Quantification of bacteriocin gene expression in

Carnobacterium maltaromaticum ATCC PTA-5313

by

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Abstract

Carnobacterium maltaromaticum ATCC PTA-5313, which produces bacteriocins carnocyclin A, piscicolin 126 and carnobacteriocin BM1, has been approved for use on meat products to control the growth of *Listeria monocytogenes*. This combination of bacteriocins is very effective as a biopreservative. It was unclear which of the three bacteriocins is responsible for the antimicrobial effect on meat. The aim of this research was to develop a novel method for detection of bacteriocin gene expression and to use this method to determine the expression of bacteriocin genes when *C. maltaromaticum* was inoculated onto a ready-to-eat meat product. A novel method to detect bacteriocin gene expression *in vitro* and on a low sodium ready-to-eat meat product was developed using a modified RNA extraction protocol and quantitative polymerase chain reaction. When *C. maltaromaticum* ATCC PTA-5313 was grown in broth in the presence of lactate and acetate preservatives, there was no change in gene expression during growth. When *C. maltaromaticum* ATCC PTA-5313 was inoculated onto ham and stored for 28 days at 4°C, the structural genes for all three bacteriocins were expressed. During storage, gene expression decreased relative to a culture grown in broth at the same temperature. Gene expression was monitored every 4 to 7 days up to 56 days of storage on vacuum packaged low sodium ham formulated with or without preservatives and inoculated with *C. maltaromaticum* ATCC PTA-5313 and a 5-strain cocktail of *L. monocytogenes*. There was an increase in expression of the piscicolin 126 and carnobacteriocin BM1 genes during the stationary phase of growth of the cultures on ham formulated with preservatives. Overall, bacteriocin gene expression from *C. maltaromaticum* ATCC PTA-5313 is decreased over storage time when inoculated on vacuum packaged ham, and the presence of preservatives results in higher expression levels during stationary phase than that of *C. maltaromaticum* ATCC PTA-5313 on ham without preservatives.

To my family, for making me the person I am today.

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Thank you, dear reader, for taking the time to read my thesis. I hope my work provides you with enjoyment, assistance, or amusement.

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List of symbols/abbreviations

APT	All Purpose Tween
ATP	adenosine triphosphate
cbnBM1	carnobacteriocin BM1 structural gene
cclA	carnocyclin A structural gene
CFU	colony forming units
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
log	logarithmic
OD_{600}	optical density measured at 600 nm
pisA	piscicolin 126 structural gene
RNA	ribonucleic acid
TS	Tryptic Soy

1 Introduction

1.1 Food Safety

Throughout Canada there is an estimated 4 million cases of foodborne disease per year (Thomas et al., 2013). These illnesses can result in not only increased medical expenses, but also loss of productivity and lowered earning potential. In the United States the cost of medical expenses associated with foodborne illness is estimated at an astonishing 152 billion per year (Scharff, 2010). This does not take into account the cost of recalls and reduced sales for the food industry, which are also large economic losses. In 2012, there was a recall of contaminated beef from XL foods in Alberta that resulted in 1800 recalled products and an estimated loss of 16 to 27 million dollars for the beef industry in Canada (Lewis et al., 2013). Together, the public health and economic consequences of foodborne illness represent a huge challenge for society and prevention of foodborne illness is a very important area for government, industry, and academia.

Consumers are more concerned with food safety now than ever before. A survey done by Brewer and Rojas (2008) reported that 45% of consumers expressed a "moderate to strong" concern with microbial contamination of food. This was increased from 12% in 1984 (Brewer & Prestat, 2002). With highly publicized outbreaks and increased information available to consumers through social media, there is an increased demand for the assured safety of food products.

As a further challenge to the food industry, consumers now desire products that are deemed "clean label" or have a minimal of chemical preservatives, but still adhere to high safety standards (Sofos, 2008). In addition, the Canadian government has called for a reduction of sodium levels in processed foods in response to public demand as well as

demand from public health officials (Health Canada, 2012). This creates new challenges for the food industry to offer a product with lower sodium but that maintains the level of quality and safety that customers expect.

1.2 Antimicrobial interventions for meat

There are numerous antimicrobial interventions that are currently used to ensure the safety of ready-to-eat (RTE) meat products. When numerous interventions are used in combination they can have synergistic effects resulting in a hurdle that is larger than the sum of the antimicrobial effects of the individual interventions. Sodium chloride is among one of the most widely used preservatives as it has an effect on taste and the desirable property of binding water. Nitrites and nitrates are used to control the outgrowth of *Clostridium botulinum* and are used in combination with lactate and acetate preservatives to control *L. monocytogenes*. These preservatives also reduce the growth of undesirable spoilage organisms. High hydrostatic pressure processing is an emerging method used to control microbial survival on processed meat products. The pressure disrupts bacterial membranes, and when used after packaging can be an effective method to reduce the overall microbial load on products and extend the shelf life. (Chen et al., 2012; Matser et al., 2004)

Curing and cooking have been used for centuries as a means to preserve meat, and continue to be important methods to ensure safety of products. Modified atmospheric packaging is also used. By storing products in vacuum packaging or in a modified atmosphere with limited oxygen available, it will select for anaerobic gram positive lactic acid bacteria instead of gram negative aerobic bacteria, such as *Pseudomonas* spp. which are associated with spoilage. Reducing the pH of a product through the addition of lactic acid is another effective antimicrobial hurdle, and can be obtained through the presence

of a lactic acid producing bacteria or the addition of lactate or acetate preservatives. Currently in the Canadian meat industry sodium lactate and sodium diacetate are some of the most widely used preservatives. Potassium lactate use is increasing as companies aim to comply with the new low-sodium goals put in place by the Canadian government (Health Canada, 2012).

1.3 *Listeria monocytogenes*

Listeria monocytogenes is a gram positive, non-sporeforming rod that is facultatively anaerobic. It is catalase positive and oxidase negative, with flagella production optimized at 20-25°C and reduced at 37°C (Peel et al., 1988). The optimal growth is at 37°C but *L. monocytogenes* can grow at temperatures as low as -0.4°C (Junttila et al., 1988). Due to its capability to grow at refrigeration temperatures, *L. monocytogenes* is a pathogen of concern for the food industry. Although the infectious dose is estimated at 10^8 CFU, the infectious dose for children, immunocompromised, pregnant and the elderly is estimated at 10^5 CFU. The mortality rate is high at 20-30%, and can cause spontaneous abortion during pregnancy (Public Health Agency of Canada, 2012). In 2008, there was an outbreak of listeriosis that originated from a contaminated slicer at Maple Leaf Foods Canada. There were 20 confirmed deaths (Health Canada, 2011). With reductions in sodium levels in processed foods, it is increasingly important to find new methods to prevent the growth of *L. monocytogenes* on ready-to-eat meat products. *Carnobacterium maltaromaticum* ATCC PTA-5313 has been found to have listeriocidal effects with its combination of organic acid production and bacteriocins, which may be a highly effective method to control *L. monocytogenes* in meat products (Martin-Visscher et al., 2011). The current CFIA guideline for an acceptable antimicrobial used on a ready to eat meat against *L. monocytogenes* is that "the agent allows no more than 2 log CFU/g

increase in *L. monocytogenes* throughout the stated shelf-life of the product." (Canadian Food Inspection Agency, 2013).

1.4 Biopreservatives

Lactic acid bacteria have been tied to food preservation throughout history, with their production of organic acids, carbon dioxide and alcohol playing a role in numerous fermentations (Axelsson, 2004). They have been used in fermented foods such as alcoholic beverages and cured meats for hundreds of years and continue to be an integral part of our food system (Deegan et al., 2006). Not only do the metabolites play a role in preservation by inhibiting the growth of spoilage organisms, but the growth of lactic acid bacteria can outcompete pathogenic bacteria by using up nutrients at a quicker rate, resulting in inhibition of undesirable cultures. They can be homofermentative, producing mainly lactic acid, or heterofermentative creating equimolar amounts lactic acid, ethanol and carbon dioxide from glucose (Axelsson, 2004). Lactic acid bacteria are non-sporeforming catalase negative bacilli, cocci or coccobacilli. They have a G + C content of 55% mol or less, and many species have GRAS status (Stiles & Holzapfel, 1997). They are ubiquitous and have been regularly isolated from plants as well as gastrointestinal tracts of animals and food products (Parada et al., 2007). Multiple species have been isolated from food products, including but not limited to *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc,* and *Oenococcus*.

As consumers and industry move towards products with lower levels of sodium, bacterial protective cultures are one way to increase the safety of ready-to-eat meat products. In addition to acids, carbon dioxide and ethanol, many LAB genera create antimicrobial compounds known as bacteriocins. These antimicrobial compounds that come from cultures regularly isolated from food are a potential way to increase the safety of ready to

eat products while also increasing consumer satisfaction in accordance with the demand for “clean label” products. (Galvèz et al., 2008). Furthermore, bacteriocins have been shown to have MICs that are lower than that of therapeutic antibiotics (Svetoch et al., 2008).

1.5 Bacteriocins

Bacteriocins are ribosomally synthesized peptides that have previously been thought to only be active against organisms similar to their producing cells; however bacteriocins that exhibit a broad range of activity have been characterized (Galvèz et al., 1986). The producing cells are immune to the toxic effects of bacteriocins and bacteriocins demonstrate low toxicity to eukaryotic cells (Cleveland et al., 2001). Due to their specificity, there is more interest in the application of bacteriocins that target specific pathogens compared to the wide range effect of antibiotics.

There are four classes of bacteriocins characterized according to size, structure and mode of action (Cotter et al., 2005). As knowledge of the structure and function of bacteriocins expanded, the classes have changed and redefined, with the Class I and class II bacteriocins being the most well-defined. Class I, the lantibiotics, contain unusual amino acid residues lanthionine and methyllanthionine. One such bacteriocin is nisin, which has been available for use in food and used since 1969 when approved by the World Health Organization (Deegan et al., 2006). Nisin was approved for use in Canada in 2013 (Health Canada, 2014). Class II bacteriocins are heat stable peptides smaller than 10 kDa. Many have been shown to have potent anti-listerial activity, and aside from cleavage of a leader peptide, they do not undergo post-translational modification (Drider et al., 2006). The class III bacteriocins are considered to be large, heat labile peptides. Whether or not this group should be considered bacteriocins has been questioned

(Cotter et al., 2005). Class IV bacteriocins are a newly emerging class of bacteriocins known as circular bacteriocins. These bacteriocins undergo backbone cyclization and have been found to be resistant to heat, pH and proteases (Maqueda et al., 2004; Maqueda et al., 2008; Martin-Visscher et al., 2008). The following review focused on class II and class IV bacteriocins only, due to their relevance to the current research.

1.5.1 Class II Bacteriocins

Class II bacteriocins are commonly divided into class IIa, which are considered "pediocin-like", class IIb, which have two peptides, and class IIc which are "other". The class IIa bacteriocins are among the most studied due to their range of activity and potential applications (Ennahar et al., 2000). The cationic and heat stable peptides that make up the class IIa bacteriocins have high anti-listerial activity (Drider et al., 2006; Ennahar et al., 2000; Quadri et al., 1994). Although the bacteriocins only require the transcription of a bacteriocin precursor, a transporter or signal peptide to export the bacteriocin out of the cell, an immunity protein and an accessory protein, they can also have regulated production. For regulated production there must be the production of induction factor and the bacteriocin transported out of the cell by an ATP binding cassette. The induction factor will interact with membrane bound histidine protein kinase and at a threshold it triggers phosphorylation of a response regulator. Once the response regulator is phosphorylated it stimulates gene transcription. (Drider et al., 2006; Ennahar et al., 2000; van Belkum & Stiles, 2000). The regulation of piscicolin 126 and carnobacteriocin BM1 are controlled by this quorum sensing method (Gursky et al., 2006; Rohde & Quadri, 2006).

