Survival of *Listeria monocytogenes* on Ready-to-Eat Roast Beef During Refrigeration Storage

by

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Abstract

Listeria monocytogenes (*LM*) is a psychrotrophic bacterium with serious consequences to human health. *Listeriosis* causes an estimated 1600 sicknesses and 260 deaths annually in the United States with the primary source of transmission being the consumption of contaminated foods, most frequently from ready-to-eat foods. Ready-to-eat (RTE) is a term applied to those products which require no cooking or heating before consumption. The lack of required heating associated with RTE products provides a unique level of food borne risk. To ensure the safety of these RTE products, antimicrobials such as diacetate and lactate are often added to limit bacterial growth. Despite strict regulations and monitoring of food borne pathogens during fabrication and packaging, contamination of RTE products can simply occur after the product has been opened. As a saprophytic organism *LM* can enter the food industry from a variety of sources and persist on food processing equipment through the formation of biofilms. These microbial communities can serve as reservoirs for pathogens and contamination of foods often occurs from contact with unclean surfaces. Although *LM* is of great concern due to its potentially serious clinical outcomes, limited information is available regarding the behavior of this pathogen under home storage conditions. Furthermore, the role of background microflora on *LM* survival is unclear. The identification and analysis of *LM* growth will be useful in generating more effective strategies to prevent future outbreaks. The objective of this research was to evaluate the survival of deli roast beef in simulated home storage.

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List of Abbreviations

LM	*Listeria monocytogenes*
RTE	ready-to-eat
LAB	lactic acid producing bacteria
GRAS	generally regarded as safe
MAP	modified atmosphere packaging
TSB	Tryptic soy broth
BHI	Brain Heart Infusion broth
sp.	species
eps	exopolysaccharide
inlA	internalin A
inlB	internalin B
prfA	positive regulatory factor A
llo	listeriosin O
hly	the gene encoding for listeriosin O
plcA	phospholipase A
mpl	metalloprotease
actA	actin A
plcB	phospholipase B
CDC	Center for Disease Control
MogR	motility gene repressor
GmaR	glycosyl transferases flagella synthesis antirepressor

QS	quorum sensing
G1P	glucose-1-phosphate
Hpt	hexose phosphate transporter

Introduction

Listeria monocytogenes is a rare but deadly opportunistic pathogen associated with food. As a psychrotrophic facultative anaerobe, this organism poses a unique threat to the food industry. Modified atmosphere packaging is commonly used to prolong the shelf life of cooked meat products. As ready to eat (RTE) products require no cooking or heating before consumption, this presents a unique level of food borne risk. Despite strict regulations and monitoring of food borne pathogens, *LM* is frequently isolated from these products and is responsible for 250 deaths each year in the United States. Although contamination can occur during fabrication and packaging, contamination of RTE products can simply occur after initial use from contact with unclean surfaces. To begin evaluating the growth of *LM* on RTE products, 4 commercially available brands of RTE roast beef were inoculated with *LM* and evaluated over a 16-day period. No decrease in *LM* levels was observed in any treatment, suggesting that currently acceptable food industry use of antimicrobials do little to eliminate an already present contaminant.

Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene revealed a dynamic microbial community within each brand and large variations in population composition between brands that changed with time. Sequencing analysis revealed multiple *Lactobacillus sp*., *Anoxybacillus flavithermus, Carnobacterium spp. Enterococcus spp*., *Weisella confusa* and many others whose genetic sequence to date has no identification. To further evaluate the role of the background microflora on *LM* growth, roast beef samples were irradiated and inoculated with *LM10403s* and monitored during refrigeration storage. No growth was observed over a 16-day period and *LM*

concentrations remained at the original inoculum concentration in each treatment. This may suggest that in the presence of antimicrobials, the background microflora may not have a large impact on overall population levels. Additionally, the metabolic activity of *LM* on commercially available brands were analyzed. This revealed differences in metabolic activity in the presence of *LM.* This information could be used to reduce this risk of *LM* on foods.

LM survived in all treatments and the presence of antimicrobials had no terminating effect on the pathogen. Surprisingly, of those products containing antimicrobials, only product B displayed a significant increase in growth over a 16-day storage period This increase in *LM* number occurred during a decrease in pH in the uninoculated control samples, likely due to a growing population of lactic acid producing bacteria. Denaturing gradient gel electrophoresis and subsequent sequencing revealed unique bacterial compositions between brands, including multiple LAB species. These data suggest that the propensity of *LM* growth on RTE products is associated with changing populations of background microflora. In products without antimicrobials, samples inoculated with the 5-strain *LM* cocktail displayed a 3log increase in growth over a 16-day period at both high (4.25log CFU/g) and low (1.70log CFU/g) levels of initial contamination. No significant differences in metabolic activity were seen by these strains in vitro.

Microbial populations can persist on food processing equipment through the development of biofilms. There are multiple methods to manage the bacterial load on foods. These include manipulation of pH, temperature, salt, and antimicrobials, as well as processes such as pasteurization, irradiation, and modified atmosphere packaging. A full understanding of microbial population and communication may present cost-effective alternatives to microbial control. The identification and analysis of *LM* growth will

be useful in generating more effective strategies to prevent future outbreaks. With an ever-increasing global population, food safety has never been more important. More research needs to be done to ensure the safety of these products during home use and storage.

Chapter I

Literature Review

1.1 Listeria biodiversity

Listeria is a genus comprised of Gram-positive, facultative anaerobic, non-spore forming rod shaped bacteria approximately 1um in length. The genus *Listeria* contains 8 species including *monocytogenes*, *ivanovii*, *seeligeri*, *innocua*, *welshimeri*, *grayi, marthii, and rocourtiaei*; of which only *Listeria ivanovii* and *Listeria monocytogenes* are pathogenic (Robinson et. al 2000). *Listeria* species belong to the phylum Firmicutes and are found in diverse environments with regards to pH (4.0-9.5), temperature (<1-45^{o}C), and salt (up to 10% NaCl) levels (Liu, 2005). As a facultative anaerobic organism, *Listeria monocytogenes* can remain prolific in a wide range of environmental conditions (Cossart, 2011).

Listeria monocytogenes (*LM*) consists of 4 evolutionary lineages (I, II, III, and IV) with overlapping ecological niches. *Listeria* species are subdivided into serotypes based on 15 somatic O-groups (I-XV) and 4 flagellar H-groups (A-D) (Seeliger and Jones, 1986). Currently, 12 serotypes are recognized for *LM:* 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7. Of these, *Listeria monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for over 98% of documented human cases. Furthermore, serotype 4b is most routinely isolated in epidemic outbreaks, while serotypes 1/2a and 1/2b are linked to sporadic infection (Liu, 2006). With a mortality rate reaching 30%, *Listeria monocytogenes* represents the deadliest foodborne pathogen. Although the genera *Salmonella, Campylobacter, and Vibrio* sickness occuring more frequently, these pathogens have mortality rates of less than 1% altogether (CDC, 2013). The risk of listeriosis is highest for the elderly, pregnant women, neonates, and the immunocompromised (Ryser, 2012). The consumption of contaminated foods remains the primary transmission route to humans. First identified a food pathogen in 1986, the Center for Disease Control

(CDC) estimates that 1600 sicknesses, 1600 hospitalizations and 260 deaths occur annually (CDC, 2013). As a saprophyte *Listeria* species are found in a variety of environments including soil, silage, sewage, and vegetation. Additionally, this pathogen has a remarkable ability to survive living hosts where it gains access to our tissues through contaminated foodstuffs (Freitag, 2009) (Vasquez-Bolland, 2011).

1.2 Biofilms

Listeria monocytogenes (LM) forms increased-resistance biofilms on the surface of food processing equipment (Chavant, 2012). A biofilm is a population of microorganisms attached to a surface encased in self-produced polymers. The formation of a biofilm increases biological fitness and promotes long-term survival in both aquatic and terrestrial environments. Microbial populations existing as biofilms display increased resistance to antibiotics, sanitizing agents, as well as other environmental conditions such as pH, water availability, and nutrient availability. This evolutionary defense mechanism allows populations of bacteria to survive in areas their planktonic counterparts could not survive. The life cycle of a biofilm can be divided into four distinct phases (Kadam, 2013). The first phase is initial contact (Stoodley et. al, 2002). Flagellar mutants form defective biofilms suggesting that motility is required for this process (Lemon et. al, 2007). This is a reversible period during which the bacteria encounter a surface either randomly or through chemical attractants (Meibom et al. 2004). The next phase includes attachment to the surface and the formation of microcolonies. This phase also includes the recruitment of subsequent bacteria and the beginning of exopolysaccharide (EPS) production. The secretion of polymers is a key feature of a biofilm as cells will embed themselves in a matrix of proteins, DNA, and

sugars (Flemming et. al 2007). The production of EPS is thought to afford the community protection from external threats, such as antimicrobial compounds and larger predators (Matz et al., 2005).In the third step, the formation of a mature biofilm can be observed. In this stage, biofilms can differentially express as much as 10% of their genomes when compared to their planktonic form (Beenken et. al, 2004). These changes in gene expression can affect metabolism, motility, virulence and EPS production. Interestingly, biofilms originating from a single colony (identical genetic composition) can have vastly different gene expression profiles dependent on location and function, a trait familiar to multicellular organisms. Biofilms may be the evolutionary precursor to multicellularity (Miller, 2001). The last phase is detachment. During this phase bacteria can either be removed by a physical force or enzymatic degradation of the EPS (Liu et al., 2007).

Flagella are very important for biofilm formation in several bacterial species (O'Toole *et al*., 2000). *Listeria monocytogenes* has four to six flagella uniformly distributed across the organism. The transcription *of flaA*, which encodes each of the thousands of flagellin monomers, is stopped at temperatures above 30°C, where *LM* strains are not motile. There are a number of known regulators in flaA expression, FlaR (flagellin regulator) (Sanchez-Campillo *et al*., 1995), PrfA (positive regulatory factor A) (Michel *et al*., 1998), DegU (degradation enzymes regulator) (Knudsen *et al*., 2004), MogR (motility gene repressor) (Gründling *et al*., 2004) and GmaR (glycosyltransferase and motility anti- repressor) (Shen *et al*., 2006). These regulators are required for complete *LM* virulence as well as playing a significant role in biofilm formation. Of interest, *LM* virulence and many aspects of biofilms formation are both controlled under PrfA (positive regulatory factor A).

Through the formation of biofilms*, Listeria monocytogenes* is able to persist in food processing environments and withstand routine cleaning due to the formation of biofilms. Although *LM* can enter the food processing chain at any point, food processing and other handling facilities likely represent the most critical points for potential contamination and dispersion. Of the 183 strains of *LM* investigated by Bestin and colleagues, all *LM* strains were able to form a biofilm and persist on stainless steel (Bestin, 2012). Additionally, the authors implicated temperature as the greatest contributor to biofilm formation and not the serotype or origin of isolation (food, animal, human etc.). Of note, Hingston and colleagues provide evidence that desiccation tolerance is proportional to the original concentration of *LM* and that no overall protective effect was seen at higher concentrations. This suggests that desiccation survival of *LM* strains may be an attribute of the cell itself and not a population dependent effect. The biofilm matrix of *LM* contains extracellular DNA (eDNA) that plays a role in initial adhesion. Enzymatically removing eDNA through DNaseI treatment significantly reduces surface attachment resulting in reduced biofilm formation. In contrast, proteases and RNases do not alter the adhesion capacity of this organism (Harmsen *et al*., 2010). The ability of biofilms to form is dependent on temperature nutrient availability and relative humidity (Kim et al., 2008). Biofilms generated in 100% relative humidity show similar levels of survival as those grown in Tryptic soy broth (TSB) (Bae, 2012). Furthermore, biofilms stored at 100% relative humidity displayed the greatest resistance to disruption (Lee,). Biofilm formation in various *LM* strains was greatest in media containing the least nutrients, highlighting the formation of *Listeria* biofilms as a defense mechanism (Bestin, 2013). The proportion of dead cells in lower nutrient media was significantly higher and plays a role in EPS biosynthesis. Initial exposure of pathogens to surfaces may take place directly with a source of contamination or indirectly through airborne particles

(Kusumaningrum et al., 2003). In a food-processing center, the slaughter and fabrication of our food animals provides pathogens with an excellent opportunity to contaminate surfaces used in processing. In these locations, *LM*-associated biofilms represent a persistent source of contamination and pose a long-term health-risk to the consumer (Wen, 2013).

