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Application of antimicrobials for the elimination of *Escherichia coli* and *Listeria monocytogenes* in brine injected beef

by

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Abstract

The application of antimicrobials to a brine-injected raw beef roast for the elimination/inhibition of heat resistant *Escherichia coli* and *Listeria monocytogenes* was investigated. The choice of antimicrobials for use in brine injected beef was based on minimum bactericidal concentration in brine solutions. Charsol® and MicocinX™, were added to the brine solution individually and in combination to evaluate possible synergy between the antimicrobials. In the brine solution, numbers of *E. coli* were not reduced by either antimicrobial; however, *L. monocytogenes* was reduced by more than 2 log CFU/mL in the presence of either antimicrobial, and no synergistic effect was detected. For the brine-injected beef, neither antimicrobial had any effect on numbers of *E. coli*, and the counts remained the same during 7 days of storage at 7°C. *L. monocytogenes* was unaffected by the Charsol®; however, counts were reduced when MicocinX™ was present and growth was inhibited during 4 days of storage.

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List of Abbreviations

APT	- all-purpose tween
BSE	- bovine spongiform encephalopathy
C.	- *Carnobacterium*
°C	- degrees Celsius
cc	- cubic centimetre
Cl	- chloride
CFC	- centrimide fucidin cephalosporin
CFU	- colony forming units
cm	- centimetre
cm^2	- square centimetre
d	- day
DNA	- deoxyribonucleic acid
E.	- *Escherichia*
EDTA	- ethylenediaminetetraacetic acid
EHEC	- enterohemorrhagic *Escherichia coli*
g	- gram
g	- acceleration due to gravity
h	- hour
HUS	- haemolytic uremic syndrome
IU	- international unit
L	- litre
L.	- *Listeria*
LAB	- Lactic acid bacteria
LB	- Luria-Bertani
m^2	- square metre
MBC	- minimum bactericidal concentration

MIC	- minimum inhibitory concentration
min	- minute
m	- milli
Na	- sodium
psi	- pounds per square inch
PCA	- plate count agar
RAPD	- random amplification of polymorphic DNA
RTE	- ready-to-eat
TBE	- tris borate EDTA
TSB	- tryptic soy broth
STPP	- sodium tripolyphosphate
µ	- micro
USA	- United States of America
v	- volume
VRBA	- violet red bile agar
VRBG	- violet red bile agar and glucose
w	- weight

1. Introduction and Literature review

In May 2003 a confirmed case of bovine spongiform encephalopathy (BSE) in a Canadian cow was recorded (Canadian Food Inspection Agency [CFIA], 2003). This single case of BSE caused the United States and other countries around the world to shut their borders to Canadian beef, resulting in a large surplus of beef in Canada; approximately 1.1 million live cattle and approximately 200 million kilograms of beef (Forge et al., 2005). Both producers and processors had to identify ways to market beef cuts traditionally destined for foreign markets. Two such methods involved further processing of certain beef cuts to increase both tenderness and moisture of these cuts (Pietrasik & Shand, 2005 and 2011). Specifically, meat tenderization technologies have been adopted by some producers. These technologies use either application of metal blades or brine injection using multiple needles that puncture the cut of beef and inject a brine solution that typically contains salt and phosphates. However, both processes potentially alter the safety of beef products. A cut of beef, either a roast or steak, is considered microbiologically safe to consume given that the outside surface is seared, which eliminates any bacteria present on the external surface, and that the sterile internal structure of the beef has not been compromised through any tenderization techniques. However, processing procedures such as mechanical blade tenderization and brine injection potentially transfer bacteria from the external surface of the meat to the internal structure, increasing the risk of foodborne illness for the consumer. As a result, novel solutions need to be found so that these products can be safe for consumption.

Escherichia coli is of considerable concern, both for consumers in terms of health and to beef processors in terms of monetary losses due to recalls. In 1993, there was an outbreak of *E. coli* O157:H7 across the western US linked to contaminated 'cooked' hamburgers. This outbreak resulted in hospitalization of 171 people; 41 of those patients developed haemolytic uremic syndrome (HUS) and four died (Centres for Disease Control, [CDC], 2001). This outbreak prompted a change in the US government's policy concerning *E. coli* O157:H7. The new policy is a "zero-tolerance" policy for *E. coli* O157:H7 in all non-intact meats; thus, if any *E. coli* O157:H7 is found in any non-intact meat product, the entire lot of product must be destroyed. When this policy change occurred, non-intact meat was generally considered to be ground meat; however, with the introduction of both mechanical tenderization and brine injection by processors, the policy was changed to include these products as non-intact meat (Food Safety and Inspection Service [FSIS], 1999). Legislation has now been changed in the USA to include any cut of beef that has gone through any type of blade tenderization or brine injection. Since 2000, there have been three outbreaks associated with mechanically tenderized beef products (CDC, 2011; Laine et al., 2005; Lewis et al., 2013). The 2012 outbreak in Canada was associated with steaks that had been blade tenderized by a retailer (Lewis et al., 2013).

Survival of *L. monocytogenes* is of great concern to the dairy (Ho et al., 2007) and ready-to-eat (RTE) (Public Health Agency of Canada [PHAC], 2008; USDA, 2003,) meat industries because these foods are generally consumed without further cooking. However, because *L. monocytogenes* can survive and grow at typical refrigeration temperatures (4°C) (PHAC, 2011) used for fresh meat, Peccio et al. (2003) and Bohaychuk et al.

(2006) reported approximately 15% of beef carcass sampled and 53% of retail ground beef samples were contaminated with *L. monocytogenes*, therefore the potential for listeriosis if heat-resistant *L. monocytogenes* survives cooking is of relevant concern. The risk of cross contamination is also a concern if fresh meat is contaminated with *L. monocytogenes* (Greer et al., 2004). The ability of *L. monocytogenes* to survive the cooking process in a raw meat product needs to be evaluated.

There is no doubt that non-intact meats, including brine injected products, pose a greater health risk to the consumer, thus interventions are needed to improve the safety of these products. When determining what interventions should be applied to control food pathogens, intrinsic factors associated with the bacteria must first be considered. Knowledge of how pathogenic bacteria survive and grow allows industry and researchers to tailor intervention steps to have the greatest impact against the pathogenic bacteria. *E. coli* is a gram-negative facultative anaerobic bacterium, commonly found in the gut micro-flora of warm-blooded animals. Its optimal growth is at 37°C but can grow at 49°C but, does not grow at 4°C or below. Transmission of *E. coli* is through the oral-fecal route which can happen in slaughter facilities when contamination is transferred from the animal hide to the carcass when the hide is removed (Fegan et al., 2005; Ramoneda et al., 2013). It has also been reported that *E. coli* O157:H7 can survive in acidic environments, such as apple juice (CDC, 1996). *L. monocytogenes* is a gram-positive facultative anaerobe that has the ability to grow at both low temperatures 1°C, across a range of pH values form 4.3 – 9.6 and can tolerate high levels of sodium chloride.

Using effective antimicrobials in the brine solution for brine-injected beef is one potential intervention that would allow the production of a safe product and minimize product recalls for processors. Hurdle technology can be used by industry when trying to provide a safe product for consumers. Hurdle technology is the application of combinations of preservation methods that eliminate or inhibit bacteria. When assessing the efficacy of antimicrobials against pathogenic bacteria it is important to determine potential synergy among preservation techniques. Synergy can be used as a hurdle as the combination of preservation techniques causes a greater reduction in bacterial counts compared to the preservation techniques alone (Cleveland et al., 2001). Synergistic effects can be observed with the use of EDTA and a bacteriocin from a gram-positive organism to inhibit gram-negative organisms. EDTA disrupts the outer membrane, which enables the bacteriocin to penetrate the cell wall (Cleveland et al., 2001). Schlyter et al. (1993) reported a synergistic relationship between sodium diacetate and the bacteriocin Pediocin AcH against *L. monocytogenes* at both room and low temperatures. It is essential that the right type of antimicrobial(s) be chosen, as it must be effective against both *E. coli* and *L. monocytogenes* while preserving the characteristics of the beef and must also be accepted by consumers, who are demanding a safe product.

1.1 Brine injection

Injection of fresh meat with brine compromises the safety of fresh meats due to the risk of translocation of pathogens into the internal tissues of the meat. Studies (Heller et al., 2007; Luchansky et al., 2008; Ray et al., 2010) demonstrated that if the surface of meat is contaminated, there is risk that the internal tissues of meat will be contaminated after the surface has been compromised. Heller et al. (2007) demonstrated that brine

injection increased the risk of translocation of *E. coli* from the surface of inside round steaks as compared to blade tenderization processes. Another concern is that the process of brine injection could result in an increase in the amount of bacterial contamination in brine, as recycled brine can be pumped into fresh meat. This increases the risk that meat that was once not contaminated may become contaminated. Bohaychuk et al. (2003) reported a 6% increase in *L. monocytogenes* contamination in moisture-enhanced pork loins in a process where brine was recycled throughout the production day. The pork loins that were sampled from the processing line were randomized and therefore the researchers were not able to determine the point in the process when the moisture-enhance loins were contaminated with *L. monocytogenes.* The re-circulating brine was not sampled during the process to determine if the brine was contaminated or when potential contamination of the brine occurred. As a result, no correlation between the contamination of the brine and the contamination of the pork loin could be made. However, Greer et al. (2004) determined that numbers of *L. monocytogenes* could increase in brine during recirculation of brines. Maximum numbers of *L. monocytogenes* (2.34 log CFU/100 mL) were reached after 2.5 h of production.

The concentration of salts in brine can have an impact on the survival of organisms that are present in meat. Alder et al. (2011) reported that a brine solution containing 5.5% sodium chloride (NaCl) and 2.75% sodium tripolyphosphate (STPP) had no impact on the survival of *E. coli* O157:H7 during storage of brine up to 24 h. Gill et al. (2009) injected steaks that had been inoculated with either *E. coli* or *Listeria innocua* with brine. The brine had a sodium tripolyphosphate concentration of 5% and contained 2 or 5% NaCl. Numbers of *E. coli* were reduced by 1 log in steaks injected with the brine

composed of 5% NaCl compared to numbers of *E. coli* in steaks injected with brine composed of 2% NaCl. The concentration of the brine had no effect on the survival of *L. innocua* in raw steaks.

1.2 Heat resistance of pathogens associated with fresh meat

Heat resistance in bacteria is generally defined as the ability of that organism to survive a particular temperature for a specific amount of time. This can be expressed as a decimal reduction time or D-value. The D-value is the thermal death time for a particular bacterium, and represents the time needed at a set temperature to reduce the counts of the bacteria by 90% (Huang, 2013). The existence of heat resistant *E. coli* is of great concern to the meat industry, as the ability of such organisms to survive cooking to the current recommended internal temperatures would result in an increased numbers of illnesses and deaths. The low infectious dose of enterohemorrhagic *E. coli* (EHEC) is a particular concern, as few cells are required to cause illness (Tuttle et al., 1999); thus there is a need for a complete kill of these bacteria in meat. Ahmed et al. (1995) determined that the D-values of *E. coli* O157:H7 strain 204P were significantly reduced as the cooking temperatures increased in a ground beef product. In addition, as the fat level in the meat increased, the D-values increased; leading to the concern that fat could act as a protectorate when determining heat lethality treatments (Ahmed et al., 1995). However, the small sample size (2 g) that was heated had a come-up time of < 1 min before timing of the experiment actually began. These two factors could have caused lower D-values to be recorded than observed in an actual hamburger patty or in brine-injected beef roast, where the rate of exposure to a thermal process is much slower.

Recent work in our laboratory demonstrated that the heat resistance among strains of *E. coli* can be highly variable. Liu et al. (2012) found that the heat resistance of 101 strains of *E. coli* was highly variable when strains were subjected to heating at 60°C for 5 min. Six of the verotoxigenic strains tested had less than a 5–log (CFU/mL) reduction when subjected to 60°C for 5 min in Luria-Bertani broth. *E. coli* AW1.7, a strain that was isolated from a beef carcass and is known to be highly heat resistant (Dlusskya et al., 2011), was reduced by less than one log (CFU/mL) when grown and heated in the presence of 1% NaCl (Liu et al., 2012). The addition of salt to brines used for injected meat products may increase the risk for consumers. As reported by Yoon et al. (2011), the addition of NaCl and STP to an *E. coli* O157:H7 inoculated ground beef sample that was heated to 65°C provided a significant increase in the heat resistance of the bacteria, up to a 2.6 log CFU/g difference in counts.

Concern with heat resistance in strains of *L. monocytogenes* is heightened with mechanically tenderized or brine-injected beef, as this process allows transfer of bacteria into the internal meat structure. With cooking to an internal temperature of ≤60°C, *L. monocytogenes* may survive and cause illness. Therefore it is important to evaluate different strains of *L. monocytogenes* at different cooking temperatures and in a variety of media to determine the heat resistance of these strains and to investigate how that heat resistance may change with changes in temperature and media.

When reviewing heat resistance of *L. monocytogenes* it is important to evaluate the reported heat resistance in not only a broth or liquid media but to also determine what the heat resistance is in both a food slurry and a food matrix, particularly a raw meat

slurry or meat matrix. Boyle et al. (1990) evaluated the heat resistance of *L. monocytogenes* Scott A, in phosphate buffer, meat slurry, and ground beef at internal temperatures ranging from 50°C to 70°C. Thermal destruction of *L. monocytogenes* Scott A at 60, 65, and 70°C, was faster in phosphate buffer than a meat slurry. The increased solids content in the meat slurry can affect the thermal destruction of bacteria (Bhaduri et al., 1991). *L. monocytogenes* was recovered in the meat slurry heated to 60°C and 65°C, although there was no recovery when ground beef was heated to either 50°C or 70°C. When a five-strain cocktail of *L. monocytogenes* in ground beef (including Scott A) was subjected to heat treatment at 55, 57.5 and 60°C, survival was observed at all temperatures (Juneja, 2003). These results show that strains of *L. monocytogenes* can survive a thermal process, and validate the concern that *L. monocytogenes* internalized through a brine injection process could survive a cooking process. Adding an extra hurdle such as antimicrobials to the brine solution could ensure that brine-injected beef cooked to a rare state would reduce the risk of survival of *L. monocytogenes* within the interior of the beef.