The class II bacteriocins create non-selective pores in the cytoplasmic membrane of target cells, resulting in depletion of ATP as well as the proton motive force (Drider et al., 2006; Ennahar et al., 2000; van Belkum & Stiles, 2000). All bacteriocin producing cells

have an immunity mechanism to protect them from their own bacteriocins. Immunity mechanisms are not fully understood but it has been found that the mannose PTS system is linked to bacteriocin resistance (van Belkum & Stiles, 2000) and that the majority of immunity proteins are found in the cytoplasm of cells, not membrane bound (Quadri et al., 1995). Some examples of class IIa bacteriocins are leucocin A, enterocin A, piscicolin 126 and carnobacteriocin BM1 (Ennahar et al., 2000).

1.5.2 Circular Bacteriocins

Circular bacteriocins are a relatively new class of bacteriocins. Although initially classified as class II bacteriocins (van Belkum & Stiles, 2000) it is now suggested that they have their own group, class IV (Maqueda et al., 2004). These bacteriocins are stable to heat, pH and proteases (Martin-Visscher et al., 2008) and have a wide range of antimicrobial activity. Currently, biosynthesis of circular bacteriocins is not well understood. A precursor peptide is formed and at some point the backbone undergoes cyclization. In the case of carnocyclin A the backbone has an amide bond linking the N and C terminus (Martin-Visscher et al., 2008). The immunity mechanism is also unknown although multiple putative immunity genes have been discovered (Maqueda et al., 2008). Enterocin AS-48 is one of the most well studied circular bacteriocins (Galvèz et al., 1986; Maqueda et al., 2004) as it was the first one discovered. Other circular bacteriocins include gassericin A (Kawai et al., 1998) and reutericin 6 (Toba et al., 1991).

The mode of action of many circular bacteriocins is not fully understood, aside from interacting with the membrane to form pores. Some of the mechanism of carnocyclin A, which is produced by *C. maltaromaticum* ATCC PTA-5313, has been elucidated. Carnocyclin A forms anion selective channels by interacting directly with the lipid bilayer. These channels are highly selective to chloride anions. Carnocyclin may have a

dual mode of activity similar to nisin, which interacts with a receptor protein as well as directly with the membrane (Gong et al., 2009).

1.6 *Carnobacterium maltaromaticum*

Carnobacterium is a genus of lactic acid bacteria that are associated with dairy products, fish, meat and shrimp (Ahn & Stiles, 1990; Franzetti et al., 2003; Milliere & Lefebvre, 1994) and have been associated with environmental sources such as soil and permafrost ice (Pikuta et al., 2005). They produce a wide variety of metabolites, including bacteriocins, at refrigeration temperatures (Leisner et al., 2007). This increases their potential for application in ready-to-eat meat products and other refrigerated food products. *Carnobacterium maltaromaticum* ATCC PTA-5313 produces the class II bacteriocins piscicolin 126 and carnobacteriocin BM1 which are regulated by quorum sensing, and the circular bacteriocin carnocyclin A, for which the regulation mechanism is unknown. *Carnobacterium maltaromaticum* UAL26 produces piscicolin 126 and carnobacteriocin BM1. *Carnobacterium maltaromaticum* UAL8C2 is a non-bacteriocin producing variant of *C. maltaromaticum* LV17.

The strain of interest for this study was *C. maltaromaticum* ATCC PTA-5313 that has been shown to have potent listeriocidal activity (Martin-Visscher et al., 2008). This strain was originally isolated from fresh pork and it is believed that the circular bacteriocin, carnocyclin A, is responsible for the majority of the antimicrobial effect of the culture. Purified carnocyclin A has activity against numerous gram-positive cultures including several strains of *L. monocytogenes, Lactococcus lactis, Enterococcus faecalis,* as well as *C. maltaromaticum* UAL26 and LV17 and is active against gram-negative organisms after the outer membrane is damaged by EDTA (Martin-Visscher et al., 2011). As bacteriocins demonstrate a selective range of activity compared to broad range

processes used in food manufacturing such as heat or high pressure, they have potential for food preservation (Galvèz et al., 2008). In Canada, *C. maltaromaticum* ATCC PTA-5313 has been approved as a protective culture on ready to eat meat products and is commercially available from Griffith Laboratories Inc. (Government of Canada, 2010); however, there is no direct method for detection of the bacteriocins that are produced by this organism.

1.7 Bacteriocin detection in food

Traditionally, bacteriocins are detected with bioassays. These can vary; however, agar diffusion, turbidometric and spot-on lawn assays (Fleming et al., 1975; Hirsch, 1950; Wu & Li, 2007) are among the most common assays for bacteriocins. The spot-on-lawn employs an agar lawn with an indicator strain and a culture supernatant spot from the bacteriocin producing strain. Turbidometric assays involve a known indicator strain growth in broth and treated with varying levels of pure bacteriocin or culture supernatant. This provides arbitrary units of bacteriocin activity, but does not differentiate between the inhibition of organic acids and bacteriocins. With the addition of proteinase K to a spot-on-lawn-assay it can be inferred whether bacteriocins that are susceptible to proteases are responsible for antimicrobial activity. However, with increasing knowledge and discovery, bacteriocins that are resistant to protease have been discovered, such as carnocyclin A. Furthermore, there can be large variability depending on the age of the culture and the diluents or media used (Hirsch, 1950). Inhibition tests done in liquid media are more sensitive, and choosing an appropriate indicator organism is crucial for increasing sensitivity of tests (Papagianni et al., 2006). To accurately quantify bacteriocins for scientific research and to provide a validated method for industry and regulators to test for production of bacteriocins by a protective culture, a more specific and sensitive method for detection of production is required. This is of

particular importance in the complex food matrices that bacteriocins and their producing organisms may be used in.

Previously liquid chromatography/mass spectrometry (LCMS) has been used to successfully detect bacteriocins in liquid media (Zendo et al., 2008). A purification step was required as the broth that the cultures were grown in resulted in many impurity peaks, as such this may not be a plausible method to use for detection in food. They were able to differentiate amongst nisin variants, but were not able to quantify bacteriocin levels. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) has been used to detect bacteriocins in culture methods (Rose et al., 2001); however, these assays are not quantitative and when a food matrix is used more issues with purity of samples arise. Enzyme linked immunosorbent assay (ELISA) has been used with limited success in food applications with most of the work done on pediocin and nisin. There has been success in dairy products but with high variation (Daoudi et al., 2001) attributed to the small size of bacteriocins and the complicated food matrix. One drawback to ELISA methods is the need for antibodies that react with the bacteriocins. Many bacteriocins are not sufficiently antigenic for production of bacteriocins due to their small size (Lewis, 2001).

Quantitative polymerase chain reaction (qPCR) is one method that has the potential to be an accurate detection method for the production of bacteriocins in a food matrix. It has been widely used for bacterial detection in food products including pathogens such as *Staphylococcus aureus* in camembert and *Salmonella* in food, and has been used with DNA isolated from chicken carcasses, minced pork, beef, fish, raw milk and camembert cheese (Hein et al., 2001; Malorny et al., 2004). Malorny and colleagues (2004) were able to obtain detection limits that were as sensitive as traditional microbiological methods; however, qPCR results can be obtained in a much shorter time. Strains that

produce gassericin K7A and K7B have been detected using DNA isolated from faecal matter, a very complex matrix (Treven et al., 2013).

Unlike detection of pathogens or bacteriocin-producing strains, to use qPCR as a method to detect production of bacteriocins, RNA must be used to determine gene expression. Using DNA provides confirmation that the producing organism is present, not that it is actively producing one or more bacteriocins. This can be overcome by using RNA; however, isolation of high quality RNA from complex matrices such as food is a challenge. Detection of bacteriocin expression in broth has been done. In MRS broth, the expression of plantaricin S by *Lactobacillus pentosus* B96 was successfully quantified and there was an increase of bacteriocin expression throughout stationary phase (Hurtado et al., 2011). The expression of sakacin T has also been studied in MRS (Vaughan et al., 2004). The research group was able to quantify the expression of the bacteriocin gene and simultaneously examined bacteriocin activity using a bioassay; however, broth cultures are not as complex and challenging as a food matrix.

Studies have accurately quantified peptide gene expression in a food matrix but there are limited reports on expression of bacteriocin genes. The enzyme responsible for converting l-tyrosine to tyramine has been quantified by Torriani and colleagues (2008). Tyrosine decarboxylase (*tdc*) expression was evaluated in broth and fresh and fermented meat. Detection was very low in fresh meat, and could not accurately be quantified; however, on fermented pork sausage they were able to compare the expression of *tdc* in the presence of different concentrations of glucose and salt. The key to detecting expression of *tdc* was a modified RNA extraction protocol for the meat matrices. Trmčić and colleagues (2011) quantified the expression and analyzed the transcription of nisin genes in a cheese-like medium, being one of the few groups to study the transcription of

bacteriocins in food. Using a model cheese medium they quantified the expression of eleven genes in the nisin gene cluster using 16S and 23S rRNA as housekeeping genes.

There are significantly more studies that report the use of qPCR with DNA isolation to quantify bacteria from food matrices; however, the use of RNA provides different answers. The studies mentioned above that used isolation of RNA from food all required a modified RNA extraction protocol to isolate high quality RNA with little organic contamination. The expression of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 has yet to be studied in broth and in meat, and it is unknown which bacteriocins are actively produced on refrigerated ready-to-eat meat products.

1.8 Research objectives

There is a large potential for bacteriocins to be effective antimicrobials in food as they have strong antimicrobial properties and have the potential to contribute to "clean labels" that consumers demand. One of the current hurdles to overcome for acceptance and regulatory approval of bacteriocins is to be able to accurately detect and quantify their presence on a food product. The methods currently available are not specific enough to determine with confidence that a particular bacteriocin is present.

As a protective culture, *C. maltaromaticum* ATCC PTA-5313 has already been shown to be effective against *L. monocytogenes*; however, it is not certain which of the three bacteriocins is responsible for the potent antimicrobial effect. The effect of different preservatives on the expression of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 is also not known.

The objectives of this study were as follows:

1. To develop a novel method to detect the expression of carnocyclin A, piscicolin 126 and carnobacteriocin BM1 genes by *C. maltaromaticum* ATCC PTA-5313 on a ready-to-eat meat product using qPCR.
2. Determine the effect of preservatives commonly used on ready-to-eat meat products on the expression of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 *in vitro* and *in situ*.
3. Quantify the expression of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 on ready-to-eat meat products over an extended storage time, as well as in the presence of *L. monocytogenes* and common preservatives.

2 Materials and Methods

2.1 Culture Maintenance

Bacterial strains used in this study were obtained from the University of Alberta Food Microbiology collection and are listed in Table 1. All stock cultures were maintained at -80°C in 33% (v/v) glycerol with All Purpose Tween broth (APT, Difco, Beckton Dickinson, Sparks, MD) for strains of *Carnobacterium* and Tryptic Soy broth (TS, Difco) for strains of *Listeria monocytogenes*.

Strains of *C. maltaromaticum* were aseptically streaked onto APT agar (1.5% w/v) agar and incubated for 48 h at 22°C. One colony was picked from the plate, inoculated into 5 mL of APT broth and incubated for 24 h at 22°C. Fresh broth was inoculated with 1% (v/v) of the 24 h culture and incubated 24 h at 22°C prior to experimental use. Strains of *L. monocytogenes* were prepared in the same manner as the *C. maltaromaticum* except they were grown with TS (Difco) agar and broth and were incubated at 37°C.