1.3 **Quorum Sensing**

Quorum sensing (QS) refers to cell density-dependent changes in gene expression that lead to a synchronous population response, such as biofilm formation. It is likely that these coordinated, population-level responses were an indispensable step toward multicellularity (Miller, 2001). As QS mechanisms can control other factors such as physiological state, metabolism, and antibiotic production, the role of quorum sensing in meat spoilage as a means to increase shelf life is of particular interest. As bacterial loads on food samples reach very high concentrations (10^8), it is highly likely that many aspects of meat spoilage are controlled by QS mechanisms. Additionally, quorum sensing responses allow intricate control of factors such as bioluminescence, virulence factor expression, sporulation and apoptosis. Many responses are only initiated at a density dependent threshold and are controlled by a family of signal molecules known as autoinducers. Quorum-sensing systems are found in a wide range of bacterial species. Gram-negative bacteria typically use signal molecules known as acyl-homoserine lactones (AHLs). These molecules are synthesized intracellularly by LuxI and accumulate outside the cell. At a minimum threshold, these molecules diffuse freely into the cell, and are recognized intracellularly by LuxR which binds to upstream elements of target genes regulating gene expression. In

contrast, Gram-positive bacteria commonly use small peptides (linear or cyclic) that initiate effects from outside of the cell. The small peptides mediate signal transduction through membrane-associated receptor kinases, which activate response regulators by phosphorylation, activating target genes (Novick and Geisinger 2008). Both of these traditional pathways display high levels of specificity ensuring private communication among highly diverse bacterial species. Specificity is afforded though (.,). Despite the name, quorum sensing can be diverse and are not limited to population density sensing (Platt, 2010). Quorum sensing allows the organism to modify their environment based on unique biotic and abiotic cues.

Additionally, the agr (accessory gene regulator) peptide mediated mechanism characteristic of most Gram-positive bacteria (Waters, 2005). The quorum sensing peptide, agrD, is likely involved in virulence as a mutant strain displayed altered expression of several virulence genes, as well as impaired biofilm formation (Garmyn, 2011). Additionally, an *in silico* comparison of the *LM* genome to that of *Staphylococcus areus* identified an open reading frame (lmo0435) with similarity to biofilm-associated protein (Bap), important for the binding of staphylococci to abiotic surfaces (Garmyn, 2011). This Bap protein also has been implicated in the virulence of *Staphylococcus aureus* (Jordan, 2008). Listeria monocytogenes mutants lacking Prfa, a master regulator of virulence, fail to form biofilms (Lemon et al., 2010). Flagellar mutants display a similar phenotype (Lemon et al., 2007).

1.4 Virulence

Infection of *Listeria monocytogenes* begins with the ingestion of contaminated foods where the organism reaches the intestine. Within the intestine, this pathogen promotes the internalization into host epithelial cells through two surface molecules, internalin A and internalin B. These surface proteins interact with their respective receptors on intestinal epithelial cells, E-cadherin and Met, leading to the changes in host cell cytoskeleton triggering bacterial engulfment. Because of this unique ability, *LM* can withstand macrophages making it a very deadly infection. From here, *LM* can cross 3 human barriers: the intestinal barrier, the blood-brain barrier, and the feto-placental barrier leading to complex and often fatal infections (Cossart, 2011) (Pizarro-Cerda, 2006).

The proteins internalin A (inlA) and internalin B (inlB), are encoded by a single gene, inlAB. This gene is regulated by PrfA, a major transcriptional activator and master regulator of virulence. PrfA is a 233 amino acid protein which binds to a 14bp palindromic sequence upstream of the target gene. Each known virulence determinant, as well at least 145 other genes, contains an upstream regulatory element under the control of the PrfA transcriptional regulator (Vasquez-Bolland, 2011). PrfA expression is normally low in broth culture (Chico-Calero, 2002). The virulence genes are arranged on two loci within the *LM* genome known as the *Listeria* pathogenicity islands 1 and 2 (LIPI-1 and LIPI-2). LIPI-1 contains prfA, plcA, hly, mpl, actA, and plcB. LIPI-2 contains inlAB. Multiple promoter regions exist and gene expression is controlled by the concentration of prfA as well as its affinity for the particular promoter region. Redundant methods of control ensure flexibility and precision. The expression of prfA is controlled by a number of environmental variables. Uniquely, prfA mRNA forms a hairpin at temperatures below 30^{o}C preventing translation of the transcript. At 37^{o}C, this secondary structure is destabilized (melts) allowing for the translation of PrfA, leading to the expression of virulence genes.

Maximal expression or prfA is seen at 37^{o}C and very limited at 30^{o}C (Johannsen, 2002). This type of translational control, known as a thermoswitch, also allows the rapid initiation of virulence in the host and prevents wasted energy expenditure in other environments (Vazquez-Boland, 2011). These highly orchestrated changes in gene expression ensure factors that promote virulence virulence are up regulated At 37^{o}C (physiological temperature), while motility and chemotaxis are down regulated (Garmyn 2011). As motility directly influences biofilm formation, temperature remains a key factor in understanding the behavior of this organism in any environment.

LM gains access to host cells though the interaction between *LM* internalin A and internalin B and host cell E-cadherin and Met. To avoid the vacuoles created upon initial entry into a cell, the pore forming toxin listeriosin O (LLO) is expressed by the hly gene and responsible for degrading the phagosomal membrane. In addition to allowing for escape from phagocyte, successful avoidance of capture provides access to the host cytosol where replication can occur. The phospholipases A and B (plcA and plcB), in conjunction with LLO, are associated with the disruption of the vacuolar membrane and avoid phagocytosis. Mutants lacking LLO fail to reach the cytoplasm and are non-virulent. Similarly, *Bacillus subtilis* mutants expressing LLO acquire phagosomal escape (Bielecki, 1990). Once free in host cell cytoplasm, the surface protein actA initiates actin based motility within the host cell and allows for cell to cell spread (Pizarro-Cerda, 2012). Mpl is a matrix metalloprotease associated with post-translational modifications of the phospholipases into their active form (Forster, 2011). The maturation of the phospholipases by mpl is dependent for intracellular infection. In vitro, mpl is upregulated at acidic pHs, consistent with physiological conditions (Forster, 2011).

Once inside host-cell cytosol, little is known about the nutritional requirements for the organism.

Of note, LM imports glucose-1-phosphate (G1P) from the host for its own metabolism. This process is mediated by PrfA-dependent expression of a hexose phosphate transporter (Dussurgent, 2004). Chico-Calero et. al. found non-pathogenic strains of *Listeria* could not use G1P while pathogenic strains of *LM* and *Listeria ivanovii* could use G1P (Chico-Calero, 2002).While, glucose is used by all *Listeria* species, the import of G1P by Hpt is required for virulence and mediates rapid intracellular proliferation as the Hpt *LM* mutant was impaired in intracellular growth. The control of virulence is also mediated by the presence of rapidly metabolizable sugars as PrfA synthesis is inhibited in the presence of glucose or fructose. The process is not mediated by the traditional catobolite control protein A (ccpA) which regulates carbon source use in a variety of Gram-positive organisms, including *LM* (Herro, 2005). Interestingly, psychrotrophs synthesize higher levels of polysaccharides at low temperatures. This is thought to support survival through the production of biofilms. Furthermore, glucosyltransferases are inactivated at higher temperatures.

The shift to becoming a human pathogen also involves changes in the expression of the flagella. The biosynthesis of flagella is also temperature dependent; controlled by repressor MogR 37^{o}C (98.6F) and anti-repressor GmaR below 30^{o}C (80F). This mechanism ensures activity in a living human host. The anti-repressor GmaR also serves as a glycosyl transferase responsible for flagellin glycosylation. GmaR's anti-repression function is independent of its glycosyl transferase function. The genetic basis for glycosylation remains ill-defined although the presence in such a diverse range of both archeal and bacterial species suggest a fundamental role to this organism's survival (Logan, 2006). Despite not expressing flagellar machinery at physiological temperatures, *Listeria monocytogenes* can polymerize host cell actin filaments to regain motility.

1.5 Meat Processing and Antimicrobials

LM can persist for extended periods in food processing centers in drains, pipes, floors, walls, and equipment of food processing centers (Ryser, 2012). A number of factors can influence LM contamination of RTE products at a facility. First, if the organism is already present in the product, improper processing time or temperature, as well as incorrect product formulation can result in transmission of the organism into the retail product. Secondly, even if a product has successfully undergone post-lethality treatments, recontamination can occur specifically by *LM*-associated biofilms on equipment or other handling surfaces. Additionally, design of facilities plays a large role in contamination of product. Path intersections between raw and finished products will foster contamination of products. Additionally, the design of the machinery itself can present difficult cleaning niches which can harbor disease causing agents.

The control of *Listeria monocytogenes* is essential to the safe production and consumption of RTE products such as deli meats. Currently, thousands of pounds of product are removed from the market each year due to risk of *LM* contamination. As a "ready-to-eat" product, these products typically aren't heated or cooked to any significant degree before consumption. As such, the addition of antimicrobials is common to both extend shelf life of these products and control the occurrence of pathogens. As a facultative anaerobe, the potential for *LM* growth is increased in modified atmosphere packaging (MAP) and vacuum packaged products. Vacuum-packaged RTE products can have CO_2 levels near 20% after just 4 hours of packaging. This number can reach as high as 30% due to residual aerobic

activity. Pseudomonas sp. (Gram-negative) are the most sensitive to high levels of carbon dioxide, while *Lactobacillus* and *Listeria* sp. (Gram-positive) are the most resistant (Mota-Meira M, 2000) . Federal regulations controlling *LM* in facilities producing and distributing RTE products have approved the use of chemical inhibitors like sodium diacetate and potassium lactate to prevent *LM* growth. Antimicrobial agents can either be added in the raw product formulation or as dip immediately prior to packaging.

The use of gamma irradiation was approved in 1990 to control for food borne pathogens. Although currently approved levels of irradiation can nearly eliminate all bacterial load, high doses can affect product quality. Furthermore, consumer perception towards "radiation" favors the use of chemical additives. Ironically, the use of irradiation in deli meat may be impractical due to the hands-on nature of distribution centers where the risk of recontamination with an unforseen population is also a concern. Controlling the microbial constituents present on the deli meats may prove a better option than a product void of microbes entirely.

1.6 **Metabolism**

With regards to traditional identification methods, *LM* produces zones of hemolysis on blood agar through the expression of a β-hemolysin called listeriolysin O (LLO). Hemolysis testing is important in the differentiation between *LM* and *L. innocua*, the most frequently occurring non-pathogenic species of *Listeria*. *LM* produces acid from L-rhamnose and α-methyl-D-mannoside but not from D-xylose or D-mannitol (Schuchat *et al*., 1991). *LM* also hydrolyzes esculin, a component of selective media incorporated into agar with ferric citrate and bile salts; components found in selective media such as

Oxford agar. Hydrolysis of esculin forms esculitin and glucose. The esculetin forms dark brown complexes with ferric citrate, indicating metabolic activity of the organism. Additionally, *LM* is catalase positive, oxidase negative, methyl red positive. Further biochemical or genetic tests are required to determine the specific species or strain. Methods such as these are time consuming, limited throughput, prone to user error. As such molecular methods are a trusted method to determine strain because of their increased accuracy, and high throughput. Pulsed field gel electrophoresis is a commonly applied technique to determine strain due to its discriminatory power. Additionally, next generation sequencing methods can be applied.