Cooking to an internal temperature of 71°C is recommended but thermal inactivation is highly dependent on cooking methods and product thickness (Shen et al., 2011). Determining the effect of the size and thickness of meat on the survival of brine injected beef is of concern as large cuts of beef, i.e. roasts, are generally cooked in contained heating systems, such as ovens, and that the entire surface is heated at the same time. In contrast, steaks are generally grilled or fried in a pan where only one side is heated at a time. This could be a factor in determining the safety of meat products as when a roast reaches its desired internal temperature it is generally allowed to rest prior

to cutting. This allows the heat from the thermal mass of the roast to continue to cook the roast and the internal temperature of the roast increases. However, when cooking a steak, there is not as much thermal mass, and therefore after a steak reaches the desired internal temperature, there may not be the same increase in internal temperature that is observed in larger cuts of beef. The endpoint cooking conditions for brine injected meats was specified by the USDA in the 1999 Food Code (USDA, 1999) based on temperature and time of holding; for example, 63°C for 180 sec. These guidelines were based on thermal destruction of *Salmonella*, which may not be adequate to ensure consumer safety in regards to brine injected beef, if *E. coli* is present. Dlusskaya et al. (2011) stated that a strain of *E. coli* isolated from a beef processing facility had a D_{60} value of 71 min. Gill et al. (2009) determined the minimum cooking conditions that would give a 6.5 log CFU reduction in steaks that were injected with broth or brines containing 2 or 5% NaCl with the same concentration of sodium tripolyphosphate and inoculated with a five strain cocktail of either non-pathogenic *E. coli* O157:H7 or *L. innocua*. They found that as the desired internal temperature of a steak increased there was a corresponding decrease the change of internal temperature during a resting period after cooking. Cooking to an internal temperature of 65°C eliminated both cocktails from the steaks. However, the steaks were heated on a laboratory hotplate covered with aluminium foil, which does not replicate conditions for cooking of steaks. This data needs to be validated under more realistic cooking conditions.

1.3 Antimicrobials as potential interventions in moisture enhanced meats

With the knowledge that pathogenic bacteria can contaminate a brine-injection system and that the presence of heat resistant pathogenic bacteria can be found on raw

meat, and that these bacteria can be internalized within the meat, a hurdle needs to be proven for the effective elimination of these pathogenic bacteria. Starting at the brine tank, this would allow industry the greatest opportunity to put in effective controls, as this would be the easiest method of application of the antimicrobials and would ensure the greatest amount of contact time between the antimicrobials themselves and the pathogenic bacteria that may be present in both the brine solution and on the meat. Therefore the application of antimicrobials to a brine solution to control the presence and growth of bacteria is critical because of the use of a re-circulating brine solution during needle injection. This process allows too great a risk of cross-contamination of bacteria from 'dirty' meat to 'clean' meat. If effective antimicrobials can be found that have the potential to not only reduce the counts of bacteria on the meat and in the brine solution, but also to impede the growth of the bacteria both in/on the meat and in the brine solution during processing, then the apparent risk that is associated with brine injected meat could be significantly reduced. It is also important that the antimicrobials that would be used in a brine-injection are or could be approved for the use in a raw meat product and that with the application of these antimicrobials there would not be any negative sensory impacts to the raw meat product. Before antimicrobials are chosen for use in brine-injected beef certain sensory characteristics need to be evaluated: 1. they must not effect the colour of beef as consumers shop with their eyes and an antimicrobial that causes the beef to turn brown or grey would deter consumers from purchasing the product; 2. the flavour of the beef must not be changed in such a way as to be unfamiliar to the consumer as a change in flavour profile may also ensure that the consumer does not re-buy the beef (Resurreccion, 2004).

Plant extracts containing phenolic compounds have been used in the food industry as both antioxidants and antimicrobials (Weiss et al., 2010). Although their applications in the food industry can be diverse, the drawback in using plant extracts is that they can impart both a flavour and colour change to the product that the consumer may find objectionable. Some commonly used extracts are herbs, spices and fruits, such as rosemary, oregano, green tea, cranberry, and cinnamon (Weiss et al., 2010). However, the use of plant extracts as antimicrobials can be difficult to assess, as the antimicrobial activity of the plant extracts can change based on; plant growth, harvest conditions, extraction method applied and bacterial species, *Bacillus* and *Staphylococcus* (gram-positive) or *Campylobacter* and *Salmonella* (gram-negative), that are being investigated, (Klančnik et al., 2009). Rosemary extract is used as a preservative in the food industry as the secondary metabolites of rosemary have been shown to have both antioxidant and antimicrobial properties (Klančnik et al., 2009). Klančnik et al. (2009) investigated the antimicrobial properties of rosemary extract, both water and oil-soluble, against both gram-positive and gram-negative foodborne pathogenic bacteria using various broth and agar dilution methods, and disk diffusion to determine the minimum inhibitory concentration (MIC) of the rosemary extract against the chosen bacteria. It was determined that the gram-positive bacteria were far more sensitive to the extract than the gram-negative bacteria, and that the oil-soluble rosemary extract had a lower MIC than the water-soluble extract for all methods tested. Given this information it would be important for further research to not only evaluate the efficacy of rosemary extract on inhibition of both *E. coli* and *L. monocytogenes*, but also to determine if the MIC values change when the rosemary extract is applied to a meat system. Cranberries have many

bioactive compounds such as phenolic phytochemicals, anthocyanins and organic acids. The use of cranberry concentrate against pathogenic bacteria has shown to be effective in not only significantly inhibiting the growth of pathogenic bacteria but also in the significant reduction of bacterial counts in ground beef. Qiu and Wu (2007) investigated the application of cranberry concentrate 10% (w/w) add to a ground beef sample that was inoculated (5 log CFU/g) with either; *Salmonella* Typhimurium, *E. coli* O157:H7, *L. monocytogenes*, or *Staphylococcus aureus*, and stored for 7 days at either 21°C or 7°C. At 7°C the cranberry concentrate significantly inhibited the growth of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* over the 7 days and significantly reduced counts of *Salmonella* Typhimurium after 3 days, below the detection limit (log 2 CFU/g). At 12°C storage Qiu and Wu (2007) reported that after one day of storage the cranberry concentrate had significantly reduced counts of all tested bacteria and counts of *Salmonella* Typhimurium and *E. coli* O157:H7 were below detection limit after one and five days of storage, respectively. Wu et al. (2009) further confirmed the efficacy of the cranberry concentrate against *E. coli* O157:H7 when they investigate the application of three different concentrations of cranberry concentrate (2.5, 5, 7.5% [w/w]) in ground beef that was inoculated at 6 log CFU/g, and stored for five days at 4°C. Researchers reported that 5% + 7.5% cranberry concentrate significantly reduced *E. coli* O157:H7 counts after three and five days of storage, at a concentration of 2.5% there was a significant reduction in *E. coli* O157:H7 counts only after five days of storage. Green tea is widely consumed across the world and a bioactive compound in the tea, catechin, has a beneficial effect on human gut microflora and an inhibitory effect against harmful bacteria (Juneja et al., 2009). Lee et al. (2009) investigate the application of green tea

extract (1% w/v) in TSB that was inoculated with either; *Bacillus cereus*, *Salmonella* Typhimurium, *Cronobacter sakazakii*, *E. coli* O157:H7, *S. aureus* or *L. monocytogenes*, and then incubated for 24 h at 22°C, samples taken at 0, 6, 12, and 24 h. Although the inoculum levels were not stated, based on 0 h samples, counts of the bacteria ranged from 1.6 log CFU/mL to 3.3 log CFU/mL. Researchers reported that the green tea only inhibited the growth of *B. cereus* over the 24 h incubation. Enumeration of the other bacteria showed that the green tea extract had no effect, and all bacteria had significantly increased in cell counts after 24 h. Juneja et al. (2009) investigated the effect of green tea on the D-values of *E. coli* O157:H7 inoculated (8 log CFU/g) into ground beef and heated to four separate temperatures (55, 58, 60, and 62.5°C). As the temperature increased there was a corresponding decrease in the D-values of *E. coli* O157:H7 compared to the control samples and the reduction in D-values were 40 – 70% depending on the temperature that was tested. Antimicrobials used for this research project were chosen based on regulatory approval for use in food and the active compounds generally found within these antimicrobials are listed in Table 1-1.

The inclusion of acids in a brine solution as antimicrobials has been thoroughly tested, examining the efficacy of the acids in not only fresh brine solutions but also in re-circulated brine solutions containing meat particulates. Alder et al. (2011) examined a variety of acids including lactic acid, acetic acid, citric acid, and hops beta acids, and their salt derivatives, sodium diacetate and potassium lactate, in combination with varying brine solutions (sodium chloride, sodium tripolyphosphate, and sodium pyrophosphate) in both fresh brine and a "meat slurry brine" to determine their efficacy for inhibition of *E. coli* O157:H7 at different temperatures and storage times. They

concluded that lactic, acetic, citric, and hops beta acids all significantly reduced *E. coli* O157:H7 over 24 h, in both the fresh brine and the meat slurry brine, at storage temperatures of 4°C and 15°C. Sodium diacetate and potassium lactate or the

Table 1-1 Antimicrobials and their corresponding active compounds.

Antimicrobial	Active compounds	Reference
Cranberry	Phenolic compounds - low-molecular weight phenolic acids - condensed tannins - proanthocyanidins - flavonoids - flavonols	Caillet et al., 2012, Puupponen-Pimiä et al., 2005
Green tea	Phenolic compounds - catechin	Lee et al., 2009
Rosemary	Phenolic diterpenes - carnosic acid - carnosol - rosmaridiphenol - rosmariquinone	Klančnik, et al., 2009, Georgantelis et al., 2007
Charsol	Carbonyl compounds	Red Arrow Products Company LLC, www.redarrowusa.com
	Acids - acetic acid Carbonyl compounds - 2-butanone Phenolic compounds - 2,6-dimethoxy phenol	Milly et al., 2005
MicocinX	Bacteriocins - carnocyclin A - carnobacteriocin BM1 - piscicolin 126	Martin-Visscher et al., 2011
PuraQ	Organic acid salts Acetic acid Sodium End products of fermentation (undefined – acids, bacteriocins)	Purac www.purac.com
EDTA	Chelator	Gill & Holley, 2000

combination of the two inhibited the growth of *E. coli* O157:H7 in both the brine and the meat slurry with brine at both temperatures over 24 h of storage; however, there was only a significant reduction of *E. coli* O157:H7 in the meat slurry with brine at 15°C when a combination of sodium diacetate and potassium lactate was used. They also found that although there was no decrease in numbers of *E. coli* O157:H7 in the brine solutions, the brine solutions containing sodium chloride and sodium tripolyphosphate did inhibit the growth of the *E. coli* O157:H7 over the same time and temperature. This means that the application of an acid in a brine solution has the potential to significantly decrease potential *E. coli* O157:H7 contamination that may occur in a brine solution, and that if a brine solution does become contaminated, the brine solution itself will inhibit the growth of *E. coli* O157:H7. However, this study only looked at stains of *E. coli* O157:H7, but it is not known what the impact of these brine solutions would have on other strains of EHEC. In addition, the experiment was done using small volumes of brine, and a prepared meat slurry versus a piece of brine injected meat. In addition, the experiments with the brine solutions were only replicated twice, which makes the significance of the results and the statistical analysis presented questionable. Application of needle-injected acid salts for the elimination of *E. coli* K12 on surface inoculated beef striploin steaks was carried out by Wicklund et al. (2007). Researchers wanted to determine if there was any synergy between the sodium lactate and sodium diacetate combination in eliminating the *E. coli* K12, and to determine if there was any significant difference in the growth of *E. coli* K12 on the steaks over the 28 day storage period among the three brine treatments: control (salt and phosphate), SL (salt, phosphate and sodium lactate), or SLDA (salt, phosphate, sodium lactate, and sodium diacetate). Brine solution that

contained the combination of both sodium lactate and sodium diacetate lowered counts of *E. coli* K12 below the detection limit for all sampling times (0-28 d), the SLDA solution decreased counts of *E. coli* K12 by at least 2 log CFU/g, given a 4 log inoculation and a detection limit of 2 log CFU/g. *E. coli* K12 counts increased during storage in samples treated with brine and brine plus sodium lactate solutions. Further studies evaluating at a variety of strains of *E. coli,* including those normally associated with foodborne outbreaks, would need to be done to validate if the addition of SLDA to brine solutions would be as effective on a broader range of strains of *E. coli*.

Ponrajan et al. (2011) evaluated the efficacy of sodium citrate plus sodium diacetate, and buffered vinegar in brine solutions to eliminate *E. coli* O157:H7 in brine injected beef. Beef round or sirloin were surface inoculated with a four strain cocktail of *E. coli* O157:H7 (6.4 log CFU/cm^2), injected with pre-contaminated (4.3 log CFU/mL *E. coli* O157:H7) brine solutions, vacuum packaged and stored for 10 days prior to enumeration and cooking to an internal temperature of 60°C. None of the brine solutions had any inhibitory effect on the *E. coli* O157:H7 in the re-circulating tank. However, sodium diacetate and the buffered vinegar solutions did significantly reduce the counts of *E. coli* O157:H7 in the top round beef, but only the buffered vinegar solution had a significant inhibitory effect on *E. coli* O157:H7 in the top sirloin. The top 1/3 of both cuts of beef had significantly higher counts compared to the lower 2/3, regardless of the brine solution. *E. coli* O157:H7 was not recovered after cooking regardless of the brine treatment. It cannot be ruled out that there is potential for heat resistant strains of *E. coli* to contaminate brine solutions and subsequently survive cooking. In this case, antimicrobials may be necessary to ensure safety.

Bacteriocins are peptides produced by strains of bacteria that have inhibitory effects against other similar bacteria. Examples include nisin produced by *Lactococcus lactis* subsp. *lactis*, pediocin produced by *Pediococcus* spp., and MicocinX™ which is a bacteriocin preparation containing the bacteriocins from *Carnobacterium maltaromaticum* UAL307. Bacteriocins have been used in conjunction with other antimicrobials to have a synergistic effect on the inhibition of pathogenic bacteria.

Solomakos et al. (2008) evaluated the efficacy of both nisin and thyme essential oil and their combination against strains of *L. monocytogenes,* Scott A and Lmk, inoculated into both tryptic soy broth (TSB) and minced ground beef stored under refrigeration storage conditions. Researchers used three concentrations of thyme essential oil (0.3, 0.6, 0.9%) and two concentrations of nisin (500, 1000 IU/g), and combinations of both antimicrobials in the inoculated TSB. For each concentration of either antimicrobial, the antimicrobial concentration significantly decreased the numbers of *L. monocytogenes* after 32 h of incubation compared to the control. The thyme essential oil at 0.6% and 0.9% caused significantly more inhibition against the *L. monocytogenes* cocktail than the 0.3% thyme essential oil. Both concentrations of nisin significantly reduced counts of the *L. monocytogenes* cocktail compared to the control and 1000 IU/g was significantly more effective than 500 IU/g nisin or 0.3% thyme essential oil. Solomakos et al. (2008) determined that the inclusion of thyme essential oil in minced beef at 0.9% caused "unacceptable organoleptic properties" and therefore was not used in later experiments. Experiments were done with 0.6% thyme essential oil in minced beef, individually and in conjunction with either concentration of nisin. Samples were stored at either 4°C or 10°C for 12 days. Solomakos et al. (2008) reported that 500 IU/g nisin had

no inhibitory effect on either strain of *L. monocytogenes* or the cocktail. The higher concentration of nisin had a significant inhibitory effect against both strains and the cocktail of *L. monocytogenes* compared to the control for both storage temperatures over the 12 days. They also found that the thyme essential oil at 0.6% was more effective against all strains of *L. monocytogenes* compared to nisin at 1000 IU/g. When both antimicrobials where evaluated together, a significant synergistic effect occurred for both concentrations of nisin with the thyme essential oil compared to the essential oil alone, and the essential oil with 1000 IU/g of nisin had a significantly lower population of all strains of *L. monocytogenes* compared to the combination of 0.6% and 500 IU/g of thyme essential oil and nisin, respectively, throughout storage at both temperatures. Future studies could also evaluate if the brine solution had any synergistic effect on the efficacy of either the thyme essential oil or nisin.