Table 1 - Cultures used in this study

Strain	Characteristics	Reference
C. maltaromaticum ATCC PTA-5313 (also referred to as UAL307 or CB1)	Isolated from fresh pork, produces carnocyclin A, piscicolin 126, carnobacteriocin BM1	(Martin-Visscher et al., 2008)
C. maltaromaticum UAL26	Isolated from vacuum packaged ground beef, produces piscicolin 126, carnobacteriocin BM1	(Gursky et al., 2006)
C. maltaromaticum UAL8C2	Derived from *C. maltaromaticum* LV17, no bacteriocin production	(Ahn & Stiles, 1990)
L. monocytogenes FSL J1-177	Human isolate, sporadic case	(Fugett et al., 2006)
L. monocytogenes FSL C1-056	Human isolate, sporadic case	(Fugett et al., 2006)
L. monocytogenes FSL N3-013	Food isolate from an outbreak in the UK associated with pate	(Fugett et al., 2006)
L. monocytogenes FSL R2-499	Human isolate from an epidemic in the US associated with sliced turkey	(Fugett et al., 2006)
L. monocytogenes FSL N1-227	Human isolate from an epidemic in the US associated with a ready-to-eat meat product	(Fugett et al., 2006)

2.2 Inhibition assays

To confirm that the five strains of *L. monocytogenes* were sensitive to the bacteriocins produced by *C. maltaromaticum* ATCC PTA-5313, the activity of the culture supernatant was determined with spot-on-lawn assays. The sensitivity of *C. maltaromaticum* UAL26 was also tested to verify if carnocyclin A was being produced. In assays where *L.*

monocytogenes was used as an indicator, TS agar was used and APT agar was used when *C. maltaromaticum* was the indicator strain. To prepare the indicator lawn, the appropriate broth was prepared with 0.75% (w/v) agar and cooled to 55°C after autoclaving, and 1% (v/v) of culture was added. This was poured into a sterile plastic petri dish and allowed to dry. A fully-grown culture of *C. maltaromaticum* ATCC PTA-5313 was centrifuged (7000 x *g* x 10 min), the supernatant transferred to a new microcentrifuge tube and the pellet discarded prior to a second centrifugation. The supernatant was heated for 20 min at 75°C to destroy viable cells, and after cooling to 22°C, 20 μL was spotted on the soft agar lawn containing the indicator organism. Plates were incubated for 24 h at 37°C for *L. monocytogenes* and 22°C for *C. maltaromaticum* prior to being evaluated for zones of inhibition.

2.3 Growth of *C. maltaromaticum* ATCC PTA-5313 in the presence of preservatives

2.3.1 Preparation of media and inoculation

To determine if the expression of the bacteriocin genes in *C. maltaromaticum* ATCC PTA-5313 would be altered by the presence of preservatives commonly used in ready-to-eat meat products, four different preservative preparations were made. Tryptic soy broth was prepared according to manufacturer instructions and was supplemented with no preservatives (control), 2% (v/v) sodium lactate (Sigma-Aldrich, Oakville ON), 2% (v/v) potassium lactate (JT Baker, Phillipsburg NJ), 1.4% (v/v) sodium lactate with 0.1% (w/v) sodium diacetate (Sigma-Aldrich) or 1.4% (v/v) potassium lactate with 0.1% (w/v) sodium diacetate. These concentrations were chosen based on industry standards (personal communication, David Smith, Griffith Laboratories Inc.).

The TS broth with and without preservatives was inoculated with 1% (v/v) of a fully grown culture of *C. maltaromaticum* ATCC PTA-5313 and incubated at 15°C until the

OD_{600} reached 0.601-0.656 (Ultrospec 100 Pro Spectrophotometer, Biochrom Ltd, Cambridge, UK). Once the desired OD_{600} was achieved samples were subject to RNA stabilization, isolation and qPCR as described in Section 2.6. This experiment was done in triplicate.

2.4 Growth of *C. maltaromaticum* on vacuum packaged ham

To determine if the genes for three bacteriocins from *C. maltaromaticum* ATCC PTA-5313 are expressed on a refrigerated, vacuum-packaged deli product, a study with low sodium ham and pure cultures of *C. maltaromaticum* ATCC PTA-5313 was carried out. This was done to elucidate any change of bacteriocin gene expression at refrigeration temperatures on a ham product when compared to a broth culture. As *C. maltaromaticum* ATCC PTA-5313 has listeriocidal activity related to the production of bacteriocins as well as organic acids, this experiment will determine the expression of the bacteriocins *in-situ*.

2.4.1 Preparation of ham, inoculation and sampling

Cooked ham with a final sodium concentration of 0.6 % (w/w) was made according to the formulation in Appendix A. The dimension of each ham slice was 50 cm^2 surface area and 3 mm thick. After production and cooling, the ham was sliced, vacuum packaged and stored at 0°C for approximately 6 months prior to use.

Cultures of *C. maltaromaticum* ATCC PTA-5313, UAL26 and UAL8C2 were prepared as described in Section 2.1. The overnight culture was centrifuged (7000 x *g* x 10 min) and washed twice in 0.85% NaCl prior to serial dilution to 10^5 CFU/mL in sterile 0.85% NaCl. The initial inoculum was further diluted, plated onto APT agar and plates were incubated 48 h at 22°C to confirm cell counts. Ham slices were aseptically removed from vacuum packages and 100 μL of the diluted bacterial culture was applied to each slice to

produce samples with no inoculation (negative control), or inoculated with either *C. maltaromaticum* ATCC PTA-5313, UAL26 or UAL8C2. Sterile spreaders were used to distribute the target inoculum of 10^3 CFU/cm^2 on one side of the ham. The inoculated slice was aseptically transferred to a bag [Oxygen transmission rate: 52 cc/m^2 (24 h/dry @ 23°C); Allied Pak Inc. Scarborough, ON, Canada] and vacuum packaged (Model C200, Multivac Canada, Woodbridge ON). Packaged ham slices were stored at 4°C and samples were taken after 0, 7, 14, 21 and 28 days of storage. Duplicate packages of inoculated ham slices were made for each sampling time and the entire experiment was done in triplicate.

On each sampling day, two packages of each treatment were removed from storage, the package was wiped with 70% ethanol and opened with sterile scissors. To remove the bacteria from the surface of the ham 10 mL of sterile 0.85% NaCl was added into the package, directed onto the inoculated side of the ham slice. In lieu of stomaching, the saline solution was massaged by hand against the ham for 5-10 s. An aliquot of the saline solution was serially diluted and plated onto APT agar and incubated 72 h at 22°C to confirm cell counts. Another aliquot was taken for RNA isolation following the protocol described in Section 2.6.

2.5 Growth of *C. maltaromaticum* ATCC PTA-5313 in combination with *L. monocytogenes* on vacuum packaged ham

C. maltaromaticum ATCC PTA-5313 has been shown to be effective in vitro against various strains of *L. monocytogenes* (Martin-Visscher et al., 2011) but there is no published literature on the effect of this organism *in-situ*. It was important to evaluate the expression of bacteriocin genes in *C. maltaromaticum* ATCC PTA-5313 *in-situ* in the presence of preservatives and *L. monocytogenes*, as would be found in a commercially prepared product. This information has the potential to demonstrate if there is a link

between the growth of *L. monocytogenes* and *C. maltaromaticum* ATCC PTA-5313 with bacteriocin gene expression.

2.5.1 Preparation of ham, inoculation and sampling

Cooked ham with a total sodium level of 0.6% (w/w) across all formulations was made according to the formulation in Appendix A containing either sodium chloride only as a preservative or 1.4% (w/w) sodium lactate and 0.1% (w/w) sodium diacetate. All ham formulations were made in triplicate batches on separate days. Prior to use, the ham was sliced 3 mm thick, vacuum packaged and stored at 0°C for 1-2 weeks. The surface area of each ham slice was 50 cm^2 per side.

Bacteria cultures were grown as described in Section 2.1 and incubated 24 h at 25°C prior to use in experiments to avoid a shock to the cells when stored at 4°C. To create the 5-strain cocktail of *L. monocytogenes,* a one mL aliquot from each strain of *L. monocytogenes* was centrifuged (7000 x *g* for 10 min) and resuspended in 0.85% NaCl. The resuspended cultures were centrifuged (7000g x *g* for 10 min) and washed twice with 0.85% NaCl. Five hundred μL of each culture of *L. monocytogenes* that had been resuspended in saline was combined to create the cocktail with a concentration of approximately 10^9 CFU/mL. This cocktail was serially diluted to 10^5 CFU/mL in 0.85% NaCl to achieve a 10^3 CFU/cm^2 inoculum of *L. monocytogenes* on the ham. *C. maltaromaticum* cultures were centrifuged (7000 x *g* for 10 min) and washed twice with 0.85% NaCl prior to dilution to 10^6 CFU/mL to achieve an inoculum of 10^4 CFU/ cm^2 on the ham.

For inoculation, ham slices were aseptically removed from vacuum package bags and both formulas of ham (with and without preservatives) were inoculated with either:

- No culture (negative control)
- 100 μL *C. maltaromaticum* ATCC PTA-5313
- 100 μL 5 strain cocktail of *Listeria monocytogenes*
- 100 μL *C. maltaromaticum* ATCC PTA-5313 + 100 μL 5 strain cocktail of *L. monocytogenes*

Following inoculation, sterile spreaders were used to distribute the culture evenly over the slice of ham. When both *C. maltaromaticum* ATCC PTA-5313 and *L. monocytogenes* were inoculated on to ham they were put on individually. Sterile tongs were used to transfer the ham into a bag and the ham was vacuum packaged. After vacuum packaging, the packages were stored at 4°C for 0, 4, 7, 14, 21, 28, 35, 42, 49 and 56 days.

At each sampling time, duplicate samples of each treatment were removed from storage, and the package was wiped with 70% ethanol and opened with sterile scissors. To remove the bacteria from the surface of the ham, 10 mL of sterile 0.85% NaCl was added directly into the package. The saline solution was massaged against the ham for 5-10 s. An aliquot of the saline solution was serially diluted and 100 μL of three dilutions were plated onto Cresol Red Thallium Acetate Sucrose Inulin (CTSI) agar (Wasney, Holley, & Jayas, 2001), a selective media for *C. maltaromaticum,* (incubated 48-72 h at 22°C)*,* PALCAM agar with the *Listeria* selective supplement added (Oxoid Ltd, Basingstoke, UK) for *Listeria monocytogenes* (incubated 24-48 h at 37°C) or TS agar for total cell counts (incubated 48-72 h at 22°C). Another aliquot was taken for RNA isolation as per the protocol described in Section 2.6.

To verify the presence of bacteriocins on the ham, the ham slice was transferred to a sterile petri dish after being massaged with saline. An aseptic brass corer with a 4 mm radius was used to make 50 mm^2 tokens of each ham slice. These slices were placed in TS soft agar (0.75% agar w/v) inoculated with 1% (v/v) of either *C. maltaromaticum* UAL26 or the 5-strain cocktail of *L. monocytogenes* as indicator strains. After incubation for 24 h at 22°C (*C. maltaromaticum*) or 37°C (*L. monocytogenes*), the plates were inspected for zones of inhibition.

2.6 Quantification of gene expression of *C. maltaromaticum* ATCC PTA-5313 bacteriocin production

2.6.1 RNA isolation

RNA was stabilized with RNAprotect (Qiagen, Toronto, ON). For cultures grown in broth, 500 μL of the culture was used. Cultures isolated from meat had 500 μL taken from the 0.85% saline solution after massaging the ham slice. The culture aliquot was combined with 1 mL RNAProtect, vortexed for 5 s, incubated for 5 min at 22°C and centrifuged at 20,800 x *g* for 5 min. Following centrifugation, the supernatant was discarded and the residual liquid was disposed of by inverting the tubes on paper towel. Cell pellets were stored at -80°C until RNA isolation was performed.