1.7 Lactic acid producing bacteria

There are currently 13 genera of LAB including: *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Lactosphera, Leuconostoc, Oenococcus, Pedicoccus, Paralactobacillus, Streptococcus, Tetragenococcus, Vagococcus, and Weisella (Yellow Book)*. Lactic acid producing bacteria (LAB) are commonly found in vacuum packaged products due to their growth in low temperatures, increased carbon dioxide levels (near 20%), and depleted oxygen. Due to the similar metabolic properties, *Listeria monocytogenes* and lactic acid producing bacteria are often in close association. Gram-negative organisms associated with spoilage of raw meat, such as *Pseudomonas* spp, cannot tolerate these low oxygen levels and are therefore their growth is not favored. In vacuum-packed RTE products spoilage occurs through interaction between LAB (typically around 10^8CFU/g) and the large Gram-negative family Enterobacteriaceae. Interestingly, spoilage organisms must reach 10^8 or 10^9 CFU/g before sensory

changes are detectable in these vacuum packaged products (Bruhn, 2004). LAB are a very common group of non-pathogenic fermenters in food and in the healthy human gut microflora. Major LAB sp. isolated from vacuum packaged products includes heterofermentative species of *Lactobacillus, Carnobacterium, Leuconostoc, and Weisella.*

The microflora of modified atmosphere packages are similar to that of vacuum packaged meats (20-40% CO_2:60-80% O_2). Because of the close association with *Listeria monocytogenes*, LAB bacteriocins, narrow-spectrum antibiotics, are of particular interest to the food industry (Thomas et al., 2000). Bacteriocins represent one of the many aspects of microbial defense systems and have been found within nearly every bacterial species examined. Many species produce tens to hundreds of different kinds of these unique antibiotics. Bacteriocins produced by LAB are divided into 3 general classes: the lantibiotics(I), the small non-lanthionine containing peptides (II), and the large heat-labile proteins (III) (Riley, 2002). Each class is subdivided by the method of killing. The killing range of Gram-positive bacteriocins is typically to other Gram-positive bacteria. The killing range can vary greatly from the very small range of Lactococcins A, B, and M, which have been found to only kill Lactococcus, while type A lantibiotics have been shown to be effective against multiple genera including *Clostridium*, *Enterococcus*, *Streptococcus*, *Listeria* and others (Mota-Meira M, 2000). *Enterococcus durans*, *Lactobacillus plantarum*, and *Lactobacillus lactis* were found to inhibit *LM* biofilms (Zhao et al, 2004).

Class IIa bacteriocins are of particular interest due to their high activity against *LM*, and their generally regarded as safe or “GRAS” status (Galvez, 2007). Furthermore, these naturally occurring compounds are likely to be consumer friendly as they permit the storage of foods in more water and less salt, two aspects of food production manipulated to limit bacterial growth. Other benefits may

include extended shelf life and reduction in food spoilage (Galvez et al., 2007). Class IIa bacteriocins are produced by a wide range of LAB including *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Streptococcus*, *Weisella*, and *Listeria innocua*. The specificity of these bacteriocins, when compared to broad spectrum antimicrobials, is highlighted by the production of a class IIa bacteriocin produced by *Listeria innocua* against *LM,* a closely related species. At least 4 genes have been identified for the production of class IIa bacteriocins and is regulated though quorum sensing (Cui, 2012). Bifidocin B has been shown to inhibit the growth of some species of the genera Listeria, Bacillus, Enterococcus, Lactobacillus, Leuconostoc, and Peidococcus (Cui, 2011).

Chapter 2

Methods

2.1 *LM* Strains

A total of seven strains of *Listeria monocytogenes* were used in this study (Table 1.). *LM*10403s, a streptomycin resistant strain is used by the majority of investigators in the U.S. studying *LM* pathogenesis. The parental strain, *LM*10403, was first reported by Edman et al. (1968) and further described in Bishop et al. (1987). Three strains were isolated from human cases of listeriosis: *LM*49594Scott A (serotype 4b, clinical isolate), *LM*19115 (serotype 4b), and *LM*7644 (serotype 1/2c). Two strains were isolated from food outbreaks: *LM*101M (serotype 4b, beef and pork sausage isolate) and *LM*108M (serotype 1/2b, hard salami isolate). *LM*F6214 is a mouse virulent strain. Cultures *LM*49594, *LM*19115, *LM*7644, *LM*101M, and *LM*108M were combined in equal volume and absorbance to obtain a 5-strain cocktail. Each strain was inoculated into brain heart infusion (BHI) broth and incubated overnight at 37°C. Following this, the bacteria were harvested by centrifugation, washed in PBS and resuspended in BHI broth containing 20% glycerol, and stored at −70°C. Before each experiment, an aliquot was thawed, inoculated into BHI broth, incubated at 37°C, centrifuged at 5000rpm at 4^{o}C, washed and resuspended in PBS.

2.2 Deli meats and *LM* enumeration

Growth of *LM* was assessed in 5 retail brands of roast beef. These products were selected by differing inspection labels and the presence of lactate and diacetate, two antimicrobial agents approved to regulate *LM* in deli meats. Meat was obtained from a local deli and stored at 4^{o}C until use. No meats

were used beyond the last date of All experiments were conducted using 25g samples in duplicate at two levels of contamination. With regards to microbiological enumeration, duplicate meat samples were stored at 4^{o}C or 25^{o}C and analyzed at days 0, 1, 3, 6, 8, 12, and 16. All samples were diluted in 100mls sterile 0.1% peptone water and homogenized for 2minutes. Appropriate serial dilutions were made in 0.1% peptone water and planted on modified Oxford agar (Difco, BD). Colony counts were made following 48hr incubation at 37^{o}C.

2. 3 Measurement of pH

Twenty-Five gram roast beef samples were transferred to whirl pack bags containing 100mls of sterile water and homogenized for 2min using an AES smasher and the pH was obtained using a MettlerToledo LE409 combination pH electrode.

2.4 Irradiation

Irradiation of meat was performed using a Co-60 Panoramic Irradiator. Ionizing radiation was applied at a dose of 4.32 MR over a period of 18hr.

2. 5 DNA extraction

To each 25g sample of meat, 100mls of sterile 0.1% peptone water was added and homogenized in a WhirlPak bag. To a 15ml conical tube, 10mls of the meat homogenate was added and centrifuged at 200rpm for 1 minute. The supernatant was then added to a clean 15ml conical tube and centrifuged for 3000rpm for 15 minutes at 4^{o}C. The supernatant was discarded and the pellet was placed into a 2ml tube containing .3g glass beads (⌀0.1mm), 300 µl Tris-SDS, and 500ul TE-saturated phenol. This was placed into a MiniBeadBeater (BioSpec Products) for 30 seconds and shaken at 12,000rpm for 12 minutes at 4^{o}C. Following centrifugation, approximately 400ul of the supernatant was transferred to a new 2ml tube containing 200µl of TE saturated phenol and 200µl of a 24:1 chloroform: isoamyl alcohol solution. This tube was briefly vortexed and centrifuged at 12000rpm for 12 minutes at 4^{o}C. The supernatant was transferred into a 1.5ml tube containing 300µl isopropanol and 25µl of 3M sodium acetate (pH 5.2). This tube was centrifuged at 12,000rpm for 12 minutes at 4^{o}C. The supernatant was removed and 500µl was added to the tube and centrifuged at high speed. The liquid was removed and allowed to air dry for approximately 5 minutes. The pellet was then dissolved in TE buffer and stored at -20^{o}C until later use.

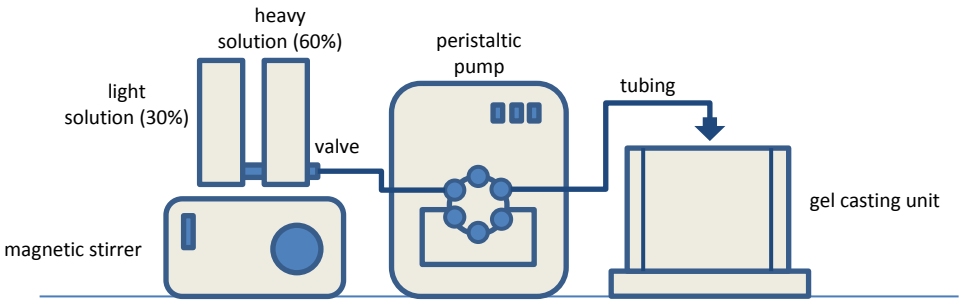
2. 6 Denaturing Gradient Gel Electrophoresis

DGGE was performed with a DCode mutation detection system (Bio-Rad, USA) by using an 8% (w/v) polyacrylamide gel with a 30% to 60% gradient of a DNA-denaturant agent for separation of the

16S rRNA genes. The 100% denaturant is defined as 7 M urea and 40% (v/v) deionized formamide. For each sample, 30ul of PCR product was loaded, and the electrophoresis was conducted at 50V for 10 minutes followed by 150V for 7 hours 100 V for 16 h at 60°C in 1×TAE buffer. The gels were rinsed in distilled water, stained with GelRed for 10 minutes, rinsed again with distilled water, and then visualized under UV. DGGE patterns were identified using the VisionWorks software (Bio-Rad, USA). Reproducibility was tested by replicate DGGE runs from all samples.

The high denaturant solution was made using 9.75mls of the 80% denaturant solution and 3.25mls of a 0% denaturant solution. The low denaturing solution was made using 4.875mls of 80% denaturant and 8.125mls of 0% denaturant solution. These were added to appropriate wells of the gradient former forming the acrylamide gel. After filtering, 50 µl of 10 % APS was added to each tube and placed on ice for 5 min. Following this, a total of 8µl TEMED was added to each tube and briefly mixed by inversion. Each solution was placed into the appropriate well of the gradient maker; spinner bar and pump were turned on. After the solution reached a designated mark, the flow was stopped and the gel was covered with 3 ml of Milli-Q water so that a level interface is made on the gel. The gel was left to sit for 40 min at room temperature to solidify. After this period, the water was poured off and excess water was removed using Whatman paper. To create the wells for the gel, 4 ml 0% denaturant solution, 24 µl 10 % APS 8 µl TEMED were combined with the gel comb to form the wells. The gel was then left at room temperature for 15 min to polymerize, sealed in a plastic bag, and stored at 4^{o}C until use.

Illustration 1. Creation of denaturing gradient acrylamide gel



2. 7 Sequencing of 16s rDNA

Using a UV light, the bands of interest were carefully removed with a scalpel and transferred into a clean 1.5ml tube. Between bands, the blade was cleaned with ethanol. 400µl ethanol was added to each tube, and placed in room temp for 15 minutes or until the gel turned white. The ethanol was then removed and 200µl of .5%NaCl solution was added to dissolve the tubes. The remaining solution was pipetted several times and placed in 4^{o}C for overnight. Each tube was boiled for 10 min, and centrifuged at 12000rpm for 10 min at 4^{o}C.The supernatant was then used as a PCR template for a 50 µl-PCR reaction using 341F/534R primers. The entire PCR product was run through gel electrophoresis and the bands were cut and purified using a Gel Extraction Kit (OMEGA). The final volume of purified product was approximately 30 µl. The PCR product was then inserted into a plasmid vector and transformed into E.Coli according to manufactures protocol. Positive colonies were sequenced using an ABI 3100 DNA Genetic Analyzer. The sequence data was inserted into VecScreen to remove the vector sequence. The remaining sequence was processed via BLASTn.

2.8 Metabolic Assay

Each *L. monocytogenes* strain was grown in 9ml BHI, at 37°C overnight. Overnight cultures were transferred (0.1 ml) to 9ml BHI and vortexed. After vortexing, 100µl volumes were transferred into eight PVC microtiter plate wells per strain, previously rinsed with 70% ethanol and air dried. Plates were made in duplicate, incubated, and covered at 25°C 24h and 48h. Each plate included eight wells of BHI without *LM* as control wells.

The metabolic activity of the samples was monitored using a microtiter plate reader at a floursescence at 595 nm (OD_{595}). Plates were made in triplicate and tested at 0, 1, 2, and 3 days. The average flouresence from the control wells was subtracted from the flouresence of all test wells. After the incubation period, 10ul of AlmarBlue was added to each well, incubated at 37C for an hour and measured. The microtiter plate metabolic assay was performed in triplicate for all *L. monocytogenes* strains, and the averages and standard deviations were calculated for all repetitions of the experiment.