Adler et al. (2011) studied the application of nisin and pediocin, in combination with ethylene diamine tetra acetic acid (EDTA) in a brine solution and a meat homogenate solution (representing a re-circulated brine), to determine the efficacy of these antimicrobials on the survival of *E. coli* O157:H7 at two storage temperatures. They found that in the brine solutions at 4 or 15°C, the nisin + EDTA led to significant reduction in the numbers of *E. coli* O157:H7; however, pediocin + EDTA only significantly inhibited *E. coli* O157:H7 at 15°C. In the meat homogenate brine solutions at 4 or15°C both antimicrobial combinations significantly reduced counts of *E. coli* O157:H7 during 48 h of storage and the addition of nisin + EDTA to meat homogenate brine solutions stored at 4°C reduced counts below the detection limit within 8 h of storage. This study shows the potential for the use of nisin or pediocin, with EDTA to

inhibit gram-negative pathogens in brine and a meat homogenate solution; however, these results need to be verified on a larger scale using actual brine injected primal cuts of beef.

The application of MicocinX™ and nisin for the control of food spoilage organisms found in liquid whole egg was evaluated (Miller et al., 2010). The addition of either antimicrobial to liquid whole egg resulted in counts of total microflora and lactic acid bacteria (LAB) below detection limit after one day and remained below detection limit for the entire 56 day storage period. In control samples without the antimicrobials, bacterial counts increased during storage. Although this experiment was carried out in an egg product and focused on food spoilage bacteria, the application of the antimicrobials into a whole beef brine injection system could be warranted to help reduce bacterial growth during product storage. The use of antimicrobials to control both food spoilage and pathogenic bacteria would be very beneficial to the beef industry to extend storage life and improve the safety of brine injected or blade tenderized fresh meat products.

1.4 Research objectives:

The objectives of the research were to:

1. measure the heat resistance of multiple strains of *E. coli* and *L. monocytogenes* in broth and ground beef and select suitable strains for use as cocktails in brine injection experiments. The criteria for selection was heat resistance as heat resistant strains would represent the "worse case" scenario for further work on survival during cooking;

2. determine a means, using molecular methods, to differentiate among strains of *E. coli* and among strains of *L. monocytogenes*;

3. select appropriate antimicrobials and their combinations that could be used in brine injection and measure their efficacy and potential synergy against *E. coli* and *L. monocytogenes*. The criteria for selection of antimicrobials was that they have regulatory approval or a history of use in meat, so that a combination could be selected and applied without significant regulatory hurdles; and,

4. assess the efficacy of antimicrobials for control of *E. coli* and *L. monocytogenes* in brines and in brine injected beef during storage.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Strains of *Escherichia coli* used in experiments were AW1.3, AW1.7, GM16.6, GM18.6, GM18.4, and MB2.1 obtained from University of Alberta Food Microbiology Laboratory collection, Edmonton, AB. Verotoxigenic strains of *E. coli* used were O103:H2, O163:NM, O120:H5, O156:H7, 0111:NM, O91:H21, and O140:H14 [Agriculture and Agri-Food Canada (AAFC), Lacombe, AB]. Strains of *E. coli* O157:H7 that did not produce shiga toxin were 02-0628, 02-0304, 02-0627, and 00-3581 were kindly provided by Health Canada, Ottawa, ON. All strains were stored at -80°C in 20% (v/v) glycerol in Luria-Burtani (LB) broth (Difco™, Becton Dickinson, Sparks, MD). Before use in experiments, each strain was streaked onto LB agar, and incubated for 24 h at 37°C. For each strain, a single colony was used to inoculate 10 mL of LB broth, and cultures were incubated at 37°C for 24 h. Each culture was serially diluted in 0.1% (w/v) peptone (Bacto™ Peptone, Becton Dickinson, Sparks, MD), streak onto LB agar plates, and incubated at 37°C for 24 h to enumerate bacterial cells at stationary phase. For replication of experiments, all strains were cultured in triplicate from frozen stock cultures for each replicate.

Listeria monocytogenes strains used were HPB 65 and 642 were kindly provided Health Canada, Ottawa, ON; List 4 were kindly provided AAFC, Lacombe AB, FS 13, 14, 15, 19, 28, 30, 33, 38, and 45 (University of Alberta Food Microbiology Laboratory Culture Collection); ATCC 7644 and 15313 (American Type Culture Collection, Manassas, VA); and CDC 7762 (Centers for Disease Control and Prevention, Atlanta,

GA) were used in experiments. All strains were stored at -80°C in 20% (v/v) glycerol in Tryptic Soy broth (TSB; Becton Dickinson). Prior to use in experiments, strains were streaked onto TSB solidified with 1.5% (w/v) agar (Becton Dickinson), and incubated for 24 h at 37°C. For each strain, a single colony was then used to inoculated 10 mL TSB, and cultures were incubated at 37°C for 24 h. Each culture was serially diluted in 0.1% peptone, streak onto TSB agar plates, and incubated at 37°C for 24 h to enumerate the number of cells at stationary phase of each strain. For replication of experiments, all strains were cultured in triplicate from frozen stock for each replicate.

2.2 Heat sensitivity of strains determined in broth

To determine a baseline of heat resistance in the 15 strains of *L. monocytogenes*, trials were performed in broth culture. Broth cultures of all strains of *L. monocytogenes* were prepared as described in Section 2.1. After incubating for 24 h, *L. monocytogenes* were mixed by vortexing, and 100 μL of each strain was separately added into individual sterile Whirl-Pak® bags, 4 mil (*Nasco*, Fort Atkinson, WI). Cultures were spread evenly across the bottom of the bags, bags were closed by folding three times, and tabs were folded to seal. For heat treatment, bags were placed into a wire mesh basket and immersed in a 60°C water bath for 30 min. After heating, bags were immediately placed into an ice-water bath until samples were plated to determine numbers of survivors (within 60 min). To determine the number of surviving cells, serial dilutions of each sample were prepared in sterile 0.1% (w/v) peptone, and 20 μL was spotted onto the surface of TSB agar for enumeration of *L. monocytogenes*. All plates were incubated at 37°C for 24 h, at which time colonies were counted. Counts were converted to log CFU/mL. The experiments were replicated three times.

2.3 Heat sensitivity of strains determined in ground beef

Fresh [15%, 24%, and 35% (w/w) fat] ground beef was received from a local processor, and transported in coolers to the University of Alberta within 1 h, where it was stored at 3°C. Ten grams of ground beef were aseptically weighed into sterile Whirl-Pak® bags, air was removed by pressing, and bags were closed and stored at -20°C until needed. Before use in trials, samples were stored at 3°C for 24 h to ensure samples were completely thawed. Once ground beef samples were thawed, they were placed into an ice bath, and moved into a biological safety cabinet (Microzone Corporation, Ottawa, ON). For inoculation of the ground beef, each 10 g sample had 9 mL sterile 0.85% (w/v) saline (Fisher Scientific, Ottawa, ON) added, along with 1 mL of one of the bacterial strains of *E. coli*; AW1.3, AW1.7, GM16.6, GM18.6, GM18.4, and MB2.1, or *L. monocytogenes;* HPB 65 and 642, List 4, FS 13, 14, 15, 19, 28, 30, 33, 38, and 45, ATCC 7644 and 15313, and CDC 7762. Uninoculated samples had 10 mL of sterile 0.85% (w/v) saline added. The meat and culture and/or saline were mixed by stomaching (Stomacher Lab-Blender400, A.J. Seward, U A C House, London UK) for 1 min, and inoculated samples were placed into a water bath (Neslab EX7, Thermo Fisher Scientific, Newington, NH) at 60°C for 30 min. The previous methods were also used for sampling of verotoxigenic strains of *E. coli* O103:H2, O163:NM, O120:H5, O156:H7, 0111:NM, O91:H21, and O140:H14 and O157:H7; 02-0628, 02-0304, 02-0627, and 00-3581, however, these strains of *E. coli* were only tested in the 15% fat ground beef. Control samples were plated directly after mixing. After heating, inoculated samples were immediately placed into an ice water bath, and serially diluted in sterile 0.1% peptone. Enumeration of *E. coli* or *L. monocytogenes* was performed by spotting 20 μL onto the surface of LB or TSB

agar, respectively. Cultures used for inoculation of the ground beef were also plated onto appropriate agars to determine the count of the initial inoculum of each strain before being added to the ground beef samples. All plates were incubated at 37°C for 24 h, at which time colonies were counted. All counts were converted into log (CFU/g), and results were subjected to ANOVA using the general linear model in SAS (Version 9.3; Cary, SC), to determine differences among heat treatments. All experiments were replicated nine times.

2.4 Random amplification of polymorphic DNA (RAPD) analyses

Analysis by RAPD was done to differentiate among strains used in the experiments. DNA was extracted from pure cultures using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Mississauga, ON) using manufacturer's instructions for gram-negative (*E. coli*) and gram-positive (*L. monocytogenes*) bacteria. The presence of DNA was determined by gel electrophoresis in a 1.5% (w/v) agarose gel in 1X TBE buffer (Sambrook & Russell, 2001) run at 80V for 30 min. The gel was stained for 30 min in a solution of SYBR® Safe (Invitrogen™, Eugene, OR), and DNA was visualized using an imager (MultiImage® II, Alpha Innotech, San Leandro, CA). For RAPD analysis of *E. coli* strains, DAF4 primer was used and methods from (Dlusskaya et al., 2011) were followed; 94°C for 3 min; 3 cycles at 94°C for 5 min, at 35°C for 5 min, at 72°C for 5 min; 32 cycles at 94°C for 30 s, at 45°C for 2 min, at 72°C for 3 min; final extension at 72°C for 7 min. For *L. monocytogenes* strains, M13 primer was used, and methods from (Cocolin et al., 2005) were followed; 40 cycles at 94°C for 1 min, 45°C for 20 s, 72°C for 2 min; final extension at 72°C for 5 min. Samples were run on 1.5% (w/v) agarose gel in

1X TBE buffer, and the gel was for 90 min at 90 V. Bands were visualized by staining in a SYBR® Safe solution and viewing using an imager.

2.5 Determination of minimum bactericidal concentration of antimicrobial preparations with potential application in meat

The bactericidal effect of several preparations were tested against *E. coli* AW1.3, AW1.7, GM16.6, O140:H14, and O157:H7 (02-0304) and *L. monocytogenes* strains FS13, 14, 19, and ATCC 7644. Strains were cultured from frozen stock into 10 mL of either LB broth or TSB, respectively, and incubated for 24 h at 37°C. For each strain, a 0.1% inoculum was subcultured into LB broth or TSB, and cultures were incubated for 24 h at 37°C. All strains were subcultured twice prior to use in experiments.

Each strain of *E. coli* was grown individually in LB broth at 37°C for 24 h and centrifuged at 5000 x *g* for 15 min. Each strain of *L. monocytogenes* was grown in TSB at 37°C for 24 h, and centrifuged at 7500 x *g* for 20 min. Pellets were washed in 1 mL sterile 0.1% (w/v) peptone and centrifuged. The pellet for each strain was resuspended in 1 mL sterile 0.1% (w/v) peptone. When preparing the cocktails the previous methods were followed and then the two cocktails were prepared, by combining each strain of either *E. coli* or *L. monocytogenes* into sterile test tubes, having a total volume of 4 mL culture, one containing four strains of *E. coli* and one containing four strains of *L. monocytogenes*. Both single strains and the two cocktails were then used to determine the minimum bactericidal concentration of antimicrobials.

Stock solutions of green tea (5% w/v), cranberry (25% w/v), rosemary extracts (10% v/v), Charsol® RA07015 (6% v/v) were prepared in a solution containing 1.6% (w/v) NaCl and 1% (w/v) sodium tripolyphosphate (STPP). MicocinX™ (6% w/v) and

PuraQ® Verdad NV55 (5% v/v) with and without 5 mM EDTA were prepared in a solution with the same concentration of NaCl and STPP.

Due to the turbidity of the antimicrobial solutions, an assay for MBC was used instead of MIC to ensure accuracy of results. A microdilution assay was used to determine antimicrobial activity of individual compounds against the bacterial strains, and a checkerboard assay was used to determine synergism when combinations of antimicrobials were used against the cocktails of *E. coli* and *L. monocytogenes* (Nuryastuti et al., 2009). For the microdilution assays the following was done for each antimicrobial against each bacterial strain of *E. coli* or *L. monocytogenes*, all assays were done in sterile 96-well microtittre plates (Costar®3595, Corning Inc., Corning NY). The first column contained 100 μL of LB broth (*E. coli*) or TSB (*L. monocytogenes*), and 100 μL of the bacterial strain that was suspended in 0.1% peptone. In the second column, within each row, 200 μL of each antimicrobial was added. From these wells containing the antimicrobials, serial twofold decreasing dilutions occurred across each row in a solution of 1.6% NaCl and 1% STPP. Next each well containing a serially diluted antimicrobial had the corresponding bacterial strain for that particular microtittre plate (100 μL) added, to give a final 6 log CFU/mL concentration in each well. For the checkerboard assay the following was done. The first antimicrobial was added (100 μL) in the first row of a 96-well microtiter plate, in a twofold decreasing concentration; the second antimicrobial was added (100 μL) to the right column of the microtittre plate in a twofold decreasing concentration. This produced a 96-well plate containing serial twofold dilutions of each antimicrobial across both row and column; each bacterial cocktail (100 μL) was then individually added to each well. Further testing of the

bacterial strains against just the brine solution was done by the addition of each individual bacterial strain (100μL) to its own well and then the brine solution (100μL) was added into each of these wells. All plates, from both the microdilution and checkerboard assay were incubated at 37°C for 24 h, and 20 μL from each well was added to 180 μL of fresh LB broth (*E. coli*) or TSB (*L. monocytogenes*) in a new sterile microtittre plate. Plates were incubated at 35°C for 24 h, at which time turbidity of each well was determined visually. Turbid wells were considered positive for bacterial growth. Three replicates were done for each experiment. Synergism between antimicrobials was determined using the Fractional inhibitory concentration (FIC) and FIC index (FICI). And were calculated as follows (Odds, 2003).

FIC = MBC (Minimum bacterial concentration) of antimicrobial A when used in combination with
antimicrobial B / MBC of antimicrobial A when used alone.

FICI = FIC of antimicrobial A + FIC of antimicrobial B

Synergy was defined as FICI ≤0.5, no interaction as >0.5 – 4.0, and antagonist as >4.0.