2.6.2 RNA extraction

RNA extraction was carried out using RNeasy Mini Kit (Qiagen) with a modified protocol for Gram-positive bacteria as well as extra washes to ensure removal of residual salts from the isolated RNA. Cell pellets treated with RNAProtect were thawed at 22°C for 5 min. The RNAProtect procedure was repeated on the cell pellets, omitting the incubation period. Pellets were vortexed (15 s) with 200 μL TE buffer containing 30 mg/mL lysozyme (Sigma-Aldrich) and incubated at 37°C for 60 min to ensure sufficient disruption of the cell wall. Following incubation 700 μL lysis buffer RLT (contains

guanidine thiocyanate) containing 1% v/v beta-mercaptoethanol was added and the sample was vortexed prior to addition of 500 μL ethanol, which was mixed by pipetting. The lysate was transferred to a spin column (Qiagen) and centrifuged (8000 x *g* for 20 s). The flow through was discarded and 350 μL wash buffer RW1 was added to the spin column and centrifuged (8000 x *g* for 20 s). The collection tube was discarded, and 20 μL of a DNase solution containing 2 μL DNase 10x reaction buffer (Promega Corporation, Madison WI), 10 μL RQ1 DNase (Promega) and 8 μL RNase-free water was pipetted directly on to the column. The column was incubated at 37°C for 30 min followed by the addition of 350 μL buffer RW1 and incubated at 22°C for 5 min, and centrifuged (8000 x *g* for 20 s). The collection tube was discarded and with a fresh collection tube, 4 washes of 500 μL buffer RPE were completed with centrifugation (8000 x *g* for 20 s) following each wash. After the initial 2-3 washes the tubes were inverted and rolled to ensure the complete removal of residual salts. Spin columns were transferred to a new collection tube and centrifuged (8000 x *g* for 3 min) to remove residual ethanol. Columns were transferred to new 1.5 mL tubes and 30-35 μL RNase-free water was used to elute RNA from the column and centrifuged (8000 x *g* for 1 min). The eluted 30-35 μL of RNA was passed through the column twice to increase yield.

The NanoDrop Spectrophotometer (Model 2000C, ThermoScientific, Waltham MA) was used to analyze quality and yield of RNA immediately after extraction.

2.6.3 DNase treatment and Reverse Transcription

As an on-column DNase treatment alone did not yield RNA free of genomic-DNA, an off-column DNase treatment was used. RNA was diluted to 40 ng/μL for experiments described in Sections 2.3 and 2.5 due to high quantity of RNA isolated, and diluted to 20 ng/μL for samples isolated from ham due to the low quantity of RNA isolated. Following

dilution, 1.25 μL of each DNase I and 10x DNase I Buffer (Ambion, Life Technologies, Burlington, ON) were added to 10 μL of the diluted RNA and the sample was incubated for 40 min at 37°C. EDTA was added to a final concentration of 5 mM prior to DNase inactivation at 75°C for 10 min.

First strand cDNA synthesis and reverse transcription were carried out according to the manufacturer's protocol for SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Burlington, ON). First strand cDNA synthesis involved the combination of 250 ng random primers (Promega), 2 μL DNase-treated RNA, 1 μL 10mM dNTP mix (Invitrogen) and 9.5 μL of RNase free water. The mixture was heated at 65°C in a thermocycler for 5 min and held at 4°C for at least one minute. The tubes were centrifuged briefly prior to the addition of the reverse transcription mixture (Invitrogen) containing 4 μL 5x First Strand Buffer, 1 μL 0.1 M DTT, 1 μL RNase Out Recombinant Inhibitor and 1 μL Superscript III RT according to the manufacturer's instructions. The mixture was put in a thermocycler at 25°C for 5 min, 50°C for 60 min, 70°C for 15 min and stored at -20°C until used for qPCR.

2.6.4 Real Time Polymerase Chain Reaction

Quantitative RT-PCR was used to evaluate the expression of the genes for the bacteriocins produced by *C. maltaromaticum*. Primers were designed to target the structural genes of the bacteriocins with *cbnBM1* for carnobacteriocin BM1, *pisA* for piscicolin 126, *cclA* for carnocyclin A and *16S* for 16S rRNA as the endogenous housekeeping gene. All primers are listed in Table 2 and were synthesized by Integrated DNA Technologies (Coralville, IO). For *cbnBM1* and *16S* genes, primers were designed using Primer 3 (Untergrasser et al., 2012) and verified using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Gene expression was measured using

QuantiFast SYBR Green (Qiagen) and 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington ON).

Each qPCR reaction contained 12.5 μL of 2x SYBR green master mix, 10.5 μL of nuclease free water, 1 μL of 5 μM forward and 5 μM reverse primer mixture and 1 μL of template cDNA. Chromosomal DNA was used as a positive control and DNase free-RNA was used as a negative control to ensure the absence of residual genomic DNA. All cDNA samples were plated with technical duplicates, with the exception of experiments with RNA isolated solely from meat (Section 2.5) due to the number of samples needed to fit on one 96-well plate. The cycle used was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 20 s, 55°C for 15 s, 72°C for 30 s. This was followed by a melt curve analysis to ensure that amplification was specific.

Primer efficiency was determined by serially diluting *C. maltaromaticum* ATCC PTA-5313 chromosomal DNA 11 times. The slope of C_T x Log_{10}[DNA] was used to calculate the primer efficiency with the following equation where E is primer efficiency.

Equation 1 $E = 10^{(-1/slope)}$

Table 2 - Primers used in this study

Gene	Primer Name	Primer Sequence (5' – 3')	Amplicon (bp)	Reference
cbnBM1	BM1-F	TGCAACAAATTAATGGTGGAGC	186	This study
cbnBM1	BM1-R	AATGTCCCATTCCTGCTAAAC		This study
pisA	pisA-F	CGGCTCCACCTGTAGTCAA	189	(Miller & McMullen, 2014)
pisA	pisA-R	AAATGCAACTAACTACAGGAGG		(Miller & McMullen, 2014)
cclA	cclA-F	GCATATGGTATCGCACAAGGTACAGC	125	(Socholotuik, 2012)
cclA	cclA-R	GCTGTGAAGACACCTGATAAACCG		(Socholotuik, 2012)
16SrRNA	16S-F	GCATGATTCTTGAAGGAAAG	239	This study
16SrRNA	16S-R	CGAAAACCTTCTTCACTCAC		This study

2.6.5 Calculations to determine relative gene expression ratio

Results were analyzed using the method proposed by (Pfaffl, 2001).

Equation 2

$$\textbf{Relative Gene Expression ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (\ control\ -sample)}}{(E_{reference})^{\Delta Ct\ reference\ (\ control\ -sample)}}$$

Where E_{target} = Efficiency of the target gene primer (*cclA, cbnBM1* or *pisA*)

$E_{reference}$ = Efficiency of *16S* rRNA primer

ΔC_t target = C_t control – C_t sample

ΔC_t reference = C_t *16S* rRNA control – C_t *16S* rRNA sample

For samples from broth cultures (Section 2.3) the reference was a pure culture grown in TS broth to an OD_{600} of 0.601-0.656 at 15°C with no preservatives. For samples from ham inoculated with *C. maltaromaticum* with no additional preservatives or cultures (Section 2.4) the reference was a pure culture grown at 4°C in APT broth to an OD_{600} of 0.503 – 0.521. The reference condition for RNA isolated from vacuum packaged ham with or without preservatives and inoculated with *C. maltaromaticum* ATCC PTA-5313 and *L. monocytogenes* was RNA isolated from vacuum packaged ham formulated without preservatives and inoculated with *C. maltaromaticum* ATCC PTA-5313. The reference ham was stored for the same time as the ham inoculated with *L. monocytogenes*.

3 Results

3.1 Inhibition assays

Spot-on-lawn assays with the heated cell-free culture supernatant of an overnight culture of *C. maltaromaticum* ATCC PTA-5313 were done to determine appropriate indicator strains for bacteriocin activity. With *C. maltaromaticum* UAL26 as the indicator strain, a clear zone of inhibition was observed as shown on panel A in Figure 1. All five strains of *L. monocytogenes* (Table 1) had clear zones of inhibition as shown on panels B through H. Occasional small colonies were observed within the zone of clearing, particularly when the five strains of *L. monocytogenes* were used as a cocktail, as shown on panel G. When proteinase K was spotted adjacent to the heated supernatant of *C. maltaromaticum* ATCC PTA-5313, the zone of inhibition was greatly reduced for the 5-strain cocktail (panel H).

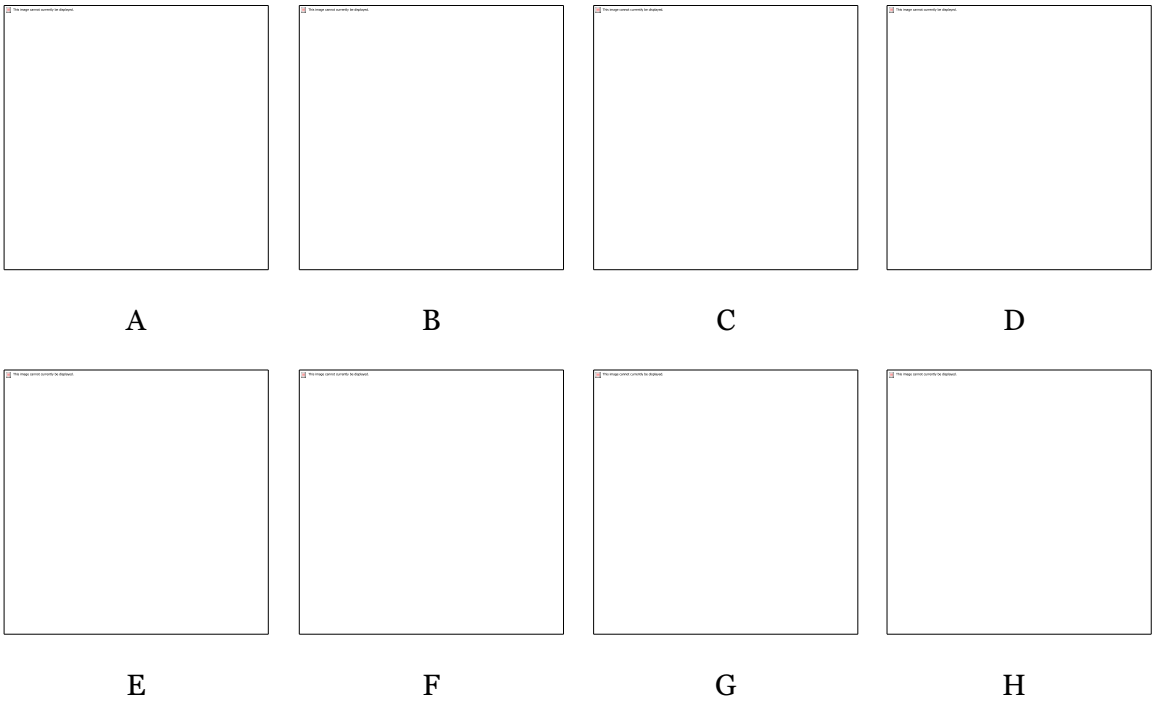

Figure 1 – Panel A – Inhibition of *C. maltaromaticum* UAL26 by the heated cell-free supernatant of *C. maltaromaticum* ATCC PTA-5313 on APT soft agar grown at 22°C for 24 h (A). Panels B – H show the inhibition of *L. monocytogenes* by the heated cell-free supernatant of *C. maltaromaticum* ATCC PTA-5313 on TS soft agar grown at 37°C for 24 h with different indicator organisms. B = *L. monocytogenes* J1-177, C = *L. monocytogenes* R2-499, D = *L. monocytogenes* C1-056, E = *L. monocytogenes* N3-013, F = *L. monocytogenes* N1-227, G = 5-strain cocktail of *L. monocytogenes* containing strains J1-177, R2-499, C1-056, N3-013, N1-227, H = 5-strain cocktail of *L. monocytogenes* spotted with proteinase K.

