli* outer membrane.

Lactate was tested at different pH values to determine if pH affected bacteriocin activity, while phenolic acids (ferulic and syringic acid) were evaluated for use as outer membrane permeants.

*C. maltaromaticum* UAL307 CFS worked synergistically with EDTA, syringic acid, and lactate buffers at pH 3.0, 3.5 and 4.0 to inhibit growth of *E. coli* in liquid medium (Figure 6). In the presence of 80 AU CFS, the concentration of EDTA required to inhibit growth of *E. coli* AW1.7 and DM18.3 was reduced by two- and four-fold, respectively. At 80 AU CFS the concentration of lactate buffer (pH 3.5) required for inhibition was reduced two-fold compared to 0 AU. For syringic acid and lactate buffer (pH 3.0), 40 AU CFS reduced the amount of outer membrane permeant needed for inhibition two-fold; however, higher AU CFS (80 AU) demonstrated no further reduction of amount of required outer membrane permeant.

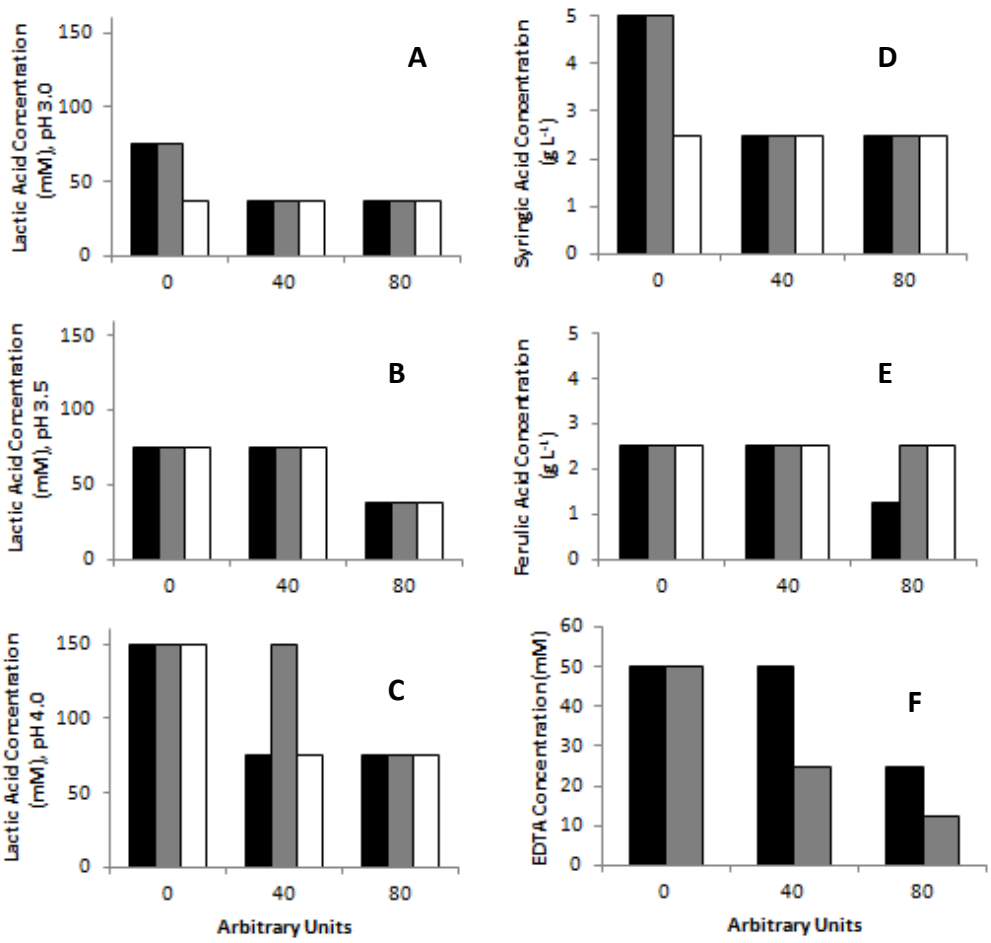


Figure 6: Minimum inhibitory concentrations of acids or EDTA required to inhibit growth of *E. coli* AW1.7 (black), DM18.3 (grey) and GGG10 (white) in the presence of *C. maltaromaticum* UAL307 cell-free culture supernatant at 0, 40 or 80 arbitrary units. (A) lactic acid, pH 3.0; (B) lactic acid, pH 3.5; (C) lactic acid, pH 4.0 (D) syringic acid; (E) ferulic acid; (F) EDTA. Optical density at 630 nm ≤ 0.1 indicated inhibition of growth. Bars represent the MIC for at least 4/6 individual trials.

### *3.6. MIC of Micocin X™ or nisin against Carnobacteria*

After exploring the antimicrobial activity of laboratory-prepared *C. maltaromaticum* UAL307 CFS against *E. coli*, efficacy of a commercially available spray-dried CFS preparation, Micocin X™, was evaluated. Another commercially available bacteriocin-containing preparation, Chrisin™ [2.5 % nisin (w/w)] was used as a positive control, as the antibacterial effects of nisin are well-

known. Initial experiments were designed to determine whether Micocin X™ was active against sensitive indicator organisms and not against the producer strain, *C. maltaromaticum* UAL307.

In APT broth, nisin inhibited the growth of all strains of *Carnobacterium* tested (Table 5). Micocin X™ inhibited sensitive strains (*C. maltaromaticum* UAL8C2 and *C. divergens* LV13) at low concentrations [0.313 % and 0.020 % (w/v), respectively], but did not retard growth of *C. maltaromaticum* UAL307 at the highest concentration tested [2.5 % (w/v)].

Table 5: Minimum inhibitory concentration of nisin or Micocin X™ required for inhibition of *Carnobacterium maltaromaticum* UAL8C2, UAL26, and UAL307 and *Carnobacterium divergens* LV13 in liquid medium. Optical density at 630 nm ≤ 0.1 indicated inhibition of growth.

| | | Micocin X™ |
|---|---|---|
| Bacterial strain | Nisin* (μg $L^{-1}$) | (g $L^{-1}$) |
| *C. maltaromaticum* UAL8C2 | 195 | 3.13 |
| *C. divergens* LV13 | 391 | 0.20 |
| *C. maltaromaticum* UAL26 | 98 | 12.5 |
| *C. maltaromaticum* UAL307 | 98 | > 25 |

* = Chrisin contains 2.5 % (w/w) nisin.

### *3.7. MIC of Micocin X™ or nisin with outer membrane permeants*

After confirming the inhibitory effect of Micocin X™ or nisin against sensitive indicator strains, the commercially available preparations were tested against heat-resistant strains of *E. coli* in liquid medium. Micocin X™ reduced the concentration of pH 3.5 lactate buffer required to inhibit *E. coli* AW1.7, but a

relatively high concentration [0.625 % (w/v)] was required. For all other assays, the outer membrane permeant alone retarded the growth of *E. coli*; nisin and Micocin X™ did not provide a synergistic effect. These results contrast with the results for *C. maltaromaticum* UAL307 CFS, although LB medium was used as a diluent in this study, whereas water was used previously. Lactate buffer at 75 mM inhibited the growth of *E. coli* AW1.7, DM18.3 and GGG10. Ferulic acid at 2.5 g $L^{-1}$ inhibited the growth of *E. coli* AW1.7 and DM18.3, while 1.25 g $L^{-1}$ was sufficient to prevent growth of GGG10. Syringic acid at 2.5 g $L^{-1}$ inhibited the growth of all strains of *E. coli.*

### *3.8. Fluorescent proteins as indicators of intracellular pH*

Strains of *E. coli* were transformed to express eGFP and RFP to examine whether pH-dependent fluorescence could be used to indicate the physiological state of *E. coli* when subjected to acid stress. Two heat-resistant strains of *E. coli* isolated from meat plants, *E. coli* AW1.7 and DM18.3, were transformed to express eGFP and RFP.

