DISSERTATION

FATE OF *LISTERIA MONOCYTOGENES* ON READY-TO-EAT MEAT PRODUCTS, TREATED WITH ANTIMICROBIALS, AND UNDER CONDITIONS SIMULATING PASSAGE THROUGH THE HUMAN STOMACH AND SMALL INTESTINE

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY IOANNA MARIA BARMPALIA-DAVIS ENTITLED FATE OF *LISTERIA MONOCYTOGENES* ON READY-TO-EAT MEAT PRODUCTS, TREATED WITH ANTIMICROBIALS, AND UNDER CONDITIONS SIMULATING PASSAGE THROUGH THE HUMAN STOMACH AND SMALL INTESTINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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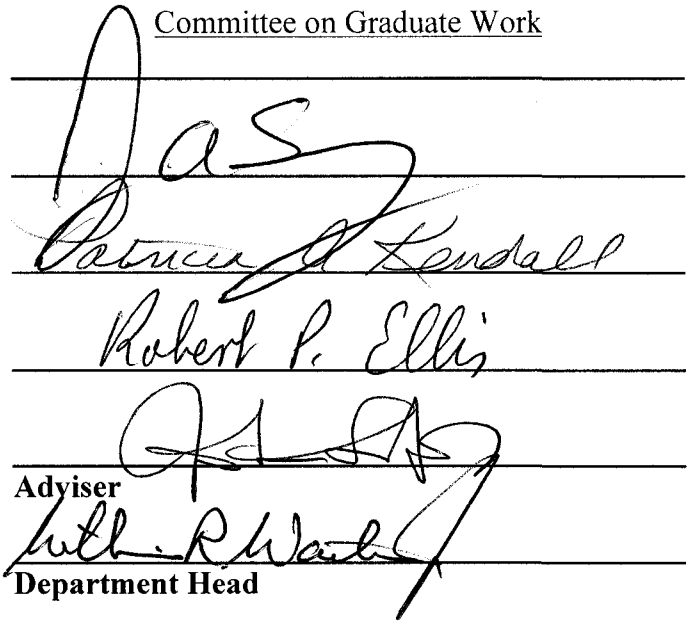

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ABSTRACT OF DISSERTATION

FATE OF *LISTERIA MONOCYTOGENES* ON READY-TO-EAT MEAT PRODUCTS, TREATED WITH ANTIMICROBIALS, AND UNDER CONDITIONS SIMULATING PASSAGE THROUGH THE HUMAN STOMACH AND SMALL INTESTINE

To cause infection, the foodborne pathogen *Listeria monocytogenes* must overcome stresses associated with food processing, storage, and preparation, as well as various defense elements of the human body. In this work, we examined factors that may affect growth and survival properties of this pathogen on foods and during passage through a simulated stomach and small intestine.

One study was conducted to evaluate the antilisterial effectiveness of the naturally occurring protein lactoferrin and its activated form (ALF), as a formulation ingredient or as surface treatment, respectively, in comparison or in combination with organic acids and salts on various ready-to-eat (RTE) meat and poultry products. Overall, findings suggested that lactoferrin used in product formulations and ALF applied as a surface treatment were not as effective as established antimicrobials. Application of ALF or lactoferrin enhanced the antilisterial activity of other antimicrobial ingredients (potassium lactate and sodium diacetate or lactoferrin) or dipping treatments (acetic acid), respectively, suggesting that appropriate combinations of these natural antimicrobials with chemical compounds may be effective in controlling *L. monocytogenes* on RTE products.

Three studies investigated survival patterns of *L. monocytogenes* during passage through the upper gastrointestinal tract in a sequential manner by utilizing a dynamic model of the stomach and small intestine. The studies tested pathogen- (e.g., strain variation, growth phase) and food- (e.g., pH, fat content) related factors that could influence the resistance of the pathogen to stresses prevailing in the gastrointestinal tract. Findings indicated that gastric survival of this pathogen was influenced by factors, including strain variation, the type (i.e., bologna vs. salami) and the fat level of the product. However, due to the gradual acidification of the gastric contents (pH 2.0 within 88 min) and the fact that gastric emptying started while the pH of the stomach was still high, populations being transferred in the intestinal compartment (pH ~6.5) were affected by the initial (0 min) contamination levels. Thus, pathogen counts in the simulated intestine depended on the growth potential of the pathogen, as affected by the strain, characteristics of the food matrix, or the length of the storage period, particularly since intestinal stresses caused slight reductions in populations.

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CHAPTER 1

INTRODUCTION

Since its initial isolation and characterization by Murray et al. (1926), the Gram-positive bacterium, now known as *Listeria monocytogenes*, has evolved into a major foodborne pathogen, due to its association with multiple food-associated outbreaks and product recalls. Foods implicated with epidemic or sporadic listeriosis have included vegetables, coleslaw, milk and dairy products, seafood products, pâté, and delicatessen meats (Farber and Peterkin, 1991). Ready-to-eat (RTE) meat and poultry products are of particular concern as they have been recognized as the culprit in numerous listeriosis outbreaks and recalls with major health and economic consequences (CDC, 1998; 2000; 2002; Porto et al., 2002; Farber et al., 2007). Taking into account the high case-fatality rate [30% (Rocourt, 1994)] associated with listeriosis and the difficulty in eliminating presence of the pathogen in food-processing environments, regulatory authorities have established several measures for control of the pathogen, managed by Hazard Analysis Critical Control Point (HACCP) systems (USDA-FSIS, 1996), establishment of a 'zero tolerance' policy (i.e., no detectable level allowed) for the organism (Shank et al., 1996) and surveillance monitoring of *L. monocytogenes* in RTE meat and poultry products (USDA-FSIS, 1990), reassessment of HACCP plans (USDA-FSIS, 1999), establishment of performance standards in the production of RTE meat and poultry products (USDA-

FSIS, 2001), and an interim final rule for *L. monocytogenes* control in RTE meat/poultry products (USDA-FSIS, 2003a).

L. monocytogenes infection is associated with various clinical syndromes such as meningitis or meningoencephalitis, septicemia, and abortion or stillbirth (Farber and Peterkin, 1991; Donelly, 1994; Low and Donachie, 1997). In addition, a mild, non-invasive form of the disease, manifested as febrile gastroenteritis has been described (Schuchat et al., 1991; Aureli et al., 2000; Lecuit et al., 2001; Gahan and Hill, 2005; Ramaswamy et al., 2007). Factors that may impact the outcome of the infection include the susceptibility of the host, levels of *L. monocytogenes* in the ingested food, virulence-associated characteristics of the specific strain and properties of the food matrix (Schuchat et al., 1991; Risk Assessment Drafting Group, 2004). Invasive listeriosis is uncommon, despite the frequent exposure of humans to the agent, suggested by studies estimating the occurrence of *L. monocytogenes* in the environment (Welshimer, 1968; Weis and Seeliger, 1975; Watkins and Sleath, 1981) and various food products (Ben Embarek, 1994; Beuchat, 1996; Jay, 1996), as well as human carriage studies (Bojsen-Moller, 1972; Kampelmacher and van Noorle Jansen, 1972; Schuchat et al., 1993; Rocourt, 1996). Nevertheless, the risk of listeriosis is evidently increased in susceptible hosts, particularly among those who are immunocompromised, neonates, pregnant women, and the elderly (Gellin and Broome, 1989; Ramaswamy et al., 2007).

Although *L. monocytogenes* infection may occur due to direct contact with the pathogen (Allcock, 1992; Regan et al., 2005) or through an airborne route (Mazzulli and Salit, 1991; Skogberg et al., 1992), most cases of listeriosis arise from consumption of contaminated food products (Hof et al., 1994; Low and Donachie, 1997). For healthy

adults, reported infectious doses vary from 10^5 to 10^7 CFU/ml, whereas for individuals at high risk, even low numbers of cells (10 to 10^4 CFU/ml) may cause disease (Maijala et al., 2001). Nevertheless, the 2003 *L. monocytogenes* risk assessment conducted by the Food and Drug Administration (FDA) of the United States (US) Department of Health and Human Services (HHS) and the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) concluded that the majority of listeriosis cases arise from foods that contain high levels of the pathogen (HHS-FDA/USDA-FSIS, 2003). Calculating a defined infectious dose, however, may be challenging due to variables, such as the food matrix, the amount of food ingested and the susceptibility of the host (King et al., 2003). Originating from the epidemiology literature, the term 'disease triangle' (host, pathogen and environment) determines the likelihood of developing illness (Coleman and Marks, 1998) and, in the case of listeriosis it may pinpoint factors (i.e., host, *L. monocytogenes* strain, and food matrix) that should be considered in the hazard characterization component of the *L. monocytogenes* risk assessment. Understanding the mode and magnitude of the role each of these factors play on the likelihood of developing listeriosis is essential, as it might help eliminate the uncertainty associated with the dose-response relationship for this pathogen.

Transmission of *L. monocytogenes* via the oral route requires survival of the pathogen under challenging conditions that may be encountered in the natural environment and at various stages of the food chain. Since *L. monocytogenes* is capable of growing at low temperatures (Junttila et al., 1988; Walker et al., 1990; Barbosa et al., 1994; Hudson et al., 1994), refrigerated storage cannot ensure the safety of RTE foods that support growth of the pathogen. Certain antimicrobial compounds have the ability to reduce or prevent

growth of *L. monocytogenes* at refrigerated, as well as at abusive temperatures and have been widely employed as means of ensuring the safety of RTE meat/poultry products, under the USDA-FSIS final rule alternatives (USDA-FSIS, 2003a).

Extended research carried out in recent years has revealed that treating meat and poultry products with antimicrobial agents creates stressful conditions that may lead to inhibition of *L. monocytogenes* growth. Several investigators have examined the antilisterial activity of antimicrobials as formulation ingredients (Schlyter et al., 1993a; 1993b; Wederquist et al., 1994; Blom et al., 1997; Bedie et al., 2001; Islam et al., 2002; Mbandi and Shelef, 2001; 2002; Samelis et al., 2002; Porto et al., 2002; Stekelenburg, 2003; Choi and Chin, 2003; Barmpalia et al., 2004; 2005; Glass et al., 2007), or as dipping or spraying solutions (Schlyter et al., 1993a; 1993b; Palumbo and Williams, 1994; Ariyapitipun et al., 2000; Samelis et al., 2001a; Glass et al., 2002; Barmpalia et al., 2004; Uhart et al., 2004; Geornaras et al., 2005; Lu et al., 2005; Luchansky et al., 2006). Although the results of these studies may assist the processed meat industry in its efforts to identify interventions for *L. monocytogenes* control in their products, evaluating the antilisterial effectiveness of other compounds may be essential, as processors may need to consider alternatives to the widely applied sodium or potassium lactate/sodium diacetate combination (Tompkin, 2002). Application of natural compounds, derived from animal, plant and microbial sources has received considerable attention in this respect (Benkerroum and Sandine, 1988; Hughey et al., 1989; Aureli et al., 1992; Nguyen-The and Lund, 1992; Hefnawy et al., 1993; Larson et al., 1996; Davies et al., 1997; Murray and Richard, 1997; Hao et al., 1998; El-Ziney et al., 1999; Aasen et al., 2003; Alzoreky and Nakahara, 2003; Dufour et al., 2003; Ransom et al., 2003; Samelis et al., 2003b;

Branen and Davidson, 2004; Samelis et al., 2005; Geornaras et al., 2006; Sivarooban et al., 2007). Research in this area, however, needs to be continued, as naturally occurring antimicrobial compounds have not provided consistent antilisterial effects (Kim et al., 1995; Hao et al., 1998).

Being able to cope with hurdles prevailing within the human gastrointestinal system, in addition to those associated with food processing and preservation methods, is strongly linked to the ability of *L. monocytogenes* to produce human infection. The digestive tract possesses a number of natural defense barriers that protect the host against foodborne infection. In healthy individuals, the acidic environment of the stomach is an effective barrier against pathogens ingested with food (Smith, 2003). Microorganisms that survive gastric passage and reach the small intestine in a viable state encounter the presence of bile and volatile fatty acids, high osmolarity, low oxygen conditions and the presence of the natural gut microflora (Kerr, 1991; Begley et al., 2002; Gahan and Hill, 2005). Other innate defenses associated with the human gastrointestinal tract include peristalsis and enterosalivary circulation of nitrate (O'May et al., 2005). Consequently, the ability of *L. monocytogenes* to survive the digestive process may be considered as an important element of its virulence. Similar to other foodborne pathogens, *L. monocytogenes* has evolved to possess numerous resistance mechanisms that may enable enhancement of its survival properties in the hostile microenvironments of the human digestive system. Among others, the pathogen possesses acid-protective mechanisms (acid-dependent and buffering systems) that may allow enhanced survival during gastric transit (Davis et al., 1996; Cotter et al., 2001a; Merrell and Camilli, 2002; Ferreira et al., 2003; Smith, 2003; Gahan and Hill, 2005), whereas reports of *L. monocytogenes* cholecystitis (infection of

the gallbladder), found in published literature (Allerberger et al., 1989; Briones et al., 1992), demonstrate the ability of the pathogen to resist the toxic effects of bile.

Numerous studies (Czuprynski et al., 1989; Farber et al., 1991; Menudier et al., 1991; Briones et al., 1992; Lammerding et al., 1992; Brosch et al., 1993; Schlech et al., 1993; Manohar et al., 2001; Czuprynski et al., 2002; Takeuchi et al., 2003) have utilized oral and intragastric animal models to address potential factors affecting the survival of *L. monocytogenes* under conditions associated with the gastrointestinal tract; however, this type of research is costly and involves ethical issues concerning animal welfare. In addition, mice, that have commonly been used to study foodborne infection with *L. monocytogenes*, lack E-cadherin, the receptor for the protein internalin A (Dramsi et al., 1995; Kathariou, 2002), which is required for invasion into specific human eukaryotic cells. Therefore, results obtained from murine bioassays may not provide accurate information regarding the disease in humans. While other animal models may be more appropriate than the murine model when studying the *L. monocytogenes* infection (ILSI Research Foundation/Risk Science Institute, 2005), data derived from animals should be cautiously extrapolated to human oral-infection information. Kararli (1995) reviewed the anatomical, physiological and biochemical differences between the gastrointestinal tracts of humans and laboratory animals and concluded that the digestive characteristics of humans cannot be simulated by any animal model.

To overcome limitations associated with animal models several researchers have examined various aspects of foodborne infection by employing artificial gastrointestinal fluids. Indeed, *in vitro* gastrointestinal challenge studies have provided important information regarding conditions and factors contributing to the gastric or intestinal

survival of various foodborne microorganisms, including *L. monocytogenes*. More specifically, studies by Roering et al. (1999) and King et al. (2003) suggested that individual *L. monocytogenes* strains may respond differently during exposure in artificial gastric fluid; reports on strain-to-stain variations, however, could have been affected by the small number of *L. monocytogenes* strains tested in these studies. King et al. (2003) also reported that exponential-phase cells of *L. monocytogenes* were more susceptible to simulated gastric fluid or bile salts, as compared to stationary-phase cells. Comparable findings have been observed in terms of bile-tolerance, as Begley et al. (2002) found that exponential-phase cells of *L. monocytogenes* strain LO28 exhibited higher susceptibility to unconjugated bile acids than cells at stationary phase. Reports on the effect of various compounds ingested as food ingredients or separately on gastric survival of the pathogen also exist. Glutamate was reported to have a protective effect against killing of wild-type *L. monocytogenes* in an *in vitro* model of gastric acid (pH 2.5; Cotter et al., 2001a), while salivary nitrite worked synergistically with gastric fluid to inactivate lactobacilli and *Escherichia coli* O157 (Xu et al., 2003). The acid tolerance of *Lactobacillus rhamnosus* in simulated gastric fluid was increased by the presence of glucose (1-19.4 mM; Corcoran et al., 2005). Smith (2003) reviewed a number of cases associated with increased incidence of listeriosis due to intake of certain drugs and concluded that patients receiving antacid or histamine H_2 antagonists were more likely to acquire foodborne listeriosis as compared to control patients. In accordance, studies employing *in vitro* gastrointestinal systems have provided similar results as they indicated that the gastric survival of *Vibrio vulnificus* and *E. coli* O157:H7 increased in oysters (Koo et al., 2001) and cooked ground beef (Tamplin, 2005), respectively, by the presence of antacids.

Results by Stopforth et al. (2005) showed that immersion of pork frankfurters, formulated with sodium diacetate (0.25%), into a 2.5% solution of lactic acid may have resulted in increased resistance of surviving cells to simulated gastric fluid as storage of the product progressed. However, it was not clear whether the increased resistance of the pathogen in simulated gastric fluid was exclusively due to the antimicrobial treatments applied on the product. Peterson et al. (1989) investigated the role of food in protecting foodborne pathogens against gastric killing. The authors reported that food exhibited a protective effect against gastric inactivation of *E. coli* and *Shigella flexneri*, but not *Salmonella* Typhimurium. Additionally, Tamplin (2005) and Gänzle et al. (1999) observed that the antimicrobial effects of gastric fluid and bile, respectively, were reduced by the presence of food.

As already stated, numerous researchers have used *in vitro* gastrointestinal models to identify factors that could affect the gastric or intestinal survival of *L. monocytogenes*. However, a large number of these studies (Roering et al., 1999; Phan-Thanh et al., 2000; Cotter et al., 2001a; Begley et al., 2002; King et al., 2003; Olier et al., 2004; Wonderling and Bayles, 2004; Stopforth et al., 2005; Formato et al., 2007) have used conventional procedures (i.e., static models), which may have compromised the predictive value of the findings. More specifically, conventional studies have overlooked the sequential stresses and the constantly shifting conditions to which ingested pathogens are subjected during transit through the human digestive tract, and/or neglected to simulate major parameters of digestion, such as pH changes, temperature conditions, or secretion of precise physiological amounts of digestive fluids and enzymes. Consideration of parameters such as gastric emptying and pH or bile fluctuations may be critical in studies designed to

evaluate survival of *L. monocytogenes* in artificial gastrointestinal environments. Simulation of the successive conditions, encountered by pathogens during gastrointestinal transit, may be important, since prior exposure to one form of sublethal stress may impart tolerance against subsequent stresses (Farber and Pagotto, 1992; Lou and Yousef, 1996; Mazzota, 2001). Consequently, exposure of pathogens to the gastric environment may affect their survival in the intestine. Indeed, Begley et al. (2002) showed that subjecting *L. monocytogenes* to sublethal levels of bile acids, acid, heat, salt, or sodium dodecyl sulfate increased its ability to tolerate bile. Similarly, acid shock offered protection against human bile in *V. cholerae*, as indicated by Alvarez et al. (2003). Studying *L. monocytogenes* survival patterns during an *in vitro* passage through the upper gastrointestinal tract, following a sequential approach, may accurately pinpoint factors that may impact the survival of the pathogen in the human digestive tract. Such an approach has previously been used in a number of studies conducted to reproduce *in vivo* data of the human gastrointestinal tract, test the absorption of environmental contaminants, establish the acceptable intake of drug residues, study the behavior of drug forms under various physiological conditions, investigate the formation of potentially carcinogenic compounds and investigate the ability of pathogenic and beneficial probiotic bacteria to withstand the unfavorable conditions in the gastrointestinal system. For these purposes, *in vitro* dynamic models of the human stomach and small intestine have been utilized in studies by Beumer et al. (1992), Nouws et al. (1994), McConville et al. (1995), Minekus et al. (1995), Hack and Selenka (1996), Marteau et al. (1997), Gänzle et al. (1999), Koo et al. (2001), Krul et al. (2004), Blanquet et al. (2004), Mainville et al. (2005), and Bernbom et al. (2006). Gänzle et al. (1999) and more recently Bernbom et al.

(2006) utilized simulated dynamic gastrointestinal models to evaluate the survival properties of *L. innocua* and *L. monocytogenes*, respectively, in the presence of bacteriocin-producing lactic acid bacteria. To our knowledge, no other studies have investigated the survival of *L. monocytogenes* in an artificial model that closely simulates the physico-chemical events prevailing in the human stomach and upper intestine.

As previously mentioned, being a successful foodborne pathogen requires increased tolerance to hurdles prevailing outside and inside the host. The goals of the studies presented here were to observe the survival patterns of *L. monocytogenes* under stressful conditions resulting from treating RTE products with antimicrobials or exposure to a simulated gastrointestinal system. Activated and non-activated lactoferrin were examined for their antilisterial activity, singly or together with other antimicrobials (i.e., organic acids and salts), using a variety of RTE products, in order to identify treatments that could replace the widely used combination of sodium or potassium lactate and sodium diacetate. Additionally, the behavior of the pathogen during an *in vitro* exposure to gastrointestinal tract conditions in a sequential manner by utilizing a simulated dynamic model of the human stomach and small intestine, and to identify factors that may influence the resistance of the pathogen to the stressful conditions encountered in the gastric and small bowel compartments. Factors assessed in the present studies included pathogen-related aspects (i.e., strain variability, growth phase, presence of the *sigB* gene) and features of the food matrix (e.g., pH and fat content) which delivers the pathogen through the gastrointestinal system.

CHAPTER 2

REVIEW OF LITERATURE

2.1. *Listeria monocytogenes*

2.1.1. Taxonomy

Both the intra- and inter-generic taxonomy of the genus *Listeria* remained unclear for many years. The genus *Listeria* was first included in the fourth edition of *Bergey's Manual of Determinative Bacteriology* as a member of the family *Corynebacteriaceae* (Bergey's Manual of Determinative Bacteriology, 1934). In the sixth and seventh editions, *Listeria* was still classified as a *Corynebacteriaceae* (Bergey's Manual of Determinative Bacteriology, 1948; 1957); however, in the next edition the genus was located in an indefinite position after the family of *Lactobacillaceae* (Bergey's Manual of Determinative Bacteriology, 1974). Finally, *Listeria* was listed with *Lactobacillus*, *Erysipelothrix*, *Brochothrix* and other genera in *Bergey's Manual of Systematic Bacteriology* in the section entitled 'Regular, Nonsporing Gram- Positive Rods' (Bergey's Manual of Systematic Bacteriology, 1986). The phylogenetic position of the genus *Listeria*, as well as the diversity within the genus has become more defined after the introduction of molecular techniques. Currently, as a result of many numerical taxonomic and chemical studies, as well as DNA homology and rRNA sequencing homology methods, it is concluded that *Listeria* is not related with the coryneform

bacteria and that it is closely related phylogenetically to *Brochothrix* (Ludwig et al., 1984; Collins et al., 1991). It was also concluded that the genus *Listeria* is comprised of six species (Farber and Peterkin, 1991): *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi* which now includes the former species *L. murrayi* (Rocourt et al., 1992).

2.1.2. Biological features

Among the six species of the genus *Listeria*, *L. monocytogenes* is the only important human pathogen, whereas *L. inanovii* is almost exclusively associated with disease in animals (Low and Donachie, 1997). Members of the genus are Gram-positive, microaerophilic, non-sporeforming, non-encapsulated bacteria. The coccoid to rod-shaped cells are small (0.4 to 0.5 μm in diameter, 0.5 to 2.0 μm in length; Seeliger and Jones, 1986) with round ends and they can be observed singly, in short chains, arranged in V or Y chains or in palisades. When cultured at 20-25°C, the cells form a few peritrichous flagella and display a characteristic tumbling motility that can aid in identifying the bacterium. *L. monocytogenes* strains become nonmotile or very weakly motile when cultured at 37°C because at this temperature they produce reduced amounts of flagellin (Peel et al., 1988; Kathariou et al., 1995). On the other hand, strains of the nonpathogenic species *L. innocua* produce large amounts of flagellin at 37°C (Kathariou et al., 1995).

L. monocytogenes is catalase-positive (although catalase negative strains have been observed; Hagen et al., 1998; Rocourt and Buchrieser, 2007), oxidase-negative and hydrolyzes esculin (Farber and Peterkin, 1991; Gahan and Collins, 1991; Schuchat et al., 1991). Aerobically, the organism can grow in the presence of glucose, lactose, rhamnose

and maltose, but not sucrose, while under anaerobic conditions only hexoses and pentoses support growth (Pine et al., 1989). Utilization of glucose, under aerobic conditions produces lactate, acetate and acetoin. Also, *L. monocytogenes* produces acid from amygdalin, cellobiose, fructose, mannose, salicin, maltose, dextrin, alpha-methyl-D-glucoside, and glycerol, while acid production from galactose, lactose, melezitole, sorbitol, starch, sucrose, and trehalose is variable (Rocourt and Buchrieser, 2007). *L. monocytogenes* expresses a β-hemolysin (58 kDa), which acts synergistically with the hemolysin produced by *Staphylococcus aureus* on sheep erythrocytes in a reaction known as CAMP test (named after Christie, Atkins, and Munch-Peterson), used to distinguish *L. monocytogenes* from *L. innocua* (Schuchat et al., 1991). Nevertheless, a few non-hemolytic isolates of *L. monocytogenes* have been observed (Kathariou and Pine, 1991). Similarly to *L. monocytogenes*, *L. inanovii* is also clearly β-hemolytic on blood agar, whereas *L. seeligeri* produces slight hemolysis.

2.2. Distribution of *L. monocytogenes*

2.2.1. Natural environment

L. monocytogenes is widely distributed in the natural environment. Soil, decaying plant material, and water, are documented sources of the pathogen (Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975; Watkins and Sleath, 1981). In addition, the pathogen has been isolated from sewage, silage, animal feces, and milk of mastitic or healthy animals (Farber and Peterkin, 1991; Sauders and Wiedmann, 2007). *L. monocytogenes* may exist as part of the normal intestinal flora in humans and several animal species (Gray and Killinger, 1966; Schuchat et al., 1991). Two studies by the same investigator indicated that approximately 1% of asymptomatic individuals excreted

the pathogen in their feces, whereas the corresponding percentage for listeriosis patients was 21.6% (Jensen, 1993a; 1993b).

According to Richmond (1990), the natural environment may serve as a source of direct contamination of foods with *L. monocytogenes*; however, more regularly, contamination of ready-to-eat (RTE) foods occurs through processing facilities that harbor the pathogen. Indeed, evidence accumulated over the last years identifies post-processing contamination as the principal source of *L. monocytogenes* in commercially processed foods (WHO, 1988; Wang and Muriana, 1994; Tompkin, 2002; Reij et al., 2004). Nevertheless, animal sources should not be underestimated as a cause of food contamination, as it was demonstrated in a listeriosis outbreak associated with consumption of coleslaw that was prepared from cabbage fertilized with untreated sheep manure from a farm with a history of ovine listeriosis (Schlech et al., 1985).

2.2.2. Foods

Although *L. monocytogenes* was recognized as a cause of human disease for more than 70 years, it was not until the 1980s that foodborne association was realized and accepted. The frequent occurrence of the pathogen in foods of animal or plant origin is expected, given the ubiquity of the pathogen in nature. *L. monocytogenes* has been isolated from a variety of foods, such as milk, soft cheeses, fruits, vegetables, fresh or frozen meat, poultry, fish, seafood, and various processed meat, dairy, fish and seafood products, while foods implicated as vehicles of human listeriosis have included raw and pasteurized milk, cheese, butter, fruits, vegetables, fresh or frozen meat and various meat products, poultry products, and seafood (Jay, 2000; Norton and Braden, 2007).

Several surveys worldwide have assessed the incidence of *L. monocytogenes* in food products. Although a number of studies have failed to detect the pathogen in pasteurized milk samples (Farber, et al., 1989; McLauchlin and Gilbert, 1990; Sharif and Tunail, 1991), Venables (1989) and Frye and Donnelly (2005) found, respectively, that 0.5% and 0.018% of pasteurized milk samples were contaminated with *L. monocytogenes*. Another study (Lewis et al., 2006) revealed that 0.4% of butter samples contained low levels (< 10 CFU/g) *L. monocytogenes*. A survey conducted by Gombas et al. (2003) in RTE foods, including luncheon meats, deli salads, various cheeses and seafood products revealed that the overall prevalence of *L. monocytogenes* was 1.82%, whereas among the product categories prevalences ranged from 0.17% (fresh soft cheese) to 4.7% (seafood salads). A survey by Mena et al. (2004) revealed that 7.0% of commercial foods in Portugal were found positive for *L. monocytogenes*, while the majority of the positive samples originated from raw products.

L. monocytogenes has been isolated from meat and meat products and according to Jay (2000), fresh meat (suspected), pork sausage, pork tongue, wieners and pâté are some of the products that have been implicated with listeriosis outbreaks or sporadic cases. Brackett (1988) showed that *L. monocytogenes* was detected in approximately 70% of ground beef, 43% of pork sausage, and 48% of poultry samples. Findings of the surveillance and monitoring activities, initiated by the USDA-FSIS, indicated that during a 26-month period of sampling (January 1987-February 1990) the pathogen was isolated from 122 out of 1726 (7.1%) samples of domestic raw beef (Farber et al., 2007). A survey conducted in Japan (Inoue et al., 2000) revealed that 12.2, 20.6 and 37% of minced beef, minced pork and minced chicken samples, respectively, contained the

pathogen. Moreover, sampling in a cattle slaughterhouse revealed that *L. monocytogenes* was present in 15.4% of carcasses and that all isolates shared the same pulsed-field gel electrophoresis (PFGE) profile (Peccio et al., 2003). A survey by Zhou and Jiao (2006) revealed that among raw food products collected from Chinese markets, meat products had the highest contamination rates.

Although *L. monocytogenes* is frequently present in raw meat, the pathogen is of higher concern for the safety of products that may be consumed without further cooking. Occurrence of the pathogen in cooked RTE products is predominantly due to recontamination during handling procedures such as slicing and packaging (Wang and Muriana, 1994; Tompkin, 2002; Reij et al., 2004). Among RTE foods, certain products, such as delicatessen meats and non-reheated frankfurters, pose a greater threat to public health as reported in the 2003 *L. monocytogenes* risk assessment for selected RTE foods (HHS-FDA/USDA-FSIS, 2003). Surveys, conducted all over the world to determine *L. monocytogenes* incidence in RTE meats have concluded that a small percentage of such foods may contain the pathogen at low levels (Farber and Peterkin, 1991). However, these surveys have also demonstrated great variations in *L. monocytogenes* prevalence among food-processing facilities that reflect the sporadic nature of contamination. For example, Wang and Muriana (1994) reported that among 20 brands of retail wieners, 19 brands had 10% incidence of *Listeria* spp. and 8% incidence of *L. monocytogenes*, while one brand had 71% incidence of *L. monocytogenes*. Governmental monitoring activities, conducted from 1990 to 1999 in 1,800 federally inspected establishments, revealed that the pathogen was isolated from as much as 5% of some RTE products, such as sliced luncheon meats (Levine et al., 2001). The same survey indicated that small diameter

cooked sausages and dry/semidry fermented sausages had a cumulative (10 and 3 years, respectively) *L. monocytogenes* prevalence of 3.6 and 3.25%, respectively. After examining refrigerated vacuum-packaged frankfurters from 12 processing operations, Wallace et al. (2003) reported that the pathogen was not found in any of the products from nine facilities whereas it was recovered at rates of 1.5%, 2.2%, and 16% from the products of the remaining producers. Additionally, a survey on *L. monocytogenes* prevalence in various RTE foods revealed that the percentage of *L. monocytogenes*-positive samples in sliced luncheon meats were 0.89% (Gombas et al., 2003). The authors, however, remarked on the considerably lower than expected prevalence rate of the pathogen. Kwiatek (2004) reported that *L. monocytogenes* was recovered from 2.38% of cooked sausage samples in Poland, whereas none of the pasteurized canned pork ham samples tested was found positive for the pathogen. A survey undertaken in Turkey (Yücel et al., 2005) demonstrated that 6.1% of the cooked meat samples tested contained *L. monocytogenes*, whereas in Greece, Angelidis and Koutsoumanis (2006) observed that 3.1% of commercial RTE meat products (not including bacon) were contaminated with *L. monocytogenes*, while 36.4% of products from a specific manufacturer contained the pathogen. In another survey performed in China, *L. monocytogenes* was detected in 21 out of the 844 RTE food samples tested (Zhou and Jiao, 2006).