Chapter 3

Results

Results

The growth of *LM10403s* on 4 different brands of deli roast beef was investigated at 4^{o}C at an initial inoculation level of 5.5logCFU/g and 1.5logCFU/g for 16 days. Samples were pulled out from the refrigerator and plated onto MOX agar. As shown in Figure 3.1, no significant change in *LM* concentrations as seen over the 16d period. Although there were antimicrobials present in the meats, *LM* survived and persist in the meat at their original inoculation level (Fig. 1).

As for the first round of study, only one strain of *LM* was used, we then tested a 5-strain cocktail containing *LM101M* (serotype 4b; beef and pork sausage isolate), LM108M (serotype 1/2b; hard salami isolate), LM19115 (serotype 4b; human clinical isolate), *LM49594* (serotype 4b; food outbreak), and *LM7644* (serotype 1/2c; clinical isolate from human) at initial inoculation levels of 6.2logCFU/g and 3.2logCFU/g. At the high level of contamination, *LM* showed no increase in population through day 8. The last 8 days of storage displayed a 2-log increase in growth in Brand B which was not present in brands A or C. Brands A and C showed no major changes in *LM* level throughout the course of experimentation. At the low level of contamination, brand B showed a 1-log increase between days 8 and 6, corresponding to the significant increase in growth at the high (6.2logCFU/g) level of contamination (Figure 3.2).

The growth of *LM10403s* was then tested on an irradiated roast beef product (X) at an initial contamination level of 7logCFU/g and 3logCFU/g. No significant changes in growth were observed over a

16 day period and *LM* growth on the irradiated product was not significantly different from brands A, B, and C.

The growth of *LM10403s* and a 5-strain cocktail on a deli roast beef product without antimicrobials was investigated at 4^{o}C at an initial inoculation level of 5.5logCFU/g (high) and 1.5logCFU/g (low). Each inoculum displayed a significant increase in growth over the 16-day incubation period. *LM10403s* displayed a 2log increase over the 16-day period at both the high and low level of contamination. The 5-strain cocktail displayed a 3-log increase in growth over the incubation period at both levels of contamination (Fig 3.4).

The pH of each product was evaluated over a 16-day period at 2 levels of *LM10403s* contamination (1.5logCFU/g and 5.5logCFU/g). The pH of brand B showed a significant drop in pH at day 12 at both levels of contamination. Brands A and C did not show a significant change in pH over the storage period (Fig 3.5).

The pH of uninoculated samples displayed a similar pattern as the inoculated samples. Brands A and B did not show any major changes over the 16-day period (Fig 3.6).

The metabolic activity of *LM10403s* on 3 brands of roast beef displayed an initial drop in activity (reduction of AlamarBlue) followed by significant increase in activity only in Brand B. The increase in reduction for Brand B was 20% higher when compared to the autoclaved control (Fig 3.7).

The metabolic activity of *the 5 strain cocktail* on 3 brands of roast beef displayed an initial drop in activity (reduction of AlamarBlue) followed by significant increase in activity only in brand B. The increase in reduction for brand B was 20% higher when compared to the autoclaved control (Fig 3.8).

The metabolic activity of *the uninoculated* 3 brands of roast beef displayed an initial drop in activity followed by significant increase in activity only in brand B, similar to that of the results obtain in Figure 3.7 and Figure 3.8. The increase in reduction for Brand B was 20% higher when compared to the autoclaved control (Fig 3.9).

Roast beef brands A, B, C, and D inoculated with 2logCFU/g *LM10403s* were evaluated for their 16s rDNA through denaturing gradient gel electrophoresis (DGGE). Each brand displayed a unique microbial population which changed with time (Fig 3.10).

Roast beef brands A, B, and C were inoculated with LM10403s at 2logCFU/g and evaluated for their 16s rDNA through denaturing gradient gel electrophoresis (DGGE). Each brand displayed a unique microbial population that changed over time. Comparison of inoculated brand B with an uninoculated sample reveals that the *Listeria* contamination directs the growth of a certain subset of bacteria. The uninoculated sample had much more variability in the microbial diversity than the contaminated sample (Fig 3.11).

Sequencing of 16 isolated bands recovered 8 known organisms, 4 organisms that have no similarity to the NCBI database, 2 organisms that are uncultured, 1 organism that is genetically identified but does not have a name in the database, and 1 band which failed to provide a sequence (yielding only the cloning vector) (Fig 3.12).

Figure 3.1 Survival of *LM*10403s on 4 brands of roast beef containing antimicrobials at 4°C

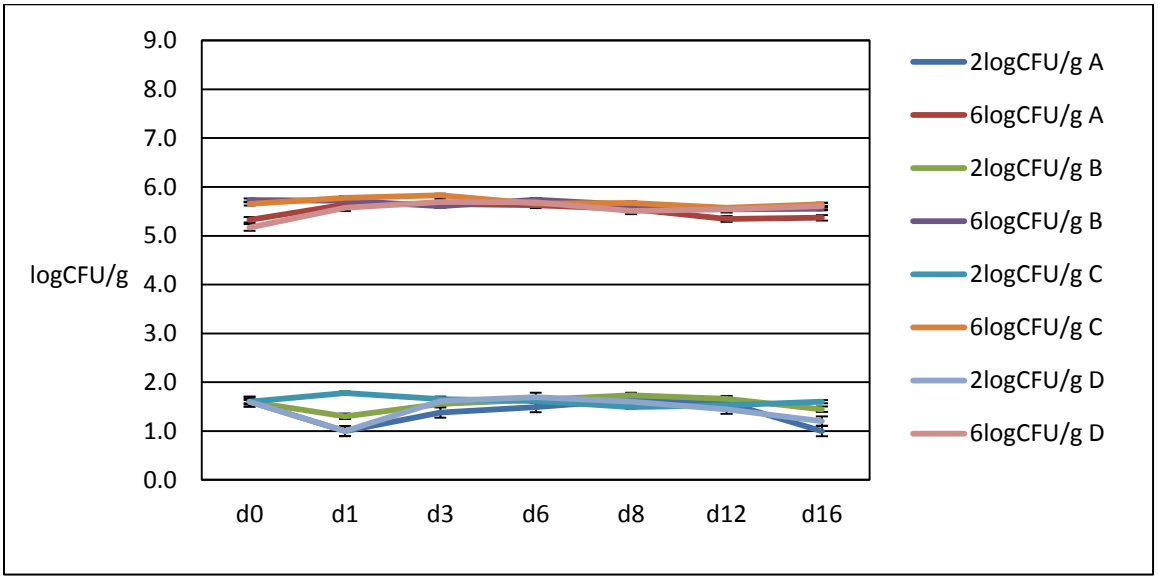


Figure 3.2 Survival of 5-strain *LM* cocktail on 3 brands of roast beef containing antimicrobials at 4C

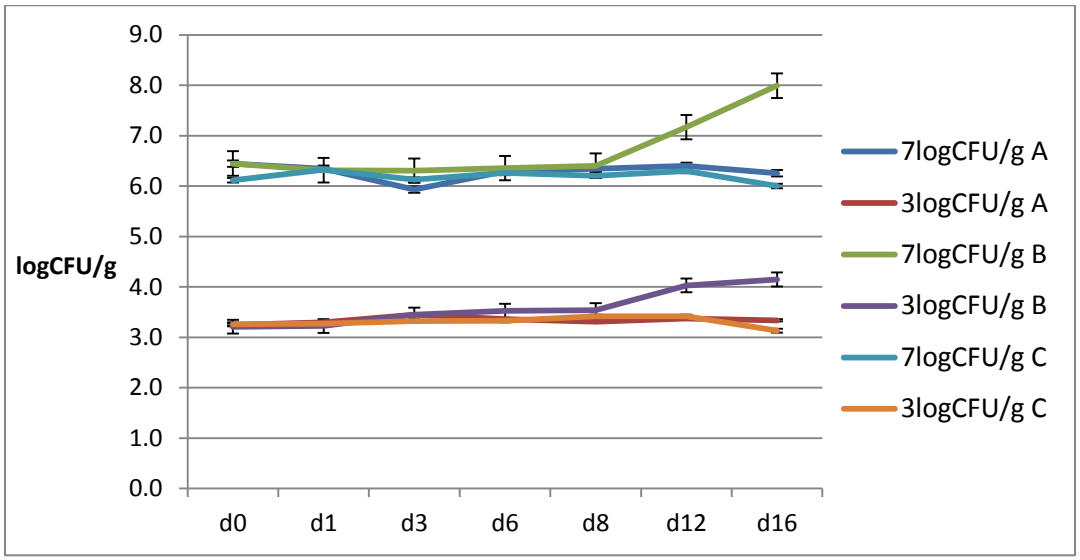


Figure 3.3 Survival of *LM* on irradiated roast beef at 4°C

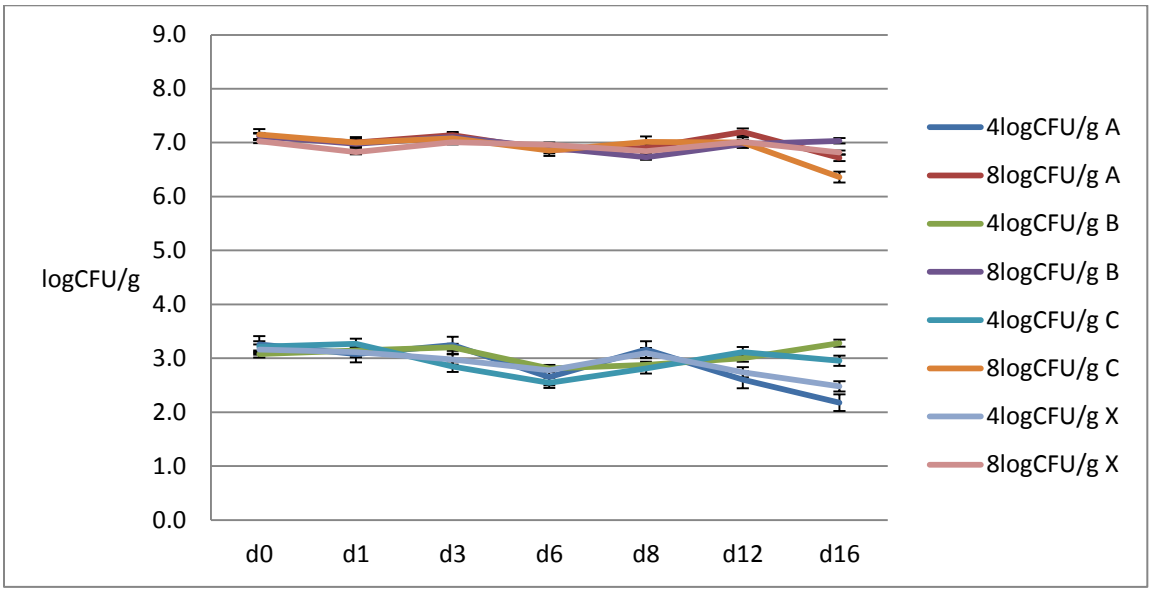


Figure 3.4 Survival of selected *LM* strains on RTE roast beef w/o antimicrobials