To determine whether transgenic strains of *E. coli* exhibited pH-dependent fluorescence and whether intact cells could be differentiated from permeabilized cells, excitation and emission spectra were run. Replicate cultures were tested and showed the same trends but data shown are for one culture as the relative fluorescence intensity varied between cultures. The same trends were observed for a given pH value within a treatment for both *E. coli* DM18.3 eGFP and *E. coli* AW1.7 eGFP or *E. coli* DM18.3 RFP and *E. coli* AW1.7 RFP. Strains expressing

eGFP were assessed first because other studies have demonstrated its usefulness for measuring changes in intracellular pH (Ehrmann et al., 2001; Killiman et al., 2005). Excitation and emission spectra for *E. coli* DM18.3 eGFP are shown in Figures 7 and 8, respectively. Fluorescence intensity within a treatment (intact, or lysed by polymyxin B or ethanol) was positively correlated to pH; the lower the pH, the lower the fluorescence intensity. At 486/507 nm (em/ex wavelengths), there was approximately a 200 unit difference in fluorescence intensity between intact cells and polymyxin B-permeabilized cells at all three pH values (Figure 7). At 488/511 nm (em/ex wavelengths), there was a difference of 208 to 247 units of fluorescence intensity between intact cells and polymyxin B-lysed cells at all three pH values tested (Figure 8). Cells lysed by ethanol showed pH-dependent fluorescence, but intensity was too low to be useful for practical applications.

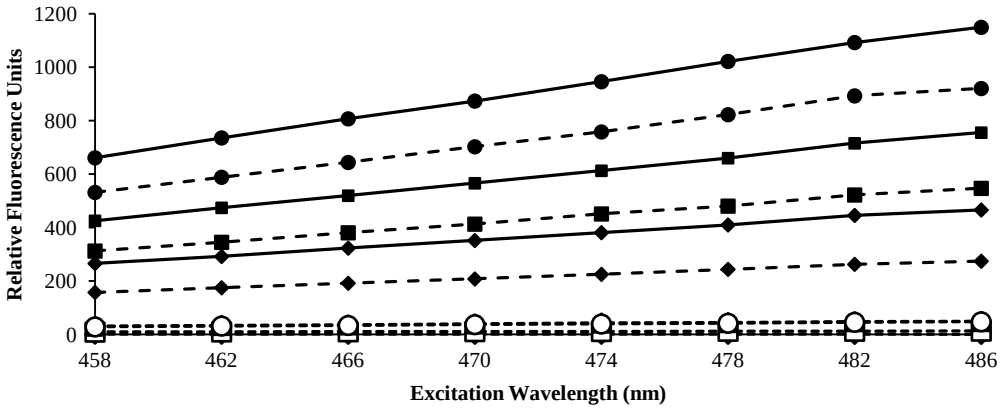


Figure 7: Excitation spectrum for *E. coli* DM18.3 eGFP cells intact (solid lines, closed symbols) or permeabilized using polymyxin B (10 mg $L^{-1}$; dotted lines, closed symbols) or 60% ethanol (v/v; dotted lines, open symbols) at pH 4 (◆), 5.5 (■), or 7 (●); emission wavelength fixed at 507 nm.

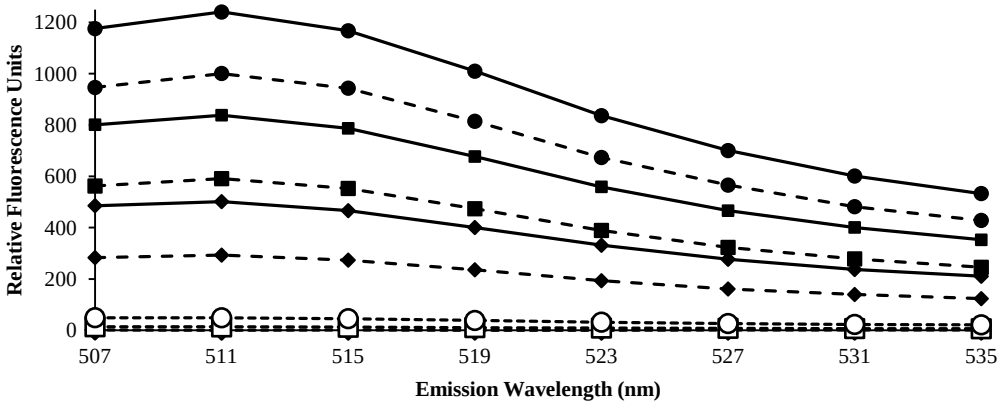


Figure 8: Emission spectrum for *E. coli* DM18.3 eGFP intact cells (solid lines, closed symbols) or cells permeabilized using polymyxin B (10 mg $L^{-1}$; dotted lines, closed symbols) or 60% ethanol (v/v; dotted lines, open symbols) at pH 4 (◆), 5.5 (■), or 7 (●); excitation wavelength fixed at 488 nm.

Emission and excitation and spectra for *E. coli* DM18.3 RFP are shown in Figures 9 and 10, respectively. Fluorescence intensity within a treatment (intact, polymyxin B-permeabilized or ethanol-permeabilized cells) was positively correlated to pH; the lower the pH, the lower the fluorescence intensity. At 557/585 nm (em/ex wavelengths), there was a 357 and 245 unit difference in fluorescence intensity between intact cells and ethanol-permeabilized cells at pH 4 and 4.5, respectively, but there was no difference at pH 7 (Figure 9). In contrast to the results for strains transformed with eGFP, there were no differences in fluorescence intensity between intact cells and polymyxin B-permeabilized cells at all pH values tested. At 555/585 nm (em/ex wavelengths), there was a difference of 1935 and 962 units of fluorescence intensity between intact cells and ethanol permeabilized cells at pH 4 and 4.5, respectively (Figure 10).

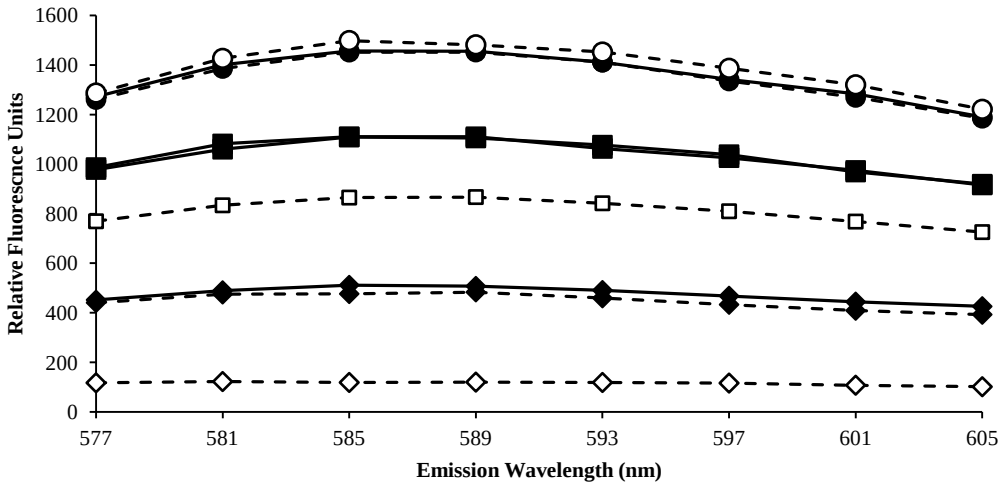


Figure 9: Emission spectrum for *E. coli* DM18.3 RFP intact cells (solid lines, closed symbols) or cells permeabilized using polymyxin B (10 mg $L^{-1}$; dotted lines, closed symbols) or 60% ethanol (v/v; dotted lines, open symbols) at pH 4 (◆), 4.5 (■), or 7 (●); excitation wavelength fixed at 557 nm.