2.2.3. Food-processing facilities

L. monocytogenes is a hardy organism that is able to withstand various cleaning and sanitizing procedures. Resistance (intrinsic or acquired by mutations) to various sanitizing agents and disinfectants, commonly used in the food industry has been increasingly documented for the pathogen during the last few years (Aase et al., 2000;

Mereghetti et al., 2000; Romanova et al., 2002). In addition, biofilms of *L. monocytogenes* are of great concern because they are more resistant to sanitizers relative to planktonic cells (Eckner, 1990; Frank and Koffi, 1990; Oh and Marshall, 1996; Lewis, 2001), rendering exclusion of the pathogen from food-processing environments challenging. Strains of the pathogen vary in their adherence and biofilm-formation ability (Chae and Schraft, 2000; Borucki et al., 2003). Increased biofilm formation by serotypes 1/2a and 1/2c was reported by Borucki et al. (2003), indicating differences among serotypes. Food-processing operations have been found contaminated with both transient and persistent strains of the pathogen as demonstrated with subtyping methods (Senczek et al., 2000; Norton et al., 2001; Kathariou, 2002). Persistent strains of *L. monocytogenes* may become established in food production environments for several months or even years (Unnerstad et al., 1996; Tompkin, 2002). For example, the strain associated with the outbreak caused by deli turkey meat products in 2000, appeared to have persisted in the processing facility for several years (Kathariou, 2002). According to Jay (1996) and Thévenot et al. (2005a) meat- processing plants are commonly contaminated with *L. monocytogenes* strains of serotypes 1/2a, 1/2b, and 1/2c.

As already noted, numerous published reports have suggested that *L. monocytogenes* and other *Listeria* spp. contaminate food products after cooking and before or during packaging (Tompkin, 2002; Kathariou, 2002; Reij et al., 2004), suggesting that the food-processing environment serves as the main source of contamination. Post-heating contamination of foods with the pathogen may also be supported by the fact that non-thermally injured cells have been isolated from various thermally processed products (Kornacki and Gurtler, 2007). According to Harvey and Gilmour (1992) the incidence of

the pathogen in milk processing facilities (33.3%) was much higher than in samples from dairy farms (5.3%). Accordingly, Van den Elzen and Snijders (1993) observed that during porcine slaughter only 2-7% of the carcasses and 0-10% of the environmental samples were found positive for *L. monocytogenes*. However, after chilling and cutting the incidence of the pathogen reached 11-36% in primal cuts and 71-100% in the environmental samples. Similarly, other reports suggest higher incidence of *Listeria* spp. on ground meat as compared to carcasses or meat cuts, indicating spreading of contamination during processing (Lowry and Tiong, 1988).

The incidence of the pathogen in the processing environment was a major concern among meat processors, even before the initiation of the governmental sampling program (Kornacki and Gurtler, 2007). A survey conducted in June 1987 in 40 meat-processing operations revealed that approximately 21% of the environmental samples tested were positive for listeriae (Anonymous, 1987). Contaminated areas included floors, drains, trenches, product-contact areas (conveyors, slicers, peelers), cleaning aids, exhaust hoods and washing areas. Despite efforts to control presence and growth of *L. monocytogenes* in the processing environment, its elimination is extremely difficult, if not impossible. According to Tompkin et al. (1992) a three-year survey in 100 packing lines indicated that the percentage of *Listeria*-negative samples increased from 44 in 1989 to 64 in 1991. Problematic sites included areas of the equipment that are difficult to clean and sanitize, such as hollow rollers for conveyors, rubber seals around doors, on/off switches, etc. Also, according to this study, packing lines may become contaminated from the floor, during processing or cleaning procedures. Sampling in a swine meat-processing plant revealed that the pathogen was isolated from a kneader and a presumably 'clean' mincer,

but not from stuffers, tables or floor drains (Peccio et al., 2003). A review by Tompkin (2002) indicated that common niches in RTE meat and poultry processing operations are sites that are difficult to clean and sanitize adequately, including hoses and spray nozzles, on/off valves for steam and water lines, rubber seals around doors, and hollow rollers on conveyors.

2.3. Outbreaks and sporadic cases of listeriosis

L. monocytogenes has been known as an agent of human disease for more than 70 years (Gray and Killinger, 1966); however, food transmission was first convincingly demonstrated with a large listeriosis outbreak that occurred in Nova Scotia between March and September 1981, involving 41 cases (34 perinatal and seven adult) (Schlech et al., 1985). Of the 34 perinatal cases, nine were stillbirths, whereas 23 of the infants who were born alive died. The mortality rate in adult cases was 28.6%. Coleslaw was epidemiologically associated with the disease onset and the causative strain (*L. monocytogenes* serotype 4b) was recovered from coleslaw in the refrigerator of one of the patients. The pathogen was also isolated from two unopened packages of coleslaw from the specific manufacturer; however, it was never found in the implicated plant. Investigation of the sources of raw vegetables used in the manufacturing plant pointed to a farm that had experienced several cases of ovine listeriosis a year earlier.

Pasteurized milk was responsible for a listeriosis outbreak in Massachusetts (1983), which involved 49 cases (42 adult and seven perinatal) with a mortality rate of 29% (Fleming et al., 1985). The outbreak strain (serotype 4b) was never isolated from the incriminated milk or the processing facility. However, the milk associated with the

disease had been obtained from a group of dairy herds on which listeriosis had been known to have occurred at the time of the outbreak.

Another large outbreak, which was also the reason for raising the pathogen to a higher level of concern, occurred in 1985 in Los Angeles, California (Linnan et al., 1988). Of the 142 cases, 93 were perinatal and 49 adults with predisposing conditions. Forty-eight deaths were recorded, resulting to an overall mortality rate of 34%. Consumption of a Mexican-style soft cheese (queso fresco), manufactured from a combination of raw and pasteurized milk, was epidemiologically linked to the disease and the outbreak strain, *L. monocytogenes* serotype 4b, was isolated from unopened cheese packages.

Processed meat and poultry products are considered of high-risk for transmission of listeriosis, as they have been associated with a number of outbreaks of the disease. Pâté has been the infectious vehicle in two outbreaks of invasive listeriosis that occurred in Europe (England, Wales and Northern Ireland) and the US (McLauchlin et al., 1991; Gilbert et al., 1993; Anonymous 1999). Consumption of hot-dogs and luncheon meats was linked to a multistate outbreak that occurred during the period of August 1998 to February 1999 with 101 cases involved (CDC, 1998). The overall mortality rate was 21% (15 adults and six miscarriages). Consumption of deli turkey meat was associated with the outbreak that occurred in 2000 and caused 53 illnesses and 11 deaths in nine states (CDC, 2000). Also, an outbreak that occurred in 2002 in the Northeastern states was linked to contaminated deli poultry products (CDC, 2002). The outbreak caused 46 illnesses, seven deaths and three stillbirths or miscarriages and it was followed by a massive recall of 27.4 million pounds of turkey products, due to possible contamination with the pathogen.

It is believed that most cases of human listeriosis are not associated with an outbreak but occur in the form of sporadic illness. Nevertheless, it is not known whether sporadic cases are truly confined to a single patient or are actually single-source epidemics that remain undocumented because of the widespread distribution of the culprit food (Linnan et al., 1988). A sporadic listeriosis case that occurred in Oklahoma in 1989 was the first documented illness linked to consumption of a RTE poultry product (microwave heated turkey frankfurters) and resulted in the death of a breast cancer patient, followed by a recall (Anonymous, 1989). To obtain an accurate estimate of the incidence of listeriosis and identify potential dietary risk factors for the disease, the Centers for Disease Control and Prevention (CDC) conducted a population-based, active surveillance for *L. monocytogenes* infections in the US (September 1986 until June 1987) which revealed that unheated hot dogs and undercooked chicken were the only foods significantly associated with sporadic cases of listeriosis (Schwartz et al., 1988). A subsequent and larger case-control study conducted by CDC involved 18 million individuals from five geographic regions in the US and aimed to define potential sources of sporadic illness (Schuchat et al., 1992). This study identified 301 cases of sporadic listeriosis, whereas high-risk foods included primarily foods purchased from delicatessen counters and soft cheeses. More recently, a case-control study was performed by CDC from 2000 to 2003 to identify sources of sporadic listeriosis and concluded that case-patients were more likely to have consumed melons at a commercial establishment and hummus prepared at a commercial establishment, foods that are not traditionally characterized of high risk for the disease, creating the need for the implementation of additional control measures for the pathogen directed to retail environments (Varma et al., 2005). In Denmark, a study

conducted from 1989 to 1990, identified consumption on unpasteurized milk and pâté as dietary risk factors for listeriosis (Jensen et al., 1994).

2.4. Pathogenesis

2.4.1. Cellular infection

Although the clinical manifestations of systemic *L. monocytogenes* infection vary, common steps involved in disease include: (i) ingestion of contaminated food; (ii) survival of the pathogen in the gastric compartment; (iii) survival and colonization in the small intestine; (iv) penetration of cells through the M cells and/or enterocytes; (v) infection of macrophages; and, (vi) resolution or systemic spread to various organs (Orndorff et al., 2006). *L. monocytogenes* is an intracellular pathogen that has the ability to evade and replicate within phagocytic and non-phagocytic cells. In brief, following internalization into the host cell, the pathogen lyses the uptake vacuole and escapes into the growth-permissive cytosol where it multiplies. Within two hours of infection, the bacterium uses an actin-based motility to move towards the cytoplasmic membrane of the host cell. When the bacterium is in contact with the cytoplasmic membrane of the host cell, it forms an elongated double-membrane protrusion that is invaginated into the adjacent cell to form a double vacuole. The double membrane is then lysed and the pathogen is released into the next cell, thus, spreading occurs by avoiding an extracellular phase. Invasion of *L. monocytogenes* into the bloodstream may lead to dissemination of the pathogen to the central nervous system or the placenta (Farber and Peterkin, 1991; Dussurget et al., 2004).

Genes that are essential for the infection process and the specific roles of their products in pathogenesis have been described (Vazquez-Boland et al., 2001; Hain et al.,

2006). For instance, internalin A and internalin B, the products of chromosomal genes *inlA* and *inlB*, respectively, are required for the listerial entry within target cells. Specifically, internalin A is required for invasion within enterocytes, as it interacts with E-cadherin located on the surface of epithelial cells (Dramsi et al., 1995), whereas internalin B is involved in entry within a broad range of non-epithelial host cells (e.g., hepatocytes, fibroblasts). Listeriolysin O (LLO) is a pore-forming toxin, encoded by the *hly* gene, which is required for *Listeria* escape from phagosomal vacuoles (Dramsi and Cossart, 2002). Phosphatidyl-inositol-phospholipase C (encoded by *plcA*) and phosphatidylcholine-phospholipase C (encoded by *plcB*) aid LLO in lysing primary vacuoles (Vazquez-Boland et al., 2001). The actin based-, intracellular motility requires the surface protein ActA (encoded by the gene *actA*) that promotes the formation of actin filaments around the bacterial cell surface (Tilney and Portnoy, 1989). Most of the genes encoding for virulence factors of the pathogen (*prfA*, *plcA*, *hlyA*, *mpl*, *actA*, and *plcB*) are located on the chromosome in the PrfA-dependent virulence gene cluster (Kuhn and Goebel, 2007). However, other essential virulence genes, such as *inlA* and *inlB* are not located on that specific locus.

2.4.2. Serotypes and genetic structure-association with virulence

Although all *L. monocytogenes* strains are considered capable of producing disease (Brosch et al., 1993; Rocourt et al., 2000), there is evidence originating from *in vivo* and *in vitro* studies suggesting that virulence within the species is heterogeneous (Brosch et al., 1993; Wiedmann et al., 1997; Barbour et al., 2001; Olier et al., 2002). Strains of *L. monocytogenes* exhibit great serotypic and genetic diversity. Based on serological reactions, thirteen serotypes have been recognized within the species (Farber and

Peterkin, 1991), of which, however, only three (4b, 1/2a and 1/2b) are responsible for most cases of both sporadic and epidemic listeriosis throughout the world (Gellin and Broome, 1989; Schuchat et al., 1991; Rocourt et al., 2000). Most outbreaks of foodborne listeriosis in North America and Europe have been caused by strains of serogroup 4b (Piffaretti et al., 1989; Bille, 1990; Buchrieser et al., 1993; Gilbert et al., 1993). On the other hand, surveys on various foods have indicated that strains of this serotype are not the most prevalent among food isolates, suggesting that their high clinical incidence may be due to their unique characteristics of adapted physiology or differences in virulence and pathogenesis capabilities among serotypes (Kathariou, 2002). Johansson et al. (1999) observed that the vast majority (86%) of isolates from RTE fish products belonged to serotype 1/2a. McLauchlin (1996) reported that the highest proportion (60%) of human isolates represented serotype 4b, whereas among food isolates the most common serotype (32%) was 1/2a. Most of the RTE food isolates collected in a study by Gilbreth et al. (2005) belonged to serotypes 1/2a or 1/2b, whereas isolates from clinical cases were of serotypes 1/2a or 4b. Using serotyping in combination with PFGE, Lukinmaa et al. (2003) demonstrated a reduction in listeriosis cases caused by serotype 4b and an increase in those caused by serotype 1/2a during an 11-year period (1990 to 2001) in Finland. Nevertheless, although serotyping provides an efficient and convenient way of differentiating *L. monocytogenes* strains, the fact that both virulent and avirulent strains may be found within the same serotype renders this method ineffective as an indicator of virulence.

Utilization of molecular typing techniques may provide a better understanding as regards to the relationship between various subtypes of *L. monocytogenes* and virulence-

related characteristics of the organism. Several molecular methods have been utilized for subtyping of *L. monocytogenes* (Wiedmann, 2002). Among these, PFGE together with ribotyping are the most commonly used for *L. monocytogenes* typing purposes (Wiedmann, 2002). Application of such techniques, including multilocus enzyme electrophoresis, ribotyping, PFGE, and amplified fragment length polymorphism has resulted into grouping *L. monocytogenes* strains into two distinct evolutionary groups or lineages (Piffaretti et al., 1989; Graves et al., 1994; Rasmussen et al., 1995; Wiedmann et al., 1997; Gray et al., 2004). Research has indicated that correlations between antigenic types and lineages exist, as serotypes 1/2b, 3b, 4b, 4d, and 4e are found primarily in lineage 1, while serotypes 1/2a, 1/2c, 3a, and 3c belong to lineage 2 (Nadon et al., 2001; ILSI Research Foundation/Risk Science Institute, 2005). Although serotypes responsible for human listeriosis are members of both genetic groups, evidence originating from characterization of isolates, involved in human listeriosis cases, as well as cell-invasion assays has led to the suggestion that the two lineages vary in their virulence potential. Rasmussen et al. (1995) reported that all human clinical isolates characterized in their study were members of lineage 1. Accordingly, Wiedmann et al. (1997) showed that a specific ribotype within lineage 1 comprised all the foodborne outbreak-associated, serotype 4b strains, whereas less than 10% of the ruminant isolates belonged to that group. On the other hand, although lineage 2 contained human isolates, no foodborne epidemic-linked isolates were members of that group. *In vitro* cell pathogenicity assays have revealed that strains of lineage 1 have greater virulence potential than those belonging to lineage 2 (Wiedmann et al., 1997; Norton et al., 2001; Mereghetti et al., 2004; Zhou et al., 2005). According to Jeffers et al. (2001) the majority of the clinical

human strains that were characterized belonged to lineage 1, whereas another study by Gray et al. (2004) showed that lineage 2 contained most of the food isolates. Using a dose-response model, Chen et al. (2006) demonstrated great variability in the virulence properties between *L. monocytogenes* lineages 1 and 2, whereas even greater diversity in virulence was observed among subtypes and concluded that the combination of food survey data and subtyping could provide useful information to be used for a more accurate risk assessment for *L. monocytogenes*.

A third phylogenetic lineage, whose members are more commonly isolated from animals with clinical listeriosis, has also been described. Lineage 3 comprises of strains of serotypes 4a and 4c (Nadon et al., 2001); another study (Ward et al., 2004) has suggested that strains of serotype 4b may be found within both lineages 1 and 3. According to Jeffers et al. (2001), 10.5% of the animal isolates characterized in their study belonged to lineage 3, indicating a possible tropism for animals. Another explanation was provided, however, by Ward et al. (2004), who hypothesized that the low association between strains of lineage 3 and human cases of listeriosis is probably due to infrequent exposure, rather than decreased virulence to humans.

2.5. Factors affecting survival and growth

2.5.1. Effect of acidity

It is generally accepted that the optimum pH for *L. monocytogenes* growth is between 7.0 and 7.5 (neutral to slightly alkaline) (Seeliger and Jones, 1987; Petran and Zottola, 1989). According to Jay (2000), *Listeria* spp. have the ability to grow over the pH range of 4.1 to 9.6, whereas the International Commission on Microbiological Specifications for Foods (ICMSF) reports that the pH values that permit *L. monocytogenes* growth vary

from 4.39 to 9.4 (ICMSF, 1996). The minimum pH for survival and/or growth of *L. monocytogenes* in culture media or food products has been the subject of investigation of numerous studies, as the acid tolerance of a pathogen has serious implications for its survival in foods and within the human host. As an intracellular, foodborne pathogen, *L. monocytogenes* encounters high acidity and/or organic acids while passing through the digestive tract and upon uptake by the host cells. Consequently, mechanisms employed by the organism for survival under acidic conditions may be regarded as virulence factors. There is much evidence indicating that the ability of *L. monocytogenes* cells to tolerate low pH may influence the outcome of infection (Park et al., 1992; O' Driscoll et al., 1996; Marron et al., 1997; Wiedmann et al., 1998; Conte et al., 2000; Cotter et al., 2001a; Kazmierczak et al., 2003; Sue et al., 2004). In addition, effects of acidity on secretion and activity of LLO, a major virulence factor of *L. monocytogenes*, have been demonstrated, as the protein was shown to have maximum activity under acidic conditions (pH 4.0 to 5.0; McKellar, 1992). Also, studies by Kouassi and Shelef (1995a,b) have indicated that organic acids might lead to enhancement (lactate, acetate, citrate) or inhibition of LLO production by the organism. Nevertheless, as the effects of acidity on the ability of the pathogen to cause illness are still unclear, the complete understanding of the determinants involved in the relationship between acid responses and virulence remains within the listerial genome.

In general, although growth of *L. monocytogenes* has never been documented at pH values below 4.0 (Lado and Yousef, 2007), this organism is quite capable of surviving under acidic conditions. Conner et al. (1986) reported that the pathogen was able to grow in cabbage juice of pH 5.0, but did not survive when the pH was reduced to $\leq$ 4.6. Parish

and Higgins (1989) found that *L. monocytogenes* remained viable for 90 days in orange juice at pH 4.8 and 5.0. The same study indicated that the pathogen grew at pH values from 4.5 to 7.0, but it was not able to grow at pH 4.0 in tryptic soy broth, supplemented with 0.6% yeast extract during incubation at 30°C. Survival of *L. monocytogenes* was also observed in fermented salami (pH 4.4) during refrigerated storage (Johnson et al., 1988), while Glass and Doyle (1989a) observed that populations underwent slight reductions during 12 weeks of refrigerated storage of summer sausage (pH 4.86-5.19). When inoculated into trypticase soy broth acidified with hydrochloric acid, all 16 *L. monocytogenes* strains tested began to grow at pH values between 4.39 and 4.63 at 20 or 30°C (George et al., 1988). Growth of the pathogen has also been reported in tryptic soy broth of pH 4.4 at 25°C (Sorrells et al., 1989). A study by Beuchat and Brackett (1991) indicated that *L. monocytogenes* survived in commercial tomato juice (pH 4.1) for15 days at 5°C. Penteado and Leitão (2004) showed that the pathogen was able to grow in low-acid fruits, such as melon (pH 5.87), watermelon (pH 5.50), and papaya (pH 4.87) at 10, 20 or 30°C, whereas according to Han and Linton (2004) the pathogen was gradually reduced (by approximately 6 log CFU/ml) in strawberry juice (pH 3.6) during a 80-hour incubation at 37°C; however, the degree of inactivation was greatly dependent on the incubation temperature, as populations were reduced by less than 1 log CFU/ml after 3 days of storage at 4°C. The same study indicated that numbers of the pathogen decreased in tryptic soy broth plus 50% strawberry juice at pH 4.7 during incubation at 37°C for 24 hours.

Contradictory findings of research discussed above suggest that the minimum pH for growth of *L. monocytogenes* may be influenced by several variables. Indeed, research has

indicated that major factors that could affect the *L. monocytogenes* responses under low pH conditions include the type and concentration of the acidulant (Parish and Higgins, 1989; Sorrels et al., 1989; Ahamad and Marth, 1990; Conner et al., 1990; Ita and Hutkins, 1991; Young and Foegeding, 1993; Phan-Thanh and Montagne, 1998; Phan-Thanh et al., 2000), strain variability (Dykes and Moorhead, 2000; Francis and O'Beirne, 2005; Lianou et al., 2006), the physiological phase of the cells (Davis et al., 1996; O'Driscoll et al., 1996; Phan-Thanh and Montagne, 1998), and the incubation temperature (Ahamad and Marth, 1989, Parish and Higgins, 1989). In addition, Datta and Benjamin (1997) observed that nisin increased the acid sensitivity of the pathogen and that the antilisterial effects depended on the concentration of the bacteriocin.

2.5.2. Effect of low temperature

Most outbreaks of human listeriosis have involved foods kept at ambient or refrigerated temperatures (Czuprynski et al., 2002), suggesting that refrigeration should be considered as a risk factor for listeriosis (ILSI Research Foundation/Risk Science Institute, 2005). *L. monocytogenes* was originally reported to be capable of growing at temperatures between 3 and 45°C (Gray and Killinger, 1966), but subsequent studies indicated the ability of the organism to grow at even lower temperatures. According to Junttila et al. (1988), the mean minimum growth temperature for 78 strains of *L. monocytogenes* on trypticase soy agar was 1.1±0.3°C, with two strains (serotype 1/2) being able to grow at 0.5°C. Growth of *L. monocytogenes* at even lower temperatures was reported by Walker et al. (1990), who showed that the pathogen grew at 0.1°C and -0.4°C in pasteurized milk and chicken broth, respectively, whereas a later study indicated growth occurred at -1.5°C in vacuum-packaged, sliced roast beef (Hudson et al., 1994).

Nevertheless, the growth properties of the organism at low temperatures may be influenced by the nature of the food matrix, since survival, but not growth has been reported in ground beef (Johnson et al., 1988) or fresh liver (Shelef and Monte, 1988) maintained at 4°C. Barbosa et al. (1994) examined the growth variation of 39 *L. monocytogenes* strains and six other species of *Listeria* in tryptic soy broth supplemented with 0.6% yeast extract at 4, 10, or 37°C. Results indicated that growth of all *Listeria* spp. strains was faster at 37°C; however, although growth was slower at 4°C, all strains grew at this temperature. The authors also reported that growth of *L. monocytogenes* was faster than other *Listeria* spp. at all temperatures. A more recent study indicated that some of the *L. monocytogenes* strains examined grew better in brain heart infusion (BHI) broth at 4 or 7°C compared to *L. welshimeri* or *L. innocua* strains, with differences being more pronounced at 4°C (Nufer et al., 2007). The temperature history of the inoculum is another major factor affecting the growth rate of *L. monocytogenes* at low temperatures. When Grau and Vanderlinde (1990) used inoculum grown at 10°C (rather than 30-37°C), they observed rapid proliferation of *L. monocytogenes* on beef at 5.3°C. Accordingly, Walker et al. (1990) reported that the lag phase of the pathogen at 0°C was 3-18 and 13-33 days for inocula pre-incubated at 4 and 30°C, respectively. Buchanan and Klawitter (1991) observed that the temperature history of the *L monocytogenes* inoculum did not affect subsequent exponential growth rates and maximum population densities in tryptic phosphate broth; however, pre-incubation temperature at ≤ 28 and ≤ 13°C for aerobic and anaerobic cultures, respectively, decreased the duration of the lag phase. Similarly, Gay et al. (1996) observed that low inoculum and pre-incubation at 30°C rather than 14°C increased the lag phase of *L. monocytogenes* and *L. innocua*.

L. monocytogenes is capable of surviving freezing and frozen storage; however, such methods of preservation may cause cell injury, rendering the pathogen more susceptible to antimicrobial agents (Lado and Yousef, 2007). Factors that affect survival of the pathogen during frozen storage include the temperature, the type of substrate, and the rate of freezing (Lado and Yousef, 2007). According to Khan et al. (1973), *L. monocytogenes*, inoculated onto sterile lamb meat, survived at 0°C for 24 days. Also, frozen storage of *L. monocytogenes* at -18°C in carrot or chicken homogenate for 29-84 days did not cause noticeable reductions in viable counts (Oscroft, 1989). Small reductions (< 1 log) in *L. monocytogenes* populations occurred during storage of inoculated fish, shrimp, ground beef, ground turkey, frankfurters, corn and ice-cream at -18 to -20°C over a period of 3 months (Harrison et al., 1991; Palumbo and Williams, 1991).

2.5.3. Effect of high temperature

Thermal processing remains the primary means for ensuring food preservation and safety. *L. monocytogenes* is able of growing at temperatures as high as 45°C (Bacon and Sofos, 2003) and it is generally believed to possess higher resistance to heat compared to other vegetative foodborne pathogens (Mackey and Bratchell, 1989; Brown, 1991). The concern about the heat resistance of *L. monocytogenes* was raised after the 1983 outbreak of listeriosis in Massachusetts, which was epidemiologically linked to pasteurized milk (Fleming et al., 1985). This outbreak, along with experimental findings (Doyle et al., 1987; Farber et al., 1992), suggested that, unlike other non-sporeforming pathogens, *L. monocytogenes* is capable of surviving minimal high-temperature short-time (HTST) pasteurization. However, other published studies, including those by the FDA (Bradshaw et al., 1991; Bunning et al., 1992) and the CDC (CDC, 1988), conducted using freely

suspended, intracellular, or heat-shocked cells, have illustrated that the minimal HTST should be considered adequate to destroy the levels of *L. monocytogenes* usually found in milk. According to the ICMSF (1996), *L. monocytogenes* should not survive 71°C for 15 s, unless very high numbers of the pathogen are present.

Doyle et al. (2001) reviewed the heat resistance of *L. monocytogenes* in culture media and various foods. According to the authors the heat resistance of the organism is determined by several factors, including strain type, age of microorganisms, growth conditions, prior exposure to heat shock, or other stresses, recovery media, and intrinsic properties of the food, such as acidity, water activity, and additives. Effects of strain variability, food characteristics, and prior growth conditions on the heat tolerance of the pathogen were also reported in a more recent review (O'Bryan et al., 2006). The importance of the heating menstruum on the *L. monocytogenes* response to heat has been demonstrated by studies (Boyle et al., 1990; Jørgensen et al., 1999; Mazzota and Gombas, 2001), suggesting that the organism exhibits greater thermotolerance in meat products or product slurries than in culture media. A study by Mackey et al. (1990), conducted to compare the thermal resistance of the pathogen on chicken or beef, yielded inconclusive results, as effects of the meat type depended on the heating temperature. Findings by Jørgensen et al. (1999) revealed that cells at stationary phase displayed increased heat resistance relative to cells at logarithmic phase. Juneja et al. (1998) reported that the thermotolerance of *L. monocytogenes* in tryptic soy broth decreased as the pH during growth increased (5.4 vs. 7.0). On the other hand, findings by Edelson-Mammel et al. (2005) indicated that growth of *L. monocytogenes* under mildly acidic

conditions might enhance or reduce the thermal resistance of the pathogen, depending on the strain and heating menstruum.

The thermal resistance of the organism in meat and meat products has been investigated in numerous studies. Karaioannoglou and Xenos (1980) found that *L. monocytogenes* survived in meatballs, cooked to an internal temperature of 78-85°C, whereas Boyle et al. (1990) revealed that viable *L. monocytogenes* cells were recovered from ground beef, inoculated at high levels, after cooking to an internal temperature of 70°C. Zaika et al. (1990) investigated the thermal resistance of the pathogen during processing of frankfurters and observed that cooking for 70 min, until the internal temperature reached 160°F (71°C), was adequate for destruction of *L. monocytogenes* inoculated (10^3 or less/g) in the raw meat. Various studies have evaluated the effectiveness of different heating methods in reducing the levels of *L. monocytogenes* in meat products. According to Lund et al. (1989), the recommended microwave heating (temperature/time) of whole chickens caused reductions of 6 log CFU/g in the populations of the *L. monocytogenes*; however, at intermediate cooking times, large numbers of viable cells were recovered, suggesting uneven cooking or presence of 'cold spots'. D'Sa et al. (2000) showed that a rapid-high-temperature, double-sided grilling-broiling system appeared more effective than single-sided broiling in reducing *L. monocytogenes* numbers in ground beef patties, cooked to the same target internal temperature (60 or 68°C).

Considering that *L. monocytogenes* is mainly a post-processing contaminant, a number of studies, reviewed by Houben and Eckenhausen (2006), have focused on the efficacy of thermal pasteurization methods applied on the surface of vacuum-packaged RTE meat

products. The authors concluded that the numbers of *L. monocytogenes* that were thermally destructed on product surfaces were fewer than those anticipated based on results of volumetric thermal resistance studies, due to surface irregularities that may protect the pathogen against the heating treatments (Houben and Eckenhausen, 2006). Excessive production of purge by the product may also reduce the antimicrobial efficacy of heat treatments, as reported by Muriana et al. (2002). Findings of another study (Schultze et al., 2007) indicated that the thermal inactivation of a serotype 4b *L. monocytogenes* strain on frankfurter surfaces was not affected by the type of the preinoculation medium (tryptic soy broth, 8.5% fat slurry or tryptic soy broth plus quaternary ammonium) or the fat level (15 or 20%) of the product. However, the thermal destruction of the pathogen increased with the fat level and previous growth in tryptic soy broth supplemented with quaternary compounds, when cells were heated in frankfurter slurries.

The presence of sodium chloride, curing salts and other additives may affect the ability of *L. monocytogenes* to tolerate heat. Consequently, a better understanding of the impact that food additives may have on *L. monocytogenes* thermotolerance may guide food processors in designing more effective interventions for control of this pathogen. Additives that may lead to protection of the pathogen against thermal destruction include sodium chloride, curing salts, dextrose, sodium lactate, and sodium diacetate (Yen et al., 1991; Yen et al., 1992; Juneja and Eblen, 1999; Juneja, 2003). Stephens and Jones (1993) showed that exposure to high salt levels increased the temperature required for denaturation of the 30S ribosomal subunit of the organism. It should be noted, however, that the thermoprotective effects of salt may be overcome by cooking products at

temperatures higher than 60°C (Yen et al., 1991; Juneja and Eblen, 1999). Sodium pyrophosphate decreased the heat resistance of *L. monocytogenes* in beef gravy (Juneja and Eblen, 1999) and in pork slurry, but not in ground pork (Lihono et al., 2001). Results by Juneja (2003) showed that although sodium lactate and sodium diacetate, used individually in ground beef increased the thermal tolerance *L. monocytogenes*, the application of the two compounds together rendered the pathogen more sensitive to heat. Contradictory results were reported by Porto et al. (2004), as addition of potassium lactate in the formulation of frankfurters did not influence the thermal killing of the pathogen.

2.5.4. Effect of water activity and salt

Similarly to most bacteria, the optimum water activity for *L. monocytogenes* growth is ≥ 0.97 (Petran and Zottola, 1989). However, in contrast to most foodborne pathogens, *L. monocytogenes* has the ability to grow at water activity values as low as 0.90 (Lado and Yousef, 2007). That brings the pathogen second to the staphylococci in being able to grow at low water activity. When glycerol, sodium chloride, and propylene glycol were used as humectants in BHI broth, the lower water activity limits for *L. monocytogenes* growth were 0.90, 0.92, and 0.97, respectively (Miller, 1992). In addition, Farber et al. (1992) found that the minimum water activity for *L. monocytogenes* growth at 30°C was 0.90 when glycerol was used to lower the water activity. According to Johnson et al. (1988) the organism survived in fermented hard salami of water activity between 0.79 and 0.86 for at least 84 days at 4°C, despite the presence of 5.0-7.8% NaCl, 156 ppm sodium nitrite, and pH of 4.3-4.5. Calicioglu et al. (2002) reported that *L. monocytogenes* populations decreased noticeably during aerobic storage (25°C for 60 days) of untreated

or treated (prior to drying) with a traditional marinade jerky, especially during the first 10 to 30 days, while the pathogen was not detectable after 15 days of storage on product pre-treated with marinades containing Tween 20 and/or acetic acid. Nevertheless, although significant declines in bacterial counts were observed during storage, the pathogen was still detectable even after 60 days in untreated jerky, suggesting inhibitory effects by the pre-drying treatments.