3.2 Primer design for endogenous qPCR controls

The primers chosen for the endogenous housekeeping gene targeted the *16S* rRNA; however, multiple other housekeeping genes were tested prior to deciding decision to use the *16S* rRNA as the housekeeping gene for qPCR. Multiple primers that targeted *rpoD,* which is a sigma factor involved with the transcription of most genes, were tested. However, *C. maltaromaticum* had two loci for *rpoD,* thus the expression was not

consistent. The gene responsible for maintenance and repair of DNA, *recA,* was also used and multiple primers were designed. When expression of *recA* was detected, it was very consistent and would only vary within 1 cycle to threshold; however, regardless of the primers used, the amplification was inconsistent. The expression was either very constant or there was no amplification. Previous studies have used *16S* rRNA and although it is not the ideal housekeeping gene (Desroche, Beltramo, & Guzzo, 2005) due to the anomalies with *recA* and *rpoD, 16S* rRNA was the best candidate.

3.3 Expression of bacteriocin genes in *C. maltaromaticum* ATCC PTA-5313 grown in broth with preservatives

Cultures were harvested in the late exponential growth and RNA extracted. All samples had a 260/280 nm ratio greater than 1.85. Out of 15 samples for all three replicates, the 260/230 nm ratio was greater than 1.9 for 11 samples, between 1.59-1.88 for 3 samples, and 1.12 for one sample.

Quantitative qPCR was used to determine if the presence of preservatives affected the expression of the structural genes of carnocyclin A (*cclA*), piscicolin 126 (*pisA*) and carnobacteriocin BM1 (*cbnBM1*). Gene expression for all three bacteriocins was quantified in in the presence of antimicrobials (Table 3). The expression of *cclA* and *cbnBM1* was increased in all treatments relative to the control whereas the expression of *pisA* decreased relative to the control sample except in the presence of sodium lactate and sodium diacetate.

Table 3 - Relative gene expression of *cclA*, *pisA*, *cbnBM1* in broth cultures grown with preservatives. n = 3. Means with different letters are significantly different ($p<0.05$).

Antimicrobial	Relative gene expression for each bacteriocin structural gene		
	cclA	*pisA*	*cbnBM1*
Sodium lactate	1.34 ± 0.63	0.38 ± 0.18	1.15 ± 0.03[b]
Potassium lactate	1.50 ± 0.86	0.65 ± 0.42	1.57 ± 0.56[b]
Sodium lactate + sodium diacetate	1.11 ± 0.44	1.12 ± 0.21	2.79 ± 0.67[a]
Potassium lactate + sodium diacetate	1.43 ± 0.50	0.53 ± 0.27	1.36 ± 0.32[b]

3.4 Expression of bacteriocin genes in strains of *C. maltaromaticum* on vacuum packaged ham

The bacteriocins produced by C. maltaromaticum ATCC PTA-5313 have been evaluated in different broth cultures; however, it is unknown which bacteriocins are produced at refrigeration temperatures in broth and on meat products. To evaluate the expression of bacteriocins during storage, C. maltaromaticum ATCC PTA-5313, UAL26 and UAL8C2 were inoculated independently onto ham, vacuum packaged and gene expression was evaluated during storage. The growth of C. maltaromaticum ATCC PTA-5313, UAL26 and UAL8C2 during 28 days of storage was determined by plate counts (Figure 2). Exponential growth occurred between 0-14 days of storage and stationary phase was reached after 14 days of storage for all three strains. The background microflora was less than 2 log CFU/cm^2 on the ham, as confirmed by plating ham samples without inoculation onto APT agar.

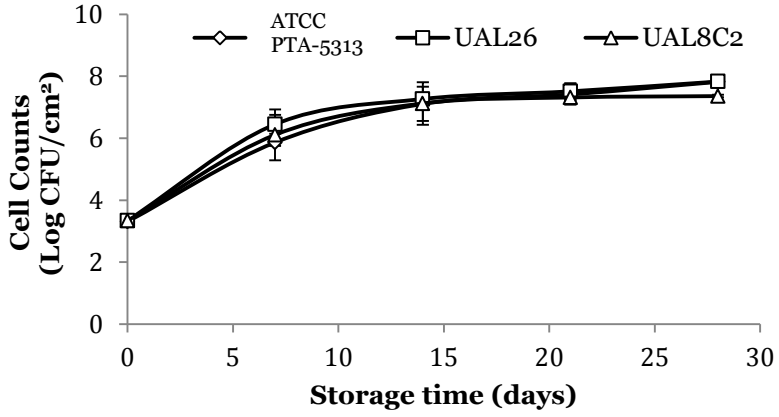

Figure 2 – Mean (± SD) counts of *C. maltaromaticum* ATCC PTA-5313, *C. maltaromaticum* UAL26, and *C. maltaromaticum* UAL8C2 during 28 days of storage on vacuum packaged ham stored at 4°C. Counts were enumerated on APT agar incubated at 25°C. n=3.

For comparison of gene expression on vacuum packaged ham to broth cultures, the reference condition was *C. maltaromaticum* ATCC PTA-5313 grown in APT broth at 4°C to an OD_{600} of 0.503-0.521. Figure 3 shows that in *C. maltaromaticum* ATCC PTA-5313, the expression of *cclA* was unchanged after 7 days of storage, while the expression of *pisA* and *cbnBM1* was decreased. After 14-28 days of storage, the expression of all three bacteriocins was decreased relative to the broth culture. There was no expression of any bacteriocins detected at the time of sample inoculation.

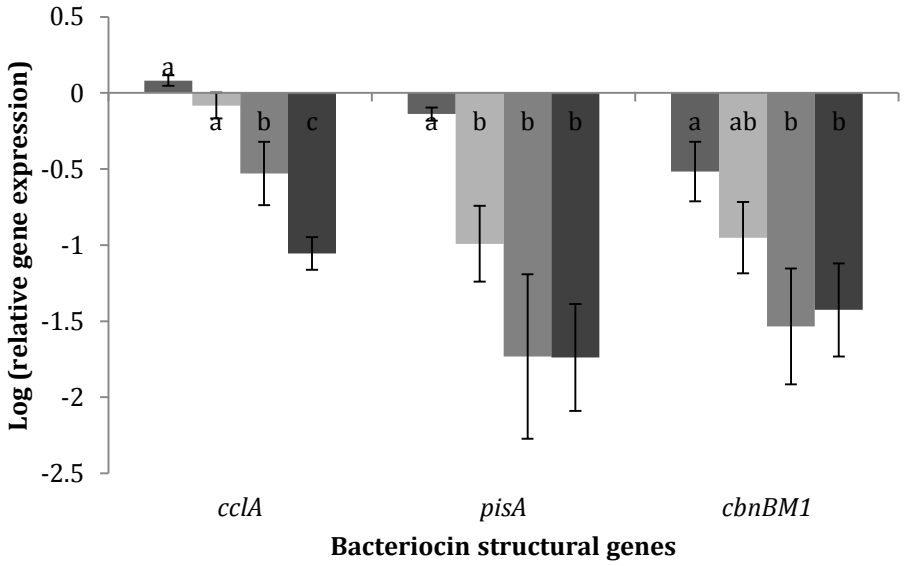

Figure 3 - Mean relative expression (± SD) of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 on vacuum packaged ham stored at 4°C for 7 (■), 14 (■), 21 (■) and 28 (■) days. The reference condition was *C. maltaromaticum* ATCC PTA-5313 grown in broth at 4°C to an OD_{600} of 0.503-0.521. Targeted genes were carnocyclin A (*cclA*), piscicolin 126 (*pisA*) and carnobacteriocin BM1 (*cbnBM1*). n=3. Data were analyzed with one-way ANOVA for each individual gene across storage times. Means with different letters are significantly different ($p<0.05$).

For *C. maltaromaticum* UAL26 on vacuum packaged ham, *cclA* was not expressed and *pisA* and *cbnBM1* were downregulated in comparison to the broth culture of *C. maltaromaticum* ATCC PTA-5313 (Figure 4). The expression of *pisA* and *cbnBM1* decreased throughout storage with the largest change in expression observed between 7 and 14 days of storage. There was no expression of bacteriocins at the start of the experiment (0 days of storage). There was no expression of any bacteriocins in UAL8C2 throughout the 28 days of storage (data not shown).

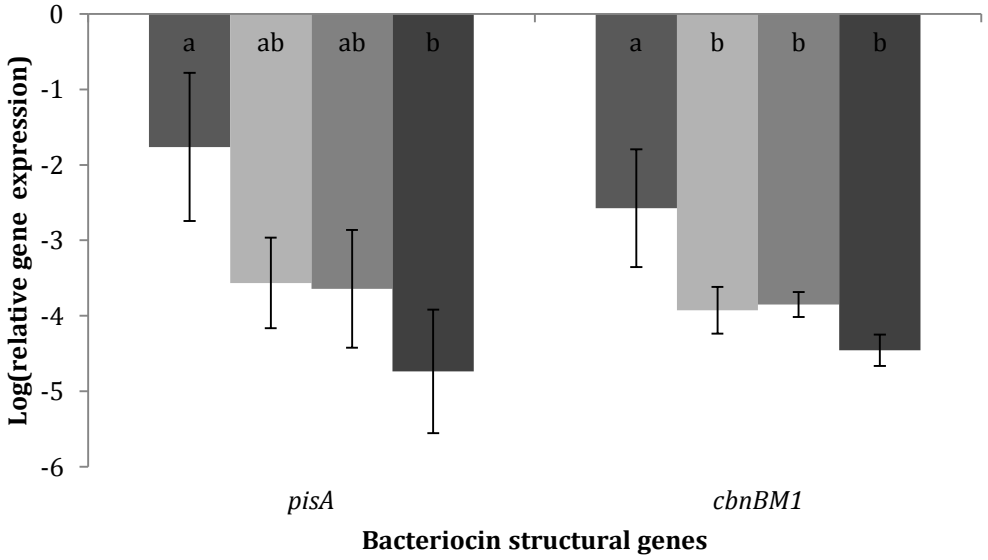

Figure 4 - Mean (± SD) relative expression of bacteriocin genes by *C. maltaromaticum* UAL26 on vacuum packaged ham stored at 4°C for 7 (■), 14 (■), 21 (■) and 28 (■) days. The reference condition was *C. maltaromaticum* ATCC PTA-5313 grown in broth at 4°C to an OD_{600} of 0.503-0.521. Targeted genes were carnocyclin A (*cclA*), piscicolin 126 (*pisA*) and carnobacteriocin BM1 (*cbnBM1*). Carnocyclin A expression was not detected. n=3. Data were analyzed with one-way ANOVA for each individual gene across storage times. Means with different letters are significantly different ($p<0.05$).

3.5 Expression of bacteriocin genes by *C. maltaromaticum* ATCC PTA-5313 with *L. monocytogenes* on ham with and without preservatives

C. maltaromaticum ATCC PTA-5313 has listeriocidal effects (Carlson et al., 2005); however, the expression of bacteriocin genes has not been studied in the presence of *L. monocytogenes* and *in-situ*. To investigate the change in bacteriocin gene expression, *C. maltaromaticum* ATCC PTA-5313 was inoculated onto ham formulated with or without preservatives, and with or without a 5-strain cocktail of *L. monocytogenes*. The growth of *C. maltaromaticum* ATCC PTA-5313 was not affected by the presence of *L. monocytogenes* (Figure 5); however, growth was slower on the ham with preservatives, and the stationary phase was reached later than the culture on ham formulated without preservatives. When *L. monocytogenes* was grown on ham with no antimicrobials or preservatives, it reached stationary phase after 35 days of storage. When it was in the presence of preservatives there was no increase from the initial inoculum of 3 log CFU/cm^2. When *L. monocytogenes* was grown in the presence of *C. maltaromaticum* ATCC PTA-5313 there was an initial rise in cell numbers after 4 days of storage followed by a 2 log decrease during the 56 days of storage. When it was grown in combination with *C. maltaromaticum* ATCC PTA-5313 and preservatives there was a 1.5 log decrease of *L. monocytogenes* during the 56 days of storage.