2.6 Brine injection of beef roasts

A combined cocktail containing equal concentrations of four strains of *E. coli* (AW1.7, AW1.3, GM16.6, and O157:H7 type 02:0304) and four strains of *L. monocytogenes* (FS 13, FS 14, FS 19, and ATCC 7644) was prepared. Strains were chosen based on their heat resistance and the ability to differentiate among them using RAPD analysis. After each strain was grown in 10 mL of appropriate broth (Section 2.1), the larger volume required for inoculation into the roasts was obtained by successive sub-

cultures of a 0.1% culture into 100 mL and 4 L of appropriate broth, with incubation at 20°C for 24 h between each sub-culture. All strains were centrifuged in 1-L centrifuge bags (Beckman Coulter, Fullerton, CA) inserted into centrifuge bottles. *E. coli* strains were centrifuged at 5000 x *g* for 15 min at 6°C, while *L. monocytogenes* strains were centrifuged at 7500 x *g* for 20 min at 6°C. Following centrifugation, the cell pellets were re-suspended in 100 mL of sterile cold 0.85% (w/v) NaCl, and decanted into a sterile 1 L bottle. After the addition of each strain of *E. coli* (4 total) and each strain of *L. monocytogenes* (4 total) a further 200 ml of saline was added to the 1 L bottle and the bottle was thoroughly shaken to ensure the even distribution of the strains. This resulted in 4 x 1 L bacterial cocktails, each of which contained all eight strains of bacteria (four strains of *E. coli* and four strains of *L. monocytogenes*) each containing approximately log 8 CFU/mL. Culture cocktails were stored at 2°C until used in injection experiment within 4 h.

2.6.1 Preparation of brine containing antimicrobial treatments

To test the efficacy of different antimicrobial treatments in a brine injection system, five treatments were prepared in a cold-water brine containing 4.8% (w/w) NaCl and 3% (w/w) STPP. The five treatments were: brine-only (B); brine containing 1 L of bacterial cocktail, log 8 CFU/mL (BC); brine containing bacterial cocktail and MicocinX™ [BCM; 6% (w/w)]; brine containing bacterial cocktail and Charsol® RA07015 [BCC; 0.25% (v/w)]; and brine containing bacterial cocktail, MicocinX™ (6%) and Charsol® RA07015 (0.25%) (BCMC). Brine solutions were prepared 48 h prior to injection of meat, and were stored at 4°C; however, antimicrobials and bacterial cocktails were added just prior to injection of roasts. The pH (Beckman Coulter PHI 350

pH/Temp/mV Meter, Fullerton CA; with a Fisher Scientific Accumet® electrode; model 13-620-287A) and the concentration of bacteria in the brines were determined. For the brine, a 10 mL of solution of brine was removed just prior to injection and another sample was collected 10 min after brine injection of the roasts. For the brines containing bacterial cultures 10 mL aliquots were taken just prior to the addition of the bacterial cocktail, directly preceding the addition of the bacterial cocktail and then ten minutes post injection of roasts. For the two individual antimicrobial treatments and the treatment containing both antimicrobials, 10 mL samples were taken at time zero and then at each subsequent addition of either the bacterial cocktail or antimicrobial, and again at ten minutes post injection of the roasts. The 10 mL solutions were stored in sterile test tubes for determination of pH and then all samples were immediately buffered in 90 mL of cold (4°C) sterile 0.1% (w/v) peptone, and microbial analyses were performed as described in section 2.6.6.

2.6.2 Preparation of beef roasts

Vacuum packaged boneless beef striploins were received from a local supplier and stored at 4°C upon receipt. Each bag was opened aseptically, the loins were cut in half and the surface fat was trimmed to expose the longissimus muscle (roast), which was used in the injection trial. The roasts were vacuum-packaged in nylon/poly (7 layer) coex vacuum bag (35.56 cm X 45.72 cm, 4 mil, O_2 transmission rate of 48 cc/m^2; Allied Pak Inc., Scarborough, ON) using a vacuum packaging machine (Multivac model C200, Multivac Inc., Kansas City, MO), and stored at 0°C until injection (3 d).

2.6.3 Injection of beef roasts

Prior to injection, each roast was aseptically removed from its packaging and its weight and pH (Beckman Coulter, Fullerton, CA; using a Fisher Scientific accumet® electrode model 13-620-289) were recorded. For each brine solution, temperature was measured prior to injection (time 0), and pH was determined at time 0, after the addition of the culture cocktail and the addition of each antimicrobial, and 10 min post-injection.

Brine injection experiments took place in a biosafety level 2 production facility at an ambient temperature of 6°C, (three for each treatment). Roasts were injected using a brine injector (IMAX 350, Schröder, Werther, DE) with the conveyor speed set to 7 and injection pressure at ~2.2 psi, which gave an approximate injection percentage of 120 over green weight, based on preliminary work. After injection, the weight and pH of each roast were recorded. Roasts were vacuum packaged in two separate vacuum pack bags (one inside the other) using a vacuum packing machine (Multivac C200), and tumbled using a tumbler (Universal Machine VM 150, Stephan Machinery, Hameln, DE) for 20 min in an ice-water bath. Following tumbling, roasts were aseptically cut into three steaks, approximately 5 cm thick, and assigned to a storage time of 0, 4, or 7 days. Steaks that were stored for future samplings were individually vacuum-packaged in nylon/poly coex vacuum bag (30.48 cm X 35.56 cm, 4 mil, O_2 transmission rate of 48 cc/m^2; Allied Pak Inc., Scarborough, ON). Steaks were stored at 7°C until sampling after 4 or 7 days of storage.

2.6.4 Sampling of steaks for microbiological analyses

An estimate of background microflora was determined by aseptically removing a 10-cm^2 surface sample from each roast prior to injection. Samples were individually

placed in sterile filtra bags (Filtra-Bag®, Fisher Scientific), 90 mL of sterile 0.1% (w/v) peptone was added and samples were stomached for 2 min. To determine microbial counts, samples were plated as described below.

Samples were taken from packaged streaks on 0, 4, and 7 days of storage. On each sampling day, one core sample was aseptically removed using a steel corer, with a diameter of 25 mm. Samples were weighed, placed into a sterile Stomacher bag, and 90 mL of sterile 0.1% (w/v) peptone was added. Samples were stomached for 2 min, and serial dilutions were prepared in sterile 0.1% (w/v) peptone for microbial analyses.

2.6.5 Bacterial enumeration

For determination of *Enterobacteriaceae,* 1 mL of each appropriate dilution was pipetted into a sterile petri plate, and ~30 mL of molten Violet Red Bile Agar (Difco™ Becton Dickinson, Sparks, MD) containing 1% (w/v) glucose (Fisher Scientific, Ottawa, ON) (VRBG) was added. Plates were gently rotated to mix the inoculum evenly, and allowed to solidify before incubation. For all other agars, 100 μL of appropriate dilutions were surface plated. Coliform and *E. coli* O157:H7 and *Proteus* sp. counts were enumerated using CHROMagar™ O157 (CA) (CHROMagar Microbiology, Paris, FR), while total aerobic bacteria were enumerated on Plate Count Agar (PCA) (Difco™). Numbers of *L. monocytogenes* were determined using supplemented PALCAM (SR0150E; Oxoid, Hants, UK), and lactic acid bacteria were enumerated using All-purpose Tween (APT) agar (Difco™). *Pseudomonas* spp. were enumerated on Pseudomonas agar supplemented with, Cetrimide, Fucidin, and Cephalosporin (SR0103E; Pseudomonas C-F-C supplement, Oxoid Ltd., Basingstoke, UK), VRGB, CA, and PALCAM plates were incubated at 35°C for 24 h, APT plates were incubated at

20°C for 48 h under anaerobic conditions, using GasPak™ anaerobe container system (Becton Dickinson, Sparks, MD), CFC plates were incubated at 25°C for 48 h, and two sets of PCA plates were inoculated and incubated; one at 20°C for 48 h and one at 4°C for 10 d. After incubation, plates were enumerated, and counts were converted into log CFU/g before data analysis. The experiment was repeated three times.

2.7 Data analysis

Data were subject to ANOVA using the general linear model in SAS (Version 9.3; Cary, SC). Student Newman Kuels multiple range test was used to differentiate among means.

3. Results

3.1 Heat resistance of *L. monocytogenes* in broth

To determine the heat resistance of strains of *L. monocytogenes* strains were heated in TSB. When strains of *L. monocytogenes* were heated for 30 min at 60°C in TSB broth, only *L. monocytogenes* FS 13 and FS 45 had counts above the detection limit of 2 log CFU/mL (Figure 3-1). These two strains decreased by 5.5 log CFU/mL during heating. All other strains tested decreased by a minimum of 5.75 log CFU/mL during heating, which lowered all counts below the detection limit for this experiment.

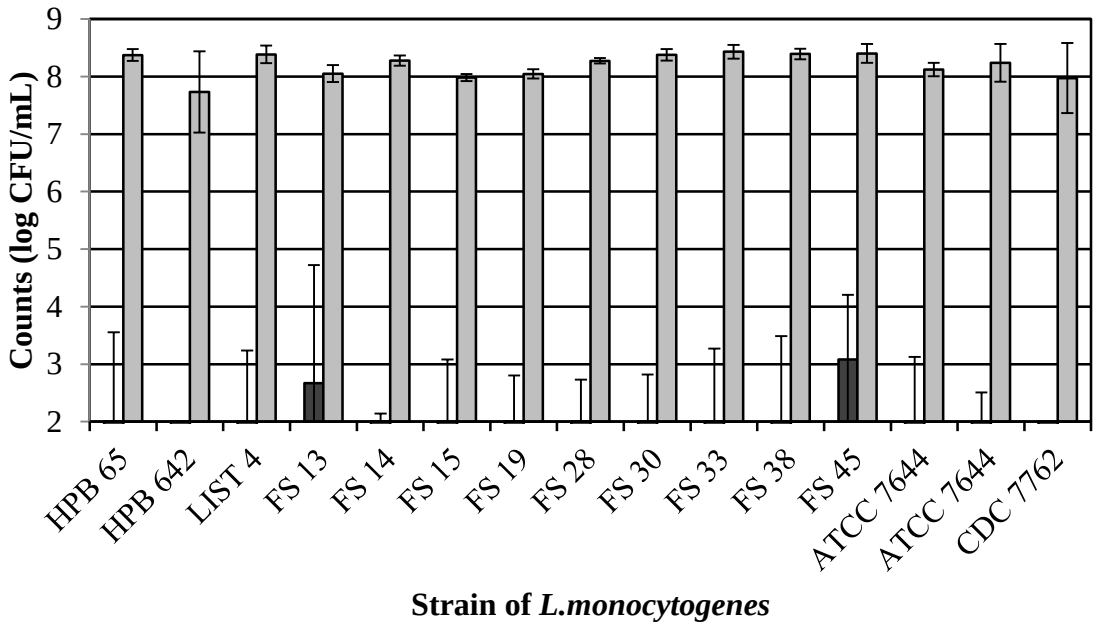

Figure 3-1. Mean log CFU/mL of *L. monocytogenes* prior to (▨) and after heating (■) in TSB broth 60°C for 30 min. n=6.

3.2 Heat resistance of *L. monocytogenes* and *Escherichia coli* in meat

To determine the effect of fat on the heat resistance of strains of *E. coli*, strains were inoculated into ground beef with 15, 24 or 35% fat and heated to 60°C. *E. coli* AW1.7, AW1.3, and GM16.6 were significantly more heat resistant compared to the other strains of *E. coli* regardless of percentage of fat in the ground beef (Figure 3-2). *E. coli* GM18.4, GM18.6, and MB2.1 were reduced by 5 to 6.5 log CFU/g during heating in ground beef whereas the more heat resistant strains were only reduced by 4 log CFU/g. There was no significant difference in the heat resistance among the strains of non-shiga toxin producing *E. coli* O157:H7 with ≥5 log CFU/g reduction in cell counts when strains were heated in lean ground beef (15% fat) (data not shown). Of the six strains of verotoxigenic *E. coli* tested, *E. coli* O163:NM was the least heat resistant with a 6 log CFU/g reduction during heating for 30 min and the most heat resistant strain was *E. coli* O140:H14 which was reduced by 4 log CFU/g during heating (Figure 3-3). For use in the meat injection experiments, *E. coli* AW1.7, AW1.3, GM16.6, O140:H14, and O157:H7 strain 02-0304 (non-shiga toxin producing strain) were used in the mixed strain cocktail.

The reduction in counts of strains of *L. monocytogenes* varied among the strains and among fat concentrations (Figure 3-4). The reduction in counts was greatest in ground beef with 35% fat for some strains but other strains had the greatest reduction in ground beef with the lowest percentage fat, 15%. Reduction in cells counts varied from a maximum of greater than 8 log CFU/g to a minimum of 4.5 log CFU/g. *L. monocytogenes* FS 13, FS 14, FS 15, FS 19, FS 30, and ATCC 7644 were chosen for use as a cocktail in the meat injection experiments.

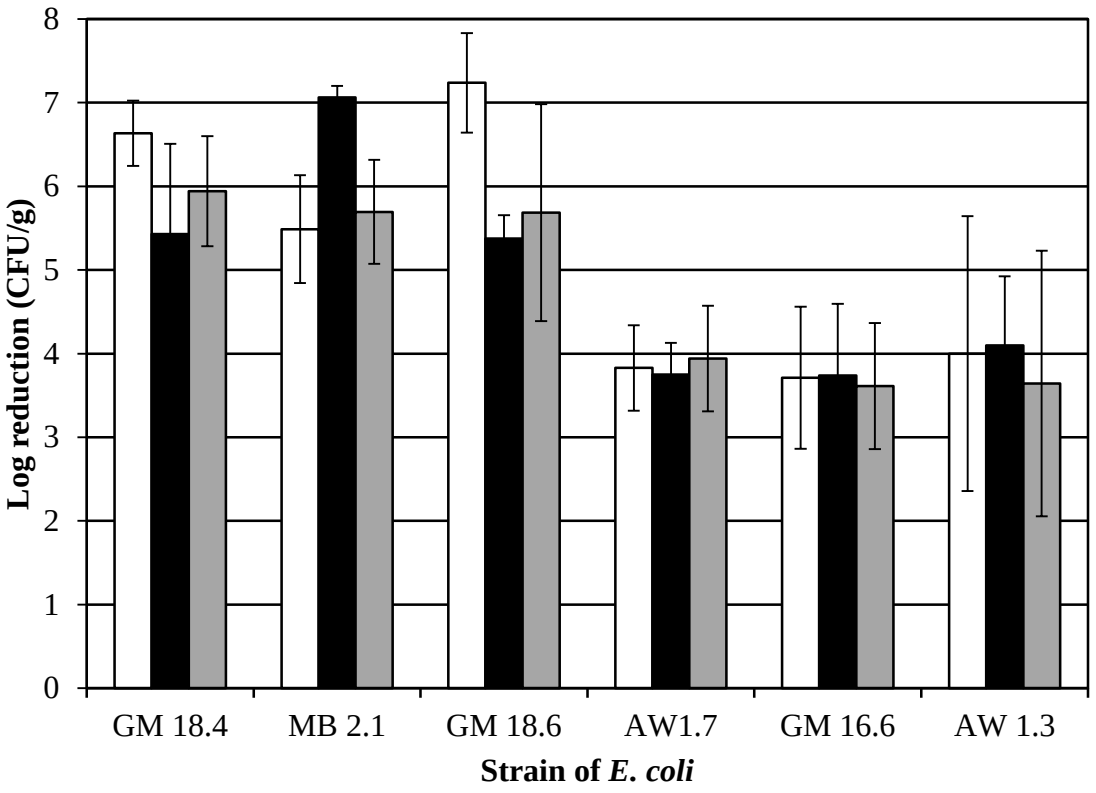

Figure 3-2. Mean reduction of counts of strains of *E. coli* inoculated into ground beef with 35 (□), 24 (■) or 15% (▒) fat and heated at 60°C for 30 min. n=9.