Salt (sodium chloride) is the humectant most widely used to reduce the water activity of foods; however, *L. monocytogenes* is quite tolerant to salt. Growth of the pathogen has been reported in broth media supplemented with 10 (Seeliger and Jones, 1987; McClure et al., 1989) or 6.5% (Hudson, 1992) NaCl. Survival of the pathogen at high levels of salt was reported by Stenberg and Hammainen (1955), as they found that 10 strains of the organism survived in nutrient broth containing 1% glucose and 10% NaCl for more than a year (20-24°C), and in nutrient broth with 12 and 24% NaCl for 34-68 and 24 days, respectively. Also, Seeliger and Welshimer (1974) reported that the pathogen remained viable in 20% NaCl for 8 weeks at 4°C. The ability of the organism to survive and grow in salted foods has also been the subject of studies. According to Kukharkova et al. (1960), *L. monocytogenes* survived for more than 60 days at 4°C in meat stored in 30% NaCl brine, which also contained nitrite. Similarly, survival of the pathogen was observed in cheese brine (23.8% NaCl, 4°C) for 249 days (Larson et al., 1999). Conner et al. (1986) showed that *L. monocytogenes* LCDC 81-861 and Scott A were capable of growing in cabbage juice supplemented 1% NaCl at pH 6.1 (30°C). Apart from the survival and growth properties of *L. monocytogenes* in the presence of salt, food processors should also consider that addition of NaCl in food products might harden the

pathogen to other stresses, such as heat (Yen et al., 1991; Juneja and Eblen, 1999) or hydrogen peroxide (Lis-Balchin and Deans, 1997).

2.5.5. Effect of food additives/antimicrobials

Various compounds may be included in the formulation of food products in order to prolong their shelf life and improve their safety. Some of these additives (e.g., salt, phosphates) have been traditionally used as ingredients in various products, primarily because of their contribution to the characteristics of the product, while others are added almost exclusively in certain food products to inactivate target pathogens or suppress their growth. In general, traditionally used additives (i.e., salt, sodium nitrite, sodium phosphates) have limited activity against *L. monocytogenes* (Stenberg and Hammainen, 1955; Kukharkova et al., 1960; Shahamat et al., 1980; Doyle, 1988; Junttila et al., 1989), unless they are used in combination with other stresses, such as low temperature, high acidity, or other additives (Buchanan et al., 1989; Zaika and Kim, 1993; Buchanan et al., 1994).

After taking into account the severity of listeriosis (Rocourt, 1994), the numerous outbreaks linked to RTE products (CDC, 1998; 2000; 2002), and the difficulty associated with producing *L. monocytogenes*-free foods, the USDA-FSIS announced that manufacturers of RTE meat and poultry products need to incorporate strategies to control the pathogen in their products. According to the 2003 USDA-FSIS interim final rule, facilities that produce RTE meat or poultry products that support *L. monocytogenes* growth are required to utilize one of three alternatives for *L. monocytogenes* control: Alternative 1- Establishments are required to apply a post-lethality treatment (may be an antimicrobial agent) to reduce or eliminate *L. monocytogenes* and an antimicrobial agent

or process to limit or suppress growth of the pathogen. Establishments selecting alternative-1 are not required to perform testing on food contact surfaces. Alternative 2- Establishments are required to employ either a post-lethality treatment or a growth inhibitor. Under this alternative, establishments selecting a post-lethality treatment are not required to perform testing of food contact surfaces. However, if an establishment selects to employ an inhibitory agent or process then its sanitation program must include testing of food contact surfaces. Alternative 3- Establishments would rely on sanitation measures only to avoid post-processing contamination of their products with the pathogen. Establishments that choose this option, however, are required to conduct regular environmental sampling in order to confirm the efficacy of sanitation procedures in the post-lethality processing environment and to develop product-holding procedures when positive tests are obtained. Moreover, establishments that rely only on sanitation measures are subject to the most frequent FSIS verification activity (USDA-FSIS, 2003a).

An expert panel, assembled by the Food and Agriculture Organization of the United Nations and the World Health Organization stated that the most efficient way of reducing the incidence of listeriosis involves preventing the occurrence of high contamination levels on foods at the time of consumption (Buchanan et al., 2004). Thus, utilization of generally-recognized-as-safe antimicrobial agents such as various organic acids and salts, in the right concentrations and/or combinations may contribute to the safety of RTE foods by preventing proliferation of this pathogen during product storage. Due to the importance of *L. monocytogenes* as a foodborne pathogen, several studies have screened the ability of various antimicrobial agents to control the pathogen in broth media or actual

food systems. Although minimizing the likelihood of product contamination with *L. monocytogenes* is essential in the effort of preventing human listeriosis, the complete exclusion of the pathogen from food-processing operations and, thus, the avoidance of cross-contamination may be unfeasible for the industry to achieve with current technology (Tompkin et al., 1999; Tompkin, 2002). To minimize the risk to public health, therefore, manufactures of high-risk products need to implement post-process interventions that limit growth of *L. monocytogenes* to high numbers, in addition to their efforts for exclusion of the pathogen from the processing environment (Chen et al., 2003). In products that allow growth of the pathogen, addition of antimicrobials in product formulations or treating (dipping or spraying) the finished product with preservative agents, mainly short chain organic acids or their salts may be of great assistance in preventing growth or inactivating *L. monocytogenes* in foods. Currently, the most widely used antimicrobials in RTE foods for *L. monocytogenes* control include sodium/potassium lactate and sodium diacetate, used usually in combination (2% SD and 0.1-0.15% SD; Tompkin, 2002). Extensive research conducted in recent years has revealed that incorporation of lactate and/or diacetate directly into product formulations may control *L. monocytogenes* growth (Bacus and Bontenbal, 1991; Schlyter et al., 1993a; 1993b; Wederquist et al., 1994; Blom et al., 1997; Bedie et al., 2001; Mbandi and Shelef, 2001; 2002; Stekelenburg and Kant-Muermans, 2001; Samelis et al., 2002; Seman et al., 2002; Porto et al., 2002; Stekelenburg, 2003; Choi and Chin, 2003; Barmpalia et al., 2004; 2005; Geornaras et al., 2006; Luchansky et al., 2006; Vogel et al., 2006; Glass et al., 2007), particularly at low temperatures (Barmpalia et al., 2005). Lactate levels currently used in RTE product formulations vary from 2 to 3%, whereas

diacetate is commonly added at 0.1% to 0.15% (Porto et al., 2002; Tompkin, 2002). Application of antimicrobial compounds as dipping or spraying solutions for surface decontamination may also assist in controlling growth of the pathogen in food products (Schlyter et al., 1993a; 1993b; Palumbo and Williams, 1994; Ariyapitipun et al., 2000; Samelis et al., 2001a; Glass et al., 2002; Islam et al., 2002a; 2002b; Barmpalia et al., 2004; Nuñez de Gonzalez, 2004; Uhart et al., 2004; Geornaras et al., 2005; Lu et al., 2005; Singh et al., 2005; Luchansky et al., 2006), whereas complete inhibition of *L. monocytogenes* growth or even reductions in the populations of the pathogen may be achieved when dipping treatments are used together with appropriate combinations of formulation additives (Barmpalia et al., 2004; Geornaras et al., 2006). Nevertheless, further research might be necessary for the identification of additional antilisterial compounds to be used as substitutes to the lactate/diacetate combination, as food processors need alternatives in order to maintain acceptable sensory characteristics in their products and meet the consumer demand for 'natural' foods.

Naturally occurring and derived antimicrobial compounds originate from animal, plant and microbial sources, and have gained increased interest due to the increased consumer demand for 'fresh', 'minimally processed' foods. Various spices and plant extracts may demonstrate antilisterial action, due to specific chemicals and/or essential oils (Aureli et al., 1992; Nguyen-The and Lund, 1992; Hefnawy et al., 1993; Larson et al., 1996; Hao et al., 1998; Alzoreky and Nakahara, 2003; Ahn et al., 2007; Oussalah et al., 2007). The antimicrobial mode of action of plant oil aromatics has been investigated by Gill and Holley (2004; 2006). Results suggested that cinnemaldehyde, but not eugenol resulted in depletion of cellular ATP in *L. monocytogenes* cells previously grown in the presence of

glucose (Gill and Holley, 2004). Also, treatments with eugenol and carvacrol led to disruption of the cellular membranes of *L. monocytogenes*, *E. coli*, and *L. sakei* (Gill and Holley, 2006). Antimicrobials originating from animal sources, such as lysozyme (derived from eggs), lactoferrin and the lactoperoxidase system (both derived from milk), have also been screened for their effectiveness against *L. monocytogenes* in culture media or various foods (Hughey et al., 1989; Bellamy et al., 1992; Wang and Shelef, 1992; Kihm et al., 1994; Payne et al., 1994; Dufour et al., 2003; Ransom et al., 2003; Branen and Davidson, 2004; Elliot et al., 2004). Finally, the effects of ribosomally-synthesized antimicrobial peptides (i.e., bacteriocins) of lactic acid bacteria (e.g., nisin, pediocin, reuterin etc) on the survival/growth responses of *L. monocytogenes* or other species of *Listeria* in bacteriological media (Benkerroum and Sandine, 1988; Bhunia et al., 1988; Nielsen et al., 1990; Uhlman et al., 1992; Buncic et al., 1995; Dufour et al., 2003; Sivarooban et al., 2007) or actual food products (Benkerroum and Sandine, 1988; Nielsen et al., 1990; Cutter and Siragusa, 1996; Davies et al., 1997; Murray and Richard, 1997; El-Ziney et al., 1999; Aasen et al., 2003; Samelis et al., 2003b; 2005; Geornaras et al., 2006; Hampikyan and Ugur, 2007; Sivarooban et al., 2007) have been illustrated in the literature. Bacteriocins exert their antilisterial effects on target cells by depleting the proton motive force (Bruno and Montville, 1993) and causing leakage of cellular materials (Abee et al., 1994). Nevertheless, the practical application of bacteriocins for control of *L. monocytogenes* in foods is limited due to the loss of their antimicrobial activity over time (Muriana, 1996; Benech et al., 2002; Samelis et al., 2005; Geornaras et al., 2006). Aasen et al. (2003) demonstrated that more than 80% of the added sakacin P

and nisin was absorbed in the food matrix (salmon or chicken), whereas the bacteriocins were also susceptible to degradation due to proteolytic activity.

Findings of published studies regarding the antilisterial efficacy of naturally occurring compounds are conflicting (Kim et al., 1995; Hao et al., 1998). In addition, the employment of natural preservatives in food products has certain practical restrictions, such as the high cost of application, the need for high levels for antimicrobial effectiveness, and the negative impact on the sensory attributes of foods (Sofos et al., 1998; Mazzotta and Montville, 1999). Those restrictions may be overcome, however, by the employment of appropriate combinations of natural or chemical antimicrobial agents in accordance with the 'hurdle concept' (Leistner, 2000; Chen and Hoover, 2003). Specifically, combinations of preservatives, used at low concentrations, may result in improved antimicrobial activity, due to multiple modes of action against the bacterial cell (Roberts, 1989; Kabara, 1993), without sacrificing the sensory quality of foods. Accordingly, several publications have illustrated that the antilisterial effects of natural antimicrobials can be enhanced when employed together with other natural (Kato and Shibasaki, 1976; Oh and Marshall, 1993; Blaszyk and Holley, 1998; Zapico et al., 1998; Pol and Smid, 1999; Boussouel et al., 2000; Ettayebi et al., 2000; Singh et al., 2001; Dufour et al., 2003; Yamazaki et al., 2004; Murdock et al., 2007; Sivarooban et al., 2007) or synthetic compounds (Schlyter et al., 1993b; Blaszyk and Holley, 1998; Nykänen et al., 2000; McEntire et al., 2003; Gill and Holley, 2003; Branen and Davidson, 2004; Geornaras et al., 2006; Garcia et al., 2007). Attention has been paid also on the combined use of synthetic or natural antimicrobial compounds with physical treatments (e.g., steam, hot water-immersion/showering, irradiation, high pressure) for *L. monocytogenes* control

(Samelis et al., 2001a; Garriga et al., 2002; Sommers et al., 2003; Sommers and Fan, 2003; Aymerich et al., 2005; Murphy et al., 2005; 2006; Chung et al., 2005; Vurma et al., 2005; Zhu et al., 2005; Luchansky et al., 2006; Marcos et al., 2008).

2.5.6. The gastrointestinal barrier

2.5.6.1. The stomach

The human stomach is a large capacity organ situated between the end of the esophagus and the beginning of the small intestine (duodenum), and serves three functions: (i) storage of food until it can be accommodated in the intestinal tract; (ii) mincing and mixing of food with digestive juices to create a murky semi-fluid mixture, called chyme; and, (iii) propelling the chyme into the lower part of the gastrointestinal tract (gastric emptying) at a rate that allows proper digestion and absorption by the small intestine (Guyton, 1986; Low, 1990). Gastric emptying is a strictly regulated process, controlled primarily by the osmotic effect and calcium binding of the digestion products in the duodenum (Hunt, 1983). Several nutritional factors, including the physical form, the energy density, the volume, and the composition (i.e., fat, protein, carbohydrate, and acid contents) of the meal have also been demonstrated to affect the rate of gastric emptying (Hunt and McDonald, 1954; Hunt and Knox, 1968; Malagelada et al., 1979; Hunt, 1983; Notivol et al., 1984; Fisher et al., 1987). In addition, to its digestive and transport functions, the stomach acts as a barrier against foodborne illness, as it is the primary site for hydrochloric acid secretion (Low, 1990). The electrolytic solution secreted by the parietal cells contains hydrochloric acid at a concentration of approximately 160 mEq/L (Guyton, 1986) and the extremely low pH of this acid solution (approximately 0.8) provides gastric juice with antimicrobial properties.

Secretion of gastric acid by parietal cells is a complicated and energetically costly procedure (Johnson, 2001) considering that at high rates of acid secretion, the pH of gastric juices may be less than 1, and that parietal cells need to produce hydrogen ions against a 2.5 million-fold concentration gradient (pH of blood is 7.4). The secretion of gastric acid is regulated by endogenous hormonal and neural agents, mainly acetylcholine, gastrin, and histamine that bind on surface receptors located on parietal cells stimulating them to secrete hydrogen ions (Helander and Keeling, 1993; Johnson, 2001). The meal-related secretion process takes place in three phases, depending on the location of receptors that start the secretory responses: cephalic, gastric and intestinal (Guyton, 1986; Low, 1990; Johnson, 2001). Acid production during the cephalic phase takes place before the food enters the stomach and is initiated by the thought, sight, smell or taste of appetizing food (Feldman and Richardson, 1986; Low, 1990; Guyton, 1986). In the gastric phase, acid secretion is stimulated by the presence of food in the stomach as food components (mainly protein) neutralize the small volume of acid existing in the stomach and, thus activate the gastrin mechanism (Johnson, 2001).

Acid secretion during the intestinal phase occurs once the food is emptied in the upper portion of the intestine, stimulating the release of small levels of gastrin by the duodenal mucosa and causing the stomach to produce small amounts of gastric acid (Johnson, 2001). Nevertheless, various intestinal factors, such as the enterogastric reflex, initiated by the presence of food in the small intestine, as well as hormones released from the duodenal mucosa may inhibit secretion of gastric acid during the intestinal phase (Johnson, 2001). The presence of fat or fatty acids in the intestine has also been shown to inhibit gastric acid secretion (Christiansen et al., 1976; 1979).

Human studies (Mojaverian et al., 1988; Dressman et al., 1990; Russell et al., 1993) have shown that the level of gastric acidity may vary greatly between individuals and age groups. More specifically, Mojaverian et al. (1988) reported that the gastric pH, 30 min after ingestion of a meal was higher in elderly males than in young males (pH of 5.6 and 3.6, respectively). Overall, the median fasting pH for a group of elderly (1.3) was lower than that of young individuals (1.7); however, the time required for the gastric pH to return to 2.0 was 100 and 150 min for the young and elderly group, respectively (Dressman et al., 1990; Russell et al., 1993). The authors (Russell et al., 1993) concluded that the delayed acidification of stomach contents in older individuals could have been the result of decreased acid production and/or slow gastric emptying. A study by Vanzant et al. (1932) showed that the incidence of achlorhydria (i.e., absence of hydrochloric acid in gastric juices) was considerably increased with old age. Reduced gastric acidity with increasing age is considered as one of the factors contributing to the increased morbidity and mortality from foodborne disease observed in the geriatric population (Klontz et al., 1997; Smith, 1998).

Apart from hydrochloric acid, whose role is discussed in a following paragraph, other functional components of gastric fluid include the intrinsic factor, pepsin and mucus (Johnson, 2001). The intrinsic factor, a glycoprotein secreted by the parietal cells is required for the absorption of vitamin B_{12} in the intestine (Guyton, 1986). The proteolytic enzyme pepsin is formed from its inactive precursor called pepsinogen (Langley and Edkins, 1886). Pepsinogen is converted to active pepsin when the pH in the stomach is reduced to < 5, with the conversion being catalyzed by previously formed pepsin (Guyton, 1986; Johnson, 2001); thus, apart from creating a bactericidal environment in

the stomach, the formation of hydrochloric acid is also a critical element for protein degradation by pepsin. Mucus is a viscous secretion, produced by specialized epithelial cells, the goblet cells, in the columnar epithelium that lines organs exposed to the outer environment, including the surface of the digestive tract (Bansil and Turner, 2006). Water is the main constituent of mucus (approximately 95%), whereas other components include salts, lipids, and the glycoprotein mucin that provides mucus with its characteristic viscoelastic properties (Bansil and Turner, 2006). Functions of mucus include maintenance of hydration over the epithelium and lubrication to facilitate passage of objects (Allen, 1981). In addition, mucus forms a protective coating that prevents the gastric contents from inducing chemical damage to the cells of the mucosa (Grant et al., 1953).

2.5.6.2. Relationship between gastric acid and infection

The physiological functions of gastric acid include (Howden and Hunt, 1986): (i) activation of pepsinogen for digestion of proteins; (ii) augmentation of dietary calcium and iron absorption; and, (iii) protection of the cells of the lower gastrointestinal tract against pathogenic microorganisms. The extreme acidity within the gastric compartment is a major natural defense mechanism against infection by ingested pathogens (Howden and Hunt, 1986; Smith, 2003). The negative association between gastric secretions and gastrointestinal disease has been assumed for more than 100 years, since the high acidity of the normal stomach is expected to inactivate a large number of pathogens ingested with food (Knott, 1923; Bartle and Harkins, 1925; Garrod, 1939; Howden and Hunt, 1987). Although, early reports have attributed antimicrobial activity to various components of gastric juice, such as mucus (Goldsworthy and Florey, 1930), organic

acids (Knott, 1923), and lysozyme (Thompson, 1940), Giannella et al. (1972) concluded that the gastric bactericidal effects are mainly pH-hydrochloric acid-dependent, as saline and nutrient broth exhibited bactericidal activity equal to that of gastric fluid at comparable pH values. A subsequent study by Peterson et al. (1989) confirmed the acid-dependency of the gastric bactericidal properties. Nevertheless, whether hydrochloric acid is the sole antimicrobial agent in gastric juice remains a controversial issue as a more recent study (Koo et al., 2000) indicated that simulated gastric fluid exhibited greater antimicrobial effects against *V. vulnificus* than acidified broth.

According to Giannella et al. (1972), inactivation of 1×10^9 *Serratia marcescens* in the stomach of healthy individuals occurred within 30 min when the pH was 3 or below. Rotimi et al. (1990) reported that subjecting intestinal bacteria (*Campylobacter jejuni*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Yersinia enterocolitica*, *Salmonella* spp., and *Shigella* spp.) to hydrochloric acid at pH 2 or 4 resulted in their complete inactivation within 60 min. Nevertheless, microorganisms may vary in their ability to survive under acidic conditions. Roering et al. (1999) showed that *Salmonella* Typhimurium survived for 5 min in simulated gastric fluid (pH 1.5), whereas *E. coli* O157:H7 remained viable for at least 2 hours.

The protective effect of gastric acidity against bacterial or parasitic foodborne infections has been evidently demonstrated in achlorhydric and hypochlorhydric individuals or patients that have undergone partial gastrectomy (Waddell and Kunz, 1956; Gray and Shiner, 1967; Drasar and Shiner, 1969; Gray and Trueman, 1971; Greenlee et al., 1971; Giannella et al., 1972, 1973; Howden and Hunt, 1986). Furthermore, treatments that prohibit acid secretion or neutralize the intragastric contents

may protect pathogens against gastric killing (Cash et al., 1974; Arnow et al., 1984; Guerrant, 1995; Asha et al., 2006). The protective effect of antacids against the acid destruction of pathogenic bacteria has been shown in experiments using simulated gastric fluids (Koo et al., 2001; Tamplin, 2005), whereas the possible association between gastric acid suppressive therapy and *L. monocytogenes* asymptomatic fecal carriage or invasive disease has also been suggested by two case-control epidemiological studies (Ho et al., 1986; MacGowan et al., 1991). Accordingly, an oral-feeding experiment, using Sprague-Dawley rats, showed that treating animals with H_2-antagonists increased their susceptibility *to L. monocytogenes* invasive infection (Schlech et al., 1993).

Introduction of food into the gastric compartment may also protect pathogenic microorganisms in the gastric chyme by producing a temporary rise of the pH. Accordingly, studies by Conway et al. (1987) and Peterson et al. (1989) have suggested that the presence of food reduces the antimicrobial properties of gastric juice. Certain foods, especially those high in fat are thought to interfere with the gastric killing of *Salmonella* (Waterman and Small, 1998). Tamplin (2005) concluded that *E. coli* O157:H7 was more resistant in simulated gastric juice when inoculated in cooked ground beef as compared to saline, while Drouault et al. (1999) reported that presence of food had a protective effect against the gastric inactivation of *Lactococcus lactis*, providing the explanation that food acts as a buffer that protects bacteria in the stomach by neutralizing the acidic gastric contents. Peterson et al. (1989), however, reported that food acted in a manner other than a buffer to elevate intragastric pH and decrease the antibacterial activity of gastric fluid against *E. coli* and *Shigella flexneri*, but not *Salmonella* Typhimurium. Kos et al (2000) revealed that presence of mucin and milk proteins

(particularly whey proteins concentrate) protected *L. acidophilus* in simulated gastric and intestinal fluids. The ability of microorganisms to survive gastric exposure depends on the length of time they remain within the stomach. As expected, pathogens that are transferred to the small intestine shortly after food consumption (while the gastric pH is still high) remain unaffected by gastric acidity and exhibit high survival rates (Takumi et al., 2000). Consequently, the initial levels of pathogens present in foods may greatly affect the number of cells that reach the intestine in a viable state.

Various compounds ingested as food ingredients may also affect the ability of microorganisms to survive gastric secretions. Gastric fluid and salivary nitrite worked synergistically to inactivate *Salmonella* and *E. coli* O157 (Xu et al., 2003; Smith, 2003). The acid tolerance of *L. rhamnosus* in simulated gastric juice was increased by the presence of metabolizable sugars (i.e., glucose) (Corcoran et al., 2005). The authors reported that glucose contributes to cell viability under acidic conditions by providing ATP to the F_0F_1-ATPase proton pump. Little information exists regarding the ability of *L. monocytogenes* to survive the transit through the gastrointestinal tract, as affected by the food matrix properties or ingredients. The fat content of the food has been suspected as a factor affecting the dose-response relationship (Buchanan et al., 2000), as high-fat foods have been frequently implicated in listeriosis outbreaks (Linnan et al., 1988; McLauchlin et al., 1991; CDC, 1998; Lyytikäinen et al., 2000). An animal study by Smith et al. (2003) showed that the oral infectivity of the pathogen in pregnant primates increased when high-fat foods were used. Another study (Mytle et al., 2006) reported that the fat content of the delivery vehicle did not affect *L. monocytogenes* colonization in the murine gastrointestinal tract. The authors reported, however, that the high numbers of the

pathogen in the food probably overwhelmed any effects of the food matrix. Glutamate was found to have a protective effect against the killing of wild-type *L. monocytogenes* in artificial gastric fluid (Cotter et al., 2001a). Moreover, results by Stopforth et al. (2005) showed that immersion of pork frankfurters, formulated with sodium diacetate (0.25%), into a 2.5% solution of lactic acid may have resulted in increased resistance of surviving cells to artificial gastric fluid as storage of the product progressed. However, it was not clear whether the increased resistance of the pathogen in gastric fluid was exclusively due to the antimicrobial treatments applied on the product.

It has been long thought that drinking alcoholic beverages may help prevent foodborne infection due to the bactericidal effects of ethanol and other constituents (e.g., organic acids and sulfur dioxide in wine). Consumption of alcohol was associated with reduced incidence of illness in a *Salmonella* Enteritidis outbreak linked to sandwiches containing tuna, boiled eggs and vegetables (Blasco et al., 1996). *In vitro* data by Just and Daeschel (2003) showed that addition of wine in an artificial stomach model that contained gastric fluid and food (vegetable turkey dinner for infants) caused dramatic reductions in *Salmonella* spp. numbers, but had little effect against *E. coli* O157:H7. Wine led to higher reductions of *L. innocua* populations than those caused by a combination of ethanol with organic acids (malic and lactic) in a simulated stomach model (Fernandes et al., 2007). Nevertheless, both these studies (Just and Daeschel, 2003; Fernandes et al., 2007) did not account for the increased secretion of gastric acid resulting from wine consumption (Lenz et al., 1983; Peterson et al., 1986), which creates the possibility that the antibacterial activity of wine could have been underestimated by their findings.

2.5.6.3. The small intestine

The small intestine is the portion of the gastrointestinal tract located between the stomach and the large intestine. It consists of three parts: the duodenum, the jejunum and the ileum (Guyton, 1986). The small intestine is the site where the majority of digestion and absorption of digestive end products takes place (Guyton, 1986). Secretions of the small intestine include mucus and intestinal digestive juices, which consist primarily of chloride and bicarbonate ions (Guyton, 1986; Banks and Farthing, 2002). In addition to its own, the small intestine also receives secretions from the pancreas (i.e., pancreatic juices) and the liver (i.e., bile). Pancreatic juices contain enzymes necessary for the digestion of all types of foods; trypsin, chymotrypsin, carboxypolypeptidase, ribonuclease, and deoxyribonuclease are required for the digestion of proteins, pancreatic amylase, is used for starch and glycogen hydrolysis, whereas pancreatic lipase, cholesterol esterase and phospholipase constitute the main enzymes for fat digestion (Guyton, 1986). Pancreatic juices also contain bicarbonate ions that neutralize the acidic gastric digesta once they enter the duodenum (King and Schloerb, 1969). Bile is a hepatic secretion necessary for the emulsification and solubilization of fats (Begley et al., 2005a). It is synthesized continuously by liver cells and concentrated in a sac-like organ, the gallbladder, until it is needed in the duodenum for digestion of dietary fat (Gyuton, 1986). Primary components of bile include, bile acids (12% by weight), cholesterol, phospholipids, and the green pigment biliverdin (Johnson, 1998; Begley et al., 2005a, 2005b). The most important bile acids are cholic acid and chenodeoxycholic acid; they are synthesized in the liver from cholesterol, and secreted as amino acid conjugates with either glycine or taurine (Hofmann and Mysels, 1992). In the intestinal tract, the bile

acids serve two major functions (Green and Riley, 1981; Gyuton, 1986; Begley et al., 2005a): (i) they act as detergents on fat particles to reduce their surface tension and break-down fat globules into minute sizes; and, (ii) they act as 'lipid-carriers' to transfer the products of lipolysis (e.g., fatty acids, monoglycerides and cholesterol) from the emulsion surface to the mucosa, where they are absorbed.

In addition to its nutritional function, bile also serves as a defense barrier against pathogen colonization and invasion in the intestinal tract. It is the ability of bile acids to emulsify and solubilize fats that provides bile with its bactericidal properties. Bile has the ability to interact with phospholipids on cell membranes and lead to the disruption of cellular homeostasis (Begley et al., 2005a). In addition, bile salts are capable of inducing cellular death by causing DNA damage and inducing oxidative stress via the production of oxygen free radicals (Payne et al., 1998; Bernstein et al., 1999). Antimicrobial effects may vary according to the type of bile. For instance, porcine bile that consists of dihydroxyconjugated bile acids has superior antimicrobial effects than bovine bile that contains trihydroxyconjugated bile acids (Grill et al., 2000). Sung et al. (1993) demonstrated that hydrophobic bile salts exhibited greater antimicrobial activity against *E. coli* and *Enterococcus fecalis* than hydrophilic salts, whereas the inhibitory effects of all salts was considerably reduced by the addition of lecithin. In general, Gram-negative bacteria are considered to be quite bile-tolerant, as implied by the addition of bile salts in selective media used for their isolation (Mac Conkey agar, *Salmonella-Shigella* agar etc). The inherent resistance of *Salmonella* and *Campylobacter* to bile can also be demonstrated by their ability to colonize the gallbladder (Darling et al., 1979; Prouty et al., 2002). Using a dynamic model of the gastrointestinal tract, Gänzle et al. (1999)

showed that *E. coli* was able to grow at high levels of porcine bile extract, whereas Gram-positive organisms were promptly inactivated in the presence of bile. The protective role of meat against inactivation of *L. curvatus* during bile-exposure was also reported in this study. Kos et al. (2000) showed that milk protein protected *L. acidophilus* in simulated intestinal fluid that contained bile salts and pancreatin. The species- or strain-dependency of the toxic effects of bile has been demonstrated in studies with lactic acid bacteria (Walker and Gilliland, 1993; Chateau et al., 1994; Gupta et al., 1996).

The intestinal tract is the portal of entry of *L. monocytogenes* into the host (Berche et al., 1988) and, therefore, bile resistance plays a major role in pathogenesis. Studies by Allerberger et al. (1989) and Briones et al. (1992) have demonstrated that *L. monocytogenes* has the ability to colonize the gallbladder, suggesting that the pathogen is inherently resistant to high concentrations of bile. The gene for bile hydrolase activity (*bsh*; discussed later), required for the survival of *L. monocytogenes* in bile, was essential for the oral infection of guinea pigs and the systemic infection of mice with the pathogen (Dussurget et al., 2002). Begley et al. (2002) showed that *L. monocytogenes* strain LO28 was capable of tolerating higher levels of human, porcine, or bovine bile than those encountered *in vivo*. Similarly, 50 isolates of the pathogen, originating from various sources, were able of tolerating high concentrations of bile, as they grew on agar and in broth supplemented with 2 and 5% of porcine bile salts, respectively (Olier et al., 2004). In addition, results of the same study suggested that bile tolerance was a strain-dependent characteristic. Nevertheless, as with resistance to other stresses, *L. monocytogenes* bile resistance may greatly depend on the experimental conditions of each study. King et al. (2003) observed that four exponential phase *L. monocytogenes* strains were inactivated

within 2 min in 0.3% oxbile. The same study also revealed that exposing *L. monocytogenes* to 100% CO_2 or 40% CO_2 : 60% N_2, but not 100% N_2, rendered the pathogen susceptible to bile salts, suggesting that CO_2 may interact with the listerial cell membrane making it more permeable to bile.

Apart from the presence of bile acids in the small intestine, other protective responses of the host against foodborne infection include high osmolarity (0.3 M NaCl) conditions, presence of volatile fatty acids and the established intestinal microorganisms (Kerr, 1991; Sarker and Gyr, 1992; Dunne et al., 1999; Phan-Thanh et al., 2000; Gahan and Hill, 2005). As discussed previously, *L. monocytogenes* is capable of tolerating high concentrations of salt (Seeliger and Jones, 1987; Hudson, 1992). Additionally, osmolyte uptake systems, utilized for the transport of solutes into the cell, have been recognized in *L. monocytogenes* (Sleator and Hill, 2002). Such systems may promote enhanced survival of the pathogen in environments with elevated osmolarity, such as the small bowel. Regarding the ability of the pathogen to withstand volatile fatty acids encountered upon entry in the small intestine, Phan-Thanh et al. (2000) observed that *L. monocytogenes* exhibited a survival of 96, 42, and 31% in the presence of hydrochloric acid, a mixture of fatty acids, simulating the composition of the human gut, and acetic acid, respectively. The contribution of gut microflora as a protective barrier, acting against colonization of harmful microorganisms in the intestinal tract has been long known (Collins and Carter, 1978; Kennedy and Volz, 1985; Kerr, 1991). The protective role of the natural intestinal microflora against infection with *L. monocytogenes* was reported in a study by Manohar et al. (2001), which revealed that gnotobiotic mice were more susceptible to oral inoculation with the pathogen when compared to normal mice.