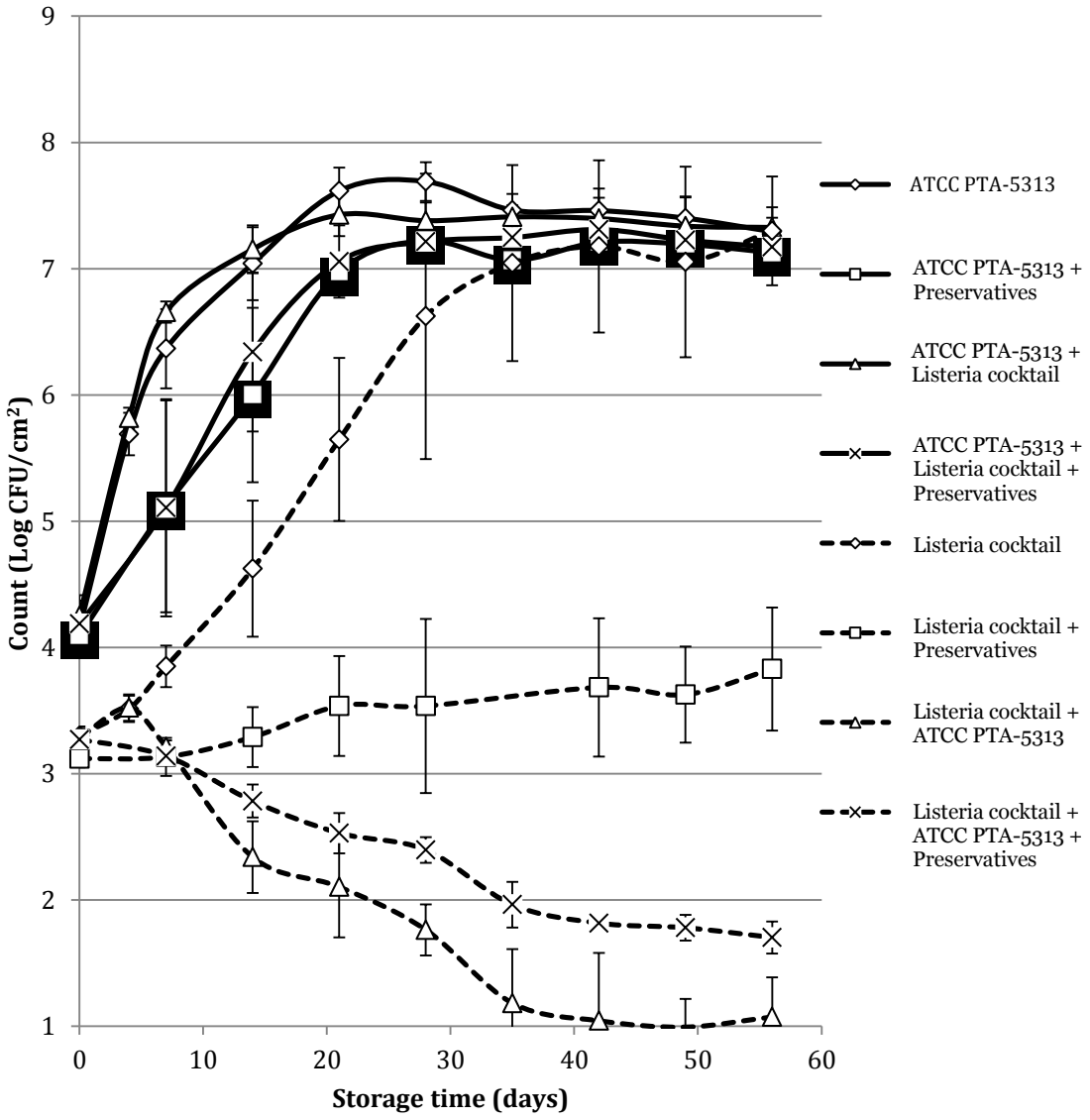

Figure 5 - Growth of *C. maltaromaticum* ATCC PTA-5313 and of the 5-strain cocktail of *L. monocytogenes* on vacuum packaged ham stored at 4°C with and without preservatives. Dashed lines represent the counts of *L. monocytogenes* enumerated on PALCAM agar and solid lines represent the growth of *C. maltaromaticum* ATCC PTA-5313 enumerated on CTSI agar. n=3.

Throughout storage the ham was tested for bacteriocin activity on a lawn of soft agar inoculated with either *C. maltaromaticum* UAL26 or a 5-stain cocktail of *L. monocytogenes* (Figure 6). When *C. maltaromaticum* UAL26 was used as an indicator, the zone of inhibition was indicative of carnocyclin A activity, as the indicator strain produces the same organic acids and two of three of the bacteriocins produced by *C. maltaromaticum* ATCC PTA-5313. When the 5-strain cocktail of *L. monocytogenes* was used as an indicator, the zone of inhibition is indicative of the antimicrobial effect of all metabolites of *C. maltaromaticum* ATCC PTA-5313. Similar results for the size of zones were obtained from all samples, regardless of storage time or treatment (data not shown). There were no zones of inhibition surrounding uninoculated ham cores overlayered with *C. maltaromaticum* UAL26 or *L. monocytogenes*.

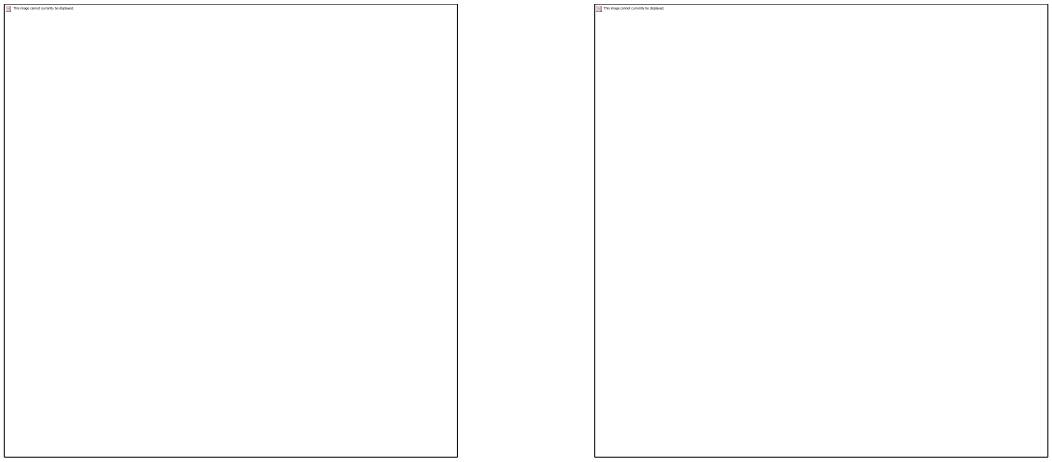

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Figure 6 - Antimicrobial activity of ham cores after storage at 4°C for 35 d overlayered with 1% (v/v) inoculum of *C. maltaromaticum* UAL26 (i) or ham stored 14 days and overlayered with 1% (v/v) of a 5-strain cocktail of *L. monocytogenes* (ii). Ham cores A and B are from two different slices of ham that had the same inoculation and had been stored for the same time.

The expression of carnocyclin A in *C. maltaromaticum* ATCC PTA-5313 is shown in Figure 7. There was no significant change of expression of carnocyclin A among different growth environments with the exception of days 14 and 28 where the expression of

carnocyclin in the presence of preservatives was downregulated. Over storage, the expression of carnocyclin A fluctuated and was statistically different on days 4 and 14, with day 7, 21 and 28 the same as each other and day 0 and 35 through 56 the same as each other. Carnocyclin A was consistently the bacteriocin that was expressed at the highest levels based on the C_T values (data not shown).

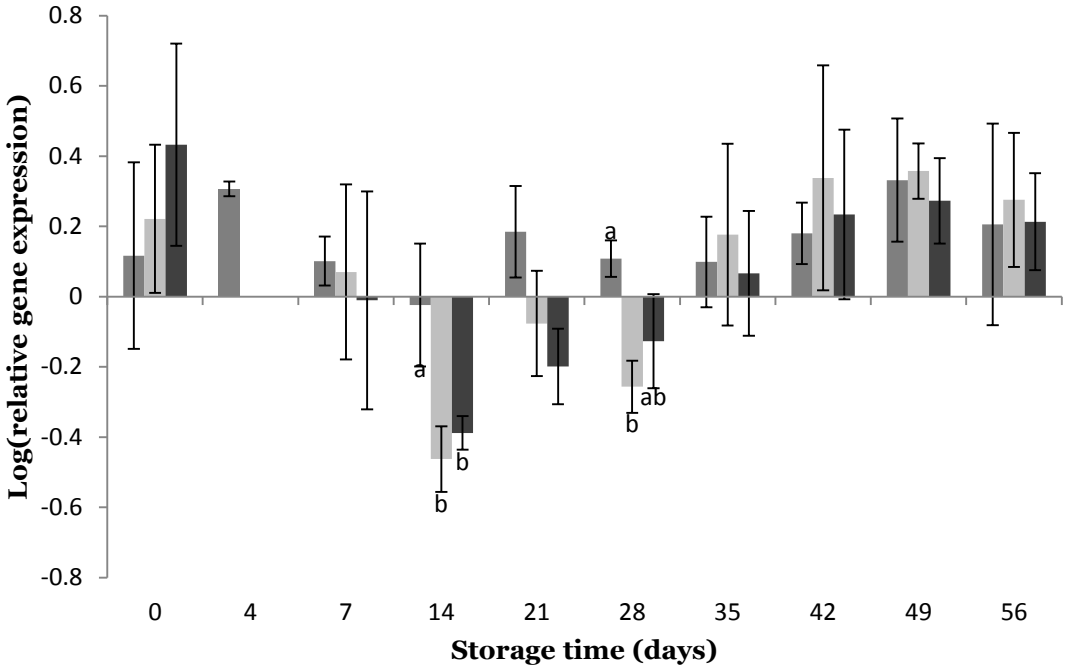

Figure 7 - Mean (± SD) of relative bacteriocin gene expression of carnocyclin A structural gene *cclA* by *C. maltaromaticum* ATCC PTA-5313 on vacuum packaged ham produced with or without preservatives and stored at 4°C with or without *L. monocytogenes*. *C. maltaromaticum* ATCC PTA-5313 in the presence of *L. monocytogenes* (■), on ham with 1.4% sodium lactate and 0.1% sodium diacetate (■), with both preservatives and *L. monocytogenes* (■); at each sampling time the reference condition was *C. maltaromaticum* ATCC PTA-5313 grown on vacuum packaged ham at 4°C without *L. monocytogenes* or preservatives. n=3. At each storage time, means with different letters are significantly different (p<0.05).

The relative expression of piscicolin 126 by *C. maltaromaticum* ATCC PTA-5313 grown on ham with and without *L. monocytogenes* and preservatives is shown in Figure 8.

There was no significant difference in expression of piscicolin 126 among treatments from 0 to 35 days of storage, and there was up-regulation of the gene expression in the presence of preservatives on day 42. With both *L. monocytogenes* and preservatives, compared to a culture with only preservatives, the increase of the expression of piscicolin 126 was not as large as with preservatives alone on day 42. The change in gene expression corresponded to the stationary growth phase of *C. maltaromaticum* ATCC PTA-5313. Across storage time, statistically days 0, 7 and 14 had the same gene expression levels, day 21 was unique, days 28 and 35 were the same, days 42 and 49 were the same and day 56 was unique.