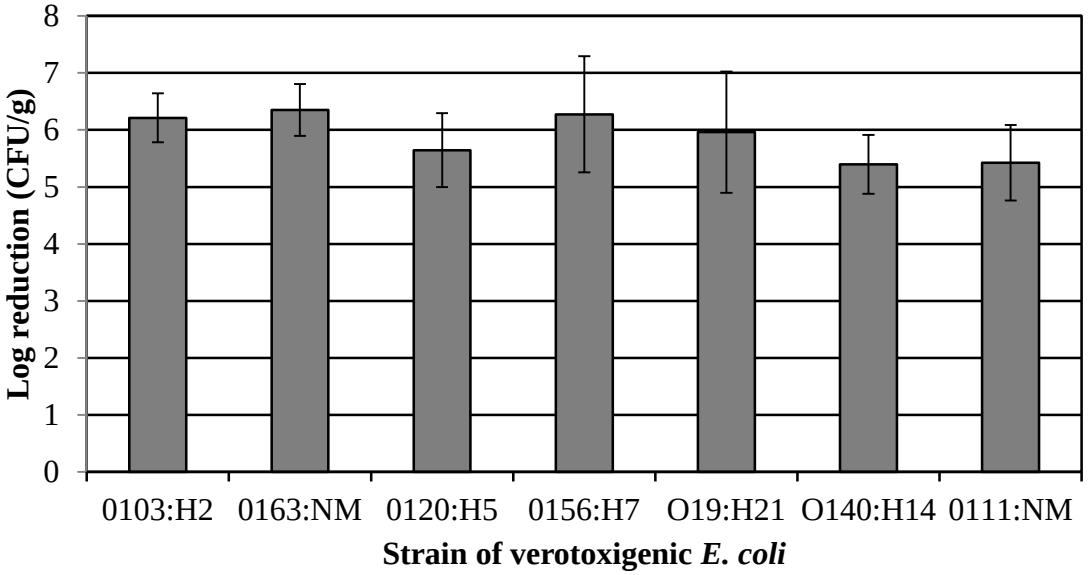

Figure 3-3. Mean reduction of counts of strains of verotoxigenic *E. coli* inoculated into ground beef with 15% fat and heated at 60°C for 30 min. n=9; SEM <1.0.

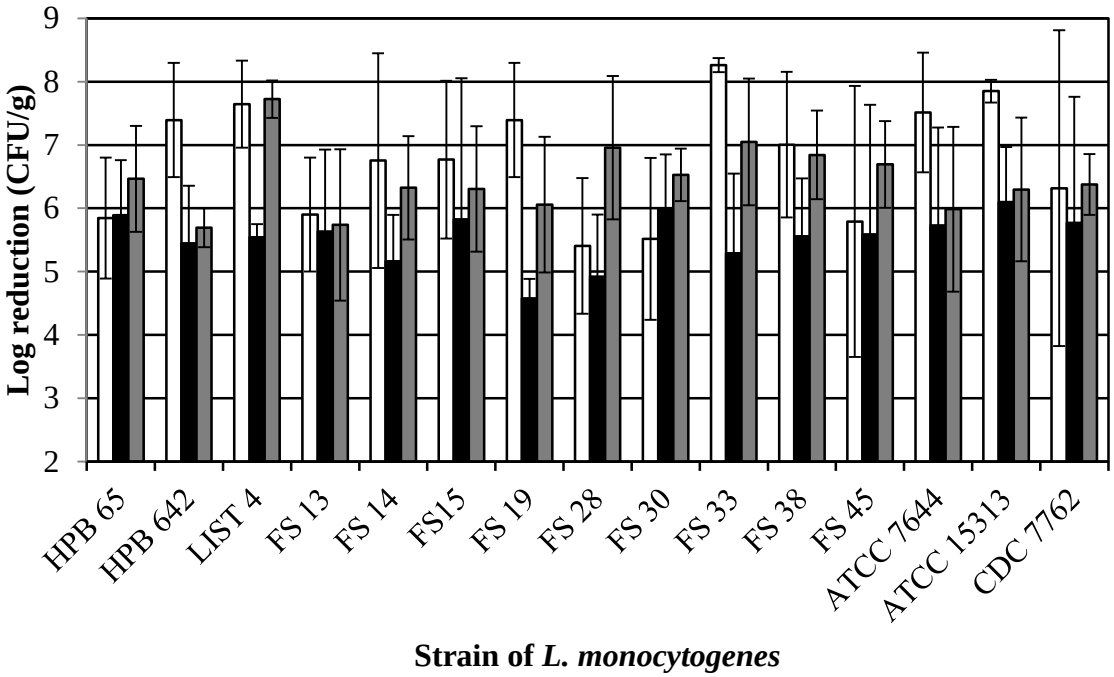

Figure 3-4. Mean reduction of counts of strains of *L. monocytogenes* inoculated into ground beef with 35(□), 24(■) or 15%(■) fat and heated at 60°C for 30 min. n=3 for 35 and 24% fat; n=12 for 15% fat.

3.3 Differentiation of strains with random amplification of polymorphic DNA

To differentiate among strains in the mixed strain cocktails used for injection experiments, individual strains were subjected to random amplification of polymorphic DNA. Results of RAPD analysis for *E. coli* (Figure 3-5) showed that the five strains of *E. coli* each had a different banding pattern when the DAF4 primer was used. *E. coli* GM16.6 and AW1.7 are the only two strains that had numerous matching bands; however, both strains could be differentiated from each other.

Results for strains of *L. monocytogenes* showed that FS 14 and FS 19 were the only two strains that could be differentiated from the other strains of *L. monocytogenes* in

the cocktail (Figure 3-6). *L. monocytogenes* FS 30 and ATCC 7644 had identical RAPD banding patterns as did *L. monocytogenes* FS 15 and FS 13. Therefore strains of *L. monocytogenes* that would be used in the cocktail for the brine injection experiments were; FS 13, 14, 19, and ATCC 7644.

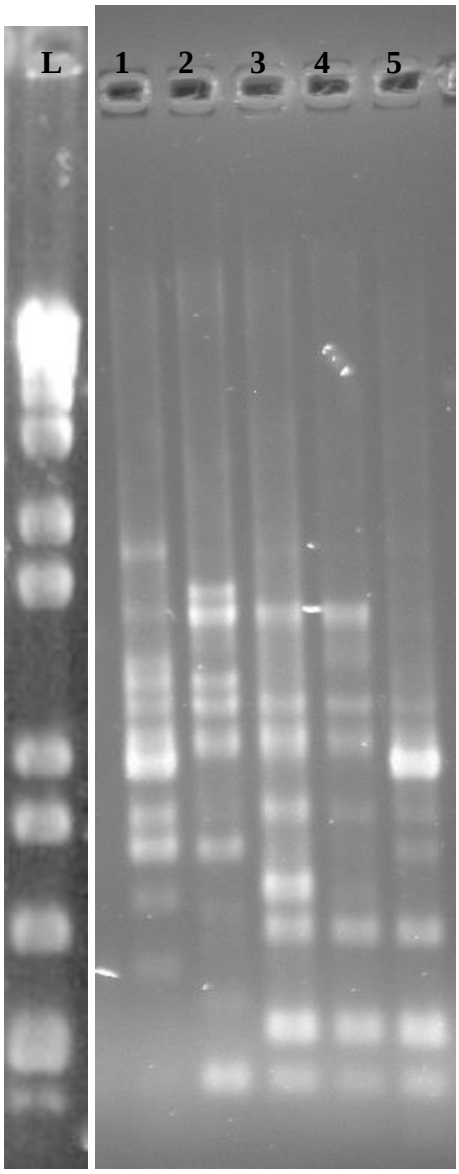

Figure 3-5. Banding patterns obtained by RAPD analysis of *Escherichia coli* strains with DAF4 primer. L, ladder; 1, O140:H14; 2, O157:H7 serotype 02-0304; 3, GM16.6; 4, AW1.7; 5, AW1.3.

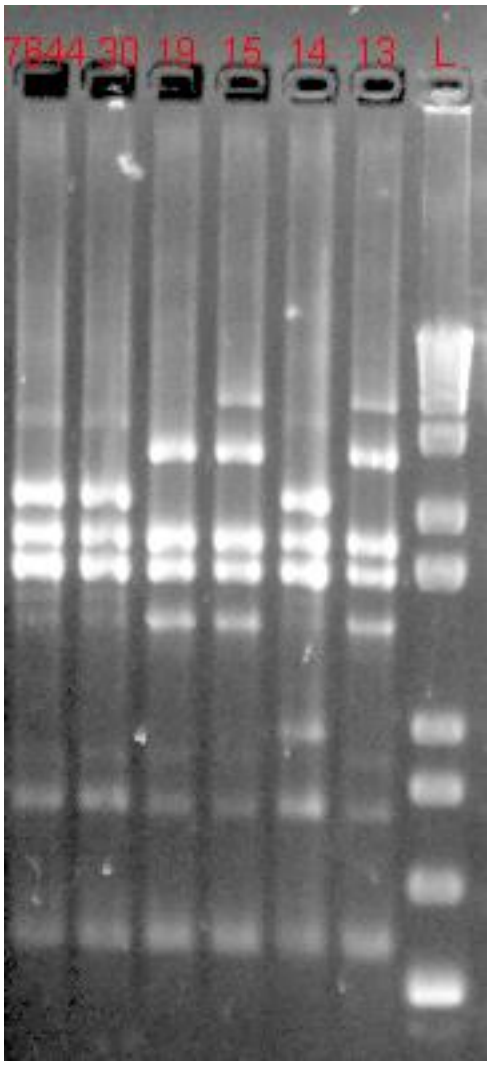

Figure 3-6. Banding patterns of *Listeria monocytogenes* strains obtained by RAPD analysis M13 primer. 1, ATCC 7644; 2, FS 30; 3, FS 19; 4, FS 15; 5, FS 14; 6, FS 13; L, ladder.

3.4 Minimum bactericidal concentration

The minimum bactericidal concentrations of different antimicrobials against individual strains of *E. coli* were determined in LB. The inhibition of the growth of *E. coli* by cranberry extract varied among strains with either 6.25 or 12.5% inhibiting the strains tested (Table 3-1). Both Charsol® and green tea inhibited the growth of all of the strains of *E. coli* at the lowest concentration of each antimicrobial tested (0.012% and

0.01%, respectively). Rosemary extract inhibited the growth of *E. coli* GM16.6 and O157:H7 strain 02-0304 at 2.5%; however, both AW1.3 and AW1.7 were inhibited by the lowest concentration of rosemary extract (0.02%) tested. *E. coli* AW1.3, AW1.7, and GM16.6 were completely inhibited by the lowest concentrations of MicocinX™ (0.006%) and PuraQ® (0.005%) in the presence of 5 mM EDTA. However, *E. coli* O157:H7 strain 02-0304 grew in the presence of both antimicrobials. Once the concentration of MicocinX™ + 5 mM EDTA reached 0.75% or the concentration of PuraQ® + 5 mM EDTA reached 0.625%, *E. coli* O157:H7 strain 02-0304 was inhibited.

Table 3-1 Minimum bactericidal concentration (MBC) of single antimicrobials against individual strains of *E. coli* at 4 log CFU/mL.

Strain of *E. coli*	Antimicrobial					
	Cranberry	Charsol®	Green tea	Rosemary	MicocinX ™ + 5mM EDTA	PuraQ® + 5 mM EDTA
AW1.3	6.25%	<0.012%	<0.01%	<0.02%	<0.006%	<0.005%
AW1.7	12.5%	<0.012%	<0.01%	<0.02%	<0.006%	<0.005%
GM16.6	6.25%	<0.012%	<0.01%	2.5%	<0.006%	<0.005%
02-0304	12.5%	<0.012%	<0.01%	2.5%	0.75%[f]	0.625%[f]

(<) denotes that no growth was observed at the lowest concentration of antimicrobial on the 96-well microtittre plate. [f] indicates the concentration of the antimicrobial at which no growth was observed at lowest concentration of antimicrobial. n = 3.

Further experiments were needed to determine MBC's for Green tea extract and Charsol® in brine solution against the strains of *E. coli*, therefore the strains were inoculated into different concentrations of antimicrobials prepared in brine solution (0.8 NaCl and 0.5% STPP) and examined for inhibition. Green tea extract inhibited the growth of *E. coli* AW1.7 and GM16.6 at 0.5% and 0.0039%, respectively (Table 3-2). However, neither AW1.3 nor O157:H7 strain 02-0304 were inhibited by the green tea

extract at the lowest concentration, which was 0.002%. Charsol® inhibited both AW1.7 and GM16.6; however, it did inhibit AW1.3 or O157:H7 strain 02-0304 at the lowest concentration of 0.002%.

Table 3-2 Minimum bactericidal concentration of green tea extract, and Charsol® prepared in a brine solution against individual strains of *E. coli* at 6 log CFU/mL.

	Antimicrobials	
Strains of *E. coli*	Green tea	Charsol®
AW1.3	<0.002%	<0.002%
AW1.7	0.5%	0.0313%
GM16.6	0.0039%	0.0039%
02-0304	<0.002%	<0.002%

(<) denotes no bacterial growth observed at the lowest concentration of the antimicrobial tested. n = 3.

To determine the impact of the brine solution on the survival of *E. coli* and *L. monocytogenes*, strains were inoculated into brine and incubated for 24 h. The growth of all strains of *E. coli* and *L. monocytogenes* tested were inhibited by the brine solution with the exception of O157:H7 strain 02-0304, which grew during incubation in brine for 24 h (Table 3-3).

The inhibitory activity of a range of antimicrobials was tested using the cocktails of cultures of either *E. coli* or *L. monocytogenes*. The 4-strain cocktail of *E. coli* was inhibited by 0.125% green tea extract, 0.125% Charsol®, and 5% PuraQ® (Table 3-4). MicocinX™ (20%) did not inhibit the growth of the cocktail of strains of *E. coli*. The growth of the cocktail of strains of *L. monocytogenes* was completely inhibited by the green tea extract and Charsol®at the lowest concentration tested which was, 0.31% for the green tea extract and Charsol®. PuraQ® inhibited the growth of *L. monocytogenes* at 2.5

% and MicocinX™ did inhibit the cocktail but not until the concentration of the MicocinX™ was below 0.31%. However, at higher concentrations of MicocinX™ growth of *L. monocytogenes* was observed.