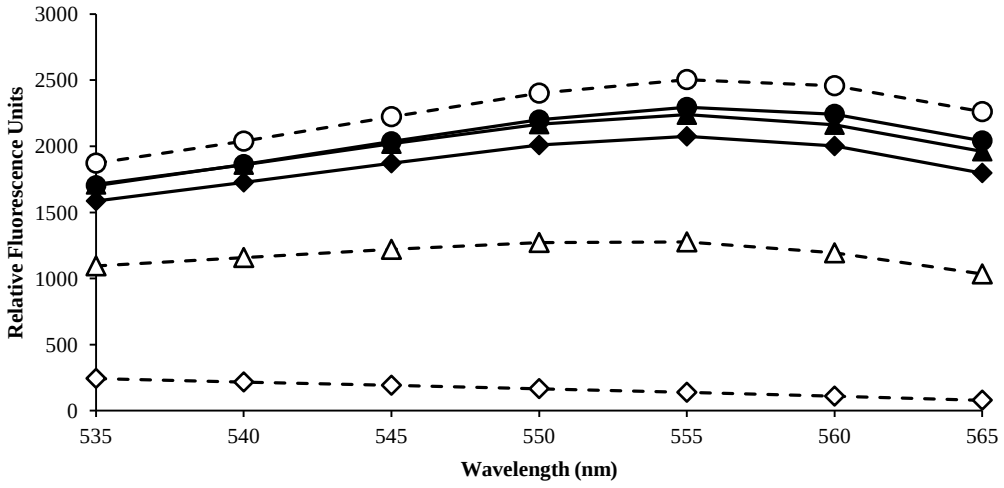


Figure 10: Excitation spectrum for *E. coli* DM18.3 RFP intact cells (solid lines, closed symbols) or cells permeabilized using 60% ethanol (v/v; dotted lines, open symbols) at pH 4 (◆), 4.5 (▲), or 7 (●); emission wavelength fixed at 585 nm.

### *3.9. pH-dependent fluorescence of RFP-transformed* **E. coli** *over time*

To investigate whether fluorescence intensity of RFP-transformed *E. coli* changed over time, cells were incubated at pH 4, 5 and 7 for 360 min, with fluorescence measurements taken every 30 min. After 360 min of incubation, fluorescence intensity of intact cells decreased by ~163 units at pH 5 and 7, and by 102 units at pH 4 (Figure 11A). Fluorescence intensity of intact cells was not markedly different at pH 5 versus pH 7 [maximum difference was 40 relative fluorescence units (RFU)]. At each time point, the RFU at pH 4 was always lower than the other two pH values tested, but the maximum difference was only 115 RFU. For permeabilized cells (Figure 11B), RFU at pH 5 and 7 were not remarkably different, with ~40 units difference in fluorescence intensity and a decrease in intensity of ~150 units at each pH value over 360 min of incubation. In contrast to the results for intact cells, permeabilized cells incubated at pH 4 showed a greatly reduced fluorescence intensity compared to the other two pH values tested.

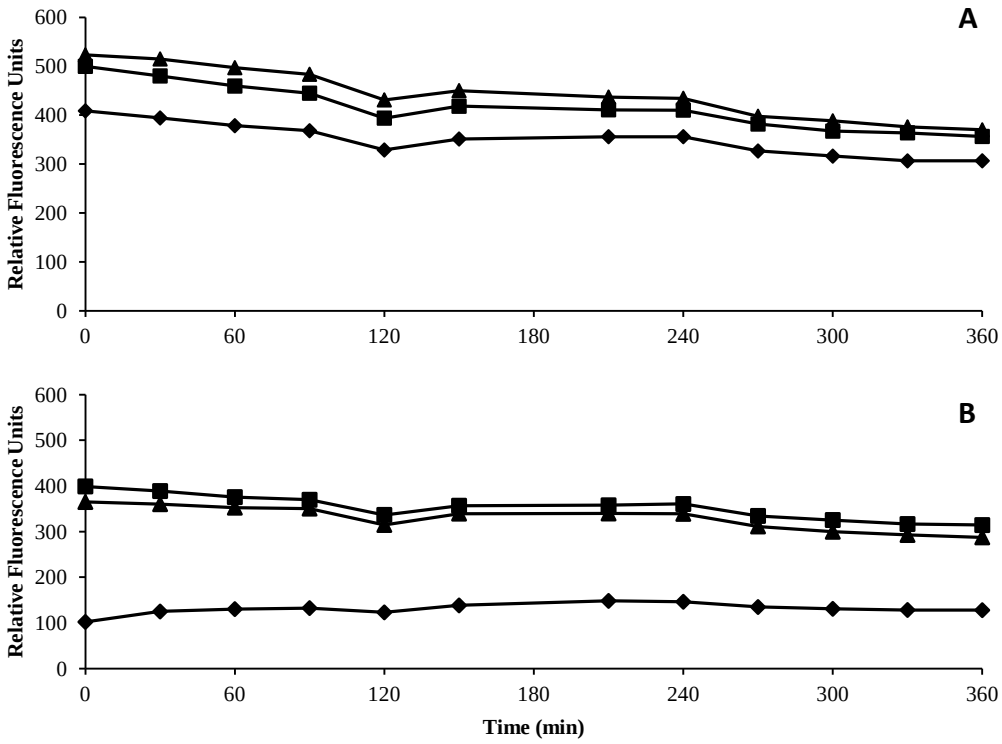


Figure 11: Relative fluorescence of intact and permeabilized cells of *E. coli* AW1.7 RFP. Cells were incubated at 37°C for 360 min. Relative fluorescence measurements were taken using excitation/emission wavelengths set at 557/585 nm. (A) Intact cells in pH 4 (◆), 5 (■), and 7 (▲) citrate phosphate buffers (30mM); (B): ethanol-permeabilized cells in pH 4 (◆), 5 (■), and 7 (▲) citrate phosphate buffers (30mM).

### *3.10. Efficacy of RFP as an intracellular pH probe*

The effect of lactate buffer (pH 3.5) on the relative fluorescence intensity of RFP-transformed *E. coli* AW1.7 was tested to determine if fluorescence could be used in place of time-consuming optical density measurements that require up to 24 h of incubation prior to measurement. Lactate buffer at 75 mM reduced the fluorescence intensity more than treatment with 37.5 mM lactate buffer (Figure 12). The greatest reduction in fluorescence intensity was observed when cells of *E. coli* were treated with 75 mM lactate buffer and 80 AU of *C. maltaromaticum* UAL307 CFS. By the end of 6 h incubation, the greatest difference between

treatments was 92 RFU, too small of a difference to allow for accurate estimation of true intracellular pH.