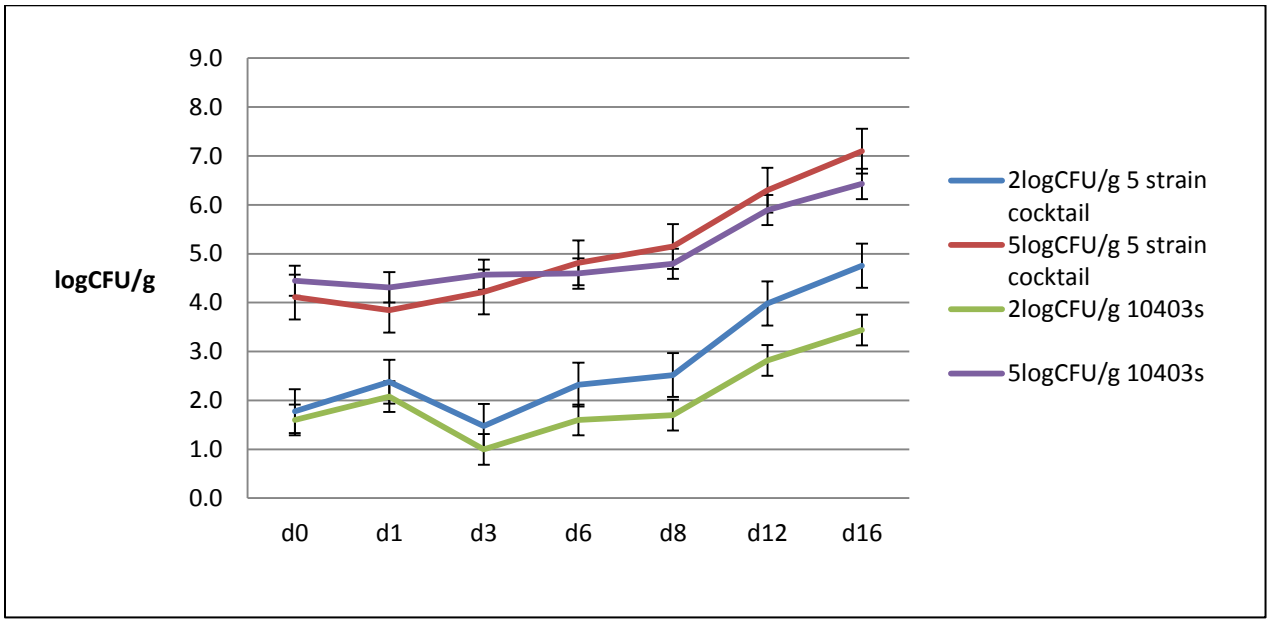


Figure 3.5 pH of *LM* inoculated RTE roast beef w/ antimicrobials at 4°C

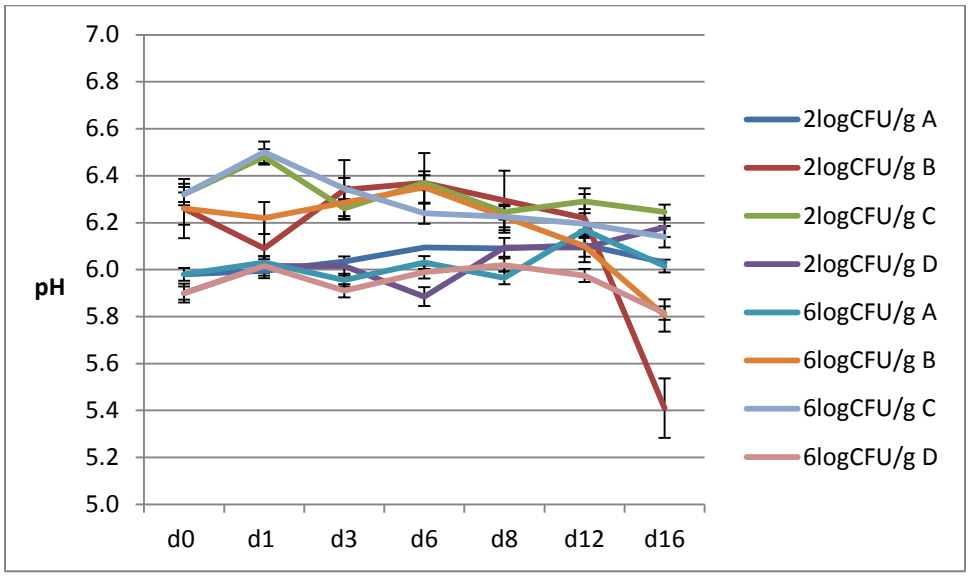


Figure 3.6 pH of uninoculated RTE roast beef w/ antimicrobials at 4°C

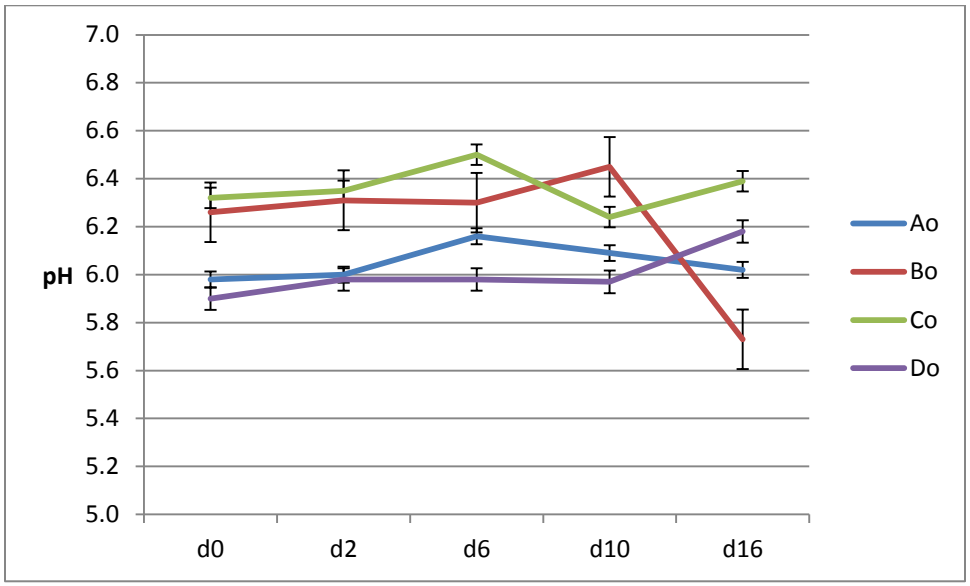


Figure 3.7 Metabolic activity of *LM10403s* and background microflora on 3 brands of roast beef juice

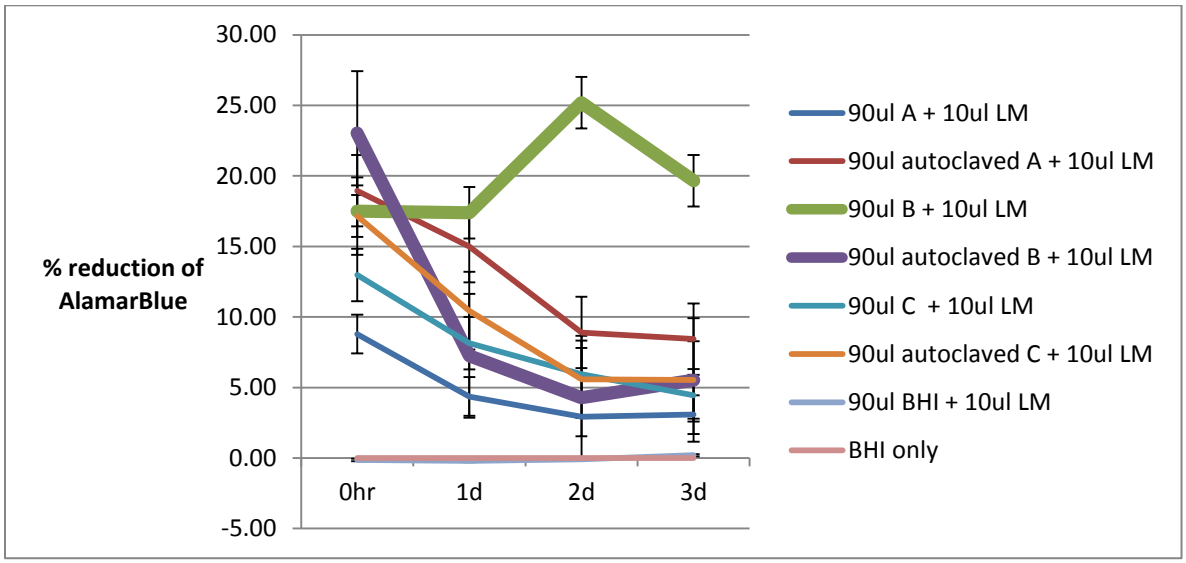


Figure 3.8 Metabolic activity of 5-strain *LM* and background microflora on 3 brands of roast beef juice

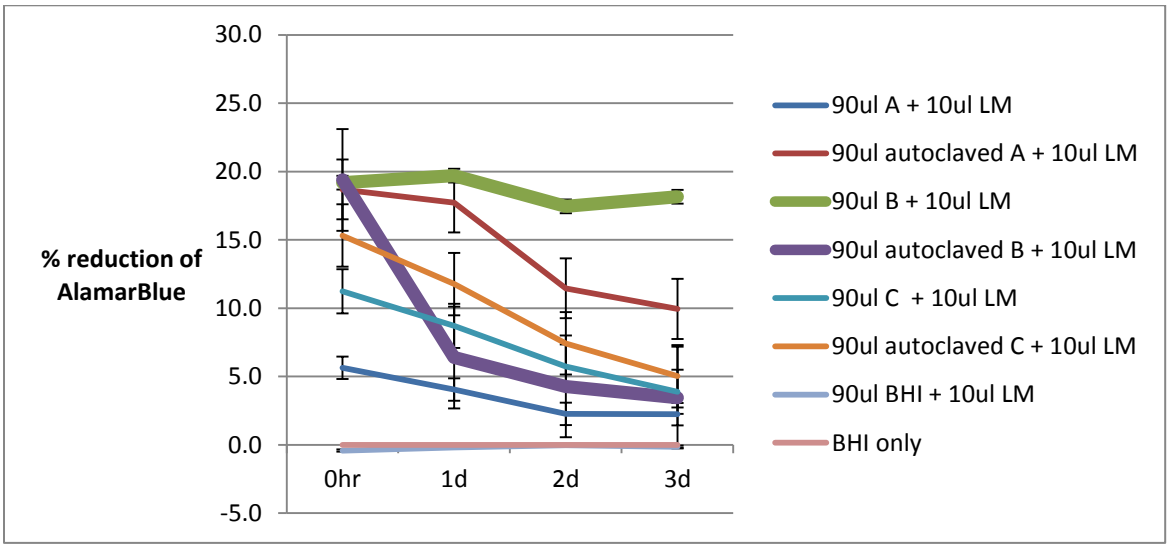


Figure 3.9 Metabolic activity of 3 uninoculated brands of roast beef juice

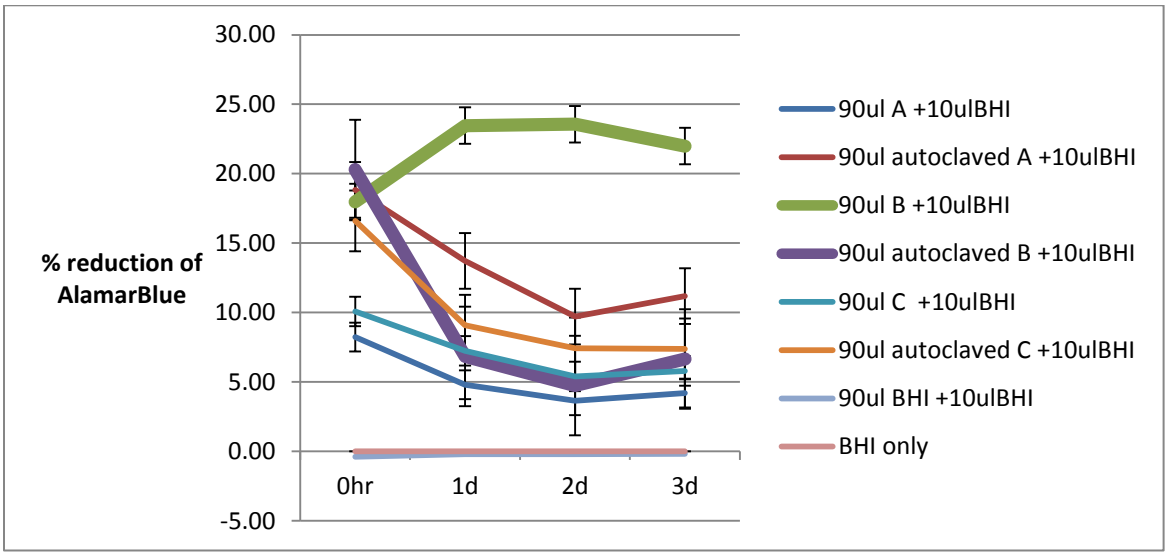


Figure 3.10 Microbial diversity of *LM*10403s contaminated roast beef brands containing antimicrobials over a 16-day period