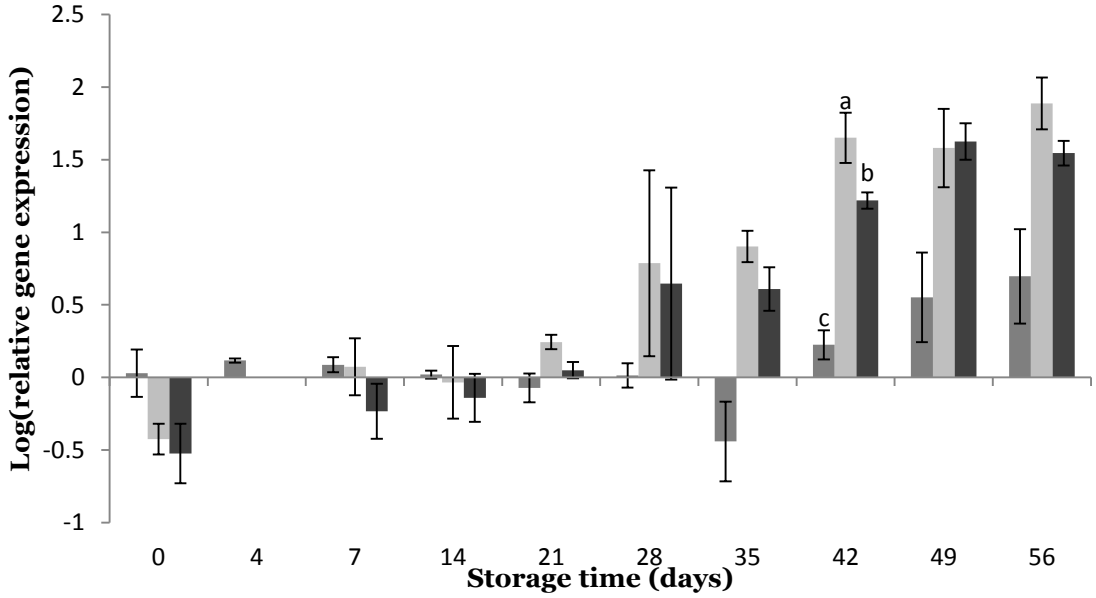

Figure 8 - Mean (± SD) of relative gene expression of the piscicolin 126 structural gene (*pisA*) by *C. maltaromaticum* ATCC PTA-5313 on vacuum packaged ham produced with or without preservatives and stored at 4°C with or without *L. monocytogenes*. *C. maltaromaticum* ATCC PTA-5313 in the presence of *L. monocytogenes* (■), on ham with 1.4% sodium lactate and 0.1% sodium diacetate (■), with both preservatives and *L. monocytogenes* (■); at each sampling time the reference condition was *C. maltaromaticum* ATCC PTA-5313 grown on vacuum packaged ham at 4°C without *L. monocytogenes* or preservatives. n=3. Means with different letters are significantly different (p<0.05).

Carnobacteriocin BM1 expression by *C. maltaromaticum* ATCC PTA-5313 grown on ham with and without *L. monocytogenes* and preservatives is shown in Figure 9. There was no significant difference among treatments from 0 to 35 days of storage, and there was up-regulation of carnobacteriocin BM1 in the presence of preservatives on day 42. The change in carnobacteriocin BM1 gene expression corresponded to the stationary growth phase of *C. maltaromaticum* ATCC PTA-5313.

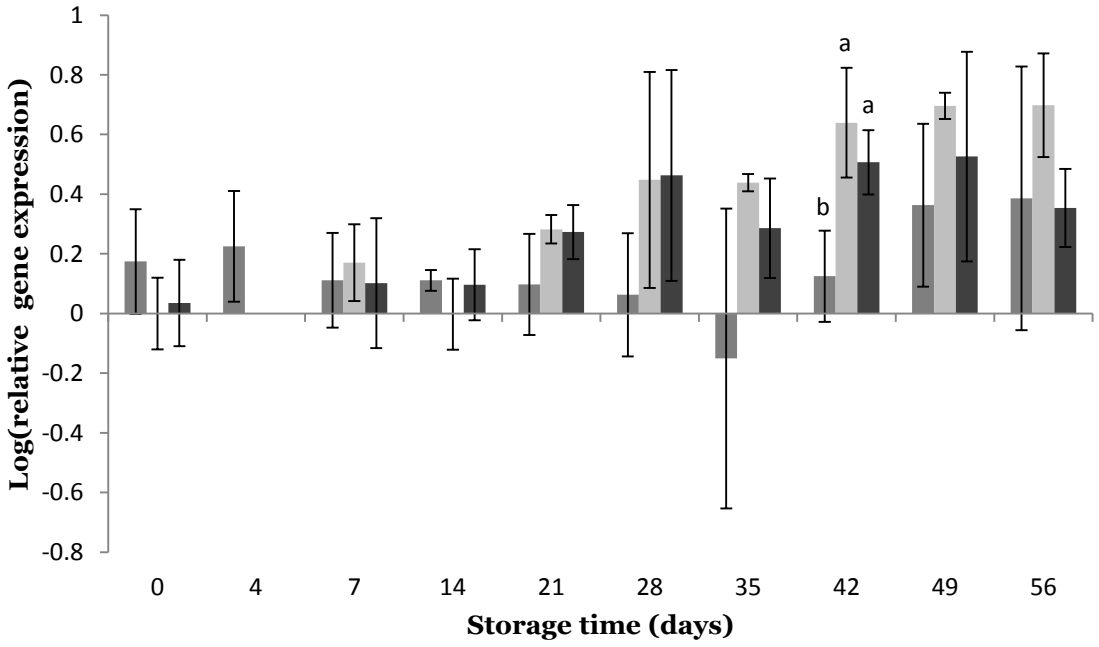

Figure 9 - Mean (± SD) of relative gene expression of the carnobacteriocin BM1 structural gene *cbnBM1* from *C. maltaromaticum* ATCC PTA-5313 on vacuum packaged ham produced with or without preservatives and stored at 4°C with or without *L. monocytogenes*. *C. maltaromaticum* ATCC PTA-5313 in the presence of *L. monocytogenes* (■), on ham with 1.4% sodium lactate and 0.1% sodium diacetate (■), with both preservatives and *L. monocytogenes* (■); at each sampling time the reference condition was *C. maltaromaticum* ATCC PTA-5313 grown on vacuum packaged ham at 4°C without *L. monocytogenes* or preservatives. n=3. Means with different letters are significantly different (p<0.05).

4 Discussion

The aim of this study was to develop a novel method to detect the expression of bacteriocin structural genes produced by *C. maltaromaticum* ATCC PTA-5313. Once such a method was developed, the research aimed to determine the effect of sodium and potassium lactate, and acetate preservatives on expression of the bacteriocin genes. Furthermore, the method for detecting gene expression was validated *in situ* on refrigerated meat products, and the impact of the presence of *L. monocytogenes* or preservatives on gene expression *in situ* was determined.

4.1 Inhibition Assays

C. maltaromaticum ATCC PTA-5313 and the isolated bacteriocin, carnocyclin A, have strong antimicrobial effect against numerous strains of *L. monocytogenes,* and inhibits the growth of *C. maltaromaticum* UAL26 (Carlson et al., 2005; Martin-Visscher et al., 2008). The bacteriocins produced by *C. maltaromaticum* ATCC PTA-5313 are effective against gram-negative pathogens, but only after the outer membrane has been disrupted (Martin-Visscher et al., 2011). The inhibitory effects shown in the current study mirrored that of previous studies, as *C. maltaromaticum* ATCC PTA-5313 inhibited the growth of five strains of *L. monocytogenes* and *C. maltaromaticum* UAL26. The addition of proteinase K reduced the activity of *C. maltaromaticum* ATCC PTA-5313 but did not completely eliminate it, indicating that the circular bacteriocin carnocyclin A is resistant to proteases (Martin-Visscher et al., 2008).

4.2 qPCR to determine gene expression

Quantitative polymerase chain reaction is a useful tool for food science, particularly with its applications for pathogen detection. It is a very sensitive technique able to detect as

few as 6 gene copies (Hanna et al., 2005; Hein et al., 2001). Food systems and feces are both complex matrices, and qPCR has been effective in both systems to study the transcription of genes or evaluate gene expression. The gene expression of various strains of lactic acid bacteria and pathogens has been studied by qPCR in foods including milk, soft cheese, Emmental cheese, cheddar cheese, fermented sausage, fresh sausage, minced beef, ready-to-eat meat products, ground beef, and sourdough (Bae et al., 2011; Desfosses-Foucault et al., 2013; Falentin et al., 2010; Falentin et al., 2012; Huefner et al., 2008; Rantsio et al., 2008; Taibi et al., 2011) as well as feces (Mokhtari et al., 2013).

One of the defining factors in qPCR is isolation of RNA of high quality, which is challenging from food matrices (Postollec et al., 2011). All of the studies mentioned above where food was involved had to use a modified RNA extraction protocol to achieve appropriate quality and integrity of RNA, with many of them requiring multiple purification steps. Furthermore, RNA degrades rapidly (Kennell, 2002) and it must be worked with quickly and carefully. Quality as measured by absorbance ratios at 260/280 and 260/230 nm indicates protein contamination and organic contamination, respectively. The standard of obtaining a 260/280 ratio greater than 1.8 and a 260/230 ratio greater than 2.0 (Thermo Scientific, 2008) was not fully achieved in this study. The 260/280 ratios were consistently higher than 1.9 and exceeded the standard. The majority of 260/230 ratios were higher than 1.8 with some samples between 1.5-1.8 and approximately 7% of samples lower than 1.5. As the majority of samples had ratios higher than 1.8 with duplicate samples and the experiment done in triplicate, it was deemed acceptable.

To achieve this level of quality, a modified RNA extraction protocol was required. Previous work (Socholotuik, 2012) used a Trizol reaction based on the work of Torriani and colleagues (2008) but was not able to achieve DNA-free RNA; however, Socholotuik

(2012) had success with the Qiagen kit. In this study, the Qiagen RNeasy protocol was modified to obtain higher quality RNA. In both broth and in cultures isolated from meat there was contaminating genomic DNA in the RNA after an on-column DNase treatment and 260/230 nm ratios were below 0.6. After the addition of extra Buffer RPE washing steps and an off-column DNase treatment, 260/230 ratios greater than 1.1 and as high as 2.1 were obtained with little to no contaminating DNA. Some groups have suggested that the RNA integrity number is more pertinent to qPCR quality, and that the RNA integrity will greatly affect the results for qPCR products that are greater than 400 base pairs (Fleige & Pfaffl, 2006). All products in this study ranged from 125-239 base pairs, and products ranging from 70-250 base pairs are not as influenced by RNA integrity (Fleige & Pfaffl, 2006). As a result, integrity number was not analyzed in the current research.

Choosing the appropriate housekeeping gene is essential for meaningful results in experiments using qPCR to measure relative gene expression. Previous studies with qPCR and *C. maltaromaticum* ATCC PTA-5313 have used the *16S* rRNA gene as an endogenous housekeeping gene (Gursky et al., 2006; Miller & McMullen, 2014). As rRNA is more stable than mRNA (Kennell, 2002), it can be preferable to use mRNA as a housekeeping gene, such as *recA*, *rpoD* or *ldhD* (Desroche et al., 2005; Schwab & Gänzle, 2006). Due to inconsistencies with primers, *16S* rRNA was chosen as the housekeeping gene, and it is still widely used in qPCR studies to study gene expression (Desfosses-Foucault et al., 2013; Falentin et al., 2010; Falentin et al., 2012; Huefner et al., 2008; Taibi et al., 2011). Further work could use mRNA as a housekeeping gene, and potentially use multiple housekeeping genes (Marco & Kleerebezem, 2008). Multiple housekeeping genes account for the difference in expression from exponential growth to stationary, as it has been found to be challenging to pinpoint a good endogenous control for the stationary phase of growth (Vandecasteele et al 2001). Ideally numerous

housekeeping genes should be evaluated for the specific bacterial strain of interest (Desroche et al., 2005).

4.2.1 Bacteriocin gene expression in broth with preservatives

C. maltaromaticum ATCC PTA-5313 was approved for use in meat in Canada (Health Canada 2010) and shows promise as an antimicrobial against *L. monocytogenes* (Carlson et al., 2005; Martin-Visscher et al., 2008). The current research evaluated if the presence of preservatives would affect the expression of bacteriocin genes *in vitro*. A temperature of 15°C was chosen as the cultures grow more rapidly than at 4°C; however, it is more indicative of how the cells may react at a refrigeration temperature compared to the 25°C used for optimal growth.