2.6. Stress responses

To establish infection, *L. monocytogenes* must overcome a variety of hurdles, associated with food processing, storage, and preparation (e.g., high acidity, salt, high or low temperature, preservatives) and various elements of the host defense system, including high acidity, low oxygen levels, volatile fatty acids, and bile. Therefore, to succeed as a pathogen, *L. monocytogenes* has evolved to possess a variety of protective systems that allow survival under suboptimal conditions. A variety of environmental stresses or antimicrobial hurdles applied during food processing may result in increased resistance of the surviving bacterial cells to otherwise lethal processes; a phenomenon termed 'stress hardening' (Lou and Yousef, 1997; Samelis and Sofos, 2003). Pre-exposure of the pathogen to low levels of a given stress may offer protection, not only against lethal levels of the same stress, but also against different stresses (cross-protection), since several antimicrobial factors may have similar effects on cell physiology and activate the synthesis of common sets of stress-related proteins. Several researchers have shown that *L. monocytogenes* may acquire increased thermotolerance following exposure to sublethal doses of heat (Farber and Brown, 1990; Linton et al., 1990; Linton et al., 1990; 1992; Stephens et al., 1994; Jørgensen et al., 1996) or other stresses (Yen et al., 1991; Farber and Pagotto, 1992; Jørgensen et al., 1995; O' Driscoll et al., 1996; Lou and Yousef, 1996; Mazzota, 2001). Conflicting findings were reported by Bunning et al. (1990) since the authors were unable to detect significant increases in heat resistance after subjecting *L. monocytogenes* to four different temperatures (35, 42, 48, or 52°C) for various times. Similarly, prior exposure to cold temperatures may decrease (Pagan et al., 1997) or increase (Miller et al., 2000) the thermal sensitivity of the

pathogen. Miller et al. (2000) suggested that exposure to cold suppresses the production of proteins that are necessary for heat protection.

The effects of osmoadaptation on the ability of the pathogen to withstand high salt levels or other stressful conditions have been demonstrated only in a limited number of publications. Exposure to 7% salt increased the survival properties of the pathogen to 1% hydroxide (Lou and Yousef, 1997), whereas pre-incubation in 3.5% salt protected most of the *L. monocytogenes* isolates studied against osmotic (20% NaCl) and acid (pH 3.5) shock (Faleiro et al., 2003). Begley et al. (2002) showed that previous exposure of *L. monocytogenes* strain LO28 to sublethal levels of bile acids, heat, salt, or sodium dodecyl sulfate enhanced its ability to tolerate bile. The ability of *L. monocytogenes* to mount an adaptive acid tolerance response (ATR), allowing bacterial cells, previously exposed to moderately acidic conditions, to withstand extreme acid exposure, as well as factors affecting the induction of ATR have also been reported (Kroll and Patchett, 1992; Davis et al., 1996; Gahan et al., 1996; O'Driscoll et al., 1996; 1997; Phan-Thanh et al., 2000; Faleiro et al., 2003; Ferreira et al., 2003; Koutsoumanis et al., 2003; Samelis et al., 2003a). In contrast to the above studies, however, Phan-Thanh and Montagne (1998) demonstrated that prolonged exposure of *L. monocytogenes* at moderately acidic pH rendered cells more susceptible to subsequent lethal pH, highlighting that experimental differences in terms of growth conditions may influence the mechanisms of acid adaptation and resistance.

A major consequence of exposure to mildly acidic conditions is the modification of protein synthesis patterns as demonstrated by Davis et al. (1996), O'Driscoll et al. (1997), Phan-Thanh and Montagne (1998), Phan-Thanh and Mahouin (1999) and Phan-Thanh et

al. (2000). Pre-exposure of the pathogen to sublethal acid may also confer cross-protection against other stresses, such as high osmolarity (Farber and Pagotto, 1992; O'Driscoll et al., 1996; Faleiro et al., 2003), heat (Mazzotta, 2001), and bile (Begley et al., 2002). On the contrary, findings reported by Lou and Yousef (1997) showed that acid adaptation of *L. monocytogenes* Scott A did not provide substantial protection against high osmolarity, while Sharma et al. (2005) revealed that acid-adapted *L. monocytogenes* did not display increased survival to heat. Acid-adapted and non-adapted *L. monocytogenes* displayed similar survival patterns on jerky treated with modified marinates, formulated to contain acetic acid alone or combined with sodium lactate plus ethanol or Tween 20 (Calicioglu et al., 2003). However, on certain days of the 25°C storage period, the survival ability of non-adapted cells was increased on untreated or treated with a traditional marinade jerky when compared to that of acid-adapted cells. Bayles (2004) demonstrated that the ability of acid adaptation to induce thermotolerance in *L. monocytogenes* greatly depended on the physiological state of the cells and the presence of nutrients during heating, whereas Edelson-Mammel (2005) showed that the occurrence of this type of cross-protection varies among different strains of the pathogen. Ravishnkar et al. (2000) reported that exposure to acid cross-protected *L. monocytogenes* against the lactoperoxidase system in tryptic soy broth, although no such effect was seen in a previous study, in which skim milk was employed (Ravishnkar and Harrison, 1999). Induction of ATR in *L. monocytogenes* was also capable of inducing resistance to nisin and other bacteriocins (Okereke and Thompson, 1996; van Schaik et al., 1999; Bonnet et al., 2006). The ability of *L. monocytogenes* to develop an ATR was inhibited by the presence of the natural flora in non-acidic fresh meat washings, as shown by Samelis et

al. (2001b), suggesting that nonacid meat decontamination treatments may sensitize the pathogen to subsequent acid stresses.

In accordance with the stress adaptation and cross-protection concepts, microbial resistance to antibiotics is believed to have originated from the frequent exposure of pathogens to sublethal levels of these agents, due to the extensive use of antibiotics in animal husbandry and human medicine. Antibiotic resistance in *L. monocytogenes* is an emerging issue in recent years. Resistance to one or more antibiotics was reported for approximately 10.9% of *Listeria* isolates from retail foods (Walsh et al., 2001), whereas Prazak et al. (2002) found that 20 out of 21 *L. monocytogenes* isolates from food, water or environmental samples were resistant to two or more antibiotics. Development of *L. monocytogenes* resistance to bacteriocins has also been reported (Gravesen et al., 2002; Martinez et al., 2005). Bacteriocins such as nisin act by binding to the bacterial cell, inserting into the cytoplasmic membrane, and forming pores, leading to efflux of accumulated amino acids and depletion of the proton motive force (Bruno et al., 1992; Bruno and Montville, 1993; Abee, 1995). Mazzotta and Montville (1997) showed that nisin-resistance in *L. monocytogenes* resulted from alterations in the fatty acid composition of the cell membrane. Other investigators (Ming and Daeschel, 1995; Crandall and Montville, 1998) observed that exposing the organism to the bacteriocin induced changes in membrane phospholipids.

Understanding the specific mechanisms that allow *L. monocytogenes* to adapt and resist adverse conditions may aid in the continuing efforts to control the pathogen in foods. Systems involved in survival of the pathogen under stressful conditions have been thoroughly investigated (Gahan and Hill, 2003). A two-component signal transduction

system in *L. monocytogenes*, designated LisR-LisK, that consists of *lisR* (response regulator) and *lisK* (histidine kinase) was shown to be involved in pH homeostasis (Cotter et al., 1999), as well as in the ability of the pathogen to tolerate nisin and cephalosporins (Cotter et al., 2002). Other strategies utilized by *L. monocytogenes* for survival under low pH conditions include the acquired ATR (discussed above), the F_0F_1-ATPase proton pump to expel excess protons from the cytoplasm (Cotter et al., 2000), and the glutamate-dependent acid resistance system or glutamate decarboxylase (GAD) system (Cotter et al., 2001a; 2001b; Cotter and Hill, 2003; Gahan and Hill, 2005), which has been shown to protect the pathogen from exposure to gastric fluid (Cotter et al., 2001a). Genes encoding the GAD system include *gadA*, *gadB* (decarboxylases) and *gadC* (antiporter). Under low pH conditions, glutamate dexarboxylases GadA or GadB convert a molecule of extracellular glutamate to γ-aminobutyrate, resulting to the loss of an intacellular proton. The intracellular γ-aminobutyrate is then exported from the cell via the GadC antiporter, located on the cell membrane, while consuming another proton (Small and Waterman, 1998). Elimination of the *gadB* gene rendered *L. monocytogenes* cells susceptible to acid, while deletion of *gadA* had minor effects in the acid resistance of the pathogen (Cotter et al., 2001a). According to Cotter et al. (2001b) the GAD system is strictly dependent upon the presence of glutamate in the external environment, which may suggest that use of glutamate as a food ingredient may result in increased *L. monocytogenes* survival in low-pH environments, such as the human stomach.

Osmolyte uptake systems in *L. monocytogenes* are utilized for transport of protective compounds, termed compatible solutes, within the bacterial cell in order to counterbalance turgor pressure and maintain homeostasis during osmotic stress (Ko and

Smith, 1999; Sleator et al., 1999; Sleator and Hill, 2002). Bayles and Wilkinson (2000) indicated that compatible solutes, serving as osmoprotectants in *L. monocytogenes* include glycine betaine, proline betaine, acetyl carnitine, carnitine, γ-butyrobetaine, and 3-dimethylsulphoniopropionate. *L. monocytogenes* possesses at least three solute uptake systems (O'Byrne and Fraser, 2000). Among these, a secondary transporter, BetL, (encoded by *betL*), and a substrate binding protein-dependent ABC transporter, Gbu (encoded by the *gbu* operon that consists of genes *gbuA*, *gbuB*, *gbuC*), are utilized by the pathogen for the accumulation of glycine betaine (Ko and Smith, 1999; Sleator et al., 1999). The uptake of carnitine is mediated by a third uptake system, a substrate binding protein-dependent ABC transporter, namely OpuC (encoded by the *opuC* operon; genes *opuCA*, *opuCB*, *opuCC*, *opuCD*) (Verheul et al., 1995). Osmolyte accumulation may promote enhanced survival of the pathogen in environments with elevated osmolarity, such as the small bowel. The ability of *L. monocytogenes* to accumulate compatible solutes is also associated with its ability to tolerate cold stress, as demonstrated by Gerhardt et al. (2000), Angelidis and Smith (2003) and Wemekamp-Kamphuis et al. (2004).

Little information is available regarding the mechanisms underlying the bile-resistance properties of *L. monocytogenes*. Changes in protein expression patterns after exposure to various stresses, including deoxycholate were observed by Phan-Thanh and Gormon (1997). Specific systems involved in the resistance of the pathogen against the detergent action of bile acids have been recently identified. Specifically, Dussurget et al. (2002) located the *bsh* gene (encoding a bile salt hydrolase) in the listerial genome (strain EGD-e) and reported its involvement in bile resistance by demonstrating that the MICs for

porcine bile and bile salts were 2-fold lower for a deletion mutant when compared to the parental strain. Furthermore, sequence analysis of the *bsh* gene revealed that it is positively regulated by PrfA, the transcriptional activator of numerous virulence genes in *L. monocytogenes*, and a PrfA DNA binding site was found in the *bsh* promoter (Dussurget et al., 2002), suggesting that bile salt hydrolase may be regarded as a virulence factor of this pathogen. Begley et al. (2005b) investigated the contribution of three loci (*bsh*, *pva*, and *btlB*) in the bile tolerance of *L. monocytogenes*. The authors reported that the *bsh* gene encodes for an enzyme that has the ability to hydrolyze both glyco- and tauroconjugated bile salts, whereas both *bsh* and *btlB* appeared to have a major role in the persistence of the pathogen in the murine intestinal tract. Disruption of the *btlA* (bile tolerance locus) locus rendered *L. monocytogenes* susceptible to lethal levels of bile and impaired its ability to grow rate at sublethal levels of acid, salt, ethanol, bile, SDS, ampicillin, and phosphomycin (Begley et al., 2003).

In addition to responses that confer protection against specific stresses, *L. monocytogenes* possesses a general stress response that facilitates cell survival under a variety of adverse conditions. The general stress response of *L. monocytogenes* is regulated by the alternative sigma factor σ^B, a subunit of RNA polymerase, which regulates the transcription of several stress response genes. The contribution of the σ^B regulon in the resistance of *L. monocytogenes* towards various environmental, energy and intrahost stresses has been thoroughly described in the literature (Becker et al., 1998; Wiedmann et al., 1998; Gahan and Hill, 1999; Becker et al., 2000; Ferreira et al., 2001; 2003; Kazmierczak et al., 2003; Moorhead and Dykes, 2003; Chaturongakul and Boor, 2004; Begley et al., 2005b; 2006). Mutations in the gene encoding for the alternative

sigma factor (*sigB*) in *L. monocytogenes* rendered the pathogen susceptible to acid (Wiedmann et al., 1998; Gahan and Hill, 1999), high osmolarity (Becker et al., 1998), low temperature (Becker et al., 2000), and antimicrobial agents such as bacteriocins and antibiotics (Begley et al., 2006). In addition, disruption of *sigB* reduced the starvation stress response of the pathogen (Herbert and Foster, 2001). Gardan et al. (2003) demonstrated that σ^B is involved in the activation of the *ctc* gene, associated with response to osmotic stress, whereas expression of the *bsh* gene was shown to be partially σ^B-dependent (Sue et al., 2003). Other σ^B-regulated genes, utilized by *L. monocytogenes* for survival under adverse conditions, include *gadB*, *opuC*, and *lmo*1433 (Fraser et al., 2003; Kazmierczak et al., 2003); the transcription, of *betL*, however, was found to be σ^B-independent (Fraser et al., 2003). The association of σ^B with the logarithmic or stationary phase ATR in *L. monocytogenes* has been proposed, since exposure to acidic conditions led to increased *sigB* transcription in exponentially growing cells (Becker et al., 1998), whereas Gahan and Hill (1999) indicated that deletion of the *sigB* gene sensitized stationary phase *L. monocytogenes* cells to acid.

2.7. Stress variation among strains

Strains of *L. monocytogenes* are quite heterogeneous in terms of their serological and molecular characteristics, whereas as previously discussed, epidemiological data, invasion assays, and food surveys have suggested that different serotypes and genetic groups display variations in virulence (Wiedmann et al., 1997; Barbour et al., 2001; Kathariou, 2002; Olier et al., 2002; Jensen et al., 2007) and environmental distribution (Kathariou, 2002; Gray et al., 2004). Since the pathogen encounters a variety of conditions, that may be optimal or stressful, both outside and inside the host, variations in

phenotypic traits (e.g., growth and survival properties and stress susceptibility) may help define the virulence and distribution variability associated with this pathogen and obtain information regarding the association among serotypes and genetic groups with growth and stress resistance properties, which may further assist in conducting more accurate *L. monocytogenes* risk assessments.

Differences in the growth behavior among four *L. monocytogenes* strains were observed by Rosenow and Marth (1987), who reported lag phases varying between 5 and 10 days in milk stored at 4°C. Junttila et al. (1988) assessed the effect of temperature on the growth behavior of 78 strains of the organism and reported that 10 strains were capable of growing at 0.8°C and 2 at 0.5°C. The authors also observed that strains of serotype 1/2 grew at lower temperatures than strains of serotype 4b. Barbosa et al. (1994) studied the growth parameters (i.e., lag phase, exponential growth rate and generation time) of 39 strains of the pathogen in culture broth under different temperatures (4, 10, or 37°C) and reported that differences were particularly noticeable at 4 relative to 37°C. Findings of this study also demonstrated that strain Scott A had the longest and one of the longest lag phase durations at 4 and 10°C, respectively. Similar results were reported by Lianou et al. (2006), who observed extensive growth variation among 25 *L. monocytogenes* strains, with differences being more pronounced at 4 rather than 30°C and slow growth of Scott A at 4°C. Furthermore, although both of these studies (Barbosa et al., 1004; Lianou et al., 2006) attempted to characterize *L. monocytogenes* serotypes by their growth potential, their results did not present a clear relationship between growth ability and serotype. Avery and Buncic (1997) correlated the source of *L. monocytogenes* strains with their growth potential, as shorter lag phases were observed for clinical

isolates as compared to meat isolates at 37°C. Vialette et al. (2003) also indicated major differences in growth kinetics between *L. monocytogenes* strains isolated from food (fish or seafood) or human clinical cases, with clinical isolates being able to adapt and grow more efficiently than food strains under acidic and osmotic conditions. De Jesús and Whiting (2003) examined the growth rates, heat tolerance and survival in modified culture broth of 21 *L. monocytogenes* strains belonging to the three distinct genetic lineages, identified by Wiedmann et al. (1997), and reported great differences in the growth kinetics among strains and, in some cases among lineages, with lineage 1 and 2 exhibiting longer lag phase durations than lineage 2. Uyttendale et al. (2004) reported strain variation in the growth responses of *L. monocytogenes* strains in BHI broth, modified to mimic conditions associated with cooked ham (pH 6.2 and a_w 0.972) or pâté (pH 6.1 and a_w 0.957). The same study concluded that the ability of the pathogen to grow under suboptimal conditions (temperature, pH, aw, NaCl, and sodium lactate) was also strain-dependant. On the other hand, 18 strains of the pathogen, representing different serotypes, randomly amplified polymorphic DNA types, and origins displayed similar growth behavior at different temperatures (5 or 37°C) or NaCl concentrations (0.5 or 5%) (Jensen et al., 2007).

Many authors have reported differences in heat resistance among *L. monocytogenes* strains (Beuchat et al., 1986; Golden et al., 1988; Gaze et al., 1989; Mackey et al., 1990; Kim et al., 1994; Sörqvist, 1994; Doyle et al. 2001; Francis and O' Beirne, 2005; Lianou et al., 2006). In an attempt to identify to identify a connection between thermotolerance and serotype, Sörqvist (1994) and Francis and O' Beirne (2005) demonstrated some serotype-related variations, however none of these studies was able to establish a clear

trend in the thermal resistance among serotypes. On the other hand, Buncic et al. (2001) observed that, on average, strains belonging to serotype 4b survived heating at 60°C better than strains of serotype 1/2a; however, no relationship between thermal tolerance and genotypic subtypes was identified in this study. Lianou et al. (2006) also found significant differences among serotypes in their ability to tolerate heat; however, on the contrary to what was found by Buncic et al. (2001), serotype 4b displayed greater heat sensitivity compared to all other serotypes examined. Lineage-dependent differences in heat resistance were identified by De Jesús and Whiting (2003), who reported that, on average, strains belonging to lineages 1 and 2 displayed higher thermal resistance properties than strains of lineage 3.

As discussed earlier, the ability of *L. monocytogenes* to tolerate acidic conditions may be considered as an element required for infection establishment, whereas variations in the ability of the pathogen to survive or grow at low pH have been documented based on the type and concentration of the acidulant (Parish and Higgins, 1989; Sorrels et al., 1989; Ahamad and Marth, 1990; Conner et al., 1990; Ita and Hutkins, 1991; Young and Foegeding, 1993; Phan-Thanh and Montagne, 1998; Phan-Thanh et al., 2000) and the growth phase of bacterial cells (Davis et al., 1996; O'Driscoll et al., 1996; Phan-Thanh and Montagne, 1998). Furthermore, strain-to-strain differences may also have an impact on the acid resistance of this organism (Dykes and Moorhead, 2000; Faleiro et al., 2003; Uyttendale et al., 2004; Francis and O' Beirne, 2005; Liu et al., 2005; Lianou et al., 2006). Dykes and Moorhead (2000) reported great differences in the ability of 30 *L. monocytogenes* isolates to tolerate extreme acidic conditions (pH 2.5), with clinical strains being less susceptible to low pH than strains isolated from foods. Liu et al. (2005)

examined the ability of three virulent and three avirulent *L. monocytogenes* strains to tolerate pH values varying from 2.0 to 5.0. The authors reported that strain-to strain variations in acid tolerance observed in this study did not depend on virulence. Variations among *L. monocytogenes* strains, relative to survival under conditions simulating those in the human stomach have been reported as well. Specifically, results by Roering et al. (1999) showed that *L. monocytogenes* CLIP 23485 and F6854 populations were reduced by >5 log CFU/ml when exposed to simulated gastric fluid at pH 1.5 for 15 min, whereas *L. monocytogenes* 101M decreased by 4 log CFU/ml after 20 min of exposure; all three strains were inactivated after 30 min of exposure to gastric juice. Variability among four *L. monocytogenes* strains, relative to survival in artificial gastric fluid was also reported by King et al. (2003). Examination of specific mechanisms that may lead to differences in the acid resistance among *L. monocytogenes* strains revealed that intracytoplasmic pH varies among *L. monocytogenes* strains, leading to different pathways of cation movement across the membrane for maintenance of homeostasis (Phan-Thanh et al., 2000).

CHAPTER 3

Fate of *Listeria monocytogenes* on processed meat and poultry products treated with lactoferrin, activated lactoferrin, and organic acids and salts

ABSTRACT

Presence of *Listeria monocytogenes* on ready-to-eat (RTE) products is a major concern to the meat processing industry and needs to be addressed in order to ensure the safety of such products. Since the pathogen enters products mainly as a post-processing contaminant, the development of antilisterial control measures in foods that support growth and may be consumed without reheating is essential. The studies presented in this chapter examined the fate of *L. monocytogenes* during storage of inoculated (composite of 10 *L. monocytogenes* strains) RTE meat and poultry products formulated with lactoferrin, surface treated with the activated form of the protein (ALF), or both, in combination or in comparison with antimicrobial agents (i.e., organic acids and salts) of proven antilisterial activity. *L. monocytogenes* and total microbial populations were enumerated during storage using PALCAM agar and tryptic soy agar supplemented with 0.6% yeast extract, respectively. In general, findings indicated that dipping products (i.e., ham, bologna, turkey breast, or frankfurters) into ALF solutions resulted in significant ($P < 0.05$) reductions (0.4-1.5 log CFU/cm^2) in initial *L. monocytogenes* levels. Subsequently, however, the ALF did not exhibit any residual antilisterial effects,

allowing pathogen populations to reach high levels (7.0-8.0 log CFU/cm^2) by the end of each storage period. Moreover, data from experiments testing sequential antimicrobial treatments (ALF followed by organic acids and salts) suggested that ALF did not enhance the antilisterial effects of other agents, as the combinations of 1% ALF with potassium lactate (3%), sodium diacetate (3%), or lactic acid (1%) provided similar ($P \geq 0.05$) antilisterial effects as single applications of 3% potassium lactate, 3% sodium diacetate, or 1% lactic acid, respectively. Similarly, lactoferrin included in the formulation of bologna resulted in slight inhibition of growth during initial stages of storage, but did not sustain its antilisterial effects as pathogen populations reached high numbers (> 7.0 log CFU/cm^2) in samples that contained lactoferrin by the end of storage at 4 or 7°C. Under the tested conditions, no substantial enhancement in the antilisterial activity of potassium lactate or sodium diacetate was observed due to addition of lactoferrin in the product. Application of ALF as a surface treatment of frankfurters containing 1.8% potassium lactate and 0.125% sodium diacetate or 0.5% lactoferrin appeared to enhance the activity of the additives against *L. monocytogenes*, suggesting that combined incorporation of antimicrobials in the formulation of the product and dipping in ALF solutions may provide more effective control of *L. monocytogenes*. In addition, incorporation of lactoferrin in the formulation of frankfurters enhanced considerably the antilisterial effectiveness of acetic acid applied as a surface treatment as it resulted in significant reductions of *L. monocytogenes* populations during storage. Overall, the results of these studies suggested that non-activated or activated lactoferrin used individually as formulation or surface treatments, respectively, were not as effective as currently used antimicrobial agents in these products. However, under certain conditions, application of

these natural antimicrobials enhanced the antilisterial effects of organic acids or salts, suggesting that lactoferrin or ALF combined, as appropriate, with other compounds could be considered as antilisterial treatments for RTE products.

3.1. Introduction

Contamination of RTE products with *Listeria monocytogenes* presents a major public health hazard, because of the pathogen's ability to proliferate at refrigeration temperatures and to cause serious illness, or even death to high-risk populations. According to a quantitative *L. monocytogenes* risk assessment that was based on data collected through 2003, among 23 categories of RTE foods, the highest relative risk for listeriosis on both per serving and per annum bases was attributed to delicatessen meats and non-reheated frankfurters (HHS-FDA/USDA-FSIS, 2003). Consequently, an expert panel, assembled by the Food and Agriculture Organization and the World Health Organization concluded that the most efficient way of minimizing the incidence of listeriosis involves preventing the occurrence of high contamination levels in foods at the time of consumption (Buchanan et al., 2004). Based on these findings and considering the complexity and uncertainty associated with the production of *Listeria*-free foods (Tompkin, 2002), utilization of generally-recognized-as-safe antimicrobial agents may be valuable in reducing consumer risk by reducing levels of post-lethality treatment contamination and preventing growth of this pathogen during storage of RTE meat and poultry products.

Extensive research has been performed on the antilisterial effects of a wide range of antimicrobial agents, with findings demonstrating that inclusion of sodium or potassium lactate and/or sodium diacetate into product formulations may provide effective control

in *L. monocytogenes* growth (Bacus and Bontenbal, 1991; Schlyter et al., 1993a; 1993b; Wederquist et al., 1994; Blom et al., 1997; Bedie et al., 2001; Mbandi and Shelef, 2001; 2002; Stekelenburg and Kant-Muermans, 2001; Samelis et al., 2002; Seman et al., 2002; Porto et al., 2002; Stekelenburg, 2003; Choi and Chin, 2003; Barmpalia et al., 2004; 2005; Geornaras et al., 2006; Luchansky et al., 2006; Vogel et al., 2006; Glass et al., 2007). Use of organic acid or salt solutions for surface decontamination (spraying or dipping) may also assist in controlling proliferation of the pathogen in foods (Schlyter et al., 1993a; 1993b; Palumbo and Williams, 1994; Ariyapitipun et al., 2000; Samelis et al., 2001a; Glass et al., 2002; Islam et al., 2002a; 2002b; Barmpalia et al., 2004; Nuñez de Gonzalez, 2004; Uhart et al., 2004; Geornaras et al., 2005; Lu et al., 2005; Singh et al., 2005; Luchansky et al., 2006). Although several naturally antimicrobial compounds have also been tested for their ability to control *L. monocytogenes* (Aureli et al., 1992; Bellamy et al., 1992; Hefnawy et al., 1993; Kihm et al., 1994; Payne et al., 1994; Cutter and Siragusa, 1996; Larson et al., 1996; Davies et al., 1997; Murray and Richard, 1997; Hao et al., 1998; El-Ziney et al., 1999; Aasen et al., 2003; Alzoreky and Nakahara, 2003; Dufour et al., 2003; Samelis et al., 2003b; 2005; Geornaras et al., 2006; Ahn et al., 2007; Oussalah et al., 2007; Sivarooban et al., 2007), their application is limited due to issues associated with stability, inconsistent activity, costs, and adverse sensory characteristics (Muriana, 1996; Hao et al., 1998; Sofos et al., 1998). Among the numerous compounds that have been screened for their antilisterial activity, only sodium or potassium lactate and sodium diacetate (Tompkin, 2002) are currently used in a large scale for *L. monocytogenes* control in commercially produced processed meats, despite the quality issues that may arise from their use in product formulations. Thus, since presence of

lactates and diacetates in foods may lead to unacceptable sensory properties and may be perceived negatively by consumers that prefer 'natural' additives, the identification of alternative antilisterial treatments is very important. For this purpose, utilization of low levels of multiple antilisterial agents that act additively or synergistically may help enhance the safety of RTE foods and reduce the levels of chemical ingredients without sacrificing product quality.

Lactoferrin is a glycoprotein found in milk that exerts its antimicrobial activity by sequestering iron ions (Bullen et al., 1972; Spik et al., 1978). However, the protein may also exhibit antimicrobial activity by means independent of its iron-binding ability (Arnold et al., 1980; 1982). The activated form of lactoferrin (ALF), which is produced by a patented method developed by Naidu (2001), has the ability to prevent bacteria from attaching and growing on biological surfaces (Naidu, 2002). The Food and Drug Administration has designated ALF as a generally-recognized-as-safe compound (FDA-CFSAN-OFAS, 2003), while the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) has approved its application as a rinsing solution for beef carcasses in order to control pathogenic bacteria, including *Escherichia coli* O157:H7, *Salmonella*, *L. monocytogenes*, and *Campylobacter* (USDA-FSIS, 2003b). The antilisterial activity of lactoferrin has been shown in milk (Payne et al., 1990; 1994). However, to our knowledge, except for a preliminary study conducted in our laboratory (Ransom et al., 2003) evaluating ALF as a post-processing dipping solution in bologna, the antilisterial activity of lactoferrin or ALF has not been evaluated in RTE meat or poultry products.

Therefore, the aim of the studies presented in this chapter was to examine the behavior of *L. monocytogenes* in various RTE meat/poultry products treated with lactoferrin (as a formulation ingredient) or ALF (as a post-processing dipping solution) alone or in combination with organic acids and salts. Results could potentially identify effective antilisterial treatments to be applied in RTE products that are also acceptable by consumers that demand reduction or elimination of chemically synthesized additives in foods.

3.2. Materials and methods

3.2.1. Bacterial strains and growth conditions

A *L. monocytogenes* 10-strain composite was prepared to include Scott A (serotype 4b, human isolate), 103M (serotype 1a, pork sausage isolate), 558 (serotype 1/2, pork meat isolate), N1-227, N1-225 (serotype 4b, food and human isolate, respectively, both associated with the same outbreak), R2-500, R2-501 (serotype not known, food and human isolate, respectively, both associated with the same outbreak), and R2-763, R2-764, R2-765 (serotype 4b, human, food and environmental isolate, respectively, all associated with the same outbreak). These strains were used for product inoculation in all studies. Another 10-strain composite, consisting of strains Scott A (serotype 4b, human isolate), NA-3 (serotype 4b), NA-19 (serotype 3b), 101M (serotype 4b) and 103M (serotype 1a), all pork sausage isolates, 558 (serotype 1/2, pork meat isolate), and PVM1, PVM2, PVM3 and PVM4 (pork variety meat isolates, serotype not known), was also used in one of the experiments in order to determine potential effects of the inoculum composition on the efficacy of the antimicrobial treatments.

All strains were available as frozen stock cultures (-70°C) in tryptic soy broth (TSB, Difco, Becton Dickinson Co., Sparks, MD) supplemented with 0.6% yeast extract (YE, Acumedia, Baltimore, MD) and 20% glycerol. Each strain was resuscitated on two consecutive days using TSBYE (10 ml), followed by incubation at 30°C for 24 h.

To prepare the inoculum, TSBYE cultures were centrifuged (five strains in each conical centrifuge tube) at 4629 x *g* for 15 min (4°C). Each pellet was resuspended in 10 ml of sterile phosphate-buffered saline (PBS; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 liter of distilled water, pH 7.4) and the two mixtures were combined and centrifuged again (4629 x *g* for 15 min, 4°C). The mixed culture was resuspended in 100 ml PBS and serially diluted to achieve appropriate concentrations based on the target inoculum level on each of the products.

3.2.2. Meat products, treatments and inoculation

The meat products used in these studies were either purchased from a commercial manufacturer (i.e., turkey breast, ham) or prepared at the Meat Science Laboratory of the Department of Animal Sciences at Colorado State University (i.e., bologna, frankfurters). Non-activated lactoferrin, rather than ALF was used as a formulation ingredient, based on discussions with Verdis N. Norton, President of aLF Ventures (Salt Lake City, UT), regarding the unsuitability of ALF as an additive in processed meat products due to its potential interaction with other chemical ingredients contained in the products.