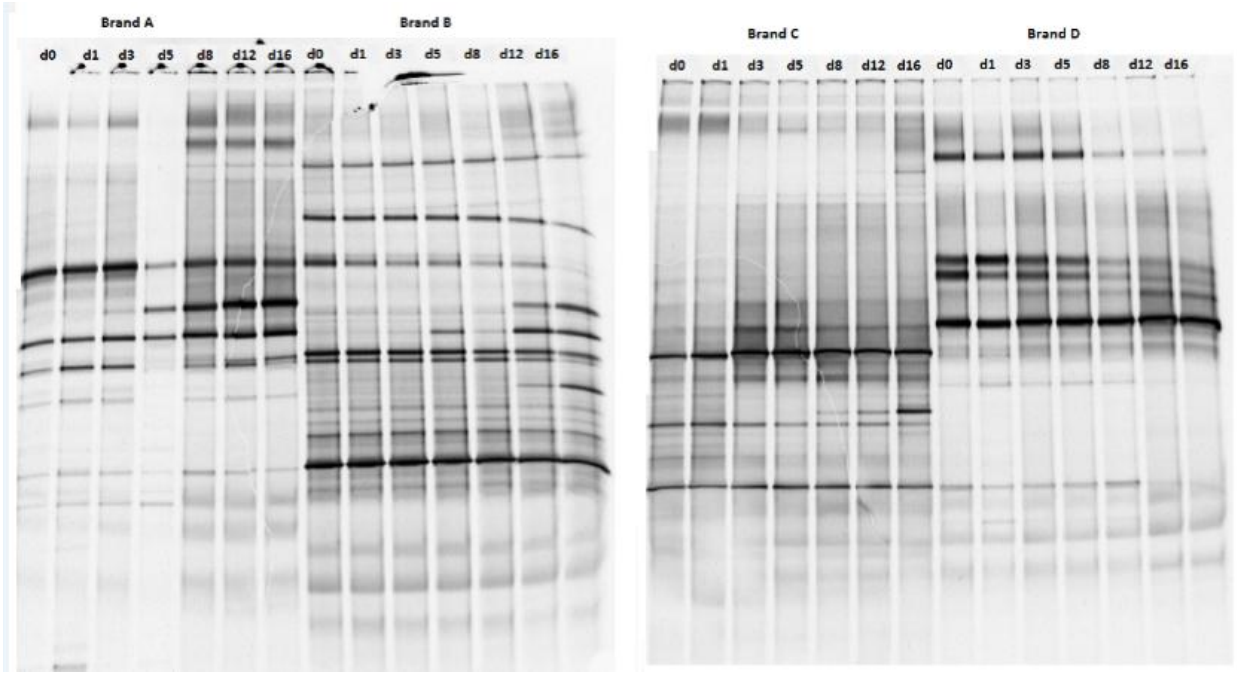


Figure 3.11 Microbial diversity of 5strain *LM* cocktail contaminated roast beef brands containing antimicrobials over a 16-day period

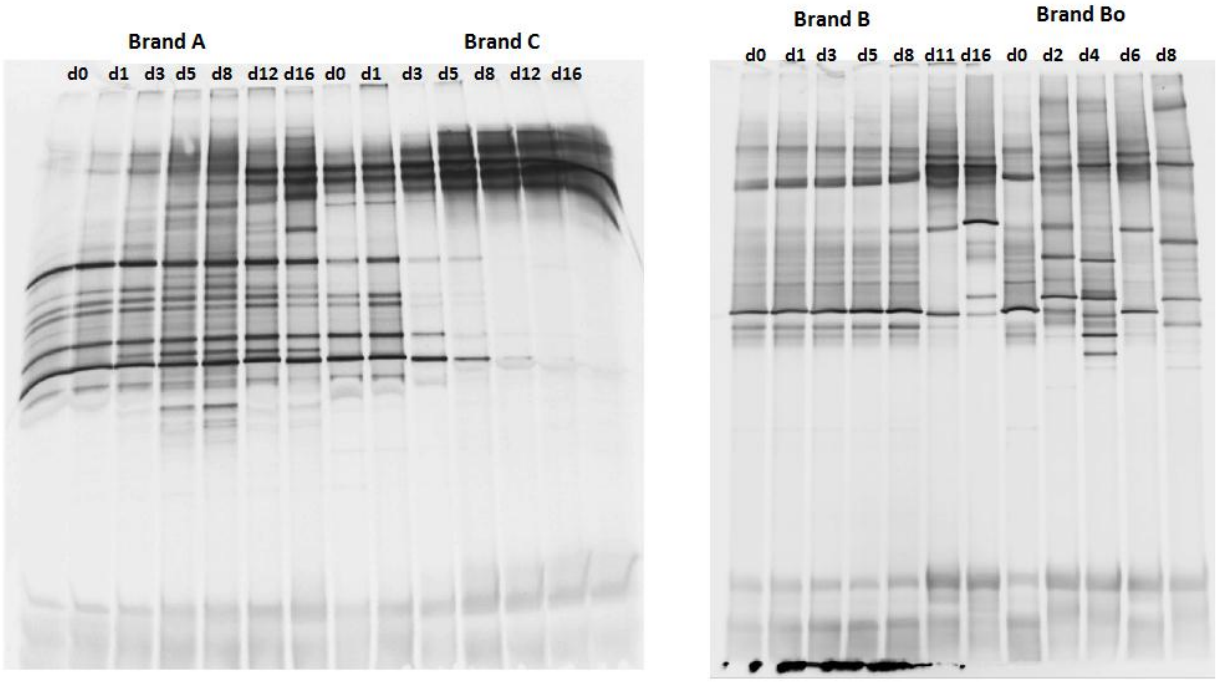
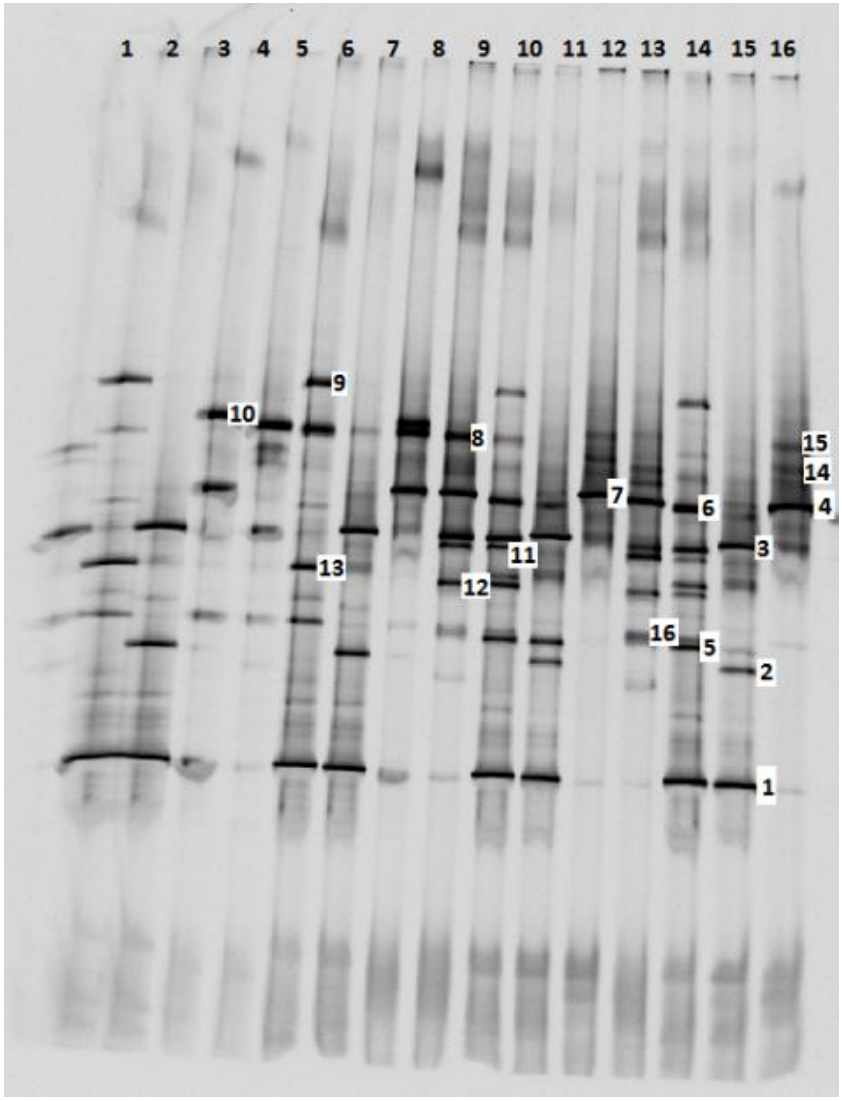


Figure 3.12 DGGE bands isolated for sequencing



lane Identification

lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
brand	A	A	A	A	B	B	B	B	C	C	C	C	D	D	D	D
day	0	0	0	0	0	0	0	0	16	16	16	16	16	16	16	16
inoculated	no	no	no	no	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	no
temp	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4°C

Chapter 4

Discussion

A major determinant of listeriosis risk is the ability of the organism to survive on food during storage. Because the minimum infectious dose is dependent on strain and host health, any level of *LM* on a product renders the product unsafe for human consumption and represents a high level of risk. In this study, *Listeria monocytogenes* survived in all treatments in this research and the presence of antimicrobials had no reductive effect beyond the initial level of contamination. Using strain *LM10403s*, the initial contamination levels persisted throughout the duration of experimentation at 4^{o}C. This data is consistent with that of previously published reports in which those products that contained antimicrobials and stored aerobically maintained their concentration throughout experimentation (Ryser, 2012). Of note, different production lots can produce products with marked variation in the concentration of antimicrobials contained within the product (Ryser, 2012). This suggests that despite similar identical branding, LM growth due to contamination may vary from package to package of the same product. Of the 4 meat products tested by Ryser (uncured turkey, cured turkey, ham, and roast beef) roast beef displays the lowest levels of *LM* growth followed by ham, cured turkey and uncured turkey (Ryser, 2012). All of the previously mentioned products stored aerobically at 4^{o}C support the survival of *LM* until the last day of sale.

In earlier work, background microflora has inhibited the growth of *LM* (Ryser 2012). Sequencing data revealed multiple *Lactobacillus sp*., *Anoxybacillus flavithermus*, *Carnobacterium spp*. , *Enterococcus spp.*, *Weisella confusa*, and still others that remain unidentified/unnamed on the NCBI database. Despite undetectable levels of anaerobic plate counts (data not shown), many of the identified bacteria were unculturable. As such, the exact levels of these microbes on these products are unknown and may be present on the product in appreciable levels. Furthermore, the metabolic activity of these products

suggests there are differences in total microbial load between products. Previous research has indicated that many lactic acid producing bacteria are already used in several products to inhibit the growth of *Listeria* species. This includes 10/20 LAB species sampled by Ibarecche et al. (Ibararreche, 2012). Through the production of bacteriocins and competitive inhibition, many of these bacteria can be used to further reduce the risk of *Listeria* growth on retail deli meats. The most common Lab of vacuum packaged products are lactobacillus, Carnobacterium, and Leuconostoc.

Listeria monocytogenes represents a unique challenge to the food industry due to its ability to persist in food processing equipment through the formation of biofilms. The persistent adhesion of unwanted organisms can represent a chronic source of contamination to the food industry and a potentially life threatening. The Jensen Farms Colorado outbreak of 2011 claimed 33 lives and 147 sicknesses and was attributed to a *LM* biofilm on food processing equipment (CDC, 2012). The vast major *Listeria* outbreaks in the have surrounded soft cheeses and cantaloupe. In the United States, 1,651 cases of listeriosis occurring during 2009–2011 were reported with a case-fatality rate of 21% (CDC, 2012). Most cases (58%) occurred among adults aged ≥65 years. Of these, approximately 3 in 4 individuals were immunocompromised. During the same period, pregnancy associated sicknesses accounted for 14% of the total 1,651 cases. The average annual incidence was 0.29 cases per 100,000 population with nearly all cases occurring in high-risk groups. Twelve reported outbreaks affected 224 patients in 38 states. A total of 5 major outbreaks were reported in this time (CDC 2012).