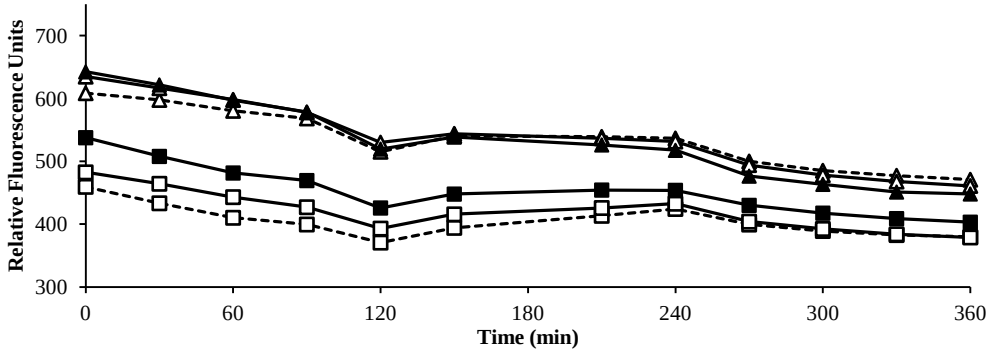


Figure 12: Fluorescence measurements of *E. coli* AW1.7 RFP treated with either 75 mM (■) or 37.5 mM (▲) pH 3.5 lactate buffers and *C. maltaromaticum* UAL307 cell-free supernatant (80 AU; dotted lines, open symbols), (40 AU; solid lines, open symbols) or (0 AU; solid lines, closed symbols). Cells were incubated at 37°C for 6 h and relative fluorescence was measured using excitation/emission at 557/585 nm.

# *4. DISCUSSION AND CONCLUSIONS*

## *4.1. General summary*

This study explored novel ways to inhibit the growth of pathogenic bacteria using biopreservatives on meat and in model systems. *L. monocytogenes* and heat-resistant *E. coli* were the organisms targeted. Lactic acid bacteria and bacteriocin-producing lactic acid bacteria were used as biopreservatives on meat, alone or in combination with antimicrobial treatments. Non-bacteriocinogenic *Lb. sakei* FUA3058 inhibited growth of *L. monocytogenes* on fresh beef, but it did not enhance the antimicrobial effect of steam and lactic acid treatments against *E. coli* on beef. In contrast, bacteriocinogenic *C. maltaromaticum* UAL307 was more effective than steam and lactic acid treatments at reducing the growth of *E. coli* on fresh meat. Fluorescent proteins were evaluated as tools for monitoring bacterial viability and they have potential for use as intracellular pH probes for cells of bacteria on meat. Green and red fluorescent proteins may be particularly useful for determining the efficacy of antimicrobial treatments that involve the use of acid to destroy *E. coli* on beef. Bacteriocin-containing CFS produced by *C. maltaromaticum* UAL307 worked synergistically with syringic acid to inhibit growth of *E. coli* in a model system, indicating that some phenolic acids may permeabilize the outer membrane of gram-negative bacteria. Three bacteriocins are produced by *C. maltaromaticum* UAL307. Prior to this study, it was not known which bacteriocin(s) were produced at sub-optimal temperature or on fresh meat. The bacteriocins produced by *C. maltaromaticum* UAL307 and the other

novel antimicrobial agents tested in this work should be further investigated for use as biopreservatives on fresh meat.

### *4.2. Green and red fluorescent proteins as intracellular pH probes*

Variants of green fluorescent protein (GFP) exhibit pH-dependent fluorescence (Patterson et al., 1997; Kneen et al., 1998; Ehrmann et al., 2001; Olsen et al., 2002; Kilimann et al., 2005). Thus, cells transformed to express GFP could potentially be useful as pH-probes to measure intracellular pH of cells *in situ*. They would be particularly useful in acid-challenge studies because there are no major changes in secondary structure over a pH range where fluorescence intensity changes by a factor of ~15 (Kneen et al., 1998).

The plasmid pEGFP has been successfully transformed into laboratory and wild-type strains of *E. coli* (Ehrmann et al., 2001; Cabrera Diaz, 2007; Pierce, 2009). In this study, the plasmid pEGFP was successfully transformed into *E. coli* AW1.7 and DM18.3, but there was some difficulty in obtaining strong fluorescence in liquid cultures. Heim et al. (1994) discovered that wild-type GFP displays two forms: the properly-folded, soluble, fluorescing native protein and the improperly-folded, insoluble, non-fluorescing protein. Thus, it is likely that the protein was not folding correctly, but when aeration was increased during propagation of cultures, fluorescence intensity and stability increased. Patterson et al. (1997) observed inefficient chromophore folding with wild-type GFP and one GFP mutant, but found that fluorescence of eGFP was consistent. Detailed culture conditions were not provided in the methods section of the paper written by

Patterson et al. (1997), but based on their findings, it is assumed that aeration must have been high to obtain strong, stable fluorescence of eGFP-transformed cells.

Enhanced GFP (eGFP) has a pKa of 5.5 - 6 (Patterson et al., 1997; Shaner et al., 2005). In this study, *E. coli* AW1.7 eGFP and *E. coli* DM18.3 eGFP showed pH-dependent fluorescence, with higher fluorescence produced by intact cells compared to permeabilized cells at a given pH. Polymyxin B permeabilized eGFP-transformed *E. coli* cells. Similarly, high pressure and a mixture of polymyxin B, nigericin and valinomycin have been effective at permeabilizing the outer membrane of *E. coli* for measuring intracellular pH (Ehrmann et al., 2001; Kilimann et al., 2005). The fluorescence intensity of polymyxin B-permeabilized cells were ~200 units lower than that for intact cells at a given pH. Thus, if a proper calibration curve is constructed for a culture of eGFP-transformed cells, it should be possible to determine whether cells are permeabilized or intact at a given pH based on the fluorescence intensity. Ethanol [60 % (v/v)] (Smigic et al., 2009) was an effective permeabilizing agent for *E. coli* AW1.7 eGFP and DM18.3 eGFP but fluorescence intensity of cells permeabilized with ethanol at pH 4, 5.5 and 7 was extremely low. Ethanol likely denatured eGFP and the loss of fluorescence may have been due to quenching as the chromophore became exposed to the aqueous environment (Ward et al., 1980). Tertiary structure must remain fully intact for the protein to shield the chromophore from being quenched (Reid and Flynn, 1997). Calculating the ratio of relative fluorescence intensities at wavelengths that exhibit pH-independent and –dependent fluorescence (Olsen et

al., 2002; Kilimann et al., 2005) may provide more reliable information in future experiments.

The pKa values of red fluorescent proteins, pH 4.5 to 5 (Shaner et al., 2005), are lower than those for GFPs and are lower than the pH of meat (5.4 to 5.5), which makes RFP useful for determining the intracellular pH of acid-treated bacteria on meat. From pH 5.0 – 9.0, DsRed exhibits stable fluorescence (Gross et al., 2000; Heikal et al., 2000; Vrzheshch et al., 2000). Two groups found that fluorescence intensity of DsRed is reduced at pH ~4.0 (Baird et al., 2000; Vrzheshch et al., 2000), but another group found fluorescence to be unaffected by pH values as low as 3.9 (Heikal et al., 2000). In this study, the plasmid pDs-REx was transformed into *E. coli* AW1.7 and DM18.3. RFP-transformed *E. coli* behaved differently than eGFP-transformed *E. coli*. RFP-transformed cells treated with polymyxin B did not differ in fluorescence intensity from intact cells at the same pH, suggesting that polymyxin B was not effective at permeabilizing the outer membrane of RFP-transformed *E. coli*. This was unexpected, as polymyxin B is known to disrupt gram-negative outer membranes, providing access to cell membrane active agents (Rosenthal and Storm, 1977; Vaara and Vaara, 1983; Vaara, 1992) and it effectively permeabilized eGFP-transformed *E .coli*. Ethanol is effective at permeabilizing gram-negative cell walls (Smigic et al., 2009), and thus was chosen as a permeabilizing agent for RFP-transformed *E. coli*. The results of the current study found that cells treated with ethanol had greatly reduced fluorescence intensity compared to intact cells at pH 4 and 4.5, but there was no difference between intact and permeabilized cells at pH 7. This indicates

that ethanol did not denature RFP as it may have done to GFP. The structures and topology of GFP and RFP are similar. The internal chromophore is surrounded by an α-helix which is enclosed in a β-barrel (Wall et al., 2000), but it is possible that RFP is more stable or more resistant to denaturation by ethanol than eGFP. For RFP-transformed cells, 60 % (v/v) ethanol was more effective at disrupting the outer membrane than polymyxin B.