Table 3-3 Bactericidal effect of a brine[a] solution against strains of *E. coli* and *L. monocytogenes.*

Strains of *E. coli*	Effect of Brine on Growth
AW1.3	-
AW1.7	-
GM16.6	-
O157:H7 strain 02-0304	-
O140:H14	+
Strains *L. monocytogenes*	
FS 13	-
FS 14	-
FS 15	-
FS 19	-
ATCC 7644	-

[a]Brine solution: 0.8% NaCl and 0.5% sodium tripolyphosphate.
- no growth; + growth. n = 3.

Table 3-4 Minimum bactericidal concentration of green tea extract, Charsol®, MicocinX™, and PuraQ® against the cocktail of strains of *E. coli* and against the cocktail of strains of *L. monocytogenes*.

Cocktail	Antimicrobial			
	Green tea extract	Charsol®	MicocinX™	PuraQ®
E. coli	0.125%	0.125%	>20%	>5%
L. monocytogenes	<0.31%	<0.31%	0.31%[c]	2.5%

(>) the highest concentration of the antimicrobial which still had no bactericidal effect against the cocktail; (<) represents no growth of the bacterial cocktail at the lowest concentration of the antimicrobial tested.
[c] the concentration that the antimicrobial needed to be diluted to before no growth was observed. n = 3.

3.5 Effect of antimicrobials on survival of *E. coli* and *L. monocytogenes* in brine injected beef

3.5.1 Bacterial counts in the brine pre- and post-injection

No bacteria were detected either pre- or post-injection in the brine solution used for the uninoculated control samples (Table 3-5). Counts of *Enterobacteriaceae* and *L. monocytogenes* in the brine prepared for use in the positive control were as expected at 7.9 log CFU/mL and 7.7 log CFU/mL, respectively. Counts for *Enterobacteriaceae* and *L. monocytogenes* decreased during the injection process and had decreased by more than 1.5 log units 10 minutes after injection was completed. The addition of Charsol® to the brine had no significant effect on the counts of *Enterobacteriaceae*, but did reduce counts of *L. monocytogenes* counts by 2.2 log CFU/mL during the injection process. Similar results were observed for both the brine with MicocinX™ and the brine containing both Charsol® and MicocinX™. None of the treatments had any significant effect on any of the other bacterial counts throughout the injection process.

3.5.2 Effect of antimicrobials on the pH and microbial counts in brine injected beef

To determine the effect of the addition of Charsol®, MicocinX™ or both antimicrobials on the pH and bacteriology of brine injected beef, roasts were injected with brines without any bacterial cocktail, brine with a cocktail of *E. coli* and *L. monocytogenes* with or without antimicrobials added. Roasts were injected, cut into steaks and the steaks were stored for up to 7 days at 7°C to determine the impact of antimicrobials during vacuum packaged storage of steaks from brine-injected roasts. The pH of the brine solution was not affected by the addition of Charsol but the addition

of Micocin X reduced the pH of the brine solution (Table 3-6). The addition of the brine to the meat increased the pH of the meat regardless of the addition of antimicrobials.

The addition of antimicrobials to the brine solution used for injection had no impact on the growth of the total population of bacteria present, the psychrotropic aerobic bacteria or presumptive *Pseudomonas* spp. (Figure 3-9). The total aerobic population in meat injected with brine without cocktails of *E. coli* and *L. monocytogenes* was significantly lower than any of the samples injected with brine containing the cocktails of organisms; however, after 7 days of storage, the counts in the control samples were the same to those found in meat injected with bacterial cocktails (Figure 3-9a). Psychrotrophic aerobic bacteria and presumptive *Pseudomonas* counts were the same in all treatments (Figure 3-9b and c). The addition of antimicrobials had no effect on the counts of total aerobic bacteria, psychrotrophic bacteria or presumptive *Pseudomonas* spp.

Figure 3-10 shows the counts for *Enterobacteriaceae*, coliforms and *E. coli* O157:H7 in the injected meats. The antimicrobial treatments had no effect on the counts for *Enterobacteriaceae*, coliforms and *E. coli* O157:H7. The control that was injected with brine without any bacterial cocktails added had significantly lower *Enterobacteriaceae* and coliform counts (Figure 3-10a,b) than samples injected with brine containing the bacterial cocktail. *E. coli* O157:H7 were not detected in samples injected with brine without bacterial cocktails (data falls below the detection limit of log 2 CFU/g; Figure 3-10c). Similar trends were observed for counts of presumptive lactic acid bacteria (Figure 3-11). Addition of antimicrobials had no impact on counts of lactic acid bacteria.

Table 3-5 Mean log counts (CFU/mL) of brine solutions pre- and post-injection of raw beef roasts.

Bacteria	Injection Solution												
	Brine solution		Brine + Cocktail		Brine + Cocktail + Charsol®			Brine + cocktail + MicocinX™			Brine + cocktail + Charsol® + MicocinX™		
	Sample[1]		Sample		Sample			Sample			Sample		
	0	10	0	10	0	1	10	0	1	10	0	1	10
Aerobic bacteria	0.0 ±0.0	0.0 ±0.0	8.2 ±0.3	7.8 ±0.1	8.2 ±0.2	8.1 ±0.2	7.8 ±0.1	8.2 ±0.1	8.0 ±0.1	7.6 ±0.5	8.2 ±0.2	7.8 ±0.04	7.9 ±0.1
Psychrotrophic aerobic bacteria	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1.0 ±1.7	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1.2 ±2.0
Pseudomonas spp.	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Enterobacteriaceae	0.0 ±0.0	0.0 ±0.0	7.9 ±0.3	6.3 ±1.2	6.9 ±1.4	7.3 ±0.6	6.9 ±0.7	7.6 ±0.3	7.8 ±0.1	7.7 ±0.1	7.7 ±0.2	7.5 ±0.3	7.4 ±0.6
Coliforms	0.0 ±0.0	0.0 ±0.0	8.2 ±0.5	7.5 ±0.2	7.9 ±0.2	7.7 ±0.2	7.3 ±0.4	7.8 ±0.03	7.8 ±0.2	7.4 ±0.5	7.8 ±0.1	7.6 ±0.1	7.6 ±0.1
E. coli O157:H7	0.0 ±0.0	0.0 ±0.0	7.7 ±0.3	7.6 ±0.3	7.5 ±0.2	7.5 ±0.4	7.4 ±0.3	7.5 ±0.2	7.5 ±0.2	7.2 ±0.6	7.7 ±0.2	7.5 ±0.2	7.5 ±0.2
Presumptive lactic acid bacteria	0.0 ±0.0	0.0 ±0.0	7.9 ±0.5	7.4 ±0.8	7.1 ±0.7	6.6 ±0.9	7.2 ±1.0	7.1 ±0.3	7.3 ±0.6	6.8 ±0.8	8.0 ±0.1	7.1 ±0.8	6.4 ±1.5
L. monocytogenes	0.0 ±0.0	0.0 ±0.0	7.7 ±0.2	5.5 ±0.3	7.6 ±0.7	6.2 ±0.3	5.4 ±0.03	7.6 ±0.2	5.4 ±0.2	5.3 ±0.02	7.9 ±0.5	5.0 ±0.6	4.8 ±0.4
Proteus sp.	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	2.0 ±3.5	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

[1]Sample: 0, pre-injection after the addition of the culture cocktail (with the exception of the negative control); 1, pre-injection following the addition of antimicrobials; 10, samples taken 10 minutes post-injection. n=3.

Table 3-6 pH of the brine solution, pre- and post injection of beef samples, and after the addition of antimicrobials and pH of the beef roast pre- and post injection.

Sample	Injection Solution													
	Brine solution		Brine + Cocktail		Brine + Cocktail + Charsol®			Brine + Cocktail + MicocinX™			Brine + Cocktail + MicocinX™ + Charsol®			
	Sample[1]		Sample		Sample			Sample			Sample			
	0	10	0	10	0	1	10	0	1	10	0	1	2	10
Brine	8.0 ±0.16	8.2 ±0.05	8.0 ±0.24	8.2 ±0.03	8.1 ±0.29	8.2 ±0.13	8.1 ±0.25	8.2 ±0.12	7.5 ±0.71	7.2 ±0.07	8.2 ±0.12	7.2 ±0.06	7.1 ±0.03	7.1 ±0.1
Beef	5.6 ±0.03	6.4 ±0.17	5.6 ±0.09	6.4 ±0.24	5.6 ±0.07	NA	6.3 ±0.08	5.6 ±0.13	NA	6.4 ±0.20	5.6 ±0.06	NA		6.2 ±0.26

[1]Sample: 0, pre-injection after the addition of the culture cocktail (with the exception of the negative control and all beef samples); 1, pre-injection following the addition of antimicrobials; 2, pre-injection following the addition of second antimicrobial; 10, samples taken 10 minutes post-injection. NA, not applicable, as beef pH samples were only taken, pre- and post injection. n=3.

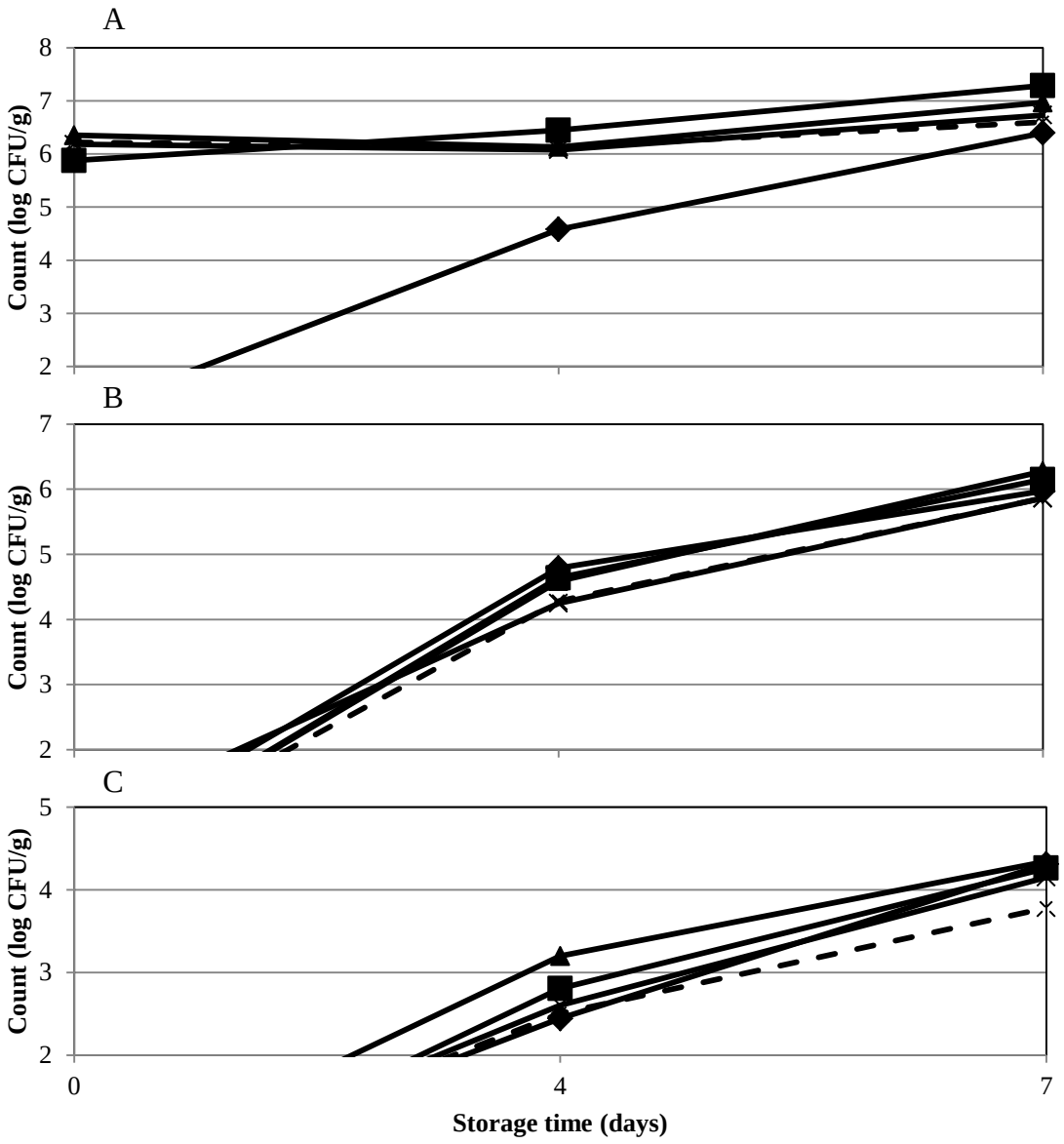

Figure 3-7. Mean log CFU/g counts of total aerobic bacteria (A), psychrotrophic aerobic bacteria (B) and presumptive pseudomonads (C) on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- -X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per storage time); SEM <0.13.

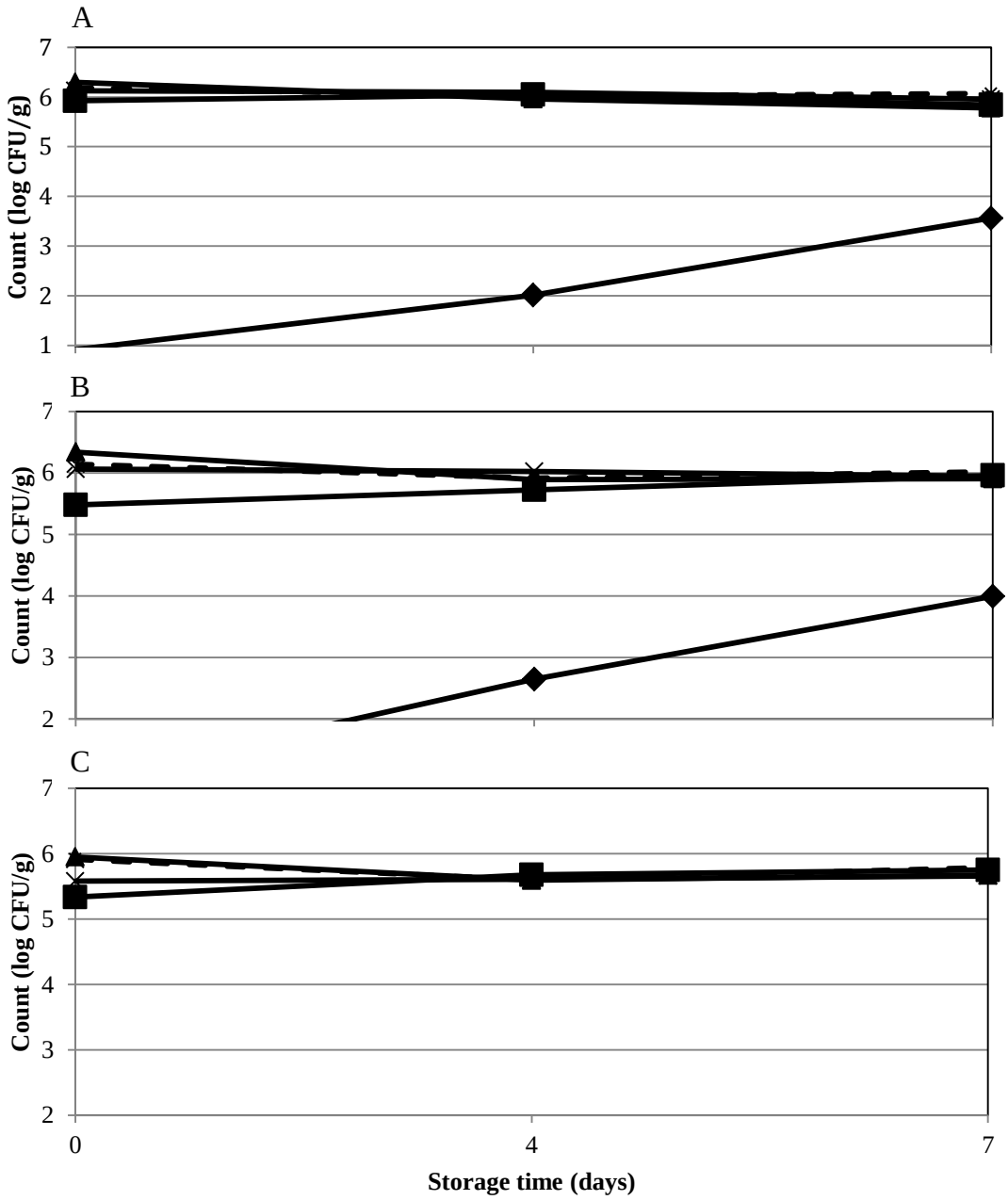

Figure 3-8. Mean log CFU/g counts of *Enterobacteriaceae* (A), coliforms (B) and *E. coli* O157:H7 (C) on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- - X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per replicate); SEM <0.23.