In summary, *Listeria monocytogenes* in ready to eat meat remains a problem for the food industry. *Listeria monocytogenes* survived the duration of storage at 4^{o}C in all experiments and those

products without antimicrobials displayed higher levels of growth. Brand B displayed higher levels of growth of *LM* during the storage period, which is also associated with a drop in pH. The metabolic activity of brand B was also increased during this time. Sequencing analysis revealed multiple differences between brands with regards to the specific microflora associated with each product. New intervention strategies that can be applied during the post-processing stages are needed to reduce risk.

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Appendix 1. Sequence Data

>JWB-1-M13F VecScreen result: nt77-247

TTAGAACGATCACTATAGGGCGATTGGGCCCGACGTCGCATGCTCCGGCCGCCAGGGCGG
CCGCGGGAATTCGATTATTACCGCGGCTGCTGGCACAGAGTTAGCCGATGCTTATTCCCC
AGATACCGTCATTGCTTCTTCTCCGGGAAAAGAAGTTCATGACCCGTAGGCCTTCTACCT
TCACGCGGCATTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCGCTGCTGCCTC
CCGTAGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCC
AACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATC
ATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTA

BLASTn accession/identity: JX183818.1 **No identity to known organism**

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>JWB-2-M13F VecScreen result: nt74-267

ATGGTACGATCATATAGGGCGATTGGGCCCGACGTCGCTGCTCCGGCCGCCAGGGCGGCC
GCGGGATTCGATTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGG
AAGAACAAGTAACGTAGTAACTGGCGTTACTGTGACGGTACCTAACGAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTC
GACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTGTAGTGTCAC
CTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTA
CC

BLASTn result: KC503890.1 **Anoxybacillus Flavithermus**

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>JWB-3-M13F VecScreen result: nt57 to 274

ATTGGTAACGATCATATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCGGGG

GCGGCCGCGGGAACTCGATTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG

AAAGTCTGACGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT

GTTAGAGAAGAACAAGGATGAGAGTAACTGCTCATCCCCTGACGGTATCTAACCAGAAAG

CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCT

GCAGGTCGACCATATGGGAGAGTCCCAACGCGTTGGGATGGGTAGCTTCAGTATTCTATA

G

BLASTn accession/identity: NR_074964.1 **Carnobacterium** (same as JWB-11)

___

>JWB-4-M13F VecScreen result: nt26-334

TTGAAATACGGATCACTATAGGGCGGGTTGTCGCCCGACGTCGCAAGATAGGCGCCATGA

ACGGCGCGGGAAAGATAAGAAGGGAAAGGCGCAGCAGTAATTTCACGATGGGAAGGAAAA

ATCTGAAGGACCACCCCGCGTGAGTGAAAAAAGGTTTTTGGATTGTAAAAATTTGGTTGT

TGGAAAAAAAAATGGATCCTGAAAGAAATTGGGCGGGGGGTGGGCCGTTTTACACCCCAA

AAAGCCCCGGGGTAATTTTTTCGGGCGGCGCCCCCCCGGGAAAATATTTTTTTTCAACCC

CGCCCCCAGAGATTTGATAGAAGAAAATTTAAAA

BLASTn result: **No significant similarity found**

___

>JWB-5-M13F VecScreen result: no vector sequence similarity found

TCGTACGATCTATAGCGATGGGCGACGTCGCAGTCGCGCGTGGCGCGATGCATCGATCTG

ACCAGCAGCAGTAGATGTCGGATGACGAGGCTGACGAGCACGCGGTAAACAAGAAAGCTT

CGGTCGTAAAGATCTTGTAGAGAACATGTGGAAGACTGTTACCCTGCGGGTTACCCAGAG

TCGCGTT

BLASTn result: **No significant similarity found**

___

>JWB-6-M13F VecScreen results: nt41-265

TAAGTAAAGATCATATAGGGCGATTGGGCCCGACGTCGCAGTACGGCCGGCAGGGAGGCC

GCGGGATTCGATTATTACCGCAGTGCTGGCACGTATCACCCGCGGCTTTCTGGTTGGATA

CCGACACTACCTGATCAGTTACTATCAGATACATTCTTCTCCAACAACAGAGTTTTACGA

TCCGAAAACCTTCTTCACTCACGCGGGGTTGCTCCATCAGACTTTCGTCCATTGTGGAAG

ATTCCCTACTGCTGCCTCCCGTAGGAATCACTATTGAATTCGCGGCCGCCTGCAGGTCGA

CCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCT

AAATAGCTTGGCGTAATCATGG

BLASTn result: KC748441.1 **Lactobacillus**

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>JWB-7-M13F VecScreen result: nt77-270

TGGGATACGGATCATATAGGGCGATTGGGCCCGACGTCGCATGTCCGGCCGGCAAGGCGG

CCGCGTGAATTCGATTCCTACGGGAGGCAGCAGTAGGGAATCCTCCACAATGGACGAAAG

TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTG

GAGAAGAATGTATCTGATAGTAACTGATCAGGTAGTGACGGTATCCAACCAGAAAGCCAC

GGCTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAG

GTCGACCATATGGGAGAGCTCCCAACGCGGTGGATGCATAGGTTGAGCATTCTATAGTGT

CACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCT

TACA

BLASTn result: KC748441.1 **Lactobacillus**

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>**JWB-8-M13F** VecScreen result: nt43 to 355

ATAAGTACAGATAACTATAGGGCGATTGGGCCCGACGTCGCAAGCCCGGCCGGAAGGCG

GCCGTGGGAATTCGATTCCAACGGGAGGCAGCAGTAGGGAATCTCCCTAATGGACGAAAG

CCGGAGGGAGAACCGCCGGGGGGAGGAAAAAGGTTTCCGAACCGAAAAGTACGGTGGTTA

TAAAAAATCAAGGTTATAAGAAACTGTTTGTCCCTTGACGGAATCAAACCAAAAAGCCCC

GGTTAATAATATTTCACAAGCCCCGGAATTAATTTTTATTGAATTCCGGGCCGCTGGCAG

GTCAACCTTATGGAGAGCCCCCCCCCTCTTTTGTGGGGGGGGGCCCCCCCCAAGG

BLASTn result: JX197377.1 **Enterococcus faecalis**

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>**JWB-9-M13F** VecScreen result: 45 to 271

ATAAGTACGAATCATATAGGGGCGATTGGGCCCGACGTCGCATGTCCGGCCGGGAAGGCG

GCCGCGGGATTTCGATTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAA

GCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTA

AGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAA

CGGCTAAATACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCA

GGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTG

TCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGGTTCCTGTGTGAAATTGTTATCC

GCTAGGGG

BLASTn result: JN792460.1 **Weissella Confusa**

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>**JWB-10-M13F** VecScreen result: nt1-163

ACCCCAAATTTTCCTCTTCTTAGGGGATGGGCGAGCGCTGTTCGGCCGGAAGGAGGCGTT

GGATTCGATTCCTACGGGAGGAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATG

GAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGA

ATGTAGGTAAGAGTAACTGTTTACCTAGTGACGGTATCTAACCAGAAAGCCACGGCTAAC

TACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACC

ATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTCCCCGTATTCTATAGTGTCACCTA

AATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTAGGC

BLASTn result: NR_028617.1 **Lactobacillus algidus**

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>**JWB-11-M13F** VecScreen result: nt44-268