In the current study, pH affected the fluorescence intensity of *E. coli*. Permeabilized eGFP- and RFP-transformed cells of *E. coli* at pH 4 had greatly reduced fluorescence intensity compared to intact cells at pH 4 and intact or permeabilized cells at pH 5 or 7. Both fluorescent proteins would be useful for measuring the intracellular pH of bacterial cells on raw meat. Although the initial pH of the meat surface would be reduced by the presence of acid, over time the pH of the meat would increase above pH 5 because of the high buffering capacity of meat (Puolanne and Kivikari, 2000). Therefore, eGFP and RFP, differing by ~ 1 pH value in pKa, could be used together to obtain a wider pH range to measure intracellular pH of bacterial cells on meat. Additionally, eGFP- and RFP-transformed gram-negative cells could be used to determine whether the outer membrane is compromised by novel antimicrobial agents or processes.

### *4.3. Combining outer membrane disrupting agents with bacteriocins*

The protective outer membrane of gram-negative bacteria prevents hydrophobic inhibitors from entering the cytoplasmic membrane; thus, effective biopreservatives must breach this membrane. EDTA and lactic acid are both

effective at disrupting the outer membranes of *E. coli* and *Salmonella* (Stevens et al., 1991; Kalchayanand et al., 1992; Vaara, 1992; Cutter and Siragusa, 1995; Scannell et al., 1997; Ariyapitipun et al., 1999; Branen and Davidson, 2004). EDTA removes $Ca^{2+}$ and $Mg^{2+}$ from the cell wall, which releases phospholipids and lipoproteins. This results in increased permeability of the cell wall that then provides membrane-active agents with access to their target (Vaara, 1992; Delves-Broughton, 1993). EDTA and lactic acid were effective outer membrane disrupting agents in this study. Lactic acid at pH 3.5 worked particularly well with *C. maltaromaticum* UAL307 cell-free culture supernatant to inhibit growth of *E. coli* in a model system. Organic acids have been combined with bacteriocins in food to increase efficacy, but most research has focused on nisin. For example, a combination of 500 IU $g^{-1}$ nisin and 2 % sodium lactate reduced *Salmonella* in broth and on fresh pork sausage (Scannell et al., 1997). Lactate and nisin reduced counts of *Salmonella* on fresh pork sausage (Scannell et al., 1997) and lactate and citrate increased the efficacy of nisin or lacticin 3147 against *Salmonella enterica* serovar Kentucky in fresh pork sausage (Scannell et al., 2000). To improve the efficacy of EDTA against gram-negative bacteria, bacteriocins produced by LAB bacteria have been used concurrently. Nisin and EDTA exhibited a bactericidal effect against *Pseudomonas fluorescens* in a model system at nisin levels of 25 mg $L^{-1}$ and 75 mg $L^{-1}$ with EDTA at 0.5 and 1 mM, respectively (Delves-Broughton, 1993). EDTA and nisin or brochocin-C resulted in a 2.1 – 4 or 2 – 3.1 log reduction, respectively, of *E. coli* and *Salmonella* in a model system (Gao et al., 1999). *E. coli* LTH1600 was destroyed by a combination of EDTA and nisin,

and low pH with nisin, sakacin P, or curvacin A (Gänzle et al., 1999). The same group also demonstrated the efficacy of low pH with nisin, sakacin P or curvacin A at destroying *Salmonella enterica* serovar Heidelberg LTH3658. In some food systems, including fresh meat, efficacy of EDTA and nisin is reduced (Delves-Broughton, 1993; Branen and Davidson, 2004) and nisin is inactivated by glutathione in fresh meat (Rose et al., 1999, 2002). In the current study, bacteriocins very different from nisin were paired with EDTA and lactate as outer membrane disrupting agents. Bacteriocin-containing cell-free culture supernatant from *C. maltaromaticum* UAL307, producing two class IIa and one class IV bacteriocin, acted synergistically with EDTA and lactate to inhibit growth of *E. coli* in a model system. Carnocyclin A, the class IV (circular) bacteriocin produced by *C. maltaromaticum* UAL307, is the most promising for use on raw meat as the cyclic structure provides more stability and protection against degradation by proteases and greater stability in a wide range of pH and temperatures (Maqueda et al., 2008). Although EDTA and lactate are useful for breaching the outer membrane of gram-negative bacteria, compounds such as phenolic acids may prove to be more useful and more desirable for use in foods.

### *4.4. Phenolic acids as natural preservatives*

Phenolic acids, including ferulic and syringic acids inhibit the growth of gram-positive and gram-negative organisms (Olasupo et al., 2003; Merkl et al., 2010; Sánchez-Maldonado et al., 2011). Syringic acid is a hydroxybenzoic acid with one hydroxyl and two methoxy groups attached to a benzene ring. Ferulic

acid is a hydroxycinnamic acid with one hydroxyl and one methoxy group attached to a benzene ring. Phenolic acids have antimicrobial activity against *E. coli* and *Salmonella*, but did not enhance the antimicrobial activity of nisin against these organisms (Olasupo et al., 2003). In the current study, the phenolic acids syringic and ferulic acid were tested as outer membrane permeants, and their efficacy when applied with bacteriocins was determined. The results indicated that nisin did not act synergistically with lactate, ferulic or syringic acid to destroy *E. coli* AW1.7, DM18.3 or GGG10 in a model system, confirming the findings of Olasupo et al. (2003). *C. maltaromaticum* UAL307 CFS acted synergistically with the phenolic acids tested, reducing the amounts of ferulic acid, and syringic acid required to inhibit growth of *E. coli* strains AW1.7, and all 3 strains of *E. coli*, respectively. Therefore, phenolic acids and *C. maltaromaticum* UAL307 CFS could potentially be used as naturally produced biopreservatives to control *E. coli* on fresh meat. In future studies, it would be beneficial to test phenolic acids at various pH values. Sánchez-Maldonado et al. (2011) determined that the antimicrobial activity of phenolic acids increased as acidity increased. The author is unaware of other studies pairing phenolic acids with the CFS produced by a multiple-bacteriocin-producing strain of LAB. Thus, it is difficult to make direct comparisons to findings in the literature, but it is quite likely that more research will be performed pairing phenolic acids with bacteriocins other than nisin and with multiple bacteriocins.