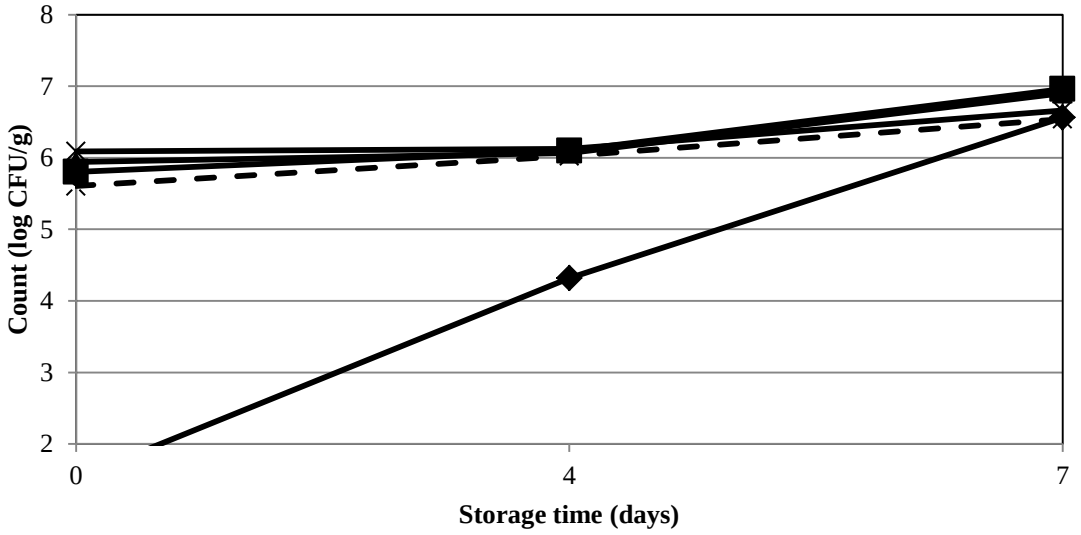

Figure 3-9. Mean log CFU/g counts of presumptive lactic acid bacteria on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- -X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per storage time); SEM <0.22.

Lactic counts in samples injected with brine with no added bacteria were significantly lower than the other samples on 0 and 4 days of storage but after 7 days counts were similar to the other treatments.

The addition of antimicrobials to the brine injected into beef had a significant impact on the counts of *Listeria* spp. (Figure 3-12). *Listeria* spp. counts for the control without any bacterial cocktail was below the detection limit until 7 days of storage. The addition of Charsol® to the brine did not significantly decrease counts of *Listeria* spp. in brine-injected steaks. However, the addition of MicocinX™ with or without Charsol®

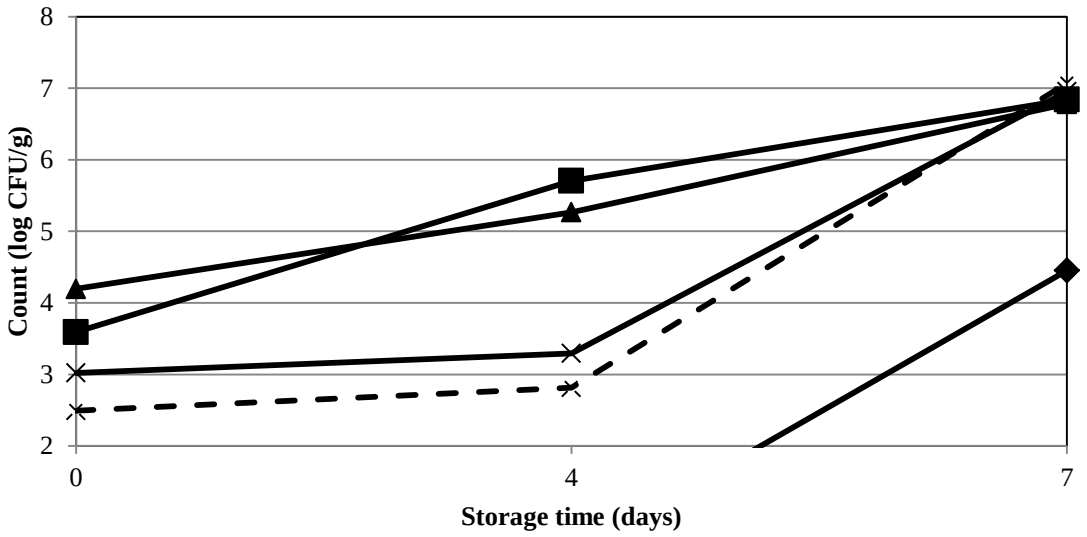

Figure 3-10. Mean log CFU/g counts of *Listeria* spp. on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- -X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per storage time); SEM <0.13.

inhibited the growth of *Listeria* spp. during 4 days of storage at 7°C. However, by day 7 all treatments except the negative control had similar counts of *Listeria* spp.

Counts of *Proteus* sp. in all treatments were below the detection limit of 2 log CFU/g at all times (data not shown).

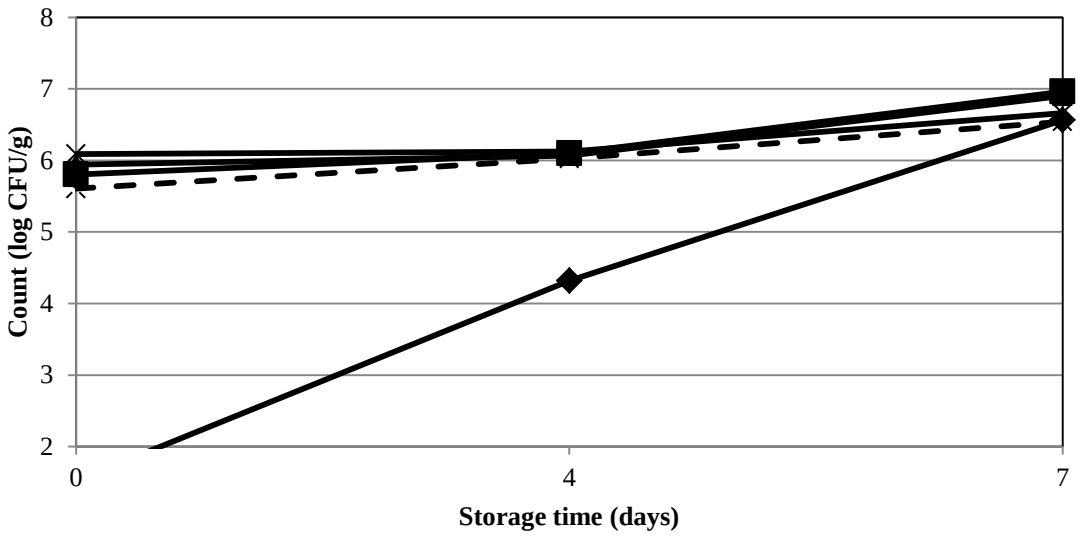

Figure 3-11. Mean log CFU/g counts of presumptive lactic acid bacteria on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- -X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per storage time); SEM <0.22.

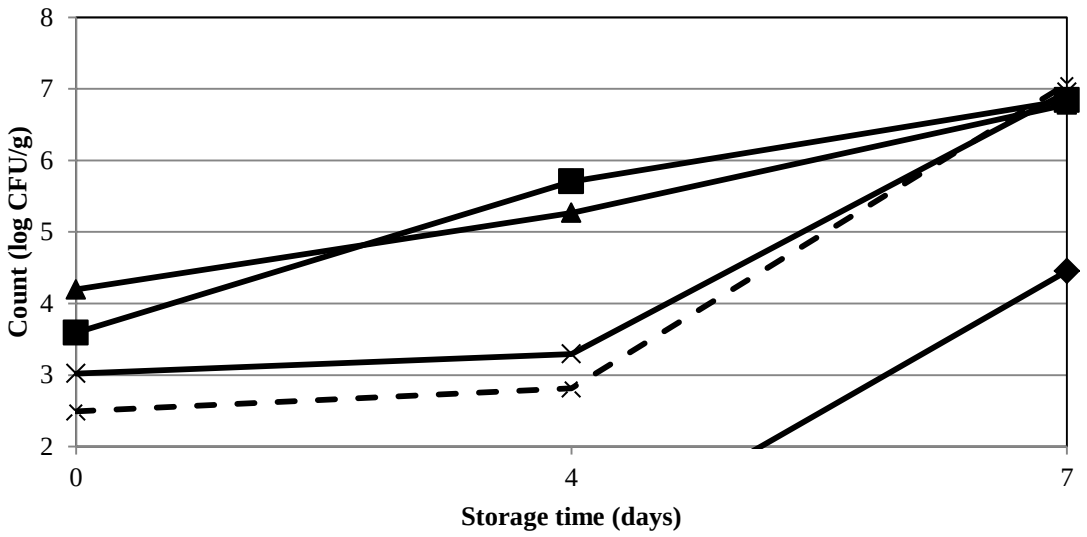

Figure 3-12. Mean log CFU/g counts of *Listeria* spp. on beef steaks injected with brine (♦), brine with a cocktail of *L. monocytogenes* and *E. coli* (■), brine with a cocktail of *L. monocytogenes* and *E. coli* and Charsol® (▲), brine with a cocktail of *L. monocytogenes* and *E. coli* and MicocinX™ (X), and brine with a cocktail of *L. monocytogenes* and *E. coli*, Charsol® and MicocinX™ (- -X- -) and stored for up to 7 days at 7°C. Means are averages of 9 samples (3 replicates x 3 steaks per storage time); SEM <0.13.

4. Discussion and Conclusion

As new processing technologies are used by the meat industry, consumer safety must remain at the forefront as to whether these new products should be made readily available without added antimicrobial hurdles. Bacterial contamination or growth that may occur needs to be effectively dealt with either by the addition of antimicrobial hurdles by the processor, or an increase in consumer awareness on how to safely prepare and store new products. The aim of this thesis was to determine which antimicrobials when added to a brine injection system would reduce or eliminate the presence of heat resistant strains of *E. coli* and *L. monocytogenes* in brine injected raw beef. Heat resistant strains of both organisms were selected as when raw meat goes through a brine injection process, the needles that deliver the brine solution pierce the muscle structure causing any bacterial contamination on the surface of the meat or in the brine solution to be transferred into the internal structure of the roast (FSIS, 2002; Luchansky et al., 2008; Heller et al., 2007; Ray et al., 2010). Because of the translocation of bacteria into the internal structure of meat during brine injection, the potential for bacteria to survive a cooking process is a significant food safety threat to consumers. This was demonstrated after four individuals became ill after consuming steak that had been needle tenderized (Lewis et al., 2013), and the use of grills for cooking meat which can result in cold spots in the meat, where a uniform temperature is not achieved, which allows the survival of *E. coli* O157:H7 even after an internal temperature of 71.1°C has been reached (Luchansky et al., 2011). Following the selection of heat resistant strains of both *E. coli* and *L. monocytogenes*, food safe antimicrobials were chosen based on their effectiveness at eliminating both bacterial species. The ability of these antimicrobials to eliminate strains

of *E. coli* or *L. monocytogenes* was important, as the antimicrobials would be in direct contact with the bacterial strains in both the brine solution during the injection process and in the vacuum packaged beef during storage. Gill et al. (2008) reported how during the injection process bacteria remained at the injection site between muscle fibres. This would allow the antimicrobials the greatest amount of time to effectively eliminate the life-threatening hazard of either of the bacterium. In this research project, once both these factors had been determined in vitro, then the effective antimicrobials were tested in real-life practice with an industrial brine injection system, based on current industry practice where it could be determined if results established in the laboratory settings could be replicated in an industrial process.

4.1 Heat resistance of *Escherichia coli*

To determine the heat resistance of different strains of *E. coli* that had been isolated from a beef slaughter facility (Aslam et al., 2004), strains were heated in ground beef with different percentages of fat. The survival of six strains of *E. coli* was not impacted by the percentage of fat present in ground beef. However, there was a significant difference observed in heat resistance among strains AW1.7, AW1.3 and GM16.6 when compared to strains GM18.4, MB2.1, and GM18.6 regardless of the percentage of fat. *E. coli* AW1.7 is significantly more heat resistant than other strains isolated from a slaughter plant, specifically MB2.1, GM18.6, GM18.4, and AW1.3 (Dlusskaya et al., 2011, Garcia-Hernandez et al., 2013). Although Dlusskaya et al. (2011), found that strain AW1.7 proved to be very heat resistant, the current research determined that it was not significantly more heat resistant when heated in ground beef than two other strains isolated from the same facility, AW1.3 or GM16.6. This indicated

that the heat resistance in a broth system could not necessarily be directly correlated to that which may be determined in meat. Although there was no significant heat resistance found among any of the four strains of non-shiga toxin producing *E. coli* O157:H7 nor the seven strains of verotoxigenic *E. coli* when heated in 15% fat ground beef, it is never the less important to note that strains from both these groups survived heating for 30 min at 60°C. Given the low infectious dose (10 cells) that is required for these types of *E. coli* to cause an illness (PHAC, 2001), and the potential for these strains to cause haemolytic uraemia syndrome (HUS) which has a mortality rate ranging from 3 – 5% (World Health Organization [WHO], 2011), it should be a concern when any of these particular strains survive a cooking process. The ramifications from these strains being consumed by the public cannot only be costly in the short term, in terms of product recall and patient hospitalization, but also in the long term, with on-going health complications and consumer perception of a particular brand or company being not as trusted to deliver a safe product.