CTTTAAAACGATCCTATAGGGCGATTGGGCCCGACGTCGCATGTTCGGCCGCCAGGGAGG

CCGCGGGATTCGATTCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTC

TGACGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGA

GAAGAACAAGGATGAGAGTAACCGCTCATCCCCTGACGGTATCTAACCAGAAAGCCACGG

CTAACTACGTGCCAGCAGCCGCGGTAATAATCACTAGTGAATTCGCGGCCGCCTGCAGGT

CGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCA

CCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTA

BLASTn result: NR_074964.1 **Carnobacterium**

___

>JWB-12-M13F VecScreen result: nt44-269

AGAAATGGAGATCATATAGGGCGATTGGGCCCGACGTCGCATGTACCGGCCGAAAGGAGG

CCGCGGGATTCGATTACTACGGGAGGTGTGGTACGGATTCTTCCGCCCCGGGCGGGAGTG

GGACGGACCGGCTCCGGGCGTGCCCTACCTGATCTTTTCTTCCCATACAACTGTTGGTAT

GGGACCCGAAATAAGGCAGGGACAGACCTTAGCCTGACCATACCGTACCCGACCGGGACG

GATAAATACCTGCCGCTGCCCCCCGTAATAATCACTATTGAATTCGCGGCCGGCTGCAGG

TCAACCATATGGGAGAGCTCCCAACGCGTTGGTTGCATAGCTTGAGTATTCTATATTGTC

ACCTAAATAGCTTGGCGTAATCATGGTCTAGCTGTTTCCTGTGGGAAATTGTTATCCGCT

AA

BLASTn result: **no significant similarity found**

___

>JWB-13-M13F VecScreen result: 77-246

ATGGAACGATCATATAGGGCGATTGGGCCCGACGTCGCATGTTCCCGGCCGGCATGGCGG

CCGCGGGATTCGATTATTACCGCGGGTGCTGGCACAGAGTTAGCCGATGCTTACTCCTCA

CATACCGACATTGCTTCTTCTCTGAGAAAAGAAGTTCATGACCCGTGGGCCTTCTACCTC

CACGCAGCATTGCTCCGACAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCC

CGTAAGAATCACTAGTGAATTCGCGGCCGCCTGCATGTCGACCATATGGGAGAGCTCCCA

ACGCGTTGGATGCATAACTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCA

TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTACC

BLASTn result: HQ781474.1 **Uncultured bacterium**

___

>JWB-14-M13F VecScreen result: no sequence similarity

GTAAGTACAGATCATATAGGGCGAATTGGGCCCGACGTCGCATGTCCCGGCCGGCATGGC

GGCCGCGTGAATTCGATATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGG

AGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTT

GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCT

BLASTn result: X65309.2 **cloning vector**

___

>JWB-15-M13F VecScreen result: no sequence similarity

CCTTCTGTAGACATCTCATCTGTAGCGCTGACGAGCAAGAGTCTATCCCGCGTGCAGGGC

CCTGTGGCTAGAGACCTGAGTCTCGGCATCTTTGGCGGGAGTG

BLASTn result: **no significant similarity found**

___

>JWB-16-M13F VecScreen result: nt78-248

AATGAAAGGATCATATAAGGGCGATTGGGCCCGACGTCGCATGTCCCGGCCGGCATGGCG

GCCGCGGGAATTCGATTATTACCGCGGCTGCTGGCACAGAGTTAGCCGATGCTTATTCCC

CGGATACCGTCATTGCTTCTTCTCCGGGAAAAGAAGTTCATGACCCGTAGGCCTTCTACC

TTCACGCGGCATTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCT

CCCGTAGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC

CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAAT

CATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTA

BLASTn result: KC700317.1 **Uncultured Bacterium**

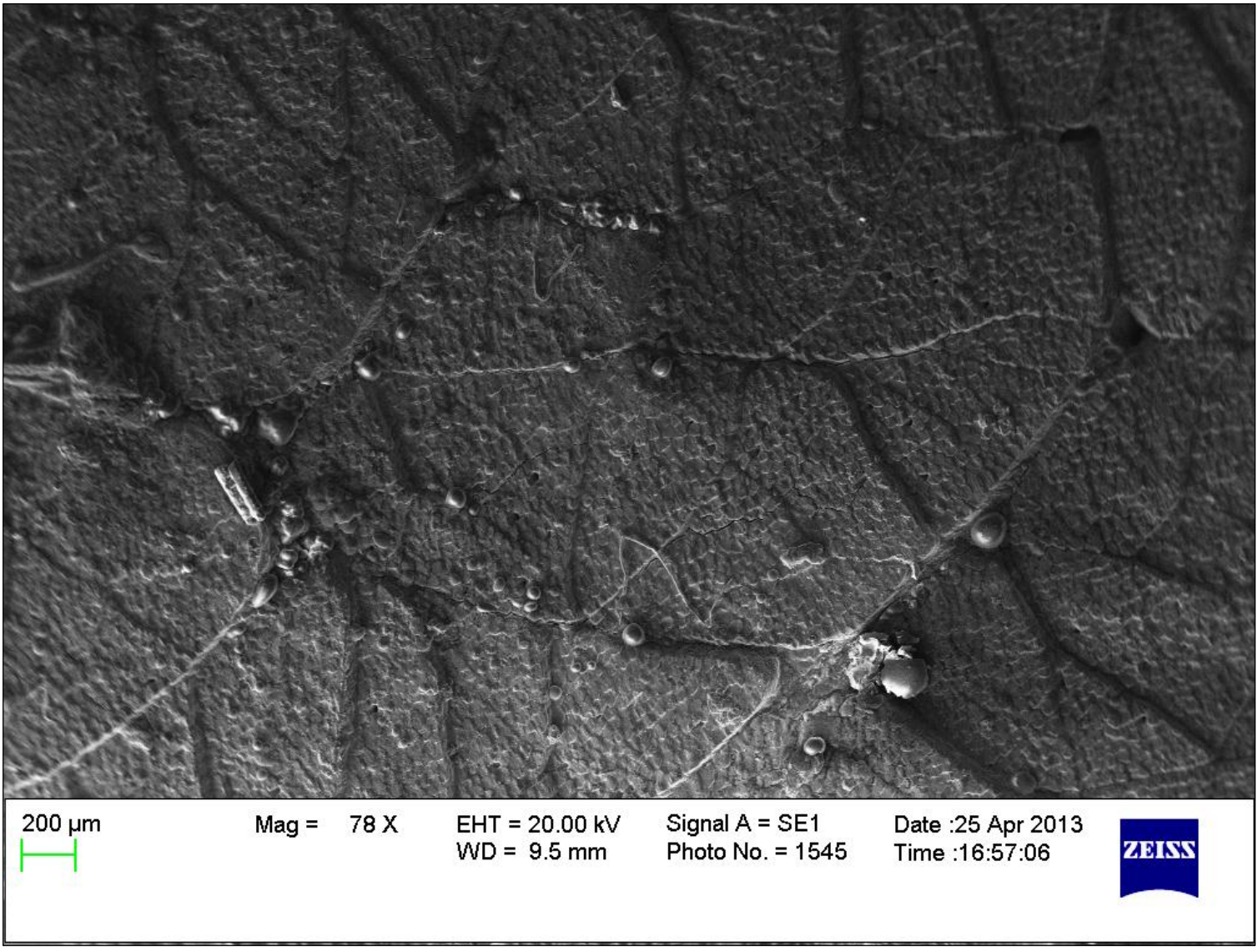
Table 1. List of primers

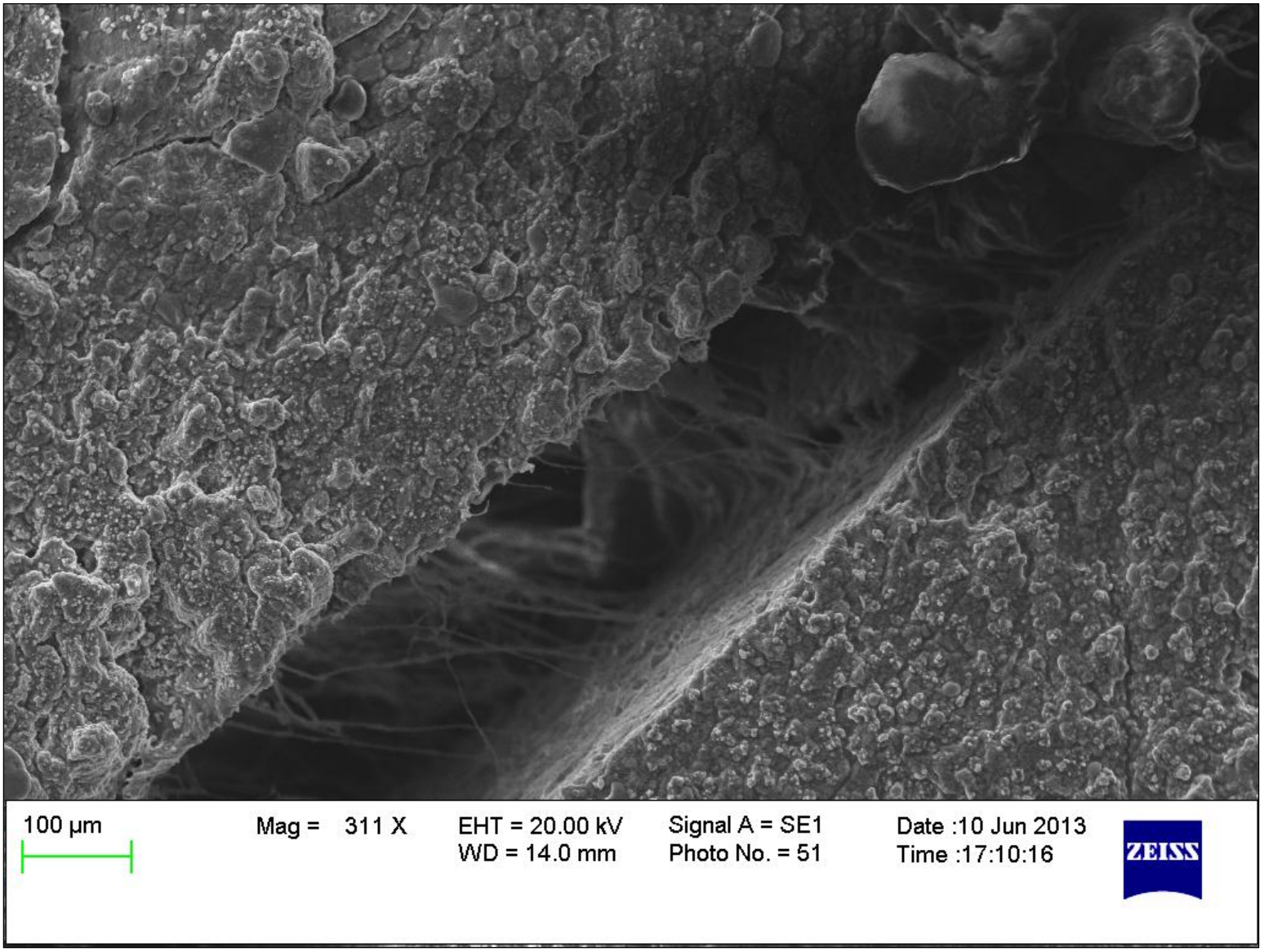
Primers	Sequence (5' to 3')	Target gene	Product size	Reference
prfA-F prfA-R	CAATGGGATCCACAAGAATA AGCCTGCTCGCTAATGACTT	prfA	186bp	Klein and Juneja, 1997
hly-F hly-R	GCAATTTCGAGCCTAACCTA ACTGCGTTGTTAACGTTTGA	hly	188bp	Klein and Juneja, 1997
16s- F 16s -R	GATGCATAGCCGACCTGAGA CTCCGTCAGACTTTCGTCCA	16s	100bp	Matilla et. al, 2011
341-FGC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG	16s (V3 region)	250bp	Muyzer et al., 1993
534-R	ATTACCGCGGCTGCTGG			

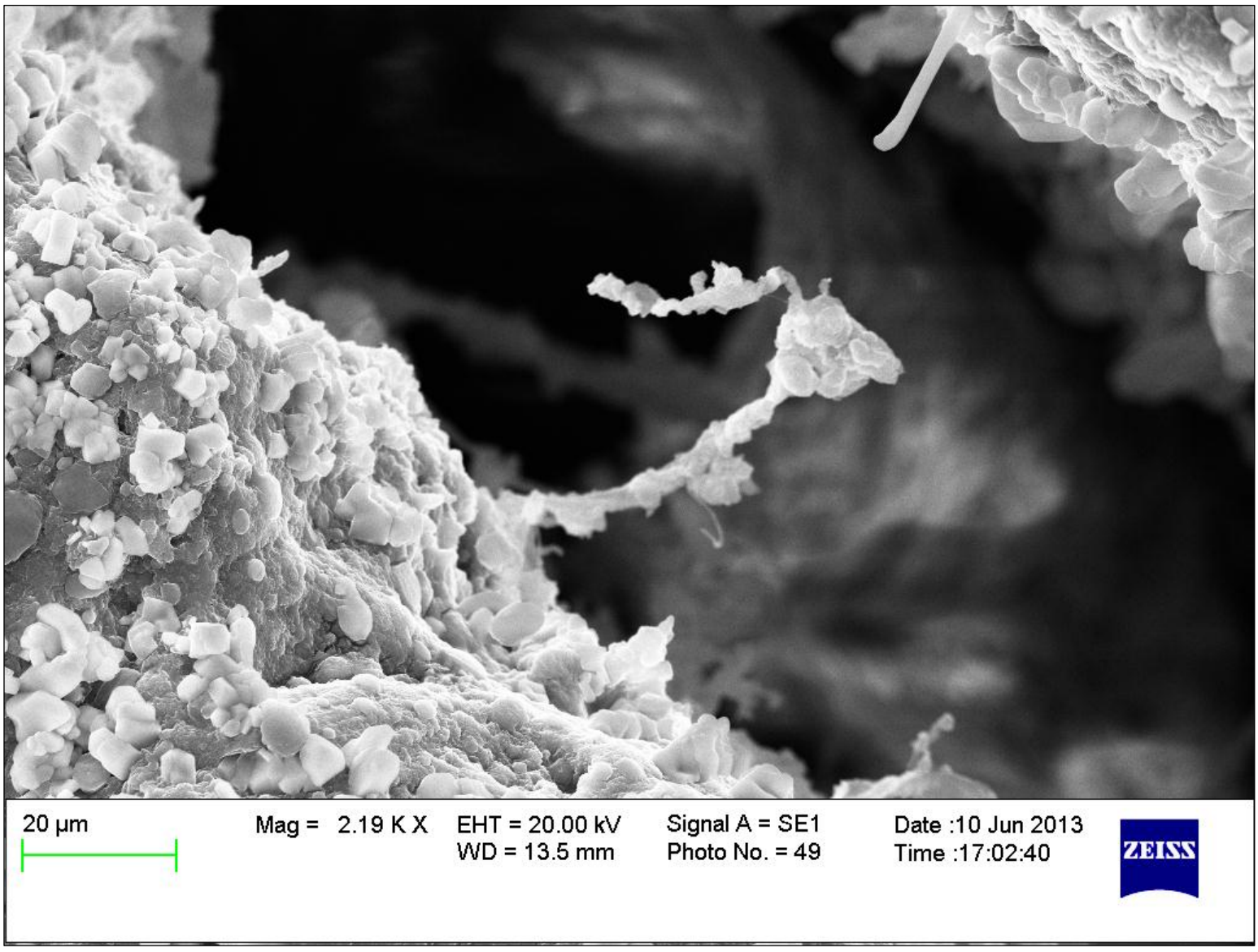
Table 2. List of strains

Strains	Description	Reference
*LM*10403s	streptomycin resistant strain of *LM*10403; laboratory strain	(Bishop, 1987)
*LM*101M	serotype 4b; beef and pork sausage isolate	(Howard, 1992)
*LM*108M	serotype 1/2b; hard salami isolate	(Kadam, 2013)
*LM*19115	serotype 4b; clinical isolate from human	(Kadam, 2013)
*LM*49594	serotype 4b; food outbreak	(Niemira, 2003)
*LM*7644	serotype1/2c; clinical isolate from human	(Gomes, 2012)
*LM*F6214	serotype 4nonb;mouse virulent strain	(Hamrick, 2003)

Appendix 2. Scanning Electron Micrographs of Roast Beef Surface




100 µm
Mag = 311 X
EHT = 20.00 kV
WD = 14.0 mm
Signal A = SE1
Photo No. = 51
Date :10 Jun 2013
Time :17:10:16
ZEISS


20 µm
Mag = 2.19 K X
EHT = 20.00 kV
WD = 13.5 mm
Signal A = SE1
Photo No. = 49
Date :10 Jun 2013
Time :17:02:40
ZEISS