The possibility of thermal denaturation of lactoferrin is of great interest since the compound is to be used as a component in heat-treated foods (e.g., milk). A review of the literature indicated that although it is known that lactoferrin is denatured at high temperatures (Ford et al., 1977; Rüegg et al., 1977), there is a potential for activity at

temperatures employed in processing of meat products. A study by Abe et al. (1991) indicated that at pH 2 or 3, some lactoferrin fragments produced by heat denaturation (100 or 120°C for 5 min) exhibited antimicrobial properties. Saito et al. (1994) demonstrated that the antibacterial activity of lactoferrin fragments, derived from thermal treatment at pH 2-3, was greater than that of lactoferrin. A more recent study (Uzzan et al., 2007) provided more promising results regarding the use of the compound in heat-treated products, as lactoferrin was found to be thermostable during treatment of milk at 72°C for 120 s. Nevertheless, overall results of studies discussed above suggest that heat denaturation of lactoferrin does not necessarily mean absence of antimicrobial activity, and considering that the heat stability of lactoferrin or the antimicrobial activity of its heat-induced fragments is affected by various parameters, other than temperature and time (e.g., pH, type of acidulant), the antilisterial effect of lactoferrin, as a formulation ingredient, needed to be evaluated under conditions that simulate processing and cooking of meat products. Thus, a preliminary study was conducted to investigate the effects of heat (71°C) on the antimicrobial effects of lactoferrin under different pH conditions (5, 6, or 6.5) in TSB. In summary, findings (not shown) demonstrated that treatment at 71°C did not affect the antilisterial properties of ALF (compared to unheated ALF) encouraging us to proceed with its use as an additive in processed meat products (bologna and frankfurters).

Commercial products

Commercially prepared 97% fat-free ham (cured with water, salt, sugar, sodium phosphates, sodium erythorbate, and sodium nitrite) was sliced (Globe slicer, Mozley Manufacturing, Stamford, CT) into 3-4 mm thick slices in the Meat Science Laboratory

of Colorado State University. Slices were transferred to the microbiology laboratory and cut into pieces having a surface area of approximately 30 cm^2 (5 x 6 cm). Pieces were placed separately on aluminum foil under a biological safety cabinet and 0.1 ml of the appropriately diluted inoculum (capable of giving approximately 2 log CFU/cm^2 of ham) was deposited on one side and spread over the entire surface with a sterile bent glass rod. Inoculated samples were left to stand at 5°C for 15 min for attachment, turned over and the same procedure was repeated for the other side. The inoculation procedure was conducted before or after dipping (16-17 pieces into 300 ml of solutions) into: nothing (control); distilled water (120 s); 1% acetic acid (30 s); 2% ALF (120 s). Pre- or post-inoculation-treated samples (2 pieces per treatment) were placed into plastic bags (15 cm x 20 cm, 3 mil std barrier; Nylon/PE vacuum pouch, Koch), vacuum-sealed (Hollymatic, Corp., Countryside, IL) and microbiologically analyzed (methodology described in a following paragraph) on days 0, 5, 15, 22, and 28.

Turkey breast (98% fat-free; turkey breast, turkey broth, salt, sugar, sodium phosphates, sodium erythorbate, sodium nitrite) was also obtained from a commercial manufacturer. Product was sliced, cut into 5 x 6 cm pieces (approximately 30 cm^2) and inoculated (approximately 3 log CFU/cm^2) as described above. Pieces were then subjected to dipping into one of the following treatments: nothing (control); distilled water; 3% potassium lactate; 3% sodium diacetate; 1% lactic acid; 1% acetic acid; 2% ALF; 1% ALF; 1% ALF followed by 3% potassium lactate; 1% ALF followed by 3% sodium diacetate; 1% ALF followed by 1% lactic acid; 1% ALF followed by 1% acetic acid. Treatments were applied by immersion of 20 pieces into 200 ml of the solution for 60 s, followed by draining, vacuum packaging (2 slices per bag) and storage at 7°C.

Samples were analyzed for microbiological counts on days 0, 4, 8, 12, and 16, 22, 28, and 43.

Bologna

For the preparation of bologna, fresh pork (approximately 30% fat) and beef (approximately 25% fat) trimmings were obtained from Swift Co. (Greeley, CO). The basic bologna formulation (without antimicrobials added; Samelis et al., 2001a) consisted of (%, wt/wt): meat (40% pork and 60% beef) trimmings (82.2), ice (10), sodium chloride (2), dextrose (2), dry mustard (0.9), corn syrup solids (2), polyphosphate (0.4; sodium tripolyphosphate and sodium hexameta-phosphate; Heller Inc., Bedford Park, IL), sodium nitrite (0.0156), sodium erythorbate (0.05), paprika (0.25), onion powder (0.05), garlic powder (0.05), coriander (0.05), and white pepper (0.05). Spices and seasonings were purchased from AC Legg Co. (Birmingham, AL). Separate product batches were prepared to contain: no antimicrobials (control); potassium lactate (3% of a 60% [wt/wt] commercial product; equivalent to 1.8% pure potassium lactate; Purac Inc., Lincolnshire, IL); lactoferrin (0.5%; aLF Ventures); lactoferrin (1%); potassium lactate (1.8%) plus sodium diacetate (0.125%; Niacet, Niagara Falls, NY); potassium lactate (1.8%) plus lactoferrin (0.5%); sodium diacetate (0.25%) plus lactoferrin (0.5%); potassium lactate (1.8%) plus sodium diacetate (0.125%) and lactoferrin (0.5%); and potassium lactate (1.8%) combined with sodium diacetate (0.0625%) and lactoferrin (0.25%). Bologna was processed according to procedures described by Samelis et al. (2001a). Specifically, the ingredients of each batch were emulsified in a 35-L bowl chopper (RMF, Kansas City, MO) to a final temperature of 15.5°C. The mixture was extruded (Handtmann Inc., Buffalo Grove, IL) into 65 mm diameter fibrous cellulose casings (Koch, Kansas City,

MO) and the bologna sticks were cooked in a smokehouse (Alkar, DEC International Inc., Lodi, WI) first in dry air (1 hour; smokehouse temperature 60°C), followed by hot smoking (60°C; Zesti liquid smoke, Hickory Specialties Inc., Crossville, TN) for 38 min. After smoking, the bologna was cooked with steam for 1 hour (smokehouse temperature 71°C, relative humidity 50%). Then the smokehouse temperature was increased to 88°C and the bologna sticks were cooked until an internal temperature of 70°C was reached. After cooking, the product was showered with cool tap water for 5 min and cooled overnight at 4°C. The casings were removed manually and each bologna stick was sliced into approximately 3 mm thick slices, which were then placed on pieces of aluminum foil and inoculated on one side (0.1 ml from the appropriate dilution) as previously described. The inoculum was spread over the entire surface (approximately 33 cm^2) to yield an inoculum level of approximately 2 log CFU/cm^2. After inoculation, two bologna slices from each treatment were vacuum-sealed and stored at 4 or 10°C. Microbiological analyses were conducted on days 0, 10, 20, 43, 75, and 95 for samples stored at 4°C, and on days 0, 4, 8, 12, 16, 20, 28, 43, and 57 for samples stored at 7°C.

Another experiment investigated whether the method of application (dipping or spraying) of surface treatments may influence their antilisterial activity. Bologna (without antimicrobials in the formulation) was prepared as described above, and following inoculation (approximately 3 log CFU/cm^2) slices were either left untreated (control) or dipped (30 s, 16 slices in 200 ml of solution) into or sprayed under a biological safety cabinet (0.69 bar, 2 s on each side) with: distilled water; 2% lactic acid (88%; Purac, Barcelona, Spain); 2% ALF (aLF Ventures). The spraying system consisted of a stainless steel sprayer, custom-built by Chad Co. (Lenexa, KS), a compressor (YI000, Husky

Professional Tools, Atlanta, GI), used as a vacuum pump and a spray gun (TriggerJet, MI 22650, Mfr. Spraying Systems Co., Wheaton IL). Each side of a slice was sprayed with the nozzle (H1/8vvss80015, Mfr. Spraying Systems Co.; flow rate at 1.4 bar-0.11 gmp) held at a 90° angle (approximately 10 cm distance from the product). Treated and untreated (control) slices were then vacuum packaged (2 per bag) and stored at 7°C. Microbiological analyses were conducted on days 0, 4, 8, 12, 16, 22, 28, and 43.

Bologna was also used in a third experiment that evaluated potential effects of product and inoculum composition on the antimicrobial effects of solutions applied on the finished product. Two batches of product were prepared, one formulated with meat trimmings consisting of 40% pork (approximately 30% fat) and 60% beef (approximately 25% fat) and a second formulated with meat trimmings consisting of beef (approximately 25% fat) only. Following cooking/cooling and slicing, product was inoculated with one of the two different 10-strain mixtures of *L. monocytogenes* described above. Bologna slices, inoculated with either one of the *L. monocytogenes* strain mixtures and dipped into: nothing (control); distilled water (120 s); 2% acetic acid (60 s; glacial; Mallinckrodt and Baker, Paris, KY); 2% ALF (60 s); 2% ALF (120 s); or 2% ALF (180 s). Samples were subsequently drained, vacuum packaged and stored at 10°C. Samples were analyzed for microbiological counts on days 0, 4, 8, 12, 16, and 26 of storage.

Frankfurters

The basic formulation of frankfurters used in these studies was identical to that of bologna (Bedie et al., 2001). Meat and non-meat ingredients were emulsified as described previously and extruded into 24 mm cellulose casings (Koch). The frankfurters were then cooked in a smokehouse (Alkar) first in dry air (30 min; smokehouse temperature 80°C),

followed by hot smoking (30 min; 60°C; Zesti liquid smoke). The frankfurters were cooked with steam for 30 min (smokehouse temperature 80°C, relative humidity 26%), showered with cool tap water for 5 min and cooled overnight at 4°C. After cooking, they were peeled manually and cut into 10-cm length links. Frankfurter links were inoculated by transferring into a vacuum bag, applying the mixture of *L. monocytogenes* strains (0.25 ml from the appropriate dilution on each link) under a biological safety cabinet to yield an inoculum level of approximately 3 log CFU/cm^2, and massaging in order to spread the inoculum uniformly on their surface (approximately 84.5 cm^2). The inoculated frankfurters were left to stand for 30 min at 5°C for attachment. Subsequently, frankfurter links were removed from the bags in which they were inoculated and immersed (32-34 links into 1 liter of solution) into: nothing (control); distilled water (30 s); 2% lactic acid (30 s); 3% sodium diacetate (30 s); 2% ALF (30 s); 2% ALF (60 s); 2% ALF (90 s); or 2% ALF (120 s). Samples were drained, vacuum packaged (2 links per bag) and stored at 7°C. Samples were microbiologically analyzed on days 0, 4, 8, 12, 16, 20, 24, and 32.

Frankfurters were also used in another experiment, conducted to test the antilisterial effects of lactoferrin incorporated into frankfurters individually or together with potassium lactate, and in combination with immersion of the finished product into solutions of acetic acid or ALF. The basic frankfurter formulation, described previously, was used to prepare four batches of product with: no antimicrobials (control); potassium lactate (1.8%) plus sodium diacetate (0.125%); lactoferrin (0.5%); and potassium lactate (1.8%) plus lactoferrin (0.5%). After processing and cooking/smoking, frankfurters were cut into 8 cm pieces and inoculated as described above. Subsequently, frankfurters of each formulation treatment were dipped (30 links into 900 ml; 120 s) into: nothing

(control); 2% acetic acid; or 2% ALF. Samples were then vacuum packaged (2 links per bag) and stored at 7°C. Microbiological analyses were conducted on days 0, 5, 10, 15, 20, 25, 35, and 50 of storage.

3.2.3. Microbiological analyses

Microbiological analyses were conducted during storage of the products as previously indicated. Each sample (two slices, links or pieces) was aseptically transferred to a sterile plastic bag (Whirl-Pak®, Nasco, Fort Atkinson, WI) containing 50 ml maximum recovery diluent (0.85% NaCl and 0.1% peptone) and shaken 30 times as described in the United States Meat and Poultry Inspection Regulation (USDA-FSIS, 1996). Appropriate ten-fold serial dilutions were made with buffered peptone water (Difco), followed by plating onto tryptic soy agar (Difco) supplemented with 0.6% yeast extract (TSAYE) for the enumeration of total microbial populations and PALCAM agar (Difco) for the enumeration of *L. monocytogenes*. Colonies were counted manually after incubation (25°C, 72 h for TSAYE and 30°C, 48 h for PALCAM).

3.2.4. Physical and chemical analyses

Determination of cooking yields (%) of bologna and frankfurters formulated with or without antimicrobials was based on product weight before and after cooking and chilling (Bedie et al., 2001). Fat and moisture contents were determined in triplicate for each product following the AOAC International Official methods 960.39 and 950.46.B (AOAC, 1998), respectively.

The water activity of treated and untreated products was determined on the day of inoculation (day-0) using an AquaLab (model series 3; Decagon Devices Inc., Pullman, WA) water activity meter. The pH of samples, using a Denver Instrument (Arvada, CO,

USA) pH meter and electrode, was determined after the microbiological analysis by homogenizing samples for 120 s at 8.0 strokes/s (Masticator, IUL Instruments, Barcelona, Spain) and measuring the pH of the resultant slurry.

3.2.5. Statistical analyses

Unless otherwise specified, three individual samples from each treatment were analyzed at each sampling day. Microbiological counts were converted to log CFU/cm^2 and the effects of treatment on *L. monocytogenes* growth or survival were determined by analysis of variance via the general linear models (GLM) procedure of SAS (SAS, 2002). Means and standard deviations were calculated and the mean differences were separated with the least significant difference procedure at the significance level of 95% (SAS, 2002). As noted above, a separate experiment was performed using two different *L. monocytogenes* strain composites for product (bologna formulated with beef and pork or beef only) inoculation. For this experiment, two samples per inoculum and product type were analyzed on each storage day; however, preliminary analysis of fixed effects using the GLM procedure of SAS indicated that log CFU/cm^2 populations were independent of the type of inoculum. Consequently, statistical analyses (described above) were applied to the pooled microbiological data, obtained for each product and on each storage day, irrespective of inoculum composition.

Growth or inactivation kinetics, including lag phase/shoulder durations (LPD/SD; days) and growth/inactivation rates (GR/IR; log CFU/cm^2/day), for each treatment and storage day were calculated by fitting *L. monocytogenes* data (log CFU/cm^2) to the model of Baranyi and Roberts (1994) using DMFit software, the in-house program of the Institute of Food Research (Norwich, UK). The Baranyi and Roberts model is a non-

autonomous, separable, first order ordinary differential equation (Baranyi et al., 1993) and it contains four parameters (Baranyi and Roberts, 1994): a parameter expressing the lag phase; μ, the potential maximum rate of the model, which can be negative for decay; y_0, which represents the low asymptote of the curve; and y_{end}, which represents the upper asymptote of the curve. Two curvature parameters, m and n, corresponding to the behavior of the curve at the "transition" regions (lag to exponential phase and exponential to stationary phase) are also included in the Baranyi and Roberts equation. Statistical analyses of calculated kinetics were performed with the GLM procedure of SAS (SAS, 2002).

3.3. Results

3.3.1. Physical and chemical properties

Percentages of cooking yield and moisture and fat content of products (bologna and frankfurters) formulated with or without antimicrobials are shown in Tables 3.1 and 3.2. The cooking yield of untreated bologna was 87.9%, while cooking yields of samples containing antimicrobials ranged between 87.2 (1% lactoferrin or 1.8% potassium lactate and 0.5% lactoferrin or 1.8% potassium lactate and 0.125% sodium diacetate and 0.5% lactoferrin) and 90.2% (1.8% potassium lactate). The moisture content of bologna ranged from 58.8 (1.8% potassium lactate combined with 0.0625% sodium diacetate and 0.25% lactoferrin) to 65.7% (0.125% sodium diacetate combined with 0.5% lactoferrin). Potassium lactate (1.8%) combined with 0.0625% sodium diacetate and 0.25% lactoferrin and 1.8% potassium lactate combined with 0.125% sodium diacetate were the treatments that resulted into the lowest (12.5%) and highest (17.4%) fat content, respectively. The cooking yield of untreated frankfurters was 79.3%. Addition of 0.5% lactoferrin in the

formulation of frankfurters caused a decrease of 1.1% in cooking yield, whereas, the remaining formulation treatments did not cause substantial changes in that product property. Moisture contents of frankfurter samples ranged between 61.0% (0.5% lactoferrin) and 68.3% (1.8% potassium lactate combined with 0.125% sodium diacetate). Control samples had the highest fat content (15.9%), whereas, the lowest fat content was observed in samples formulated with 0.5% lactoferrin (13.1%).

Water activity values of untreated (control) and treated products are presented in Tables 3.3-3.9. Overall, application of distilled water or antimicrobial solutions to the surface of products resulted in activity values that were 0.001 to 0.018 units higher than those of untreated samples. In some cases, however (e.g., treatment of frankfurters), no effects or even slight (≤ 0.005 units) reductions in water activity were caused by application of ALF as a surface treatment. Water activity values obtained for samples formulated without antimicrobials on day-0 were 0.966 (bologna) and 0.949 (frankfurters) (Tables 3.8 and 3.9). Treatments that led to the lowest water activity values of bologna samples were 1.8% potassium lactate and its combination with 0.5% lactoferrin (0.958 and 0.959, respectively). In frankfurters, the greatest reduction in water activity was achieved by the inclusion of 1.8% potassium lactate and 0.125% sodium diacetate (water activity: 0.940) in the formulation. It should be noted that potassium lactate was not used as a single formulation treatment in frankfurters.

Changes in pH during storage of products surface treated or formulated with antimicrobials are shown in Tables 3.10-3.17. In general, applying organic acid solutions on the surface of products resulted in significant ($P < 0.05$) decreases in the pH of products on day-0. Sodium diacetate, applied as dipping treatment, reduced the pH of

frankfurter or turkey breast samples by 0.08 and 0.54 units, respectively, while potassium lactate caused only minor ($P \geq 0.05$) changes in pH of turkey breast samples. Dipping in ALF solutions (for 60, 120, or 180 s) resulted in significant ($P < 0.05$) increases in pH of bologna formulated with beef and pork; however, in most experiments, the solution did not cause substantial ($P \geq 0.05$) changes in pH of products. Sequential treatments of ALF with sodium diacetate, lactic acid or acetic acid applied on the surface of turkey breast slices led to significant pH reductions ($P < 0.05$), whereas, ALF followed by potassium lactate caused only slight ($P \geq 0.05$) increases. The pH of control (no antimicrobials in the formulation) bologna samples on day-0 was 6.32 (Tables 3.15 and 3.16). Inclusion of antimicrobials in the product formulation caused reductions of 0.01 (1.8% potassium lactate) to 0.11 (1.8% potassium lactate and 0.125% sodium diacetate) units on day-0. At 4°C, significant ($P < 0.05$) reductions in pH were observed in samples that contained single lactoferrin (0.5 or 1%) or the combination of 0.125% sodium diacetate with 0.5% lactoferrin (reductions of 0.56, 0.60, and 1.25 units, respectively) by the end of the storage period (day-95), suggesting microbial growth. Similar trends were observed during storage at 7°C, with samples containing 0.5 or 1% lactoferrin and 0.125% sodium diacetate with 0.5% lactoferrin having pH values of 5.89, 5.82, and 5.42, respectively by the end of storage (day-57). The pH of frankfurters formulated without antimicrobials on day-0 was 6.04, while the pH of samples containing antimicrobials ranged from 5.95 (1.8% potassium lactate and 0.125% sodium diacetate) to 6.03 (0.5% lactoferrin used singly or together with 1.8% potassium lactate) (Table 3.17). No significant ($P \geq 0.05$) changes in pH were observed during storage of frankfurters formulated with or without antimicrobials.

3.3.2. *L. monocytogenes* populations on products surface treated with antimicrobials

Populations and growth kinetics of *L. monocytogenes* during storage of ham dipped in water or solutions of acetic acid (1%) or ALF (2%) before or after inoculation are shown in Figure 3.1 and Table 3.18, respectively. As expected, no major ($P \geq 0.05$) reductions of initial *L. monocytogenes* populations were obtained on samples that were treated prior to inoculation. Of all treatments applied before inoculation, 1% acetic acid exhibited superior residual antilisterial effects during storage, since it resulted in inhibition of *L. monocytogenes* growth for 19.9 days, while subsequent growth of the pathogen on treated samples was significantly ($P < 0.05$) slower (GR: 0.231 log CFU/cm^2/day) compared to that observed on samples treated with water or ALF which had similar GR (0.332 and 0.339 log CFU/cm^2/day, respectively).

Immersing ham samples into antimicrobial solutions or water after inoculation resulted in reductions in *L. monocytogenes* initial counts that ranged from 0.2 (acetic acid) to 0.6 (water or ALF) log CFU/cm^2. Although acetic acid caused small initial reductions of *L. monocytogenes*, it resulted in significantly ($P < 0.05$) slower subsequent growth (GR: 0.052 log CFU/cm^2/day) than that observed in samples treated with water (GR: 0.595 log CFU/cm^2/day) or ALF (GR: 0.347 log CFU/cm^2/day). Interestingly, the rate of bacterial growth on samples dipped into acetic acid before inoculation was faster ($P < 0.05$) than that on samples treated after inoculation. This effect is probably due to injury of the cells that were exposed directly to the high acidity of the 1% acetic acid solution (pH 3.02), while cells inoculated on the previously acid-treated product experienced higher pH values (pH of ham treated with acetic acid prior to inoculation: 5.92), as a result of the buffering capacity of meat. Nonetheless, rates of *L.*

monocytogenes growth were similar ($P \geq 0.05$), on pre- and post-inoculation water or ALF-dipped samples, and therefore, solutions were applied after inoculation in all subsequent experiments.

Populations of *L. monocytogenes* and growth parameters obtained for the pathogen during storage of frankfurters previously dipped into 2% lactic acid (for 30 s), 3% sodium diacetate (for 30 s), or 2% ALF (for 30, 60, 90, or 120 s) solutions are shown in Figure 3.2 and Table 3.19, respectively. Decreases in initial *L. monocytogenes* populations were the greatest (0.8-1.0 log CFU/cm^2) on samples treated with solutions of ALF (applied at 2% for 30, 60, 90, or 120 s), as compared to those resulting from other antimicrobials or water (0.5-0.7 log CFU/cm^2). During storage, however, all lactoferrin treatments permitted extensive growth of the pathogen, as suggested by GR that ranged from 0.291 to 0.328 log CFU/cm^2/day. The duration of the ALF dipping treatment did not appear to affect the inhibitory properties of the compound, since 30, 60, 90, and 120 s of dipping resulted in similar ($P \geq 0.05$) GR (Table 3.19). Moreover, all ALF treatments allowed instant proliferation of the pathogen after dipping (no lag phase was observed). Calculated LPD for controls and samples treated with water, 2% lactic acid or 3% sodium diacetate suggested that growth was inhibited for 0.7, 0.6, 5.1, or 3.6 days, respectively. Overall, treatments (including control) resulted in high GR ranging between 0.239 (control) and 0.370 (water) log CFU/cm^2/day. Interestingly, water activity values obtained for treated and untreated samples on day-0 suggested that dipping into antimicrobial solutions or water did not cause substantial increases of product water activity that could justify the faster growth of the pathogen on dipped samples.

Counts and growth kinetics of the pathogen inoculated onto slices of bologna formulated with beef or beef and pork prior to dipping in water, 2% acetic acid, or 2% ALF solutions are shown in Figure 3.3 and Table 3.20, respectively. Reductions in *L. monocytogenes* numbers achieved immediately after dipping beef or beef and pork bologna slices into distilled water or antimicrobial solutions were 0.4 (water) to 0.7 (2% ALF for 120 or 180 s) and 0.4 (water) to 0.8 (2% ALF for 180 s) log CFU/cm^2, respectively. Subsequently during storage, the pathogen grew abundantly on control samples and samples treated with water and ALF, as suggested by the high GR that varied from 0.492 (control) to 0.858 (water) log CFU/cm^2/day on beef bologna, and from 0.614 (control) to 0.876 (ALF for 180 s) log CFU/cm^2/day on bologna formulated with beef and pork. *L. monocytogenes* grew significantly ($P < 0.05$) faster on beef bologna samples treated with water or ALF for 120 s than on undipped samples; although growth on samples dipped into ALF for 60 or 180 s was faster than that observed on control samples, the difference was not significant ($P \geq 0.05$). Similarly, on bologna formulated with beef and pork, water and ALF treatments that resulted in significantly ($P \geq 0.05$) faster growth of the pathogen, as compared to growth on control samples. Acetic acid, applied at 2%, was the most effective treatment ($P < 0.05$), as it resulted in listeriocidal effects during storage of both products, with estimated IR of *L. monocytogenes* being -0.056 (beef bologna) and -0.073 (beef and pork bologna) log CFU/cm^2/day. Except for 2% acetic acid that resulted in reductions of *L. monocytogenes* populations during storage, treatments allowed immediate growth of the pathogen or caused very brief inhibition of growth, as suggested by calculated LPD (0.29-0.52 days). In general, *L. monocytogenes* GR observed during storage of untreated and treated bologna, formulated

with beef and pork, were higher than corresponding GR on beef bologna. That was probably due to the more ($P \geq 0.05$) favorable conditions for bacterial growth (i.e., higher water activity and pH) prevailing on the surface of beef and pork bologna (Tables 3.5 and 3.12). Nevertheless, growth kinetics data obtained for both products followed similar patterns, suggesting that the composition of bologna did not affect the effectiveness of the dipping treatments.

Figure 3.4 and Table 3.21 present *L. monocytogenes* counts and growth kinetics, respectively, during storage of bologna treated (dipped or sprayed) with water, 2% lactic acid or 2% ALF. Dipping into water or antimicrobial solutions caused initial reductions of 0.4 (lactic acid or ALF) to 0.6 (water) log CFU/cm^2 in *L. monocytogenes* populations, while spraying resulted in reductions ranging between 0.3 (ALF) and 0.7 (water) log CFU/cm^2. Spraying with 2% ALF was the only treatment that did not cause significant ($P \geq 0.05$) decreases in initial contamination levels. Most treatments allowed immediate growth of *L. monocytogenes* and only dipping into 2% lactic acid led to complete inhibition for 8.8 days before allowing increases in bacterial counts. Calculated GR varied from 0.158 (spraying with 2% lactic acid) to 0.552 (dipping into water) log CFU/cm^2/day. Although the rate of *L. monocytogenes* growth during storage of water-sprayed samples was also high (0.401 log CFU/cm^2/day), it was still lower ($P < 0.05$) than that on water-dipped samples. Increases in water activity resulting from dipping or spraying with water were similar (0.008-0.009 units; Table 3.6), and therefore cannot account for the differences observed in GR during storage. In a similar way, *L. monocytogenes* proliferated at a faster rate ($P \geq 0.05$) in samples dipped into 2% ALF, compared to the control, as suggested by corresponding GR values (0.348 and 0.300 log

CFU/cm^2/day); however, when the same solution was applied as a spraying treatment, the estimated GR (0.251 log CFU/cm^2/day) was lower ($P \geq 0.05$) than that of the control. Application of water or antimicrobial treatments on bologna slices by spraying (0.69 bar, 2 s on each side) resulted in lower ($P < 0.05$) GR compared to those obtained for corresponding samples that were dipped for 30 s. Results of this study show that, under the given conditions, spraying appeared more effective than dipping, as the former method of application resulted in slower growth of the pathogen during storage. Overall, changes in water activity and pH values (Table 3.6 and 3.13) and reductions in initial populations (Figure 3.4) resulting from dipping or spraying were similar for corresponding treatments, regardless the method of application and, thus cannot explain the differences in GR that were observed during storage. Perhaps, spraying each slice individually allowed more thorough surface coverage than the dipping of multiple slices in the antimicrobial solution. On the other hand, the fact that the effectiveness of spraying was greater than that of dipping even in water-treated samples leads to the assumption that the spraying application alone may have had damaging effects on the bacterial cells.

L. monocytogenes populations and growth parameters on cured turkey breast treated for 60 s with water, single (3% potassium lactate, 3% sodium diacetate, 1% lactic acid, 1% acetic acid, 1% ALF, or 2% ALF) or sequential (1% ALF followed by 3% potassium lactate, 3% sodium diacetate, 1% lactic acid, or 1% acetic acid) antimicrobial treatments are shown in Figure 3.5 and Table 3.22, respectively. Initial reductions in *L. monocytogenes* counts caused by water or single antimicrobials ranged between 0.4 (1% lactic acid) and 0.7 (water or 2% ALF) log CFU/cm^2. On the other hand, reductions caused by applying solutions sequentially were 0.7 to 0.8 log CFU/cm^2 for all treatments.

Dipping in sodium diacetate (3%) individually or after dipping in 1% ALF resulted in complete inhibition of growth and in death, respectively, during product storage. Dipping in water, 3% potassium lactate, 1% lactic acid or 1% ALF followed by 3% potassium lactate allowed instant growth of the pathogen. Other treatments led to LPD that ranged from 0.8 (1% ALF) to 42.0 (1% acetic acid) days. Calculated GR for all treatments ranged between -0.001 (IR; 1% ALF followed by 3% sodium diacetate) and 0.340 (1% ALF) log CFU/cm^2/day. Of all single treatments, dipping into 3% sodium diacetate caused the slowest growth of the pathogen (GR: 0.002 log CFU/cm^2/day), whereas, 1% ALF allowed the fastest growth (GR: 0.340 log CFU/cm^2/day). Dipping in ALF (1%) and then in potassium lactate (3%), sodium diacetate (3%), or lactic acid (1%) provided similar ($P \geq 0.05$) antilisterial effects as dipping individually single 3% potassium lactate, 3% sodium diacetate, or 1% lactic acid, respectively, as suggested by GR obtained for these treatments. However, when ALF (1%) was applied in combination with 1% acetic acid, it appeared to reduce its antimicrobial activity as suggested by calculated GR obtained for single acetic acid (0.060 log CFU/cm^2/day) and the combination treatment (0.221 log CFU/cm^2/day). The results of this study indicated that dipping turkey breast slices sequentially into ALF and sodium diacetate led to complete inhibition and even slight reduction of *L. monocytogenes* growth during storage. Nevertheless, no evidence existed that ALF increased the antilisterial effects of other antimicrobial compounds, as sodium diacetate was very effective, even when applied individually.

For all experiments described above, growth/inactivation of total microbial populations (Appendix Tables 9-13) followed similar trends to those observed on PALCAM agar. Subsequently, effective antilisterial treatments also inhibited total

microbial populations, as detected on TSAYE. Slightly higher (in most cases 0.1 to < 1 log CFU/cm^2) counts obtained on TSAYE, however, indicated presence of natural microflora and/or better recovery on the non-selective medium.

3.3.3. *L. monocytogenes* populations on products formulated with antimicrobials

Populations of *L. monocytogenes* on bologna slices that contained single or combined antimicrobials during storage at 4 or 7°C are presented in Figure 3.6. Growth or inactivation kinetics data are shown in Tables 3.23 (4°C) and 3.24 (7°C). Populations of the pathogen were 6.6 log CFU/cm^2 on control samples on day-95 of storage at 4°C. The treatment that allowed the most extensive growth of the pathogen was 0.125% sodium diacetate and 0.5% lactoferrin, as *L. monocytogenes* counts reached 8.0 log CFU/cm^2 by day-43. The pathogen also grew readily on samples formulated with 0.5 or 1% lactoferrin, exceeding 7.0 log CFU/cm^2 by the end of storage (day-95). It appears that lactoferrin added individually or in combination with sodium diacetate in the bologna formulation promoted, rather than inhibited, growth of the pathogen during storage; however, the reason for this observation is not clear, particularly since the combination of lactoferrin with potassium lactate reduced ($P < 0.05$) the potential for growth during the first 75 days of storage, when compared to growth on control samples. The most effective antilisterial treatment was the combination of 1.8% potassium lactate with 0.125% sodium diacetate, as it did not allow significant ($P \geq 0.05$) microbial increases during storage. Lag phases in samples that contained antimicrobials ranged from 1.5 (1% lactoferrin) to 70.3 days (1.8% potassium lactate); no lag phase was obtained for control samples. Calculated GR ranged from 0.047 log CFU/cm^2/day (1.8% potassium lactate combined with 0.0625% sodium diacetate and 0.25% lactoferrin) to 0.189 log CFU/cm^2/

day (0.125% sodium diacetate combined with 0.5% lactoferrin). Of all single treatments, 1.8% potassium lactate exhibited the greatest antilisterial effects, as it resulted in slower ($P \geq 0.05$) growth (GR: 0.048 log CFU/cm^2/day) and a more extended ($P < 0.05$) LPD than treatments that consisted of only lactoferrin.