4.2 Heat resistance of *Listeria monocytogenes*

Heat resistance of *L. monocytogenes* can vary among strains (Boyle et al., 1990, Monfort et al., 2012). In the current research, only two strains survived heating at 60°C for 30 min whereas in a meat matrix, a larger number of strains were able to survive the heat treatment. This is consistent with what other researchers have reported. For example, Boyle et al. (1990) determined that the thermal destruction of *L. monocytogenes* Scott A in a phosphate buffer was faster when compared to a meat slurry (20% beef and 80% water) and D-values for *L. monocytogenes* Scott A increased when inoculated into ground beef (20% fat). Boyle et al. (1990) also reported that although no cells were

recovered by direct plating onto Modified Doyle-Schoeni Selective Agar after an internal temperature of 70°C was reached, they did detect the pathogen in eight out of the nine samples when enrichment of samples was done. This is important as an internal temperature of 70°C in a steak is much higher than what is generally recommended. Bhaduri et al. (1991) reported a shouldering and tailing effect when *L. monocytogenes* Scott A was added to a liver sausage slurry, showing a thermal resistance in the liver sausage slurry. However, Bhaduri et al. (1991) asserted that holding liver sausage for 1-2 min at 65.6°C would ensure that any listeriae present would be completely eliminated based on their finding. This needs to be considered when developing a thermal intervention step in a production facility as the particular food matrix can have a significant impact on the heat resistance of the organism that is to be eliminated. Studies on heat resistant bacteria in a broth or liquid heating menstrum need to be regarded as an overly optimistic thermal inactivation of a particular species of bacteria. Even with counts below the detection limit, heating doesn't ensure that bacteria are completely eliminated from products, and given that *L. monocytogenes* is a psychotrophic organism, the survival of injured cells is a concern when evaluating thermal treatments. The current research found there was no significant difference in heat resistance among any of the 15 strains of *L. monocytogenes* in any of the three different fat levels in ground beef. This was important to demonstrate that over a wide selection of strains of *L. monocytogenes* the thermal treatment had a similar effect, as previous studies tended to examine only a few strains. Typically *L. monocytogenes* Scott A is considered a benchmark strain for heat resistance. However, it was isolated from a human and may not represent strains that can be found in meat products. It is important to evaluate a

larger cross section of strains to ensure that there is the greatest possibility of finding and using the most heat resistant strains available.

When evaluating the heat resistance in pathogenic bacteria it is important to consider not only the food matrix that the bacteria may commonly be found in but also the conditions in which that food will be stored and consumed. Only evaluating the heat resistance of the bacteria in an isolated broth system could lead to the assumption that the organism may be less heat resistant than what may actually be found in a food matrix. It cannot be assumed that just because counts fall below detection limit that the bacteria has been completely eliminated, this is of greatest concern when looking at low infectious dose pathogens such as EHEC's and psychotrophic bacteria like *L. monocytogenes* which given enough time at refrigeration temperatures could grow to the levels need to cause illness to the consumer.

4.3 Determination of the MIC and MBC of antimicrobials

The addition of antimicrobials to a brine solution could be an important step in eliminating pathogenic bacteria that may contaminate the brine solution from either contaminated meat that was injected or through faulty cleaning which may allow pathogenic bacteria to re-contaminate fresh brine when a new processing cycle starts. It is not only important to evaluate multiple antimicrobials, but to also choose antimicrobials that can be added to a meat product without causing major changes to the sensory quality of that product, and that could be added without major regulatory hurdles that would prohibit its use in a brine solution intended for meat injection. The MBC values of six antimicrobials were determined against the four strains of *E. coli* that had been previously selected as the strains that would be used in the injection experiments. From the six

antimicrobials tested only Charsol® and green tea extract completely inhibited all four strains of *E. coli*. The only antimicrobial that did not completely inhibit at least one strain of *E. coli* was the cranberry extract. Similar findings were reported by LaPlante et al. (2012) for both minimum inhibitory concentration (MIC) and MBC values. In the current study, cranberry extract had no effect on stopping the outgrowth of *E. coli*. MicocinX™ combined with EDTA completely inhibited three out of the four strains of *E. coli*. Typically bacteriocins from gram-positive organisms only inhibit the growth of other gram-positive organisms (Miller et al., 2010). However, the presence of chelating agents, such as EDTA, disrupts the outer membrane leaving the inner cell wall susceptible to bacteriocins from gram-positive bacteria (Gänzle et al., 1999; Gao et al., 1999; Lappe et al, 2009; Martin-Visscher et al., 2011; Stevens et al. 1991).

There is potential for the brine solution to reduce numbers of *E. coli* and *L. monocytogenes* without added antimicrobials. In the current study, when strains were tested in brine solutions for inhibitory effects at room temperature, only a single strain of *E. coli* survived in the brine solution and all other strains of *E. coli* or *L. monocytogenes* were killed. This observation had not been reported in previous studies. Alder et al. (2011) reported that although *E. coli* O157:H7 did not grow in the brine solution (5.5% NaCl + 2.75% STPP), it could still be recovered after 24 h at either 4°C or 15°C. Wicklund et al. (2005) reported no significant decrease in *E. coli* K12 counts when inoculated into a brine solution (4% NaCl + 4% phosphate). Gill et al. (2008) determined that *L. innocua* inoculated into a brine solution (5% NaCl + 5% STPP) and held for 24 h at 2°C did not differ from the initial inoculum by more than 0.3 log CFU/mL. It is possible that in the current study the methods used for determining bactericidal effects

could have caused the different results. In the current study, cultures were centrifuged and washed twice with 0.1% peptone prior to inoculation into microtittre plates containing brine solutions. Previous studies added the inocula directly from the broth medium that was used to grow the bacteria. The washing of the cultures, as was done in the current study, may have caused stress on the cultures, which may account for the lack of growth in microtittre plates with brine solutions.

When the cocktails of either *E. coli* or *L. monocytogenes* were tested for inhibition, Charsol® and Green tea extract, were equally effective at inhibiting *E. coli* with the same MBC values and both these antimicrobials inhibited the growth of the *L. monocytogenes* cocktail at the lowest concentration tested, which means that either of these antimicrobials could be used in the brine injection process. Charsol® was chosen for the following brine-injection trial, based on ease of addition to the brine solution, this product was liquid versus the green tea extract which was a powder. PuraQ® required a concentration of 5% to inhibit the growth of the *E. coli* cocktail and 2.5% to inhibit the growth of the *L. monocytogenes* cocktail, demonstrating that without the addition of the EDTA the PuraQ® was not nearly as effective as an antimicrobial against the bacteria tested. It is possible that a higher concentration of PuraQ® may be needed to inhibit the growth of *E. coli*, as 5% was the highest concentration tested. When the MicocinX™ was used alone, without EDTA, variable results were obtained, Martin-Visscher et al. (2011) reported similar findings, and concluded that without the presence of EDTA bacteriocins produced by *C. maltaromaticum* had no inhibitory effect against *E. coli* DH5α. The MicocinX™ had no inhibitory effect on the *E. coli* cocktail at 20%, this being the highest concentration that was tested. At this concentration the application of MicocinX™ would

be too costly and have severe negative sensory impacts on a meat product, so the use of MicocinX™ for the inhibition of *E. coli* would not be recommended. When the MicocinX™ was tested against the *L. monocytogenes* cocktail an unusual occurrence took place in that the inhibition of the strains of *L. monocytogenes* did not occur until the concentration of the MicocinX™ was less than or equal to 0.31%. For all concentrations of MicocinX™ greater than 0.31% growth of *L. monocytogenes* was verified; however, below this concentration no growth was seen. A possible reason for this occurrence could be that the MicocinX™ is a spray dried culture supernatant containing the bacteriocins produced by *Carnobacterium maltaromaticum* UAL307, which means that the supernatant may provide a protective effect for the survival of the *L. monocytogenes,* and it is not until the lower concentrations of MicocinX™ are reached that this effect is no longer found and the bacteriocins present can effectively eliminate the *L. monocytogenes*. According to the manufacturer's website the recommended concentration of MicocinX™ is 0.4 – 0.6% (www.micocin.com/en/home), which is at roughly the concentration where the inhibition of *L. monocytogenes* in the microtiter plates was observed in this study.

4.4 Injection experiments

For industry, a recycled brine solution can be used to recover the large quantities of brine solution that are not captured in the meat matrix. The recycled brine solution brine can be contaminated with pathogenic bacteria, which would be injected into fresh meat as it was being processed (Wicklund et al., 2007). Because of this, the brine solution used in this experiment was not recycled to ensure that each piece of beef that was injected had the exact same solution. In this way, the efficacy of the brine solution and the brine plus antimicrobials against the two cocktails, *E. coli* or *L. monocytogenes* could

be evaluated. With the brine only solution (positive control), a decrease in both *Enterobacteriaceae* and *L. monocytogenes* counts occurred 10 min after the cocktails were added to the brine. However, *Enterobacteriaceae* counts in the brine containing antimicrobials, regardless of which antimicrobial was present in the solution, did not decrease after the 10 min of processing. Before injection, *L. monocytogenes* counts decreased by up to 2 log CFU/mL, with the addition of MicocinX™, regardless if the Charsol® was present, in the brine tank and there was greater than 1 log CFU/mL reduction in counts with Charsol® alone. However, there was no further significant decrease in counts of *L. monocytogenes* in the brine 10 minutes post injection for all three treatments. Even though previous results in microtittre plates indicated that the Charsol® was effective at inhibiting the *E. coli*, this was not observed when it was used in brine injection experiments. If a longer processing time was used, more effective inhibition might have been observed; however, that would require recycling of the brine solution, as the brine injection process requires large quantities of brine. In these experiments, 60 L of brine was required for approximately 2 min of brine injection, therefore, to increase processing time tanks larger than1000 L tanks would be needed if recycling of the brine solution was not acceptable. Future experiments evaluating the efficacy of Charsol® against *E. coli* in a meat slurry solution may help establish if this antimicrobial could be added into a recycled brine solution on an industrial scale, as the addition of Charsol® to a beef product would not impose any adverse sensory characteristics to the beef, and therefore may be readily accepted by consumers.

Both MicocinX™ and Charsol® were able to reduce counts of *L. monocytogenes* in the brine; however, the combination of both antimicrobials did not show any

synergistic effects. Although counts of *Listeria* were still quite high in the brine, a longer process may have resulted in greater reduction in cell counts. However, to increase the time in the brine solution, the experiment would have to be changed to allow the brine solution to be recycled, although the use of recycled brine would added in additional variables.

The addition of antimicrobials to the brine had no impact on the counts of *Enterobacteriaceae* recovered from the injected beef roasts. Given that there was no killing effect observed in the brine solution from either of the antimicrobials, the fact that no additional effect was observed in the injected beef itself was not surprising. Considering that bacteria injected into meat remain at the point of injection (Gill et al., 2008) and that some antimicrobials previously tested in brine injected beef caused greater reduction of *E. coli* at 15°C compared to 4°C (Alder et al., 2011), it was anticipated that the use of Charsol® would result in a decrease in counts of *Enterobacteriaceae*. However, this was not the case. Possible reasons for this could be an interaction between Charsol® and the raw beef that has not previously been reported or that the concentration of the Charsol® was not sufficiently high to have an effect in meat. Also, the growth of *Enterobacteriaceae* in the brine only solution demonstrates that even a small abuse in refrigeration storage, 7°C, can lead to a significant increase in bacterial counts within as little as 7 days.

The use of MicocinX™ in a brine solution used for injection of beef resulted in lower counts of *L. monocytogenes* compared to either the positive control or the treatment containing the Charsol®. MicocinX™ was able to inhibit the growth of *L.*

monocytogenes in injected beef stored for 4 days at 7°C. Unlike experiments in microtittre plates with brine where Charsol® completely inhibited the growth of *L. monocytogenes*, it was not able to inhibit *L. monocytogenes* at all in beef. After seven days of storage all antimicrobial treatments including the positive control had similar microbial counts, showing that the effect the antimicrobials had at inhibiting the *L. monocytogenes* in the beef roast was lost after this length of time at this storage temperature. The fact that the MicocinX™ no longer inhibited the growth of the *L. monocytogenes* in the beef sample after day 4 is not completely unexpected, as the MicocinX™ only contains a finite concentration of bacteriocins within the solution and it would not be unreasonable to speculate that after a certain amount of time the bacteriocins would be bound to membranes and the *L. monocytogenes* would be able to grow unimpeded. It was not until the day seven storage samples that any growth of *L. monocytogenes* was observed in the uninoculated control, demonstrating the potential for growth of *L. monocytogenes* in a temperature abused raw beef product.

These results show that although the brine solution for the MBC experiments and the brine solution post injection showed either complete inhibition or a decrease in bacterial counts, once added into the injected beef the antimicrobial effects were no longer observed. This demonstrates the value in testing and evaluating the results from in vitro experiments in actual products under industry processing conditions. Future studies could investigate if increasing the concentration of Charsol® in the brine solution could have any impact on the presence of *Enterobacteriaceae* or possibly further decrease numbers or inhibit the growth of *L. monocytogenes*. Decreasing the inoculum level in the brine to a more realistic level is suggested, as 6 log CFU/mL of a pathogen in a brine

solution is quite high and it may be that the antimicrobials would be more effective at lower bacterial numbers.

4.5 Conclusion

Heat resistant strains of *E. coli* and *L. monocytogenes* were selected by inoculating ground beef samples and heating the inoculated ground beef to 60°C for 30 min. The strains with the greatest heat resistance had approximately a 4 log CFU/g reduction in counts during heating. This demonstrates the ability of these strains of *E. coli* and *L. monocytogenes* to survive a cooking process to what would represent a 'rare' state for a steak. Additionally, though all the strains of *E. coli* O157:H7 (non-shiga toxin producing) had very little heat resistance, verotoxigenic *E. coli* O140:H14 had only a 4 log CFU/g reduction in counts. The fact that heat resistant strains of both *E. coli* and *L. monocytogenes* could be determined from a small number of a variety of strains should be cause for concern when determining the risks associated with a brine-injected beef. With the evaluation of antimicrobials against both types of bacteria, green tea extract and Charsol®, showed the greatest efficacy in inhibiting the growth of both *E. coli* and *L. monocytogenes* cocktails at minimum bactericidal concentration of 0.125% for both antimicrobials. No synergy was found between any of the antimicrobials tested against either of the bacterial cocktails. When raw beef was injected with the bacterial cocktails and the antimicrobials in a brine solution, Charsol® had no effect against the strains of *E. coli* or *L. monocytogenes*. However, MicocinX™ caused a reduction in *L. monocytogenes* counts and inhibited the growth of *L. monocytogenes* during four days of storage at 7°C, with or without the addition of the Charsol®. This showed that results obtained in vitro may not be confirmed. There remains a need to find antimicrobials that are effective in

the elimination and inhibition of pathogenic bacteria as producers continue to develop value added raw beef products for consumers, who demand that these products are safe even when cooked to a rare condition. The presence of heat resistant pathogenic bacteria is a risk to the beef industry that requires further research.

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