### *4.5. Efficacy of Micocin X™ compared to* C. maltaromaticum *UAL307 CFS to inhibit the growth of* E. coli

Micocin X™ is a commercially available spray dried preparation of *C. maltaromaticum* UAL307 cell-free culture supernatant that contains the bacteriocins piscicolin 126, carnobacteriocin BM1 and carnocyclin A. It has strong anti-listerial activity and has recently been approved for use in some ready-to-eat meats in Canada (Health Canada, 2010). Micocin X™ extended the shelf life of pasteurized liquid whole eggs by 5 weeks at a concentration of 0.05 % (w/v) (Miller et al., 2010), but there is no other research presented in the literature using Micocin X™. To determine whether Micocin X™ had the same level of activity as *C. maltaromaticum* UAL307 CFS against *E. coli* AW1.7, DM18.3 and GGG10 when combined with outer membrane permeants, the bacteriocin-containing preparation was tested with lactate buffer, ferulic acid and syringic acid. Micocin X™ reduced the concentration of lactate buffer (pH 3.5) required to inhibit *E. coli* AW1.7, but not *E. coli* DM18.3 or GGG10. *E. coli* AW1.7 is extremely heat-resistant ($D_{60}$-value = 71 min) (Dlusskaya et al., 2011) and it has different genetic and physiological characteristics than *E. coli* GGG10 which may account for this difference (Ruan et al., 2011). *E. coli* AW1.7 over-expresses genes for transport proteins and porin formation compared to *E. coli* GGG10, a heat-sensitive strain. The less heat resistant strain *E. coli* DM18.3 does not express the outer membrane porin NmpC (Ruan et al., 2011); this may be related to the lack of efficacy of Micocin X™ against this strain compared to *E. coli* AW1.7. *E. coli* AW1.7 also has a higher proportion of saturated and cyclopropane

fatty acids in the cytoplasmic membrane compared to heat-sensitive strain *E. coli* GGG10; thus, the membrane has lower fluidity and that would affect the activity of membrane-active agents such as bacteriocins (Montville et al. 1995).

Micocin X™ did not act synergistically with ferulic or syringic acid against *E. coli* AW1.7, DM18.3 or GGG10. These results contrast with the results for *C. maltaromaticum* UAL307 CFS, which acted synergistically with lactate and syringic acid against all strains of *E. coli* tested. The diluent used in this experiment was nutrient-rich LB medium, which may have enhanced recovery of injured cells compared to the sterile distilled water used in the other experiment. It is also possible that the laboratory-obtained CFS contained other antimicrobial compounds produced by the growing cells which may have been destroyed by the spray-drying process (Hartmann et al., 2011). In addition to bacteriocins, *C. maltaromaticum* produces L (+)-lactic acid, ethanol and acetate (Mora et al., 2003). Specifications for the spray drying process used to obtain Micocin X™ are unknown, but industrial spray driers are commonly used on food products at temperatures ranging from 65 to 270°C (Filková et al., 2006). The boiling points of lactic acid, acetic acid, and ethanol are within this temperature range. Therefore, it is possible that these compounds were removed.

Experiments were performed to determine the MIC of Micocin X™ against *C. maltaromaticum* UAL 26, UAL307 and UAL8C2 and *C. divergens* LV13 as a quality control measure. The producer strain was immune to all concentrations tested. *C. maltaromaticum* UAL26 produces two of the same bacteriocins as *C. maltaromaticum* UAL307 and this strain required a high

concentration of Micocin X™ [2.5 % (w/v)] for inhibition. Therefore, carnocyclin A activity was likely responsible for inhibition of this strain because *C. maltaromaticum* UAL26 does not produce it (Martin-Visscher et al., 2008). *C. maltaromaticum* UAL8C2 and *C. divergens* LV13 were inhibited by low concentrations of Micocin X™. *C. divergens* LV13 is sensitive to the bacteriocins produced by *C. maltaromaticum* UAL307 (Martin-Visscher et al., 2008; Martin-Visscher et al., 2011) and *C. maltaromaticum* UAL8C2 (LV17C) is a plasmidless mutant of *C. maltaromaticum* LV17 that does not produce any immunity proteins or bacteriocins (Quadri et al., 1994; Quadri et al., 1997). Nisin activity was also tested. Nisin inhibited all strains of *Carnobacterium* tested at concentrations ranging from 98 to 391 μg $L^{-1}$. After determining the efficacy of *C. maltaromaticum* UAL307 bacteriocins in model systems, it was important to explore expression of bacteriocin genes at refrigeration temperature and on meat to determine which bacteriocin(s) produced by *C. maltaromaticum* UAL307 are active at refrigeration temperature and on fresh meat.

### *4.6. Bacteriocin gene expression in broth cultures and on meat*

*C. maltaromaticum* UAL307 may have potential for use as a bacteriocinogenic protective culture on raw vacuum-packaged meat. In particular, the bacteriocin carnocyclin A has strong activity against a wide range of gram-positive bacteria (Martin-Visscher et al., 2008). Prior to the current study, no research had been performed with this bacterial strain or its bacteriocins on fresh

meat. To determine whether efficacy is related to production of bacteriocins, expression of bacteriocin genes was quantified.

To study production of bacteriocins by strains of *C. maltaromaticum*, reverse transcription qPCR (RT-qPCR) was chosen. This is a very sensitive method that detects as few as 6 to $10^3$ gene copies, depending on the gene and the matrix in which it is present (Hanna, 2005). The usefulness of RT-qPCR to detect bacterial gene expression in food is largely dependent on the food matrix. In addition, bacterial mRNA can be particularly difficult to isolate as it is degraded rapidly (Rauhut and Klug, 1999; Steege, 2004). RT-qPCR has been used to detect and quantify bacterial gene expression in cheese, fresh meat, fermented sausages, and sourdough (Hüfner et al., 2008; Rantsiou et al., 2008; Torriani et al., 2008; Falentin et al., 2010; Slanec and Schmidt, 2011; Taïbi et al., 2011; Trmčić et al., 2011). Thus, it was chosen to quantify bacteriocin gene expression on fresh meat.

In this study, RNA isolation was a challenge. In a previous study (unpublished), bacterial RNA from *Leuconostoc gelidum* UAL187 was successfully isolated from meat using the acidified phenol chloroform method developed by Torriani et al. (2008) with minor adjustments. When this method was used to isolate RNA from liquid cultures of *C. maltaromaticum* UAL26, UAL307, and UAL8C2, it was difficult to obtain RNA samples free of chromosomal DNA. Initially, it was thought that the DNase treatment was ineffective. Therefore, DNase from different suppliers was used with different concentrations of the enzyme and RNA. Time and temperature of incubation with the enzyme were also varied and none of these attempts resulted in DNA-free

RNA. The reagents used for the RNA isolation procedure may have been expired, but instead of ordering new reagents, a commercially made RNA isolation kit was used.