Similar to findings discussed above, among the tested treatments, 0.125% sodium diacetate and 0.5% lactoferrin permitted the greatest increases in *L. monocytogenes* levels at 7°C, with populations exceeding 7.0 and 8.0 log CFU/cm^{2}on days-28 and 57, respectively, on samples containing these antimicrobials. Also, the combination of 1.8% potassium with 0.125% sodium diacetate did not permit significant ($P \geq 0.05$) increases of populations. Treatments that permitted immediate *L. monocytogenes* growth included the control, samples that contained 0.5 or 1% lactoferrin and the combinations of 0.125% sodium diacetate with 0.5% lactoferrin and 1.8% potassium lactate with 0.0625% sodium diacetate and 0.25% lactoferrin. As already mentioned, the combination of 1.8% potassium lactate with 0.125% sodium diacetate provided complete inhibition of growth, whereas, the rest of the treatments caused LPD that ranged from 6.4 (1.8% potassium lactate combined with 0.5% lactoferrin) to 28.7 (1.8% potassium lactate combined with 0.125% sodium diacetate and 0.5% lactoferrin) days. As expected, lag phases observed during storage at 7°C were generally shorter compared to those at 4°C across treatments. Estimated GR varied from -0.023 log CFU/cm^2/day (IR; 1.8% potassium lactate combined with 0.125% sodium diacetate) to 0.299 log CFU/cm^2/day (control or 1.8% potassium lactate combined with 0.0625% sodium diacetate and 0.25% lactoferrin). Lactoferrin used individually at 0.5 or 1% or in combination (at 0.5%) with 1.8% potassium lactate and/or 0.125% sodium diacetate provided inhibition of *L.*

monocytogenes growth, as these treatments resulted in lower ($P < 0.05$) GR compared to that of control. However, unlike results obtained for growth at 4°C, the *L. monocytogenes* GR on samples formulated with 0.25% lactoferrin and 1.8% potassium lactate and 0.0625% sodium diacetate was the same as that on control samples (i.e., 0.299 log CFU/cm^2/day).

Overall, these results indicate that, under the conditions of this study, lactoferrin used individually in the formulation of bologna resulted in slight inhibition of *L. monocytogenes* growth during the first days of the storage period, particularly at 4°C. The combination of 0.5% lactoferrin with 1.8% potassium lactate was more effective than 1.8% potassium lactate, as it resulted in slower ($P \geq 0.05$) growth of the pathogen during storage at 4 but not at 7°C. On the other hand, lactoferrin appeared to lessen the antilisterial effects of the combination of potassium lactate and sodium diacetate, as *L. monocytogenes* populations on samples that contained lactoferrin (0.5%) together with 1.8% potassium lactate and 0.125% sodium diacetate in bologna formulation were higher than those on samples formulated with 1.8% potassium lactate and 0.125% sodium diacetate throughout storage at 4 or 7°C(Figure 3.6).

Growth on TSAYE (Appendix Tables 14 and 15) and PALCAM agar followed similar patterns at both temperatures especially during the first days of storage, suggesting that the majority of colonies that grew on TSAYE were *L. monocytogenes*. The higher counts obtained on the nonselective medium, especially during the last days of the storage reflected growth of spoilage microorganisms.

3.3.4. *L. monocytogenes* populations on product formulated and surface treated with antimicrobials

Figure 3.7 and Table 3.25 present populations and growth parameters of *L. monocytogenes* on frankfurters formulated with or without antimicrobials and left undipped or dipped into antimicrobial solutions. *L. monocytogenes* populations increased during storage of undipped control (no antimicrobials in the formulation) samples and exceeded 7.0 log CFU/cm^2 on day-50. Overall, populations on product formulated with 0.5% lactoferrin were similar ($P \geq 0.05$) to those on undipped control samples, on corresponding days. However, the compound provided some inhibition of growth, as populations in samples that contained 0.5% lactoferrin reached lower ($P \geq 0.05$) levels (6.6 log CFU/cm^2) compared to those in control (7.1 log CFU/cm^2) samples at the end of the storage period. Inclusion of combined antimicrobials (1.8% potassium lactate and 0.125% sodium diacetate or 0.5% lactoferrin) in the formulation of frankfurters did not permit significant ($P \geq 0.05$) increases in populations during product storage.

Immediate reductions in *L. monocytogenes* populations caused by dipping into 2% acetic acid ranged from 0.8 to 1.2 log CFU/cm^2. During storage of acetic acid-dipped samples, increases ($P < 0.05$) in populations occurred only in product formulated without antimicrobials; however, final (day-50) *L. monocytogenes* counts reached in these samples were only 0.1 log CFU/cm^2 higher than initial counts. During storage of samples that contained antimicrobials and were dipped in acetic acid, *L. monocytogenes* populations either remained constant (0.5% lactoferrin) or underwent significant ($P < 0.05$) reductions (1.8% potassium lactate plus 0.125% sodium diacetate and 1.8% potassium lactate plus 0.5% lactoferrin).

Dipping into 2% ALF also had immediate killing effects (1.3 to 1.5 log CFU/cm^2), but subsequently during storage, major ($P < 0.05$) increases in populations of the pathogen were observed in ALF-dipped control and 0.5% lactoferrin-containing samples. On the contrary, dipping frankfurters that contained 1.8% potassium lactate and 0.125% sodium diacetate or 1.8% potassium lactate and 0.5% lactoferrin into ALF led to listeriocidal effects during storage. Specifically, the lowest final (day-50) populations of *L. monocytogenes* observed in this experiment (0.0 log CFU/cm^2) were reached in ALF-dipped samples that contained the combination of 1.8% potassium lactate and 0.5% lactoferrin.

Growth in undipped control samples occurred after a LPD of 12.0 days, whereas, lactoferrin added at 5% in the product formulation inhibited *L. monocytogenes* growth for 17.7 days. On the other hand, in samples containing the combination of 1.8% potassium lactate with 0.125% sodium diacetate, *L. monocytogenes* populations remained constant for 42.0 days (SD) before being reduced rapidly (IR -0.488 log CFU/cm^2/day), while decreases in *L. monocytogenes* populations in samples formulated with 1.8% potassium lactate and 0.5% lactoferrin started immediately upon storage and were less abrupt ($P < 0.05$) (IR: -0.005 log CFU/cm^2/day).

In samples containing either one of the antimicrobial formulations and were dipped into acetic acid, growth of the pathogen was completely inhibited and/or reduced during storage, suggesting that antimicrobial additives may increase the antilisterial effectiveness of the acid solution, considering that the GR on acetic acid-dipped controls was 0.172 log CFU/cm^2/day. Among the acetic acid treatments, the fastest ($P \geq 0.05$) reduction in *L. monocytogenes* populations was observed in samples that contained 1.8%

potassium lactate and 0.125% sodium diacetate (IR: -0.117 log CFU/cm^2/day), whereas 0.5% lactoferrin added individually in the product formulation before immersing into acetic acid caused complete inhibition of *L. monocytogenes* growth for 49.1 days (SD), which was followed by listeriocidal effects (IR: -0.040 log CFU/cm^2/day).

Growth of *L. monocytogenes* started immediately in 2% ALF-dipped samples that contained no antimicrobials or 0.5% lactoferrin, whereas addition of 1.8% potassium lactate and 0.125% sodium diacetate in formulation, led to inhibition of *L. monocytogenes* proliferation and even reductions in populations (Figure 3.7) for 42.5 days. The estimated GR obtained for samples formulated without antimicrobials and dipped in 2% ALF was 0.165 log CFU/cm^2/day. For the same dipping treatment, growth of the pathogen was also observed in samples that contained 1.8% potassium lactate combined with 0.125% sodium diacetate (GR: 0.180 log CFU/cm^2/day; increase of 0.7 log CFU/cm^2 by the end of storage) or 0.5% lactoferrin (GR: 0.114 log CFU/cm^2/day; increase of approximately 6 log CFU/cm^2 by the end of storage). However, as indicated above, the combination of 1.8% potassium lactate with 0.125% sodium diacetate delayed or even reduced growth for 42.5 days *L. monocytogenes* growth unlike 0.5% lactoferrin that allowed growth immediately. In samples containing 1.8% potassium lactate and 0.5% lactoferrin and that were dipped into 2% ALF, an IR of -0.011 log CFU/cm^2/day suggested slight listeriocidal activity of the treatment during storage. As already mentioned, populations (0.0 log CFU/cm^2) recovered from samples treated with the latter combination of antimicrobials (i.e., potassium lactate and lactoferrin in the formulation and dipping into ALF) on day 50 were the lowest observed among treated or untreated samples, although the *L. monocytogenes* IR in ALF-dipped, potassium lactate and

lactoferrin-containing frankfurters (-0.011 log CFU/cm^2/day) was lower ($P \geq 0.05$) than those in acetic acid-treated frankfurters that contained potassium lactate and sodium diacetate (-0.117 log CFU/cm^2/day) or potassium lactate and lactoferrin (-0.021 log CFU/cm^2/day). That was probably because of the higher initial reductions achieved by the ALF treatment as compared to those resulting from dipping into acetic acid (shown above).

Findings of this study suggested that treatments that combined incorporation of antimicrobials (i.e., potassium lactate plus sodium diacetate or potassium lactate plus lactoferrin) in the formulation of frankfurters and dipping into acetic acid or ALF provided very effective control against *L. monocytogenes* during storage. Addition of lactoferrin to the formulation of frankfurters enhanced ($P < 0.05$) the antilisterial activity of the acetic acid surface treatment during storage. In addition, comparing the effectiveness of antimicrobial ingredients against *L. monocytogenes* in undipped or dipped into ALF samples suggests that the antilisterial activity of certain combination treatments included in the product formulation (1.8% potassium lactate and 0.125% sodium diacetate or 1.8% and 0.5% lactoferrin) may be enhanced by dipping into ALF solutions.

Growth of total microbial populations on TSAYE (Appendix Table 16) presented similar patterns to populations detected on PALCAM agar, suggesting that the predominant organism on tested samples was *L. monocytogenes*. Subsequently, treatments that provided *L. monocytogenes* inhibition during storage also appeared to control growth of total microbial populations, as detected on TSAYE. Nevertheless, higher counts (< 0.5 log CFU/cm^2, in most cases) observed on TSAYE as compared to

those on PALCAM throughout storage and across treatments suggest presence of relatively low levels of spoilage organisms and/or better recovery on the non-selective medium.

3.4. Discussion

The studies described above examined the effectiveness of ALF as a post-processing treatment, alone or followed by organic acids or their salts, using different RTE meat and poultry products, obtained commercially or formulated in the Meat Science laboratory at Colorado State University. Moreover, the antilisterial effects of lactoferrin, added in the formulation of two products, bologna and frankfurters, was tested together and in comparison with known antilisterial synthetic compounds (potassium lactate and sodium diacetate). As already noted, most experiments described in this chapter were performed once, with three samples being analyzed per treatment and storage day. Nevertheless, potential issues with statistical reliability may be overcome, to some degree, by the fact that experiments had the same objective and that surface treatments or formulations consisted of the same antimicrobial compounds (i.e., ALF and organic acids or salts as surface treatments; lactoferrin, and organic salts as formulation treatments). Thus, considering the method of application of the antimicrobial treatments (dipping/spraying vs. formulation), conclusions drawn from findings of individual experiments were combined and are presented collectively.

Although the product type (sliced vs. sausage-type) and composition (formulated with beef vs. beef and pork) affected the rate of *L. monocytogenes* growth on surface treated samples, as well as final counts of the pathogen, similar trends were observed across treatments in different products. Overall, application of organic acid or salt aqueous

solutions as surface treatments provided different degrees of inhibition of *L. monocytogenes* growth. Similarly to findings of other studies (Ahamad and Marth, 1989; Sorrels et al., 1989; Samelis et al., 2001a; Barmpalia et al., 2004), acetic acid and sodium diacetate exhibited greater antilisterial effects compared to lactic acid and potassium lactate. As expected, the composition of the treated product affected *L. monocytogenes* growth, with chemical antimicrobials being generally more efficient when used on lower vs. higher pH product (i.e., beef vs. beef and pork bologna). Application of ALF, as a 1 or 2% dipping (or spraying) solution, resulted in similar or even higher ($P < 0.05$) reductions in initial *L. monocytogenes* populations than those achieved by same-duration organic acid or salt treatments. The ability of ALF to detach bacteria from tissue surfaces by removing adhesive structures of Gram-negative bacteria (i.e., fimbriae) is a major element of its antimicrobial activity (Naidu, 2002). However, to our knowledge, effects of lactoferrin on other adhesive cell components, utilized by Gram-positive bacteria, have not been described and, thus possible explanations of the high initial reductions achieved by ALF remain unknown. Unlike organic acids and salts, ALF did not provide long-term antimicrobial effects, as it allowed extensive *L. monocytogenes* growth during storage. Although Uzzan et al. (2007) observed that lactoferrin did not degrade during storage (112 days) of milk at room or refrigerated temperature, the stability of the compound, when incorporated in meat products has not been investigated. Results obtained for combination surface treatments gave no indication that ALF improved the antilisterial effectiveness of organic acids or salts. Obviously, the high pH (approximately 7.40) of the 1% ALF solution was inadequate to sensitize cells to the subsequent treatment, while

rinsing with organic acid or salt solutions after the ALF treatment possibly prevented any iron-binding effects of ALF from taking place during storage of the product.

The antilisterial activity of lactates and diacetates, applied together in product formulations, has been established (Mbandi and Shelef, 2001; 2001; Samelis et al., 2002; Barmpalia et al., 2004; 2005). Accordingly, findings of studies presented here indicated that the combination of 1.8% potassium lactate with 0.125% sodium diacetate provided substantial or complete inhibition of growth in both bologna and frankfurters. Lactoferrin added in the formulation of bologna inoculated with *L. monocytogenes* resulted in slight inhibition of *L. monocytogenes* growth at the early stages of the storage period; however, the compound did not seem to sustain its antilisterial effects, as populations on products that contained lactoferrin reached high numbers, eventually. Moreover, no considerable enhancement in the antilisterial activity of potassium lactate or sodium diacetate was observed by their combined use with lactoferrin. Nevertheless, under the tested conditions, lactoferrin provided greater control of *L. monocytogenes* growth when added as an ingredient (0.5%) in frankfurters rather than in bologna, possibly due to the nature of these products. More specifically, enhanced inhibition of *L. monocytogenes* proliferation on frankfurters compared to bologna was expected, as the surface of a sausage-like product is less supportive of bacterial growth than that of a sliced product. Consequently, the combination of 1.8% potassium lactate with 0.5% lactoferrin in the formulation of frankfurters was completely listeriostatic or even listeriocidal; however, the same combination of antimicrobials included in bologna formulation allowed growth of the pathogen. Applying ALF as a surface treatment on frankfurters formulated with 1.8% potassium lactate combined with 0.5% lactoferrin appeared to enhance the activity

of the additives against *L. monocytogenes*, suggesting that combined incorporation of antimicrobials in the formulation of the product and dipping in ALF solutions may provide effective control of *L. monocytogenes* on that product.

Under the tested conditions, ALF and lactoferrin, applied as a dipping/spraying solution and a formulation ingredient, respectively, were generally less effective than organic acids and salts. Based on findings of one study, the combined use of ALF (surface treatment) or lactoferrin (formulation ingredient) with other antimicrobial agents incorporated into the formulation (i.e., potassium lactate and sodium diacetate or lactoferrin) or applied as dipping solutions (i.e., acetic acid), respectively, for treatment of frankfurters led to the identification of very effective antilisterial treatments. More research is needed, however, to confirm and further investigate potential additive or synergistic effects of ALF and lactoferrin with other antimicrobials on various RTE products.

Table 3.1. Mean values (± standard deviation) of cooking yields, and moisture and fat content of bologna formulated with or without antimicrobials.

Treatment	Cooking yield (%)	Moisture content (%)	Fat content (%)
Control	87.9±7.7	62.7±1.3	13.4±0.0
PL (1.8%)	90.2±3.0	60.7±0.1	15.7±1.0
LF (0.5%)	89.2±5.4	65.1±0.7	12.7±0.6
LF (1%)	87.2±5.0	64.3±2.4	12.9±1.4
PL (1.8%) + SD (0.125%)	88.2±2.2	63.1±0.7	17.4±0.4
PL (1.8%) + LF (0.5%)	87.2±4.3	59.1±0.1	13.8±0.2
SD (0.125%) + LF (0.5%)	89.0±1.6	65.7±0.3	17.2±0.6
PL (1.8%) + SD (0.125%) + LF (0.5%)	87.2±2.2	61.6±0.4	13.7±0.5
PL (1.8%) + SD (0.0625%) + LF (0.25%)	88.9±3.4	58.8±1.2	12.5±0.3

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

Table 3.2. Mean values (± standard deviation), of cooking yields, and moisture and fat content of frankfurters formulated with or without antimicrobials.

Treatment	Cooking yield (%)	Moisture content (%)	Fat content (%)
Control	79.3±0.7	68.2±0.3	15.9±2.0
PL (1.8%) + SD (0.125%)	79.2±0.7	68.3±1.4	15.6±1.2
LF (0.5%)	78.2±1.1	61.0±0.1	13.1±3.1
PL (1.8%) + LF (0.5%)	79.0±0.1	62.2±0.5	13.5±0.4

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

Table 3.3. Mean a_w values (± standard deviation) of ham slices inoculated with *Listeria monocytogenes* before or after dipping into water (120 s), acetic acid (30 s), or activated lactoferrin (120 s) vacuum packaged and stored at 7°C.

Application	Treatment	Water activity on day-0
None	Control	0.972±0.003
Pre-inoculation	Water	0.981±0.000
	AA (1%)	0.976±0.000
	ALF (2%)	0.978±0.001
Post-inoculation	Water	0.981±0.000
	AA (1%)	0.978±0.000
	ALF (2%)	0.979±0.002

AA: acetic acid, ALF: activated lactoferrin

Table 3.4. Mean a_w values (± standard deviation) of frankfurters, inoculated with *Listeria monocytogenes*, dipped into antimicrobial solutions for 30, 60, 90 or 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Water activity on day-0
Control	0.962±0.006
Water (30 s)	0.963±0.001
LA (2 %; 30 s)	0.963±0.001
SD (3%; 30 s)	0.954±0.003
ALF (2%; 30 s)	0.957±0.002
ALF (2%; 60 s)	0.967±0.005
ALF (2%; 90 s)	0.960±0.001
ALF (2%; 120 s)	0.963±0.001

LA: lactic acid, SD: sodium diacetate, ALF: activated lactoferrin

Table 3.5. Mean a_w values (± standard deviation) of bologna, formulated with beef or beef and pork, inoculated with *Listeria monocytogenes* and dipped into water (120 s), 2% acetic acid (AA; 60 s), or 2% activated lactoferrin (ALF; 60, 120, or 180 s), vacuum packaged and stored at 10°C.

Meat used in bologna formulation	Treatment	Water activity on day-0
Beef	Control	0.955±0.003
	Water	0.970±0.001
	AA (2%)	0.970±0.001
	ALF (2%; 60 s)	0.967±0.001
	ALF (2%; 120 s)	0.966±0.001
	ALF (2%; 180 s)	0.973±0.001
Beef and pork	Control	0.963±0.001
	Water	0.975±0.001
	AA (2%)	0.965±0.001
	ALF (2%; 60 s)	0.970±0.001
	ALF (2%; 120 s)	0.972±0.001
	ALF (2%; 180 s)	0.974±0.004

AA: acetic acid, ALF: activated lactoferrin

Table 3.6. Mean a_w values (± standard deviation) of bologna slices inoculated with *Listeria monocytogenes* and dipped in (30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control).

Application	Treatment	Water activity on day-0
None	Control	0.966±0.001
Dipping	Water	0.975±0.001
	LA (2%)	0.970±0.001
	ALF (2%)	0.966±0.001
Spraying	Water	0.974±0.001
	LA (2%)	0.969±0.000
	ALF (2%)	0.965±0.002

LA: lactic acid, ALF: activated lactoferrin

Table 3.7. Mean a_w values (± standard deviation) of cured turkey breast slices, inoculated with *Listeria monocytogenes* and dipped into antimicrobial solutions or water for 60 s (except control).

Treatment (dipping)	Water activity on day-0
Control (undipped)	0.975±0.001
Water	0.982±0.001
PL (3%)	0.981±0.001
SD (3%)	0.981±0.001
LA (1%)	0.981±0.000
AA (1%)	0.981±0.001
ALF (2%)	0.982±0.000
ALF (1%)	0.981±0.001
ALF (1%) + PL (3%)	0.982±0.001
ALF (1%) + SD (3%)	0.980±0.000
ALF (1%) + LA (1%)	0.981±0.001
ALF (1%) + AA (1%)	0.982±0.001

PL: potassium lactate, SD: sodium diacetate, LA: lactic acid, AA: acetic acid, ALF: activated lactoferrin

Table 3.8. Mean a_w values (± standard deviation) of bologna slices formulated with or without antimicrobials and inoculated with *Listeria monocytogenes*.

Treatment (formulation)	Water activity on day-0
Control	0.966±0.001
PL (1.8%)	0.958±0.002
LF (0.5%)	0.965±0.004
LF (1%)	0.965±0.000
PL (1.8%) + SD (0.125%)	0.960±0.001
PL (1.8%) + LF (0.5%)	0.959±0.004
SD (0.125%) + LF (0.5%)	0.969±0.000
PL (1.8%) + SD (0.125%) + LF (0.5%)	0.963±0.001
PL (1.8%) + SD (0.0625%) + LF (0.25%)	0.964±0.000

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

Table 3.9. Mean a_w values (± standard deviation) of frankfurters formulated with or without antimicrobials, inoculated with *Listeria monocytogenes*, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Treatment (formulation)	Water activity on day-0
No dipping	Control	0.949±0.001
	PL (1.8%) + SD (0.125%)	0.940±0.000
	LF (0.5%)	0.951±0.001
	PL (1.8%) + LF (0.5%)	0.941±0.000
AA (2%)	Control	0.954±0.004
	PL (1.8%) + SD (0.125%)	0.942±0.000
	LF (0.5%)	0.956±0.001
	PL (1.8%) + LF (0.5%)	0.941±0.001
ALF (2%)	Control	0.955±0.001
	PL (1.8%) + SD (0.125%)	0.945±0.001
	LF (0.5%)	0.954±0.002
	PL (1.8%) + LF (0.5%)	0.940±0.000

AA: acetic acid, ALF: activated lactoferrin, PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

Table 3.10. Mean pH values (± standard deviation) of ham slices inoculated with *Listeria monocytogenes* before or after dipping into water (120 s), acetic acid (30 s), or activated lactoferrin (120 s), vacuum packaged and stored at 7°C.

		Day of storage					
Application	Treatment	0	5	10	15	22	28
None	Control	$6.32\pm0.02_{Aa}$	$6.29\pm0.01_{Aa}$	$6.29\pm0.0_{Aa}$	$6.24\pm0.00_{Aab}$	$6.13\pm0.05_{Ab}$	$5.91\pm0.02_{Ac}$
Pre-inoculation	Water	$6.38\pm0.01_{Aa}$	$6.36\pm0.01_{Aa}$	$6.32\pm0.07_{ACa}$	$6.20\pm0.03_{Ab}$	$5.96\pm0.01_{Bc}$	$5.93\pm0.01_{Ac}$
	AA (1%)	$5.92\pm0.05_{Ba}$	$5.97\pm0.03_{Ba}$	$5.91\pm0.03_{Ba}$	$5.92\pm0.06_{Ba}$	$5.91\pm0.02_{Ba}$	$5.90\pm0.06_{Aa}$
	ALF (2%)	$6.39\pm0.02_{Aa}$	$6.39\pm0.02_{Aa}$	$6.39\pm0.01_{ACa}$	$6.34\pm0.03_{Aa}$	$6.20\pm0.06_{Ab}$	$5.92\pm0.02_{Ac}$
Post-inoculation	Water	$6.38\pm0.01_{Aa}$	$6.35\pm0.02_{Aab}$	$6.34\pm0.02_{ACab}$	$6.25\pm0.06_{Ab}$	$5.96\pm0.01_{Bc}$	$5.88\pm0.05_{Ac}$
	AA (1%)	$5.91\pm0.07_{Bab}$	$5.98\pm0.02_{Ba}$	$5.89\pm0.07_{Bab}$	$5.88\pm0.06_{Bab}$	$5.93\pm0.08_{Ba}$	$5.82\pm0.05_{Ab}$
	ALF (2%)	$6.39\pm0.02_{Aa}$	$6.38\pm0.02_{Aa}$	$6.40\pm0.02_{Ca}$	$6.34\pm0.03_{Aa}$	$6.22\pm0.01_{Ab}$	$5.91\pm0.03_{Ac}$

AA: acetic acid, ALF: activated lactoferrin

ABC...: means within a column; and, abc...: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.11. Mean pH values (± standard deviation) of surface of frankfurters, inoculated with *Listeria monocytogenes*, dipped into antimicrobial solutions for 30, 60, 90 or 120 s, vacuum packaged and stored at 7°C.

	Day of storage							
Treatment	0	4	8	12	16	20	24	32
Control	$6.09\pm0.01_{ACa}$	$6.15\pm0.01_{Aa}$	$6.17\pm0.03_{Aa}$	$6.13\pm0.00_{ABa}$	$6.16\pm0.01_{ABa}$	$6.11\pm0.04_{Aa}$	$5.90\pm0.02_{Ab}$	$5.61\pm0.07_{Ac}$
Water (30 s)	$6.10\pm0.01_{ACa}$	$6.17\pm0.01_{Aa}$	$6.18\pm0.02_{Aa}$	$6.16\pm0.01_{Aa}$	$6.16\pm0.01_{ABa}$	$5.91\pm0.06_{Bb}$	$5.70\pm0.14_{Bc}$	$5.63\pm0.11_{Ad}$
LA (2 %; 30 s)	$5.80\pm0.29_{Ba}$	$6.03\pm0.00_{Bb}$	$6.05\pm0.01_{Bb}$	$6.04\pm0.01_{Bb}$	$6.06\pm0.03_{ACb}$	$6.10\pm0.02_{Ab}$	$5.87\pm0.06_{Aa}$	$5.70\pm0.07_{Bc}$
SD (3%; 30 s)	$6.01\pm0.02_{Aa}$	$6.09\pm0.01_{ABa}$	$6.10\pm0.01_{ABa}$	$6.10\pm0.00_{ABa}$	$6.10\pm0.01_{ABCa}$	$6.13\pm0.02_{Aa}$	$6.06\pm0.02_{Ca}$	$5.83\pm0.12_{Cb}$
ALF (2%; 30 s)	$6.10\pm0.01_{ACa}$	$6.18\pm0.01_{Aa}$	$6.17\pm0.01_{Aa}$	$6.14\pm0.01_{Aa}$	$6.14\pm0.02_{ABa}$	$6.07\pm0.02_{Aa}$	$5.85\pm0.07_{Ab}$	$5.65\pm0.05_{Ac}$
ALF (2%; 60 s)	$6.10\pm0.02_{ACa}$	$6.18\pm0.03_{Aab}$	$6.20\pm0.01_{Ab}$	$6.18\pm0.01_{Aab}$	$6.21\pm0.00_{Bb}$	$6.11\pm0.01_{Aab}$	$5.89\pm0.04_{Ac}$	$5.57\pm0.10_{Ad}$
ALF (2%; 90 s)	$6.11\pm0.02_{ACa}$	$6.16\pm0.02_{Aa}$	$6.21\pm0.02_{Aa}$	$6.17\pm0.00_{Aa}$	$6.20\pm0.01_{Ba}$	$6.12\pm0.07_{Aa}$	$5.94\pm0.14_{Ab}$	$5.57\pm0.12_{Ac}$
ALF (2%; 120 s)	$6.12\pm0.01_{Ca}$	$6.20\pm0.01_{Aa}$	$6.20\pm0.02_{Aa}$	$6.13\pm0.01_{ABa}$	$5.99\pm0.07_{Cb}$	$5.89\pm0.01_{Bbc}$	$5.81\pm0.07_{Ac}$	$5.44\pm0.14_{Dd}$

ABC...: means within a column; and, abc...: means within a row lacking a common letter are significantly different ($P < 0.05$)

LA: lactic acid, SD: sodium diacetate, ALF: activated lactoferrin

Table 3.12. Mean pH values (± standard deviation) of beef or beef and pork bologna slices, inoculated with *Listeria monocytogenes*, and dipped into water (120 s), 2% acetic acid (AA; 60 s), or 2% activated lactoferrin (ALF; 60, 120, or 180 s), vacuum packaged and stored at 10°C.

Meat used in bologna formulation	Treatment	Day of storage					
		0	4	8	12	16	26
Beef	Control	$6.17\pm0.04_{Aa}$	$6.12\pm0.01_{Aa}$	$6.08\pm0.02_{Ab}$	$5.24\pm0.19_{Ac}$	$5.23\pm0.13_{Ac}$	$4.99\pm0.24_{Ad}$
	Water	$6.17\pm0.03_{Aa}$	$6.17\pm0.01_{ACa}$	$5.33\pm0.08_{BCb}$	$4.87\pm0.02_{Bc}$	$4.84\pm0.01_{Bc}$	$4.62\pm0.01_{Ad}$
	AA (2%)	$5.17\pm0.13_{Ba}$	$5.20\pm0.0_{Ba}$	$5.17\pm0.01_{Ba}$	$5.22\pm0.08_{Aa}$	$5.21\pm0.06_{Aa}$	$5.18\pm0.09_{Ba}$
	ALF (2%; 60 s)	$6.19\pm0.01_{Aa}$	$6.13\pm0.05_{Aa}$	$5.92\pm0.17_{ACb}$	$5.09\pm0.08_{ACc}$	$5.00\pm0.06_{ACc}$	$4.67\pm0.08_{Ad}$
	ALF (2%; 120 s)	$6.20\pm0.01_{Aa}$	$6.17\pm0.05_{ACa}$	$6.02\pm0.01_{Ab}$	$5.09\pm0.12_{ACc}$	$4.97\pm0.07_{BCc}$	$4.70\pm0.04_{Ad}$
	ALF (2%; 180 s)	$6.22\pm0.04_{Aa}$	$6.20\pm0.02_{ACa}$	$5.99\pm0.21_{ACb}$	$5.06\pm0.09_{ACc}$	$4.95\pm0.01_{BCc}$	$4.65\pm0.04_{Ad}$
Beef and pork	Control	$6.26\pm0.02_{ACa}$	$6.21\pm0.08_{ACa}$	$5.95\pm0.23_{ACb}$	$5.10\pm0.02_{ACc}$	$5.02\pm0.11_{ACc}$	$4.73\pm0.08_{Ad}$
	Water	$6.25\pm0.01_{ACa}$	$6.25\pm0.02_{Ca}$	$5.54\pm0.53_{Cb}$	$4.91\pm0.04_{BCc}$	$4.81\pm0.08_{Bc}$	$4.59\pm0.06_{Ad}$
	AA (2%)	$5.12\pm0.04_{Ba}$	$5.16\pm0.11_{Ba}$	$5.37\pm0.23_{BCb}$	$5.16\pm0.07_{Ac}$	$5.17\pm0.07_{Ac}$	$5.14\pm0.06_{Bc}$
	ALF (2%; 60 s)	$6.28\pm0.02_{Ca}$	$6.25\pm0.01_{Ca}$	$5.69\pm0.01_{Cb}$	$5.01\pm0.05_{Ac}$	$4.91\pm0.04_{BCc}$	$4.66\pm0.00_{Ad}$
	ALF (2%; 120 s)	$6.31\pm0.05_{Ca}$	$6.26\pm0.03_{Ca}$	$5.47\pm0.10_{BCb}$	$4.92\pm0.04_{BCc}$	$4.85\pm0.06_{Bc}$	$4.62\pm0.06_{Ad}$
	ALF (2%; 180 s)	$6.31\pm0.01_{Ca}$	$6.28\pm0.03_{Ca}$	$5.71\pm0.02_{Cb}$	$4.89\pm0.04_{BCc}$	$4.85\pm0.04_{Bc}$	$4.61\pm0.04_{Ad}$

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.13. Mean pH values (± standard deviation) of bologna slices inoculated with *Listeria monocytogenes* and dipped in (30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control), vacuum packaged and stored at 7°C.