Extraction of high quality RNA is extremely important for all qPCR work. In experiments with broth cultures with preservatives, the control culture consistently had higher RNA quality, although the experimental cultures did meet the minimum standard. Initially all cultures were grown in APT; however, when the cells were grown in TS, the overall RNA quality was better and TS was used for all further work. There were difficulties acquiring consistent RNA quality with the cultures grown in the presence of preservatives compared to the control cultures. The silica gel columns used for RNA extraction bind based on optimal chaotropic salt concentrations as well as pH (Qiagen, 2014). The different components of the preservative preparations as well as the broth may have interfered with the binding capacity of the spin-column.

The presence of preservatives did not affect the expression of bacteriocin genes. Although the expression of *cclA* and *cbnBM1* increased, and expression of *pisA* decreased compared to a control with no preservatives, the margin of error was too large to make any conclusions. Hurtado and coworkers (2011) studied bacteriocin gene expression under stress and found an increase of bioactivity at 4% and 6% saline, and a

decrease with 8% saline. The maximum expression of the plantaricin S gene occurred in the stationary phase of growth in the presence of saline, regardless of the concentration, compared to lag phase when grown in MRS broth. This indicates that different levels of stress may affect the expression of bacteriocin genes. Vaughan et al. (2004) found that *L. sakei* grown in MRS had maximum expression of the sakacin X gene in the exponential phase of growth as measured by qPCR; however, expression remained steady throughout the stationary phase. In the current study, the differences in expression of bacteriocin genes among treatments with preservative were minimal, indicating that the effect of lactate and acetate preservatives on gene expression did not vary greatly among the four different preparations tested. The margin of error among replicates may be reduced if samples were collected at exactly the same cell density, or by preparing larger volumes of broth with preservatives to ensure that the preservatives were present in more consistent concentrations. In the current study, small volumes of preservatives were used and this could have impacted the ability to detect bacteriocin gene expression.

4.2.2 Bacteriocin gene expression of *C. maltaromaticum* grown on vacuum packaged ham

Using the modified RNA extraction and qPCR protocol, experiments were performed to determine bacteriocin gene expression in *C. maltaromaticum* ATCC PTA-5313, UAL26 and UAL8C2 throughout storage on a vacuum packaged ham product. *C. maltaromaticum* UAL26 and UAL8C2 were used as control cultures as *C. maltaromaticum* UAL26 produces both piscicolin 126 and carnobacteriocin BM1 whereas *C. maltaromaticum* UAL8C2 does not produce any bacteriocins. The stationary phase of growth was reached by 14 days of storage for all strains used in this study. The quality of RNA isolated was not as high as desired but was acceptable for the purposes of this experiment. The expression of all three bacteriocins genes in *C. maltaromaticum* ATCC PTA-5313 decreased throughout storage compared to the broth culture control,

and a similar trend was observed with the two bacteriocins of *C. maltaromaticum* UAL26. The decreased expression may be attributed to the change in growth phase, as the culture used as a reference condition was in late exponential growth phase when RNA was extracted whereas the culture on ham progressed through to the stationary phase. The expression of bacteriocins by *C. maltaromaticum* UAL26 is temperature dependent (Gursky et al., 2006), and although the antimicrobial activity of *C. maltaromaticum* ATCC PTA-5313 has been shown *in vitro* (Martin-Visscher et al., 2008) and *in situ* (Carlson et al., 2005), it was unknown as to which bacteriocins were actively produced on ready-to-eat meat products. Using qPCR, this study successfully detected the expression of all bacteriocins on vacuum packaged ham throughout 28 days of storage.

Gyu-Sung Cho and coworkers (2010) examined the expression of bacteriocins in *Lactobacillus plantarum* grown on raw turkey during 5 days of storage. They were able to quantify absolute gene expression on each day of storage when samples were examined. As the gene expression was absolute, it is hard to draw similarities between the this study and the current study; however, Gyu-Sung Cho (2010) did note that the expression of the bacteriocin gene was notably lower than the expression of *16S* rRNA, even when the culture was grown *in vitro*. Trmčić and colleagues (2011) investigated the expression of the nisin structural gene (*nisA)* in cheese using *16S* as the housekeeping gene. They found that the expression of *nisA* increased in stationary phase in cheese when using samples extracted from cheese as the control. This is the opposite of what was found in the current study; however, in the current study the reference was a broth culture whereas Trmčić et al. (2011) used a culture from the cheese.

4.2.3 Bacteriocin gene expression by *C. maltaromaticum* ATCC PTA-5313 with *L. monocytogenes* on ham with and without preservatives

When *C. maltaromaticum* ATCC PTA-5313 was combined with a 5 strain cocktail of *L. monocytogenes,* the growth of *C. maltaromaticum* ATCC PTA-5313 was not affected. When grown in combination with *C. maltaromaticum* ATCC PTA-5313, *L. monocytogenes* was reduced by 1-2 log over the 56 days of storage and numbers did not increase near the end of storage, similar to that reported by Carlson et al. (2005). Grown without *C. maltaromaticum* ATCC PTA-5313*, L. monocytogenes* reached stationary phase around 35 days of storage. When *L. monocytogenes* was grown on ham formulated with preservatives, and no antimicrobial culture, cell counts did not increase during storage, demonstrating that the use of the preservatives used in this study are an effective antimicrobial to prevent growth of *L. monocytogenes* on refrigerated vacuum packaged ham. However, the preservatives did not have the same ability to reduce numbers of *L. monocytogenes* as the addition of *C. maltaromaticum* ATCC PTA-5313 did. *C. maltaromaticum* ATCC PTA-5313 is an effective antimicrobial to control the growth of *L. monocytogenes* on refrigerated vacuum packaged meats.

Throughout 56 days of storage there was minimal change in the expression of carnocyclin A relative to the reference conditions. For piscicolin 126 and carnobacteriocin BM1 from 0 through 35 days of storage the expression of bacteriocin structural genes remained largely unchanged for *C. maltaromaticum* ATCC PTA-5313 grown in the presence of *L. monocytogenes,* preservatives, or the combination of both. There was an increase in expression of piscicolin 126 and carnobacteriocin BM1 in the presence of preservatives, most notably in the expression of *pisA*. The increase of expression corresponded with the stationary phase of growth of the culture with preservatives, compared to the reference culture which was in late stationary phase. This indicates that the expression of the carnocyclin A gene is stable in the presence of *L.*

monocytogenes and preservatives. The expression of piscicolin 126 and carnobacteriocin BM1 are upregulated during stationary phase in the presence of 1.4% sodium lactate and 0.1% sodium diacetate relative to a culture without preservatives. The production of piscicolin 126 is controlled by quorum sensing and carnobacteriocin BM1 uses the same transporters, whereas the regulation and production of carnocyclin A is independent. The stability of carnocyclin A and the similar changes in expression of both piscicolin 126 and carnobacteriocin BM1 may be due to the different regulatory mechanisms for bacteriocin gene expression.

When *C. maltaromaticum* ATCC PTA-5313 was grown in the presence of *L. monocytogenes* and preservatives, relative to a reference of the same bacteriocin-producing culture on ham with preservatives and no *L. monocytogenes*, there was a smaller yet significant increase of expression of piscicolin 126 and carnobacteriocin BM1 on day 42. This indicates that although the presence of preservatives increases the expression of the genes for the class IIa bacteriocins produced by *C. maltaromaticum* ATCC PTA-5313, the presence of *L. monocytogenes* reduces this effect. When not in the presence of preservatives, *L. monocytogenes* does not have any effect on the gene expression of *C. maltaromaticum* ATCC PTA-5313, indicating that the presence of preservatives had the greatest effect on gene expression of bacteriocins.

The trend of increasing expression of bacteriocin genes in the stationary phase of growth in the presence of preservatives on vacuum packaged ham observed in this study is similar to what has been noted by others who found an increase in the expression of bacteriocin genes in stationary phase when *Lactobacillus pentosus* was grown in the presence of saline (Hurtado et al., 2011) or when *Lactococcus lactis* bacteriocin gene expression was monitored during cheese production (Trmčić et al. 2011). It is possible that the increased osmotic stress of the preservatives in the ham may have resulted in the

increased expression of bacteriocin genes on RTE meats. The difference in gene expression may be partially attributed to the difference in growth phase when comparing cultures with and without preservatives, however in the presence of *L. monocytogenes* alone the growth of *C. maltaromaticum* ATCC PTA-5313 was not affected.

4.3 Conclusions

When grown in broth, of the presence of preservatives had no effect on bacteriocin gene expression in *C. maltaromaticum* ATCC PTA-5313 at 15°C.

C. maltaromaticum ATCC PTA-5313 is a LAB that effectively controlled the growth of *L. monocytogenes* on low sodium vacuum packaged ham. The presence of preservatives did not affect its ability to prevent the growth of *L. monocytogenes*. The potent anti-listerial effect has been attributed to three bacteriocins, and the current study demonstrated that all three bacteriocins are produced during storage of ham at refrigeration temperatures and the expression of all three bacteriocins decreased over storage time. During late stationary phase the presence of preservatives increased the expression of piscicolin 126 and carnobacteriocin BM1 relative to a culture without preservatives also in late stationary phase, indicating that during the overall decrease of expression of bacteriocins over storage time of a product, products formulated with preservatives may have less of a decrease of gene expression over time. Expression of the carnocyclin A structural gene remained unaffected by the presence of *L. monocytogenes* and preservatives but is expressed in the highest levels compared to the other two bacteriocins.

Using a modified RNA extraction procedure, qPCR was an effective way to rapidly quantify the gene expression of bacteriocins by *C. maltaromaticum* ATCC PTA-5313 on vacuum packaged meat products.

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Appendix A

A.1 Ham Formulation

3.8 kg total batch

- 3 kg lean meat
- 0.6 kg ice
- 19 g sodium triphosphate (STPP)
- 6.6 g sodium chloride – only for batch with preservatives
  - 3.8 g sodium diacetate (0.1% w/w)
  - 53.2 g sodium lactate (1.4% w/w)
- 36 g sodium chloride – only for batch without preservatives
- 14 g Prague powder (6% sodium nitrite, 94% NaCl)
- 3 g sodium erythorbate
- 93 g dextrose

A.2 Sodium calculations

To achieve 0.6% total sodium (Na), 22.8 g is required in a 3.8k g batch of ham.

Molecular weight of all ingredients containing sodium:

Na = 23 g/mol

Fresh pork = 55 mg /100 g

STPP = 367.84 g/mol

sodium nitrite = 69 g/mol

sodium diacetate = 142.08 g/mol

sodium ertythorbate = 198.11 g/mol

sodium chloride = 58.44 g/mol

sodium lactate = 112.06 g/mol

Total sodium in base recipe (without added NaCl or preservatives)

3 kg lean fresh pork = 3kg * 55mg/100g = 1.65 g Na

19 g STPP = 19 * 23/367.84 = 1.19 g Na

14 g Prague Powder = (14 * 0.06 * 23/69) + (14 * 0.94 * 23/58.44) = 0.28 + 5.18
= 5.46 g Na

3 g sodium erythorbate = 3 * 23/198.11 = 0.35 g Na

Total Na from base recipe = 1.65 + 1.19 + 5.46 + 0.35 = 8.65 g Na

Control batch of ham (0.6%, 22.8 g Na)

36 g NaCl = 16 * 23/58.44 = 14.17 g Na

Combined with base recipe = 8.65 g + 14.17 g = 22.8 g Na

Ham with preservatives (0.6% Na, 1.4% sodium lactate, 0.1% sodium diacetate)

53.2 g sodium lactate = 53.2 g * 23/112.06 = 10.92 g Na

3.8 g sodium diacetate = 3.8 * 23/142.08 = 0.615 g Na

6.6 g NaCl = 6 * 23/58.44 = 2.60 g Na

Combined with base recipe = 8.65 + 10.92 + 0.615+2.60 = 22.8 g Na