The Enzymatic Lysis of Bacteria protocol was followed using the Qiagen RNeasy Minikit for RNA isolation from liquid cultures. Initially, it was unsuccessful as yield and quality were low and DNA was present. A second centrifugation step was required prior to elution to remove residual ethanol because ethanol interfered with the action of the DNase. Yield remained low compared to the phenol chloroform method, but the product obtained from the kit was RNA, with little or no DNA present, whereas RNA isolated using the acidified phenol chloroform method contained a significant amount of contaminating DNA. Therefore, the Qiagen RNeasy RNA Isolation Kit was used to isolate RNA from meat samples. RNA quality was measured by assessing the ratios of absorbance readings at 260/280 and 260/230 nm. RNA concentration was determined by measuring the absorbance at 260 nm. For most meat samples, quality and concentration of RNA were low (Thermo Fisher Scientific Inc., 2008), but samples were still used for RT-qPCR. Some groups have questioned the accuracy of the A260/A280 ratio used to determine purity of nucleic acids as a value of 1.8 (Thermo Fisher Scientific Inc., 2008) corresponds to 40 % RNA in the presence of contaminating proteins (Baelde et al., 2001; Bustin and Nolan, 2004). Low quality and concentration of the isolated RNA may be explained by low viable counts in the meat samples. Viable counts of *C. maltaromaticum* UAL26, UAL307 and UAL8C2 reached a maximum of 7.4 log CFU $cm^{-2}$,

meaning that there was at most 2.2 log CFU in a 0.5 mL sample of homogenate. Therefore, very little mRNA was present, making it difficult for RNA quality and quantity to be accurately assessed. Recent studies indicate that obtaining high quality RNA with a good RNA integrity number is key to obtaining meaningful gene expression data (Fleige and Pfaffl, 2006; Jahn et al., 2008; Postollec et al., 2011), but this may be more important for longer amplicons (> 400 bp) than for shorter ones when a relative quantification method is used (Fleige and Pfaffl, 2006). In this study, amplicon size ranged from 125 to 279 base pairs and the relative quantification method was used with 16S rRNA as the reference gene. Therefore, high RNA integrity and quality determined by optical density measurements are not imperative for interpretation of gene expression results in this study.

The expression of bacteriocin genes produced by *C. maltaromaticum* UAL26 and UAL307 on meat and *in vitro* was quantified. *C. maltaromaticum* UAL8C2, a bacteriocin negative strain, was used as a negative control. Bacteriocin genes were amplified using gene-specific primers and 16S rRNA was used as the endogenous reference gene. Quantitative RT-PCR results indicated that all bacteriocins produced by *C. maltaromaticum* UAL307 and UAL26 were detected in liquid medium, with higher gene expression at 10°C than at 4°C. Temperature-dependent bacteriocin production has previously been demonstrated (Degnan et al., 1992; Katla et al., 2002; Gursky et al., 2006). At 4°C and 10°C, carnocyclin A was the only bacteriocin consistently detected on meat inoculated with *C. maltaromaticum* UAL307; piscicolin 126 was produced by

*C. maltaromaticum* UAL307 but not by *C. maltaromaticum* UAL26 on meat. Both bacteriocin genes were detected at very low levels near the detection limit. Carnobacteriocin BM1 was not detected in any of the meat samples, suggesting that it may not be active under these conditions. It is also likely that the highly unstable mRNA corresponding to the three bacteriocins was rapidly degraded (Rauhut and Klug, 1999; Steege, 2004). The high cycle numbers obtained (not shown) may have been more indicative of mRNA stability than of actual gene expression. For all strains, expression of 16S rRNA genes was detected in meat samples indicating that RNA isolation and reverse transcription were effective.

To explore the hypothesis further, future studies could include the method of Aasen and coworkers (2003) or one similar to recover adsorbed bacteriocins from the meat surface along with gene expression results. Aasen et al. (2003) used urea to release bacteriocins from the meat surface to determine whether bacteriocins were adsorbed to the surface or 'free' in the liquid surrounding the meat. They then determined the activity level of the free and adsorbed bacteriocins to test whether the bacteriocins retained activity. This would complement the findings of a gene expression study because it would enable researchers to determine if low levels of gene expression are due to true lack of expression or due to mRNA degradation.

Bacterial mRNA is very unstable (Rauhut and Klug, 1999; Steege, 2004). Ribonucleases degrade mRNA to ensure a constant supply of ribonucleotides is available for synthesis of new mRNAs for protein production, and this rapid degradation of mRNA is an integral part of the adaptation response of bacterial

cells to changes in their environment (Steege, 2004). Some researchers preferentially use mRNA reference genes rather than 16S rRNA because 16S rRNA is both more stable and has a higher copy number than mRNA (Desroche et al., 2005; Schwab and Gänzle, 2006). Desroche and coworkers (2005) determined that 16S rRNA was the most stable of seven reference genes tested, but transcript levels were 1000-fold higher than all mRNA reference genes tested. Therefore, 16S rRNA levels may not be representative of the mRNA content and may be inappropriate for use in relative quantification studies. In addition, Desroche et al. (2005) obtained more accurate results linking cell counts to RT-qPCR quantification results when using mRNA (lactate dehydrogenase gene) compared to 16S rRNA for the reference gene. Schwab and Gänzle (2006) used lactate dehydrogenase as a reference gene rather than 16S rRNA to more accurately relate gene expression to carbon metabolism. Therefore, it may be beneficial to explore other reference genes for use in future studies. However, at the time of commencement of this study, the *Carnobacterium* genome was not available. Gene expression should not be affected by experimental conditions, but often transcripts are affected by experimental conditions to some extent (Vandecasteele et al., 2001; Desroche et al., 2005; Marco and Kleerebezem, 2008). Therefore, it is best to use more than one reference gene to normalize RT-qPCR results (Marco and Kleerebezem, 2008).

### *4.7. Pathogen control on meat by protective cultures*

Lactic acid bacteria are effective protective cultures in meat (Holzapfel et al., 1995; Helander et al., 1997; Bredholt et al., 1999). While most research focuses on the antimicrobial effect of bacteriocinogenic strains, many non-bacteriocin producers have been shown to effectively inhibit growth of pathogens on meat products. To determine if growth of *Listeria monocytogenes* could be inhibited by a non-bacteriocin producing psychrotrophic strain of *Lb. sakei* on raw meat, fresh beef was inoculated with a *L. monocytogenes* strain cocktail and *Lb. sakei* FUA3058, vacuum packaged and stored at 10°C. In this study, non-bacteriocinogenic *Lb. sakei* FUA3058 inhibited the growth of *L. monocytogenes* on vacuum packaged meat stored at an abusive refrigeration temperature. *Lb. sakei* grew rapidly, reaching ~7.5 log CFU $cm^{-2}$ within 7 days. It is not clear what may be responsible for the inhibition of *L. monocytogenes*, as pH measurements and analysis of the metabolites on the meat or in the surrounding liquid were not performed. In future studies, pH of the meat samples should be measured and high performance liquid chromatography should be employed to analyze the metabolites present.

Many groups have demonstrated that bacteriocin-negative LAB can inhibit growth of other organisms on meat, confirming the results of this study. For example, Alvez and others (2006) inoculated cooked ham with *L. monocytogenes* and a bacteriocin-positive or –negative strain of *Lb. sakei* or nisin and stored at 8°C in vacuum packaging. Nisin did not reduce counts of *L. monocytogenes*, but both strains of *Lb. sakei* did, indicating that bacteriocin production was not

required for inhibition. The authors concluded that the inhibition was due to competition for nutrients, acid production or production of other antimicrobial compounds. Similar results were found by Nilsson and coworkers (1999) , who inoculated cold-smoked salmon with *L. monocytogenes* and one of four strains of *Carnobacterium piscicola (C. maltaromaticum)* or *Lb. sakei* and incubated at 5°C for 32 days. All strains were found to be anti-listerial, with bacteriocins responsible for inhibition by two *C. maltaromaticum* strains and the *Lb. sakei* strain tested. The other two strains inhibited *L. monocytogenes* growth by other means, likely acid and antimicrobial metabolite production. *C. maltaromaticum* had no influence on the sensory characteristics, but *Lb. sakei* LK5 produced unacceptable strong sulphurous off-flavours, deeming this organism inappropriate for use on cold-smoked salmon (Nilsson et al., 1999). Psychrotrophic strains of *Lb. sakei* inhibited the growth of *L. monocytogenes* and *E. coli* O157:H7 in cooked ham (Bredholt et al., 1999). Only one of the *Lb. sakei* strains tested was bacteriocinogenic. Therefore, the authors determined that the fast-growing strains of *Lb. sakei* had a selective advantage over the pathogens tested, but the exact reason for pathogen growth inhibition was not determined. Acidity of the ham was monitored over 28 days of storage and the pH remained relatively constant at pH 6, but no other metabolites were assayed. Sensory quality of *Lb. sakei* inoculated ham was deemed acceptable after 21 days of storage at 8°C. In an industrial setting on cooked ham and servelat sausage, the psychrotrophic strain of *Lb. sakei* grew to maximum numbers within 10 days and inhibited growth of $10^3$ CFU $g^{-1}$ of *E. coli* O157:H7 and *L. monocytogenes* at 4° and 8°C. The inoculum