		Day of storage							
Application	Treatment	0	4	8	12	16	22	28	43
None	Control	$6.31\pm0.01_{Aa}$	$6.31\pm0.02_{Aa}$	$6.31\pm0.01_{Aa}$	$6.28\pm0.06_{Aa}$	$6.97\pm0.03_{Ab}$	$5.80\pm0.03_{ADc}$	$5.61\pm0.02_{Ad}$	$5.48\pm0.04_{Ae}$
Dipping	Water	$6.32\pm0.02_{Aa}$	$6.33\pm0.01_{Aa}$	$6.10\pm0.01_{Bb}$	$6.05\pm0.05_{Bb}$	$5.64\pm0.01_{Bc}$	$5.57\pm0.03_{Bc}$	$5.32\pm0.08_{Bd}$	$5.20\pm0.08_{Bd}$
	LA (2%)	$5.67\pm0.01_{Ba}$	$5.70\pm0.02_{Ba}$	$5.72\pm0.02_{C}$	$5.68\pm0.02_{Ca}$	$5.69\pm0.03_{Ba}$	$5.76\pm0.03_{Ca}$	$5.65\pm0.02_{Aa}$	$5.32\pm0.05_{CDb}$
	ALF (2%)	$6.38\pm0.01_{Aa}$	$6.38\pm0.02_{Aa}$	$6.37\pm0.02_{Aa}$	$6.34\pm0.02_{Aa}$	$5.96\pm0.09_{Cb}$	$5.65\pm0.03_{Bc}$	$5.42\pm0.02_{Bd}$	$5.27\pm0.04_{BCe}$
Spraying	Water	$6.34\pm0.01_{Aa}$	$6.34\pm0.01_{Aa}$	$6.31\pm0.01_{Aa}$	$6.26\pm0.03_{Aa}$	$5.81\pm0.06_{Db}$	$5.64\pm0.01_{Bc}$	$5.42\pm0.01_{Bd}$	$5.34\pm0.04_{CDd}$
	LA (2%)	$5.88\pm0.04_{Ca}$	$5.85\pm0.02_{Cab}$	$5.88\pm0.02_{Da}$	$5.89\pm0.04_{Da}$	$5.85\pm0.04_{Dab}$	$5.90\pm0.02_{Aa}$	$5.76\pm0.04_{Cb}$	$5.40\pm0.05_{ADc}$
	ALF (2%)	$6.36\pm0.03_{Aa}$	$6.34\pm0.04_{Aa}$	$6.37\pm0.01_{Aa}$	$6.32\pm0.01_{Aa}$	$6.19\pm0.07_{Eb}$	$5.87\pm0.08_{Dc}$	$5.49\pm0.03_{Bd}$	$5.39\pm0.04_{ADd}$

LA: lactic acid, ALF: activated lactoferrin

ABC...: means within a column; and, abc...: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.14. Mean pH values (± standard deviation) of cured turkey breast slices, inoculated with *Listeria monocytogenes*, dipped into antimicrobial solutions or water for 60 s (except control), vacuum packaged and stored at 7°C.

	Day of storage							
Treatment (dipping)	0	4	8	12	16	22	28	43
Control (undipped)	$6.22\pm0.02_{Aa}$	$6.19\pm0.05_{Aa}$	$6.17\pm0.05_{Aa}$	$6.23\pm0.05_{Aa}$	$6.18\pm0.03_{Aa}$	$6.19\pm0.07_{Aa}$	$6.17\pm0.03_{Aab}$	$6.07\pm0.07_{ADb}$
Water	$6.23\pm0.02_{Aa}$	$6.22\pm0.02_{Aa}$	$6.22\pm0.03_{Aa}$	$6.25\pm0.04_{Aa}$	$6.20\pm0.02_{Aab}$	$6.14\pm0.04_{Aab}$	$6.15\pm0.03_{Aab}$	$6.10\pm0.01_{ADb}$
PL (3%)	$6.21\pm0.02_{ADa}$	$6.19\pm0.07_{Aab}$	$6.20\pm0.05_{Aab}$	$6.20\pm0.03_{Aa}$	$6.20\pm0.03_{Aab}$	$6.14\pm0.08_{Aab}$	$6.11\pm0.08_{Aab}$	$6.09\pm0.06_{ADb}$
SD (3%)	$5.68\pm0.05_{Bab}$	$5.71\pm0.13_{Bab}$	$5.80\pm0.03_{Ba}$	$5.71\pm0.05_{Bab}$	$5.67\pm0.05_{Aab}$	$5.73\pm0.04_{Bab}$	$5.78\pm0.04_{Ba}$	$5.64\pm0.10_{Bb}$
LA (1%)	$5.93\pm0.06_{Ca}$	$5.90\pm0.04_{Ca}$	$5.92\pm0.11_{Ca}$	$5.82\pm0.12_{Ca}$	$5.94\pm0.04_{Aa}$	$5.85\pm0.02_{Ca}$	$5.83\pm0.11_{BCa}$	$5.82\pm0.07_{Ca}$
AA (1%)	$5.73\pm0.03_{Ba}$	$5.70\pm0.05_{Ba}$	$5.74\pm0.10_{Ba}$	$5.71\pm0.09_{Ba}$	$5.69\pm0.11_{Aa}$	$5.67\pm0.21_{Da}$	$5.76\pm0.02_{Ba}$	$5.72\pm0.09_{BCa}$
ALF (2%)	$6.19\pm0.03_{Aa}$	$6.19\pm0.03_{Aa}$	$6.24\pm0.07_{Aa}$	$6.29\pm0.01_{Aa}$	$6.21\pm0.03_{Aa}$	$6.23\pm0.06_{Aa}$	$6.15\pm0.03_{Aa}$	$6.00\pm0.17_{Ab}$
ALF (1%)	$6.31\pm0.02_{Da}$	$6.23\pm0.03_{Aab}$	$6.27\pm0.00_{Aab}$	$6.28\pm0.01_{Aab}$	$6.20\pm0.02_{Aab}$	$6.19\pm0.05_{Ab}$	$6.18\pm0.01_{Ab}$	$6.17\pm0.01_{Db}$
ALF (1%) + PL (3%)	$6.26\pm0.00_{ADa}$	$6.23\pm0.02_{Aab}$	$6.28\pm0.03_{Aa}$	$6.28\pm0.02_{Aa}$	$6.25\pm0.02_{Aab}$	$6.23\pm0.01_{Aab}$	$6.17\pm0.01_{Aab}$	$6.16\pm0.05_{Db}$
ALF (1%) + SD (3%)	$5.81\pm0.05_{Fa}$	$5.80\pm0.04_{BCa}$	$5.82\pm0.04_{Ba}$	$5.83\pm0.10_{Ca}$	$5.84\pm0.05_{Aa}$	$5.88\pm0.05_{Ca}$	$5.89\pm0.02_{CDa}$	$5.75\pm0.02_{BCa}$
ALF (1%) + LA (1%)	$5.84\pm0.06_{Fab}$	$5.89\pm0.01_{ACab}$	$5.96\pm0.03_{Ca}$	$5.86\pm0.17_{Cab}$	$5.81\pm0.18_{Ab}$	$5.87\pm0.10_{Cab}$	$5.94\pm0.12_{Da}$	$5.62\pm0.13_{Bc}$
ALF (1%) + AA (1%)	$5.78\pm0.09_{BFa}$	$5.72\pm0.04_{Bab}$	$5.76\pm0.02_{Ba}$	$5.79\pm0.15_{Bab}$	$5.77\pm0.10_{Aab}$	$5.82\pm0.11_{Ca}$	$5.75\pm0.06_{Bab}$	$5.71\pm0.22_{BCb}$

PL: potassium lactate, SD: sodium diacetate, LA: lactic acid, AA: acetic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.15. Mean pH values (pH ± standard deviation) of bologna slices formulated with or without antimicrobials, inoculated with the pathogen after slicing, vacuum packaged and stored at 4°C.

	Day of storage					
Treatment (formulation)	0	10	20	43	75	95
Control	$6.32\pm0.04_{Aab}$	$6.34\pm0.00_{Aa}$	$6.23\pm0.02_{Ab}$	$5.81\pm0.02_{Ac}$	$5.57\pm0.04_{Ad}$	$5.52\pm0.06_{Ad}$
PL (1.8%)	$6.31\pm0.01_{Aab}$	$6.34\pm0.01_{Aa}$	$6.25\pm0.02_{Ab}$	$6.36\pm0.02_{Ba}$	$6.28\pm0.02_{Bab}$	$6.27\pm0.02_{Bab}$
LF (0.5%)	$6.26\pm0.01_{ABa}$	$6.28\pm0.00_{ABa}$	$6.20\pm0.02_{Aa}$	$6.25\pm0.05_{Ba}$	$5.84\pm0.02_{Cb}$	$5.70\pm0.02_{Cc}$
LF (1%)	$6.27\pm0.03_{ABa}$	$6.30\pm0.00_{Aa}$	$6.23\pm0.02_{Aa}$	$6.11\pm0.00_{Cb}$	$5.82\pm0.07_{Cc}$	$5.67\pm0.05_{ACd}$
PL (1.8%) + SD (0.125%)	$6.21\pm0.00_{Bab}$	$6.23\pm0.00_{Bab}$	$6.14\pm0.00_{ABa}$	$6.26\pm0.01_{Bb}$	$6.26\pm0.02_{Bb}$	$6.26\pm0.01_{Bb}$
PL (1.8%) + LF (0.5%)	$6.30\pm0.00_{Aa}$	$6.32\pm0.00_{Aa}$	$6.23\pm0.01_{Aa}$	$6.32\pm0.04_{Ba}$	$6.29\pm0.03_{Ba}$	$6.29\pm0.05_{Ba}$
SD (0.125%) + LF (0.5%)	$6.27\pm0.04_{ABa}$	$6.22\pm0.01_{Ba}$	$6.11\pm0.02_{Bb}$	$5.41\pm0.11_{Ac}$	$5.10\pm0.01_{Dd}$	$5.02\pm0.06_{Dd}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	$6.20\pm0.01_{Bab}$	$6.20\pm0.02_{Bab}$	$6.11\pm0.01_{Ba}$	$6.23\pm0.02_{Bab}$	$6.18\pm0.04_{Eab}$	$6.26\pm0.05_{Bb}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	$6.28\pm0.00_{ABab}$	$6.28\pm0.01_{ABab}$	$6.15\pm0.04_{ABa}$	$6.32\pm0.02_{Bb}$	$6.20\pm0.03_{BEa}$	$6.22\pm0.05_{Bab}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.16. Mean pH values (± standard deviation) of bologna slices formulated with or without antimicrobials, inoculated with *Listeria monocytogenes* after slicing, vacuum packaged and stored at 7°C.

Treatment (formulation)	Day of storage								
	0	4	8	12	16	20	28	43	57
Control	$6.32\pm0.04_{Aa}$	$6.32\pm0.03_{Aa}$	$6.34\pm0.02_{Aa}$	$6.32\pm0.02_{Aa}$	$6.29\pm0.01_{ABab}$	$6.21\pm0.12_{Ab}$	$5.87\pm0.06_{Ac}$	$5.66\pm0.01_{Ad}$	$5.57\pm0.06_{Ad}$
PL (1.8%)	$6.31\pm0.01_{Aab}$	$6.32\pm0.01_{Aab}$	$6.33\pm0.02_{Aab}$	$6.32\pm0.02_{Aab}$	$6.33\pm0.02_{Aab}$	$6.41\pm0.01_{Ba}$	$6.34\pm0.03_{Ba}$	$6.29\pm0.01_{Bb}$	$6.31\pm0.02_{Bab}$
LF (0.5%)	$6.26\pm0.01_{ABa}$	$6.25\pm0.03_{ABa}$	$6.29\pm0.01_{ACa}$	$6.27\pm0.02_{ACa}$	$6.30\pm0.02_{ABa}$	$6.33\pm0.01_{BCa}$	$6.28\pm0.00_{BCa}$	$6.08\pm0.07_{CDb}$	$5.89\pm0.01_{Cc}$
LF (1%)	$6.27\pm0.03_{ABa}$	$6.29\pm0.01_{Aa}$	$6.29\pm0.01_{ACa}$	$6.27\pm0.01_{ACa}$	$6.29\pm0.01_{ABa}$	$6.30\pm0.01_{BCa}$	$6.29\pm0.01_{BCa}$	$6.00\pm0.12_{Cb}$	$5.82\pm0.02_{Cc}$
PL (1.8%) + SD (0.125%)	$6.21\pm0.00_{Bab}$	$6.22\pm0.01_{Ba}$	$6.11\pm0.07_{Bb}$	$6.17\pm0.06_{Bab}$	$6.22\pm0.01_{Ba}$	$6.23\pm0.04_{ACa}$	$6.22\pm0.04_{Ca}$	$6.19\pm0.02_{Bab}$	$6.26\pm0.01_{Ba}$
PL (1.8%) + LF (0.5%)	$6.30\pm0.00_{Aab}$	$6.30\pm0.01_{Aab}$	$6.34\pm0.02_{Aa}$	$6.21\pm0.01_{BCb}$	$6.32\pm0.01_{Aa}$	$6.37\pm0.06_{Ba}$	$6.35\pm0.01_{Ba}$	$6.29\pm0.04_{Bab}$	$6.33\pm0.00_{Ba}$
SD (0.125%) + LF (0.5%)	$6.27\pm0.04_{ABa}$	$6.20\pm0.01_{Bab}$	$6.16\pm0.05_{Bbc}$	$6.06\pm0.02_{Dc}$	$6.20\pm0.00_{Bab}$	$6.26\pm0.02_{ACa}$	$6.18\pm0.01_{Dab}$	$5.68\pm0.02_{Ad}$	$5.42\pm0.04_{Dc}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	$6.20\pm0.01_{Ba}$	$6.18\pm0.01_{Ba}$	$6.20\pm0.01_{BCa}$	$6.06\pm0.03_{Db}$	$6.21\pm0.01_{Ba}$	$6.24\pm0.03_{ACa}$	$6.24\pm0.02_{BCa}$	$6.19\pm0.02_{Ba}$	$6.23\pm0.01_{Ba}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	$6.28\pm0.00_{ABa}$	$6.22\pm0.06_{Bac}$	$6.24\pm0.05_{ACa}$	$6.06\pm0.05_{Db}$	$6.25\pm0.01_{Bac}$	$6.31\pm0.01_{BCa}$	$6.30\pm0.01_{Ba}$	$6.17\pm0.02_{BDc}$	$6.08\pm0.01_{Eb}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.17. Mean pH values (± standard deviation) of frankfurters formulated with or without antimicrobials, inoculated with *Listeria monocytogenes*, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Treatment (formulation)	Day of storage							
		0	5	10	15	20	25	35	50
No dipping	Control	$6.04\pm0.02_{Aa}$	$5.05\pm0.02_{Ab}$	$6.03\pm0.02_{ABa}$	$6.02\pm0.02_{Aa}$	$6.01\pm0.03_{Aa}$	$6.05\pm0.01_{Aa}$	$5.94\pm0.05_{Aa}$	$5.94\pm0.05_{Aa}$
	PL (1.8%) + SD (0.125%)	$5.95\pm0.07_{Aa}$	$5.01\pm0.03_{Aa}$	$6.01\pm0.01_{ABa}$	$5.97\pm0.02_{Aa}$	$6.02\pm0.05_{Aa}$	$5.97\pm0.03_{Aa}$	$5.95\pm0.05_{Aa}$	$5.95\pm0.05_{Aa}$
	LF (0.5%)	$6.03\pm0.02_{Aa}$	$6.01\pm0.01_{Ba}$	$6.11\pm0.02_{Aa}$	$5.97\pm0.02_{Aa}$	$6.02\pm0.06_{Aa}$	$5.99\pm0.01_{Aa}$	$5.98\pm0.02_{Aa}$	$5.98\pm0.02_{Aa}$
	PL (1.8%) + LF (0.5%)	$6.03\pm0.01_{Aa}$	$6.03\pm0.04_{Ba}$	$6.07\pm0.02_{ABa}$	$6.05\pm0.01_{Aa}$	$6.07\pm0.02_{Aa}$	$6.03\pm0.05_{Aa}$	$6.03\pm0.06_{Aa}$	$6.03\pm0.06_{Aa}$
AA (2%)	Control	$5.40\pm0.08_{Ba}$	$5.88\pm0.04_{Cbc}$	$5.91\pm0.01_{Bb}$	$5.83\pm0.02_{Bbc}$	$5.84\pm0.05_{Bbc}$	$5.84\pm0.02_{Bbc}$	$5.79\pm0.01_{Bc}$	$5.79\pm0.05_{Bc}$
	PL (1.8%) + SD (0.125%)	$5.70\pm0.01_{Ca}$	$5.77\pm0.02_{CDab}$	$5.71\pm0.02_{Ca}$	$5.82\pm0.03_{BCbc}$	$5.90\pm0.06_{ACc}$	$5.73\pm0.04_{Ca}$	$5.80\pm0.06_{Bbc}$	$5.80\pm0.06_{Bbc}$
	LF (0.5%)	$5.06\pm0.03_{Da}$	$5.75\pm0.05_{Db}$	$5.83\pm0.03_{Db}$	$5.78\pm0.05_{Bb}$	$5.84\pm0.01_{BCb}$	$5.78\pm0.03_{BCb}$	$5.76\pm0.02_{Bb}$	$5.76\pm0.02_{Bb}$
	PL (1.8%) + LF (0.5%)	$5.84\pm0.01_{Ea}$	$5.82\pm0.01_{CDa}$	$5.84\pm0.02_{Da}$	$5.86\pm0.01_{BCa}$	$5.87\pm0.13_{BCa}$	$5.80\pm0.01_{BCa}$	$5.83\pm0.01_{Ba}$	$5.83\pm0.01_{Ba}$
ALF (2%)	Control	$6.09\pm0.03_{Aa}$	$6.09\pm0.06_{Ba}$	$5.96\pm0.03_{Ba}$	$6.05\pm0.05_{Aa}$	$6.08\pm0.03_{Aa}$	$6.03\pm0.03_{Aa}$	$5.95\pm0.01_{Aa}$	$5.95\pm0.01_{Aa}$
	PL (1.8%) + SD (0.125%)	$5.98\pm0.01_{Aa}$	$6.05\pm0.05_{Ba}$	$5.93\pm0.02_{Ba}$	$5.92\pm0.02_{ACa}$	$6.05\pm0.06_{Aa}$	$5.99\pm0.04_{Aa}$	$5.98\pm0.01_{Aa}$	$5.98\pm0.02_{Aa}$
	LF (0.5%)	$6.03\pm0.01_{Aab}$	$6.07\pm0.05_{Bab}$	$6.04\pm0.02_{ABab}$	$6.02\pm0.02_{Aab}$	$6.13\pm0.04_{Ab}$	$6.07\pm0.03_{Aab}$	$5.97\pm0.05_{Aa}$	$5.97\pm0.05_{Aa}$
	PL (1.8%) + LF (0.5%)	$6.09\pm0.01_{Aa}$	$6.09\pm0.05_{Ba}$	$6.13\pm0.03_{Aa}$	$6.03\pm0.02_{Aa}$	$6.09\pm0.04_{Aa}$	$6.06\pm0.01_{Aa}$	$6.04\pm0.03_{Aa}$	$6.04\pm0.03_{Aa}$

AA: acetic acid, ALF: activated lactoferrin, PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Table 3.18. Mean growth kinetics of *Listeria monocytogenes* growth on the surface of ham slices inoculated with *Listeria monocytogenes* before or after dipping into water (120 s) acetic acid (30 s), or activated lactoferrin (120 s) vacuum packaged and stored at 7°C.

Application	Treatment	Lag phase duration (days)	Maximum growth rate (log CFU/cm^2/day)	Y_0[3] (log CFU/cm^2)	Y_{end}[4] (log CFU/cm^2)
None	Control	1.5 $_{AC}$	0.333 $_{A}$	3.0	7.9
Pre-inoculation	Water	[1]	0.332 $_{AC}$	3.4	8.1
	AA (1%)	19.9 $_{B}$	0.231 $_{B}$	3.0	-
	ALF (2%)	3.9 $_{C}$	0.339 $_{A}$	2.9	8.1
Post-inoculation	Water	3.2 $_{C}$	0.595 $_{C}$	2.3	8.0
	AA (1%)	[2]	0.052 $_{D}$	2.8	4.1
	ALF (2%)	3.1 $_{C}$	0.347 $_{A}$	2.3	8.3

[1] no lag phase observed (growth was immediate); [2] slow growth or inactivation of *L. monocytogenes* did not allow estimation of lag phase or shoulder duration; [3] lower asymptote estimated by the Baranyi and Roberts model; [4] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

AA: acetic acid, ALF: activated lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.19. Mean growth kinetics of *Listeria monocytogenes* growth on the surface of frankfurters, inoculated with the pathogen, dipped into antimicrobial solutions for 30, 60, 90 or 120 s, vacuum packaged, and stored at 7°C for 32 days.

Treatment	Lag phase duration (days)	Maximum growth rate (log CFU/cm^2/day)	Y_0[2] (log CFU/cm^2)	Y_{end}[3] (log CFU/cm^2)
Control	0.7_A	0.239_A	2.8	7.9
Water (30 s)	0.6_A	0.370_B	2.1	8.1
LA (2 %; 30 s)	5.1_A	0.282_C	2.0	8.2
SD (3%; 30 s)	3.6_A	0.244_A	2.4	7.8
ALF (2%; 30 s)	-[1]	0.328_B	2.1	8.0
ALF (2%; 60 s)	-[1]	0.296_C	1.9	8.0
ALF (2%; 90 s)	-[1]	0.291_C	1.9	8.1
ALF (2%; 120 s)	-[1]	0.312_B	1.9	7.3

[1] no lag phase observed (growth was immediate); [2] lower asymptote estimated by the Baranyi and Roberts model; [3] upper asymptote estimated by the Baranyi and Roberts model

LA: lactic acid, SD: sodium diacetate, ALF: activated lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.20. Mean growth kinetics of *Listeria monocytogenes* growth (or inactivation) on the surface of inoculated bologna slices formulated with beef or beef and pork, dipped into water (120 s), 2% acetic acid (AA; 60 s), or 2% activated lactoferrin (ALF; 60, 120, or 180 s), vacuum packaged and stored at 10°C for 26 days.

Meat used in formulation	Treatment	Lag phase duration (days)	Maximum growth or inactivation rate (log CFU/cm^2/day)	Y_0[3] (log CFU/cm^2)	Y_{end}[4] (log CFU/cm^2)
Beef	Control	-[1]	0.492_A	3.7	8.0
	Water	-[1]	0.858_B	3.1	8.2
	AA (2%)	-[2]	-0.050_C	2.7	-
	ALF (2%; 60 s)	-[1]	0.698_{AB}	3.9	8.1
	ALF (2%; 120 s)	0.29_A	0.737_B	2.7	8.2
	ALF (2%; 180 s)	-[1]	0.694_{AB}	2.8	8.1
Beef and pork	Control	-[1]	0.614_{AB}	3.5	8.2
	Water	0.52_A	0.779_B	3.3	8.0
	AA (2%)	-[2]	-0.073_D	2.4	-
	ALF (2%; 60 s)	-[1]	0.763_B	3.0	7.9
	ALF (2%; 120 s)	-[1]	0.874_B	2.8	8.4
	ALF (2%; 180 s)	0.37_A	0.876_B	2.7	8.3

[1] no lag phase observed (growth was immediate); [2] slow growth or inactivation of *L. monocytogenes* did not allow estimation of lag phase or shoulder duration; [3] lower asymptote estimated by the Baranyi and Roberts model; [4] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

AA: acetic acid, ALF: activated lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.21. Mean growth kinetics of *Listeria monocytogenes* growth on bologna slices inoculated with the pathogen and dipped in (30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control), vacuum packaged and stored at 7°C for 43 days.

Application	Treatment	Lag phase duration (days)	Maximum growth rate (log CFU/cm^2/day)	Y_0[2] (log CFU/cm^2)	Y_{end}[3] (log CFU/cm^2)
None	Control	-[1]	0.300_{AE}	3.5	7.4
Dipping	Water	-[1]	0.552_{D}	2.6	7.5
	LA (2%)	8.8	0.266_{A}	2.7	7.5
	ALF (2%)	-[1]	0.348_{BE}	3.0	7.5
Spraying	Water	-[1]	0.401_{B}	3.1	7.6
	LA (2%)	-[1]	0.158_{C}	2.5	7.2
	ALF (2%)	-[1]	0.251_{A}	3.0	7.5

[1] no lag phase observed (growth was immediate); [2] lower asymptote estimated by the Baranyi and Roberts model; [3] upper asymptote estimated by the Baranyi and Roberts model

LA: lactic acid, ALF: activated lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.22. Mean growth kinetics of *Listeria monocytogenes* growth on the surface of cured turkey breast slices inoculated with the pathogen, dipped into antimicrobial solutions or water for 60 s (except control), vacuum packaged and stored at 7°C for 43 days.

Treatment (dipping)	Lag phase duration (days)	Maximum growth or inactivation rate (log CFU/cm^2/day)	Y_0[3] (log CFU/cm^2)	Y_{end}[4] (log CFU/cm^2)
Control (undipped)	3.7_A	0.204_A	3.0	7.2
Water	-[1]	0.240_A	2.2	7.0
PL (3%)	-[1]	0.254_A	2.4	6.9
SD (3%)	-[2]	0.002_A	2.7	-
LA (1%)	-[1]	0.118_A	2.7	-
AA (1%)	42.0_B	0.060_A	2.8	-
ALF (2%)	3.3_A	0.250_A	2.5	7.1
ALF (1%)	0.8_A	0.340_A	2.6	7.2
ALF (1%) + PL (3%)	-[1]	0.216_A	2.3	7.0
ALF (1%) + SD (3%)	-[2]	-0.001_A	2.4	-
ALF (1%) + LA (1%)	9.4_A	0.200_A	2.4	6.6
ALF (1%) + AA (1%)	5.4_A	0.221_A	2.4	-

[1] no lag phase observed (growth was immediate); [2] slow growth or inactivation of *L. monocytogenes* did not allow estimation of lag phase or shoulder duration; [3] lower asymptote estimated by the Baranyi and Roberts model; [4] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

PL: potassium lactate, SD: sodium diacetate, LA: lactic acid, AA: acetic acid, ALF: activated lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.23: Mean growth kinetics of *Listeria monocytogenes* growth on the surface of bologna slices with or without antimicrobials in the formulation, inoculated with the pathogen after slicing and stored at 4°C for 95 days.

Treatment	Lag phase duration (days)	Maximum growth rate (log CFU/cm^2/day)	Y_0[2] (log CFU/cm^2)	Y_{end}[3] (log CFU/cm^2)
Control	-[1]	0.146_A	2.5	6.8
PL (1.8%)	70.3_A	0.048_A	1.7	6.0
LF (0.5%)	3.1_B	0.077_A	2.0	7.0
LF (1%)	1.5_B	0.134_A	1.9	7.1
PL (1.8%) + SD (0.125%)	76.7_A	0.084_A	1.9	1.8
PL (1.8%) + LF (0.5%)	45.6_A	0.164_A	2.0	6.7
SD (0.125%) + LF (0.5%)	2.7_B	0.189_A	2.0	8.0
PL (1.8%) + SD (0.125%) + LF (0.5%)	69.50_A	0.114_A	2.1	-
PL (1.8%) + SD (0.0625%) + LF (0.25%)	54.0_A	0.047_A	2.0	-

[1] no lag phase observed (growth was immediate); [2] lower asymptote estimated by the Baranyi and Roberts model; [3] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin,

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.24. Mean growth kinetics of *Listeria monocytogenes* growth on the surface of bologna slices with or without antimicrobials in the formulation, inoculated with the pathogen after slicing and stored at 7°C for 57 days.

Treatment	Lag phase duration (days)	Maximum growth or inactivation rate (log CFU/cm^2/day)	Y_0[3] (log CFU/cm^2)	Y_{end}[4] (log CFU/cm^2)
Control	-[1]	0.299_A	3.1	7.2
PL (1.8%)	14.2_A	0.106_{BE}	2.1	5.2
LF (0.5%)	-[1]	0.148_{BC}	2.1	6.2
LF (1%)	-[1]	0.162_{CF}	2.2	7.2
PL (1.8%) + SD (0.125%)	-[2]	-0.023_D	2.1	1.9
PL (1.8%) + LF (0.5%)	6.4_A	0.069_E	2.1	-
SD (0.125%) + LF (0.5%)	-[1]	0.208_{FG}	2.3	7.9
PL (1.8%) + SD (0.125%) + LF (0.5%)	28.7_B	0.142_{BC}	1.9	-
PL (1.8%) + SD (0.0625%) + LF (0.25%)	-[1]	0.299_A	2.1	7.2

[1] no lag phase observed (growth was immediate); [2] slow growth or inactivation of *L. monocytogenes* did not allow estimation of lag phase or shoulder duration; [3] lower asymptote estimated by the Baranyi and Roberts model; [4] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

PL: potassium lactate, LF: lactoferrin, SD: sodium diacetate

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

Table 3.25. Mean growth kinetics of *Listeria monocytogenes* growth on the surface the surface of frankfurters formulated with or without antimicrobials, inoculated with *Listeria monocytogenes*, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Treatment (formulation)	Lag phase or shoulder duration (days)	Maximum growth or inactivation rate (log CFU/cm^2/day)	Y_0[4] (log CFU/cm^2)	Y_{end}[5] (log CFU/cm^2)
None	Control	12.0_A	0.155_A	1.9	7.9
	PL (1.8%) + SD (0.125%)	42.0_B[3]	-0.488_B	2.1	1.9
	LF (0.5%)	17.7_C	0.211_A	2.3	6.6
	PL (1.8%) + LF (0.5%)	-[2]	-0.005_C	2.0	-
AA (2%)	Control	44.6_B	0.172_A	0.8	1.9
	PL (1.8%) + SD (0.125%)	-[1]	-0.117_{BC}	1.2	0.4
	LF (0.5%)	49.1_D[3]	-0.040_C	1.0	-
	PL (1.8%) + LF (0.5%)	-[2]	-0.021_C	1.0	0.6
ALF (2%)	Control	-[1]	0.165_A	0.6	6.9
	PL (1.8%) + SD (0.125%)	42.5_B	0.180_A	0.4	-
	LF (0.5%)	-[1]	0.141_A	0.9	-
	PL (1.8%) + LF (0.5%)	-[2]	-0.011_C	0.7	0.0

[1] no lag phase or shoulder observed (growth or inactivation was immediate); [2] Slow growth or inactivation of *L. monocytogenes* did not allow estimation of lag phase or shoulder duration; [3] lag phase is regarded as shoulder period due to subsequent inactivation of *L. monocytogenes*; [4] lower asymptote estimated by the Baranyi and Roberts model; [5] upper asymptote estimated by the Baranyi and Roberts model; no y_{end} value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to stationary phase

AA: acetic acid, ALF: activated lactoferrin, PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

Means within a column lacking a common subscript letter are significantly different ($P < 0.05$)

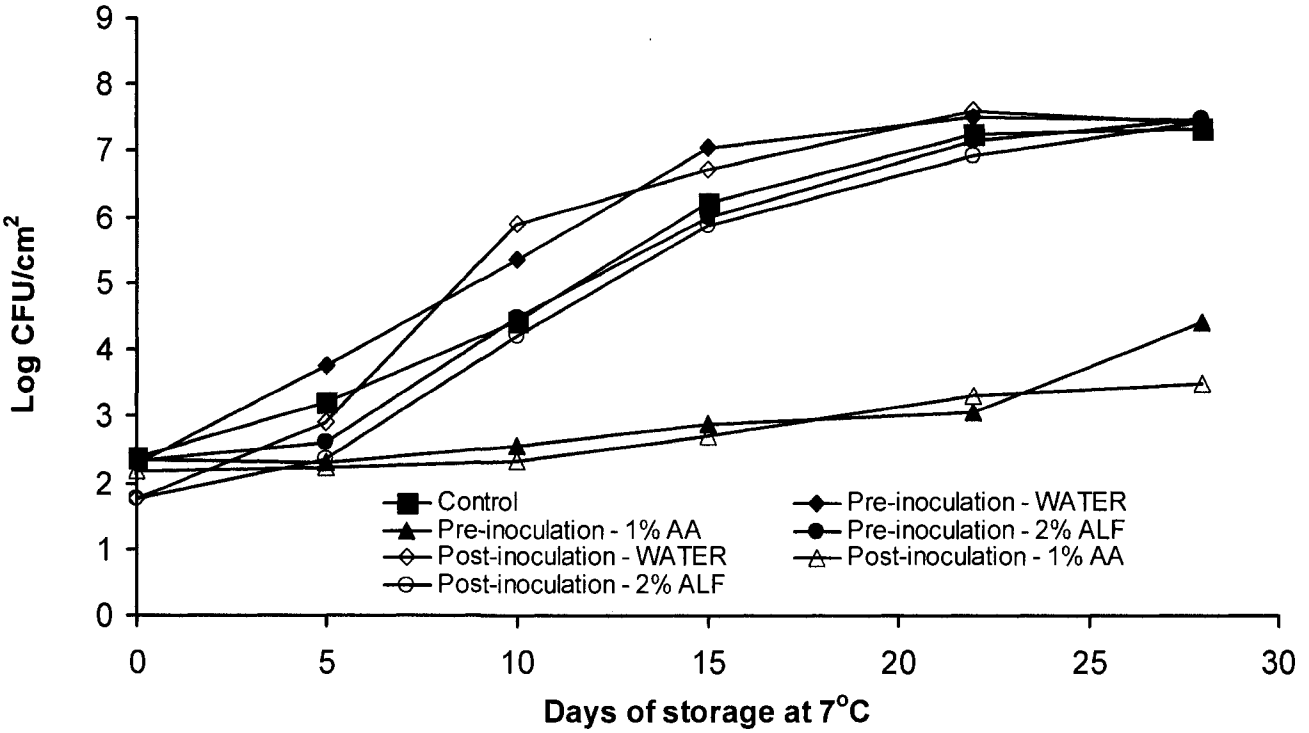


Figure 3.1 (Appendix Table 1). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of commercial ham slices, inoculated with the pathogen before or after dipping into (except control) water (for 2 min), acetic acid (for 30 s), or activated lactoferrin (for 120 s), vacuum packaged, and stored at 7°C. AA, acetic acid; ALF, activated lactoferrin.