level of *Lb. sakei* was 1 log CFU $g^{-1}$ higher than in the previous model experiment (~$10^{5-6}$ CFU $g^{-1}$) and this resulted in a drop in pH in the product from ~ pH 6 to 5 after 28 days of storage at 4°C or 8°C (Bredholt et al., 2001). Vermeiren and coworkers (2006) assessed the antilisterial ability of bacteriocin-positive and –negative *Lb. sakei* on model cooked ham. A lactocin S producing strain did not inhibit growth of the three-strain cocktail of *L. monocytogenes* cocktail under modified atmosphere or vacuum packaging. The non-bacteriocin producing strain, *Lb. sakei* inhibited growth of *L. monocytogenes* with vacuum packaging at 4°C and with modified atmosphere packaging at 7°C for 42 days. Interestingly, modified atmosphere packaging alone retarded growth of the pathogen until day 42. After day 42, counts increased by nearly 1 log CFU $g^{-1}$. The authors attributed the antilisterial effect to the competitiveness of the culture. The average decrease in pH was 0.35 and the buffering capacity of the meat was high, so reduced pH was not considered to be a factor, although titratable acidity was not measured. The group also measured the glucose content of the meat (0.21 ± 0.06 %) and concluded that the glucose content was too low to contribute to a significant amount of lactic acid production. Taste and smell of the cooked ham product was not affected by inoculation with the protective culture (Vermeiren et al., 2006), but it remains unknown what was responsible for the inhibition of growth.

Most research on the use of non-bacteriocinogenic cultures to destroy pathogens on meat has been performed using cooked, cured, or ready-to-eat meat, but it is also important to explore the use of protective cultures on raw meat to prevent foodborne illnesses in the case of improper cooking or handling, cross-

contamination, or temperature abuse. Smith and coworkers (2005) showed that four strains of *Lactobacillus acidophilus* isolated from cattle, alfalfa sprouts or cooked hot dogs reduced counts of *E. coli* O157:H7 and *Salmonella* in vacuum-packaged ground beef. These strains did not grow in ground beef during the storage period, but they produced inhibitory substances. Unlike the work of Smith et al. (2005), most of the research regarding pathogen control on fresh beef has focused on antimicrobial interventions used on carcasses, but some groups have explored the use of bacteriocins with these decontamination treatments.

Steam treatments, hot water washes, and organic acids are effective decontaminating agents for reducing counts of *E. coli* on raw beef (Prasai et al., 1991; Cutter and Siragusa, 1994; Hardin et al., 1995; Dorsa et al., 1997a, b; Nutsch et al., 1997; Phebus et al., 1997; Castillo et al., 1998; Dormedy et al., 2000; Bosilevac et al., 2006; Harris et al., 2006; Echeverry et al., 2009). Lactate or lactic acid and bacteriocins have also been used to control pathogens on fresh and cooked meat products (Scannell et al., 1997, 2000; Uhart et al., 2004).

Although many microbial inactivation studies have been performed using nisin, nisin is inactivated by glutathione in fresh meat (Rose et al., 1999, 2002). Ariyapitipun et al. (1999) combined nisin with poly lactic acid and lactic acid for use as antimicrobial agents on fresh vacuum-packaged beef and nisin did not improve the efficacy of either acid. Therefore, nisin or other lantibiotics were not used in this study on fresh meat. Bacteriocins used in this study were in class IIa and class IV (circular), and potentially more appropriate for use on fresh meat.

To determine if protective cultures could be used as an additional hurdle with steam and lactic acid to destroy heat-resistant *E. coli* on beef, raw beef was inoculated with heat-resistant *E. coli* AW1.7 and DM18.3, subjected to steam and lactic acid treatments, inoculated with LAB, vacuum packaged and stored at refrigeration temperature. Non-bacteriocin producing *Lb. sakei* FUA3058 and multiple bacteriocin producing *C. maltaromaticum* UAL307 were used and the vacuum packaged meat was stored at 4°C for 28 days. *Lb. sakei* FUA3058 was no more effective than steam and lactic acid treatments alone at reducing counts of total and injured *E. coli.* Total *E. coli* remained above the detection limit for the duration of the study and by day 21, counts of uninjured *E. coli* were below the detection limit. When paired with steam and lactic acid treatments, *Carnobacterium maltaromaticum* UAL307 reduced total counts of *E. coli* by more than 2 log CFU $cm^{-2}$ on vacuum-packaged meat after 28 days of storage at 4°C. The reduction of total *E. coli* was higher when *C. maltaromaticum* UAL307 was applied to the meat after steam and lactic acid treatments than when steam and lactic acid were used alone. To date, there have no reports of *Carnobacterium* spp. that produce multiple bacteriocins being used to control heat-resistant *E. coli* on fresh meat. Based on this the results of this study, *C. maltaromaticum* UAL307 may be a good candidate for use on fresh meat.

### *4.8. Conclusions*

Novel biopreservatives were shown to inhibit growth of pathogens *in vitro* and on meat. Non-bacteriocinogenic *Lb. sakei* FUA3058 inhibited growth of

*L. monocytogenes* on raw vacuum-packaged beef, demonstrating that this strain is able to outcompete the slower growing strains of *L. monocytogenes* and that bacteriocins are not imperative for inhibition of *L. monocytogenes* on fresh meat. Bacteriocins produced by *C. maltaromaticum* UAL307 can inhibit growth of *E. coli* when combined with lactic acid, syringic acid or syringic acid *in vitro.* On meat, *C. maltaromaticum* UAL307 reduced growth of total *E. coli* when combined with steam and lactic acid. Therefore, future studies pairing *C. maltaromaticum* UAL307 with steam and phenolic acids should be performed to determine whether phenolic acids are also effective at permeabilizing the outer membrane of *E. coli* on meat. If phenolic acids are effective permeabilizing agents, beef processors would have an additional natural preservative to use to combat pathogens on fresh meat. The combined effect of acids and bacteriocins on *E. coli* outer and cytoplasmic membranes, respectively, could be assessed non-invasively by measuring the intracellular pH via pH-dependent fluorescence of GFP and RFP transformed cells. Fluorescence microscopy could be used to visualize eGFP- or RFP-transformed cells on the meat surface. Using both green and red fluorescent proteins, a greater useful pH range could be obtained, facilitating more accurate measurements of internal pH of *E. coli* cells on acid-treated meat. It is still unclear whether carnocyclin A is the bacteriocin most strongly expressed by *C. maltaromaticum* UAL307 on meat. Further studies must be performed using mRNA reference genes for relative quantification of the three bacteriocin genes, and gene expression studies should be paired with bioassays to determine whether active bacteriocins can be recovered from the meat. It is

important to continue exploring novel natural antimicrobials and biopreservatives to control pathogens on fresh meat to better protect our food supply while providing consumers with natural alternatives to chemical preservatives.

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