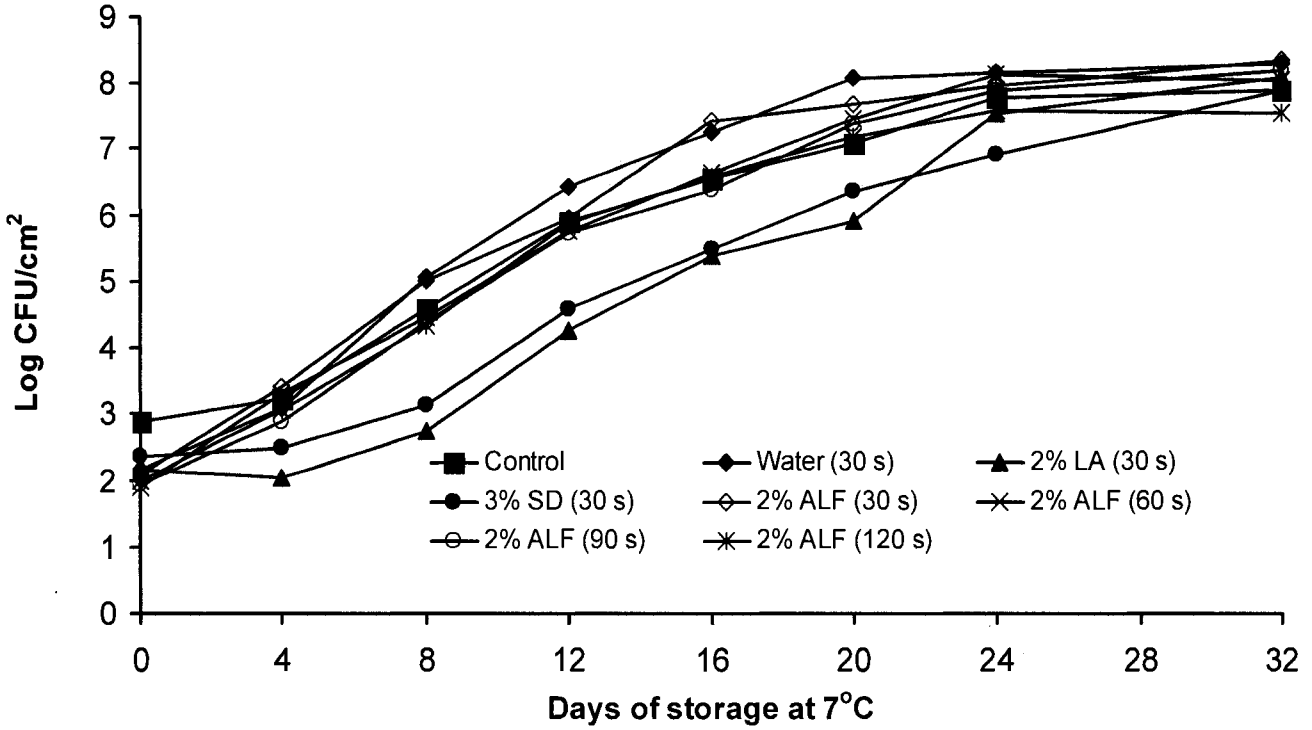


Figure 3.2 (Appendix Table 2). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of frankfurters, inoculated with the pathogen and left undipped or dipped into distilled water (for 30 s), lactic acid (for 30 s), sodium diacetate (for 30 s), or activated lactoferrin (for 30, 60, 90, or 120 s), vacuum packaged, and stored at 7°C. LA, lactic acid; SD, sodium diacetate; ALF, activated lactoferrin.

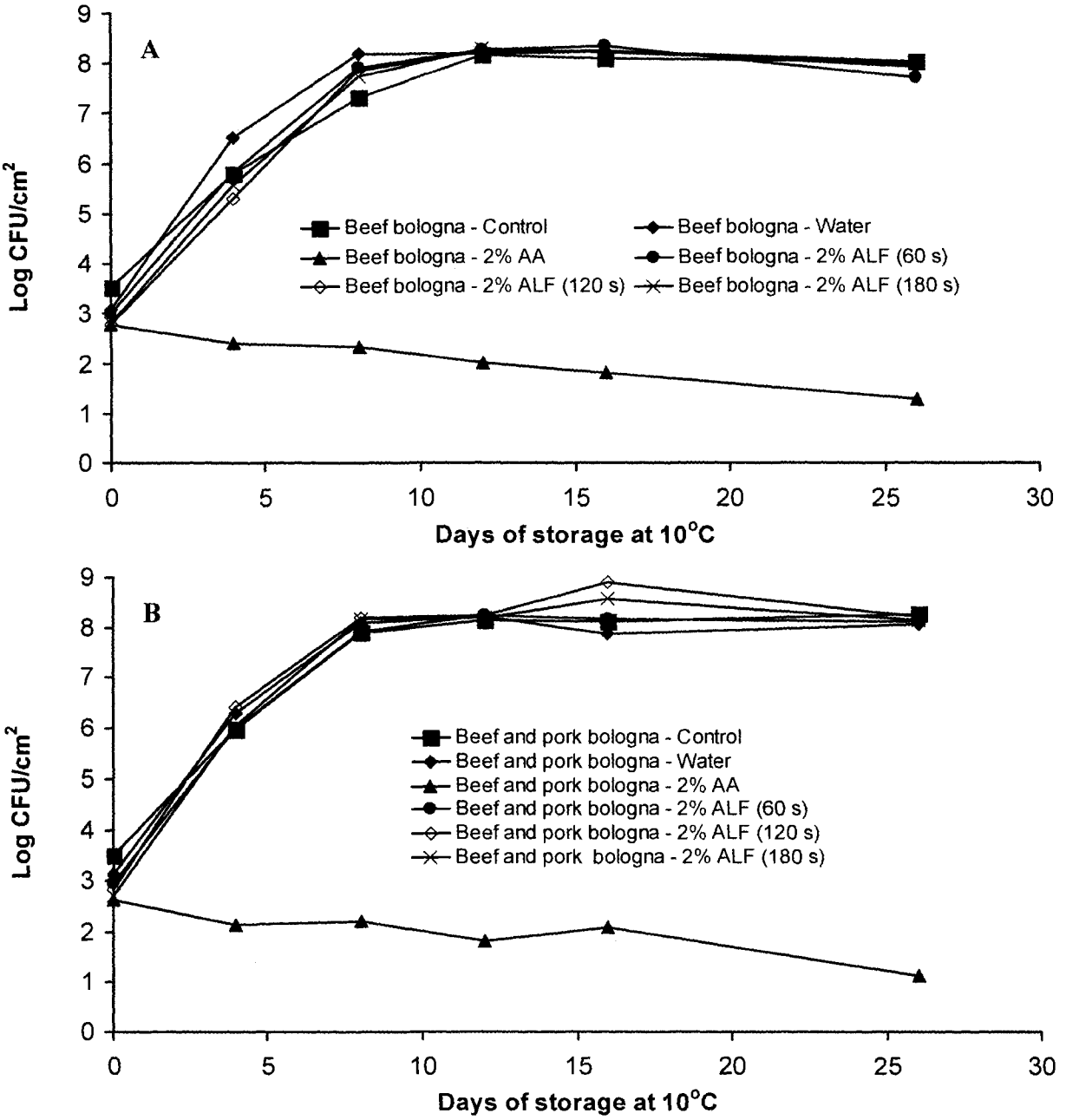


Figure 3.3 (Appendix Table 3). Mean[1] populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of bologna slices, formulated with beef (A) or beef and pork (B), inoculated with the pathogen and left undipped or dipped into distilled water (for 120 s), acetic acid (for 60 s), or activated lactoferrin (for 60, 120, or 180 s), vacuum packaged, and stored at 10°C. AA, acetic acid; ALF, activated lactoferrin.

[1] For each product, means (n=4) were calculated from pooled values (log CFU/cm^2) from two experiments, each one using a different *L. monocytogenes* inoculum composite, since preliminary analysis of fixed effects using the general linear models (GLM) procedure of SAS (SAS, 2002) indicated no significant effects of the inoculum type.

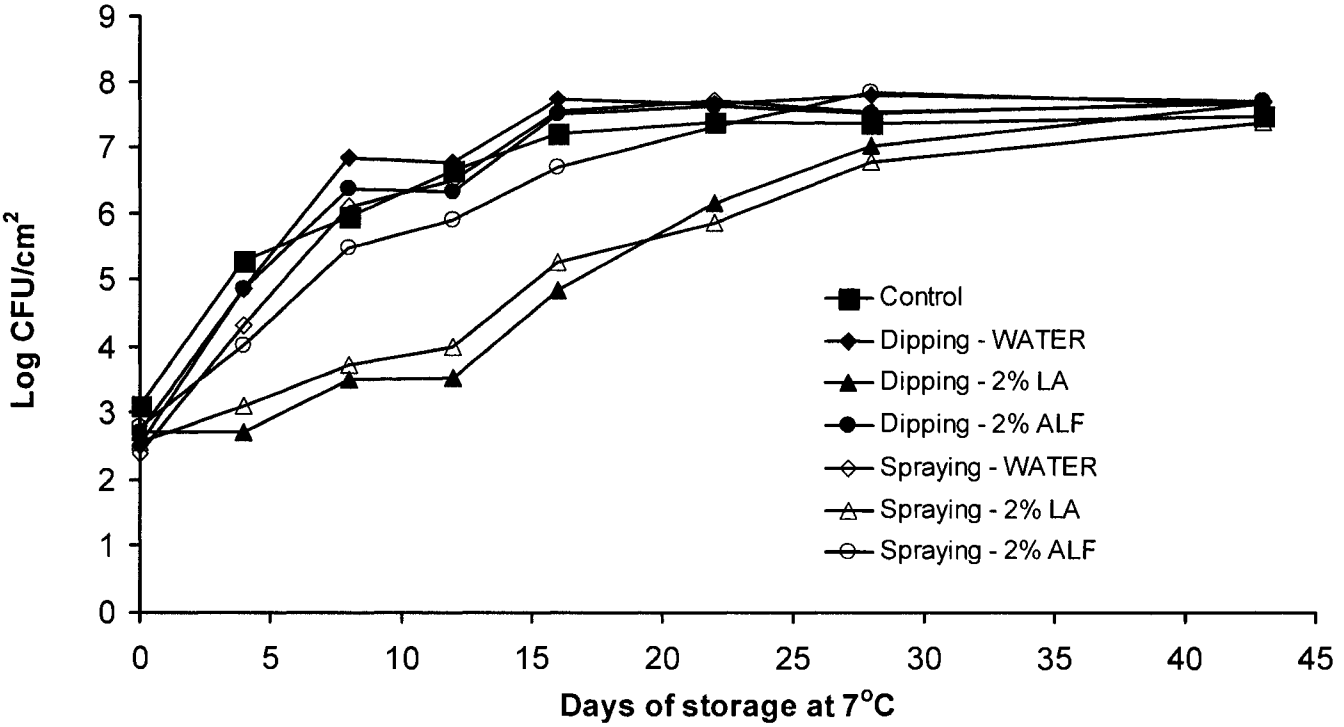


Figure 3.4 (Appendix Table 4). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of bologna slices, inoculated with the pathogen and dipped in (for 30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control), vacuum packaged and stored at 7°C. LA, lactic acid; ALF, activated lactoferrin.

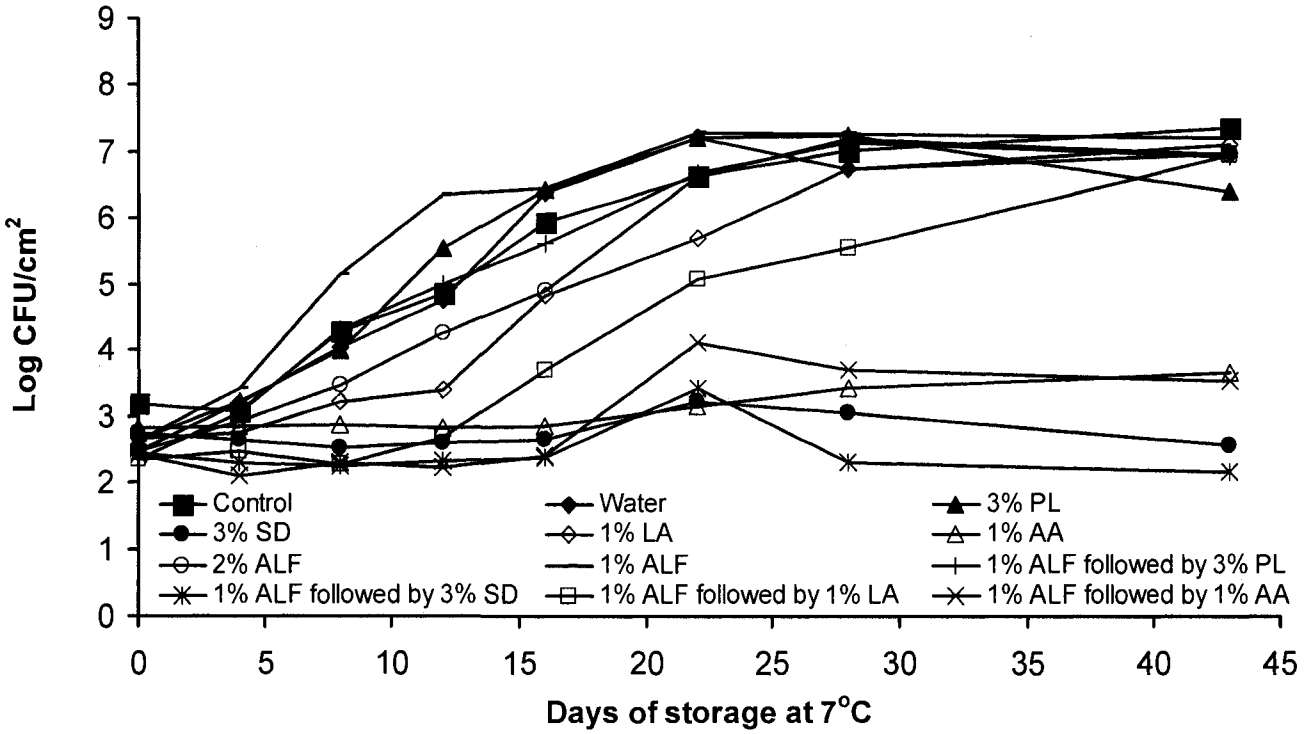


Figure 3.5 (Appendix Table 5). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of commercial turkey breast slices, inoculated with the pathogen and dipped into water or antimicrobial solutions for 60 s (except control), vacuum packaged and stored at 7°C. PL, potassium lactate; SD, sodium diacetate; LA, lactic acid; AA, acetic acid; ALF, activated lactoferrin.

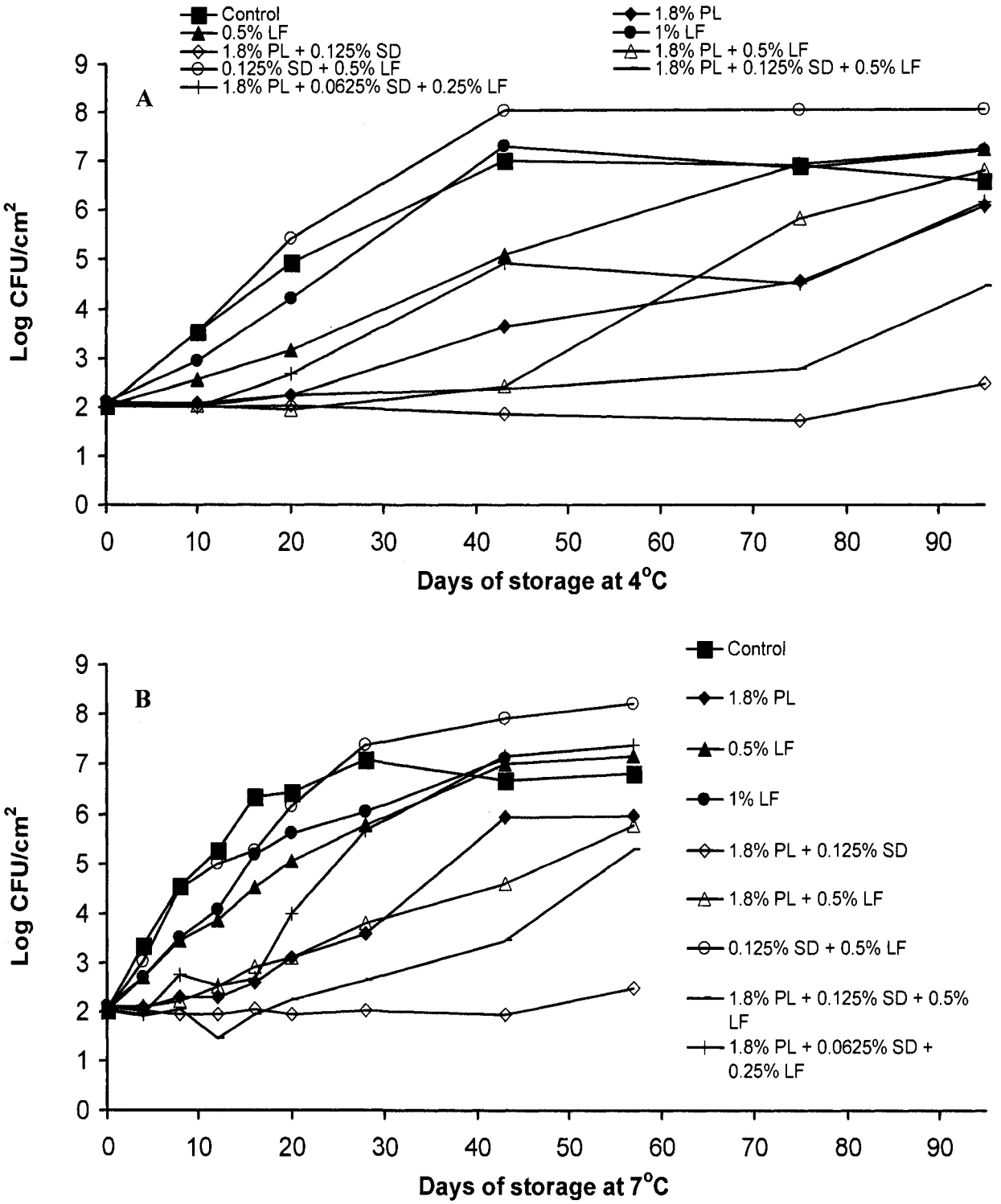


Figure 3.6 (Appendix Tables 6 and 7). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of bologna, formulated with or without antimicrobials, inoculated with the pathogen after slicing, vacuum packaged and stored at 4°C (A) or 7°C (B). PL, potassium lactate; SD, sodium diacetate; LF, lactoferrin.

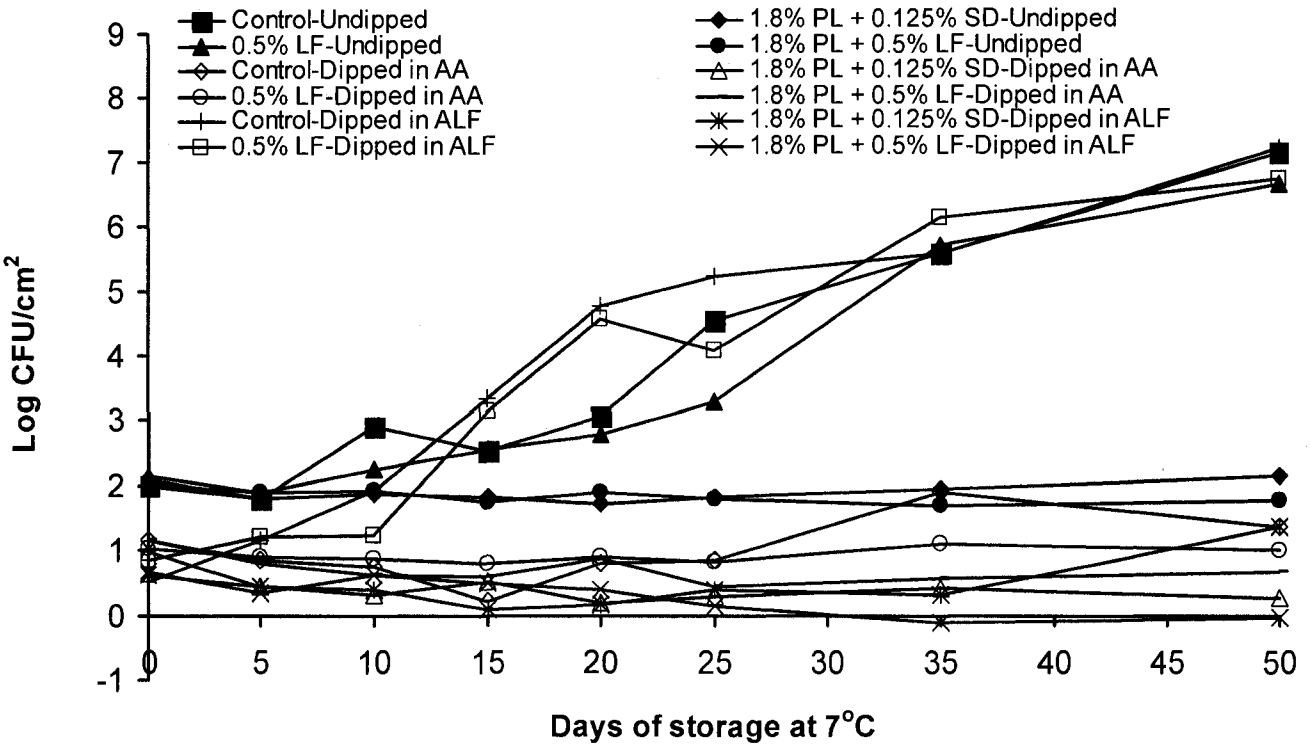


Figure 3.7 (Appendix Table 8). Mean populations of *Listeria monocytogenes* (log CFU/cm^2) on the surface of frankfurters, formulated with or without antimicrobials, inoculated with the pathogen, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 2 min, vacuum packaged and stored at 4°C (A) or 7°C (B). PL, potassium lactate; SD, sodium diacetate; LF, lactoferrin; AA, acetic acid; ALF, activated lactoferrin.

CHAPTER 4

Differences in survival among thirteen *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine

ABSTRACT

Thirteen *Listeria monocytogenes* strains (including 10403S and its Δ*sigB* derivative, A1-254), representing different serotypes (1/2, 1/2a, 4a, 4b) and three genotypic lineages, were compared for their ability to withstand transit through the gastrointestinal tract using a dynamic model of the stomach and small intestine. The survival of each *L. monocytogenes* strain was determined (PALCAM agar, tryptic soy agar plus 0.6% yeast extract) during the simulated gastrointestinal challenge (gastric exposure: 120 min; intestinal exposure: 240 min) under physiological conditions (e.g., 37°C, gastric emptying, gastrointestinal fluid secretion rates, gradual gastric acidification and intestinal pH maintenance), following mixing of 4 or 16-hour cultures (tryptic soy broth without dextrose plus 0.6% yeast extract) with artificial saliva. Inactivation curves in each gastrointestinal compartment were fitted using the Baranyi and Roberts model. Strain-to-strain differences in gastric survival were observed, as gastric inactivation rates (IR; log CFU/ml/min) ranged from 0.002 to 0.262 and from 0.009 to 0.221, for 4 and 16-hour cultures, respectively. However, most strains exhibited major ($P < 0.05$) reductions in populations mainly after 90 min of gastric challenge. Thus, *L. monocytogenes* cells

delivered to the intestine during the first 60 min of gastric exposure (pH $\geq$ 3) were virtually unaffected by gastric acidity. Subsequent intestinal IR were lower than gastric IR and varied from 0.004 to 0.016 log CFU/ml/min (4-hour cultures) and 0.000 to 0.021 log CFU/ml/min (16-hour cultures). Although clinical isolates C1-056 and Scott A displayed the highest gastric sensitivity, levels recovered from the intestine were generally > 6 log CFU/ml, even after the 240-min challenge. Significant serotype- and lineage-related effects in intestinal survival were identified (16-h cultures only), with serotype 4b isolates possessing slower ($P < 0.05$) intestinal IR than serotype 1/2 isolates, while grouped isolates of lineage 2 exhibited faster ($P < 0.05$) IR, as compared to those of lineage-1 or lineage-3 isolates.

4.1. Introduction

Listeria monocytogenes, the etiological agent of listeriosis is a ubiquitous microorganism, frequently isolated from various food products (Farber and Peterkin, 1991). Listeriosis is a relatively uncommon illness, despite the apparent frequent exposure of humans to the pathogen (Ben Embarek, 1994; Beuchat, 1996; Hitchens, 1996), and although the occurrence of foodborne listeriosis is evidently affected by the immune status of the human host (Gellin and Broome, 1989; Farber and Peterkin, 1991; Rocourt, 1994), characteristics of the particular pathogenic strain are also thought to be involved in pathogenesis (Ryser, 1999). Strains of *L. monocytogenes* are quite diverse in terms of their serological and molecular features, while epidemiological data, invasion assays, and food surveys have indicated that different serotypes and genetic groups display great diversity in virulence (Wiedmann et al., 1997; Barbour et al., 2001; Kathariou, 2002; Olier et al., 2002; Sauders and Wiedmann, 2007) and environmental

distribution (Kathariou, 2002; Gray et al., 2004; Sauders and Wiedmann, 2007). Out of the thirteen serotypes identified within the species, only three (4b, 1/2a and 1/2b) are responsible for most cases of human listeriosis throughout the world (Gellin and Broome, 1989; Farber and Peterkin, 1991; Rocourt et al., 2000); among these, serotype 4b is the serotype accountable for most outbreaks of invasive listeriosis (Farber and Daley, 1994; McLauchlin, 1997). Indications of heterogeneity among evolutionary groups or lineages with respect to their environmental occurrence and/or virulence potential have also been presented (Rasmussen et al., 1995; Piffaretti et al., 1989; Brosch et al., 1993; Graves et al., 1994; Rasmussen et al., 1995; Wiedmann et al., 1997; Norton et al., 2001; Gray et al., 2004; Ward et al., 2004; Zhou et al., 2005).

Variations among *L. monocytogenes* strains, relative to their physiological responses may contribute, to some extent, to the virulence heterogeneity described above. That is because tolerating stressful conditions is integral for bacterial survival in stressful microenvironments within the host, such as the digestive tract (Davis et al., 1996; Gahan and Hill, 2005). In healthy individuals, the acidic environment of the stomach is one of the most important defense barriers against foodborne infection (Howden and Hunt, 1986; Smith, 2003). Subsequently, pathogens that survive in the gastric environment and reach the small intestine in a viable state must withstand the presence of bile and volatile fatty acids, high osmolarity and low oxygen conditions (Begley et al., 2002; Gahan and Hill, 2005). Strain differences have been identified in terms of resistance to stresses encountered within the human host, such as acidity (Dykes and Moorhead, 2000; Faleiro et al., 2003; Uyttendaele et al., 2004; Francis and O' Beirne, 2005; Liu et al., 2005; Lianou et al., 2006), high osmolarity (Faleiro et al., 2003; Uyttendaele et al., 2004) and

bile (Olier et al., 2004). Roering et al. (1999) observed differences among three *L. monocytogenes* strains in their ability to survive in simulated gastric fluid. In addition to strain-to-strain variation, the growth phase of *L. monocytogenes* cells may also influence the ability of the pathogen to tolerate the adverse conditions of the gastrointestinal tract, as numerous studies (Rees et al., 1995; Davis et al., 1996; O'Driscoll et al., 1996; Cheroutre-Vialette et al., 1998; Phan-Thanh and Montagne, 1998; Jørgensen et al., 1999; Cheroutre-Vialette and Lebert, 2000; King et al., 2003) have shown that exponential phase *L. monocytogenes* cells are generally more stress-susceptible than cells in stationary phase. Similarly, King et al. (2003) showed that exponential-phase cells of *L. monocytogenes* were more susceptible to simulated gastric fluid or bile salts, as compared to stationary-phase cells, while Begley et al. (2002) observed that exponential-phase *L. monocytogenes* strain LO28 exhibited great susceptibility to unconjugated bile acids.

Various aspects of foodborne *L. monocytogenes* infection have been examined using artificial gastric or intestinal fluid broth systems (Roering et al., 1999; Phan-Thanh et al., 2000; Cotter et al., 2001; Begley et al., 2002; King et al., 2003; Olier et al., 2004; Wonderling and Bayles, 2004; Stopforth et al., 2005). Utilization of these static models, however, may not reflect the specific stages of the *L. monocytogenes* survival in the digestive tract, since they do not account for the successive gastrointestinal stresses and the constantly shifting conditions to which pathogens are subjected during transit through the human digestive tract, and/or neglect to simulate major parameters of digestion, such as pH changes, temperature conditions, gastric emptying or secretion of precise physiological amounts of digestive juices and enzymes. Simulation of the sequential conditions, encountered by *L. monocytogenes* during gastrointestinal transit, may be

essential, since prior exposure to one form of sublethal stress may affect the pathogen's tolerance against subsequent homologous or heterologous stresses (Farber and Brown, 1990; Yen et al., 1991; Farber and Pagotto, 1992; Davis et al., 1996; Lou and Yousef, 1996; O'Driscoll et al., 1996; Faleiro et al., 2003). Consequently, exposure of *L. monocytogenes* to the acidic conditions of the stomach may affect its survival in the intestine. Indeed, Begley et al. (2002) indicated that subjecting *L. monocytogenes* to sublethal levels of bile acids, acid, heat, salt, or sodium dodecyl sulfate increased its ability to tolerate bile. Exposing *L. monocytogenes* to conditions that mimic the physiological patterns found *in vivo* could accurately pinpoint factors that may affect the gastrointestinal survival of the pathogen, including differences among strains. Such an approach has been used previously in numerous studies conducted to reproduce *in vivo* data of the human digestive tract, test the absorption of environmental contaminants, establish the acceptable intake of drug residues, study the behavior of drug forms under various physiological conditions, investigate the formation of carcinogenic compounds, and examine the ability of pathogenic or beneficial bacteria to survive in the gastrointestinal system (Beumer et al., 1992; Nouws et al., 1994; McConville et al., 1995; Minekus et al., 1995; Hack and Selenka, 1996; Marteau et al., 1997; Koo et al., 2001; Krul et al., 2004; Blanquet et al., 2004; Mainville et al., 2005). Moreover, studies by Gänzle et al. (1999) and Bernbom et al. (2006) evaluated the survival properties of *L. innocua* and *L. monocytogenes*, respectively, in the presence of bacteriocin-producing lactic acid bacteria using dynamic gastrointestinal models. To our knowledge, however, no other studies have investigated factors that may affect the gastrointestinal survival of

L. monocytogenes in an artificial model that closely simulates the physico-chemical events prevailing in the human stomach and upper intestine.

The objective of this study was to examine differences in gastric and intestinal survival among 13 *L. monocytogenes* strains, representing different serotypes and three genotypic lineages (Wiedmann et al., 1997), using an *in vitro* dynamic model of the human stomach and small intestine.

4.2. Materials and methods

4.2.1. Bacterial strains and growth conditions

Thirteen strains of *L. monocytogenes* were used in this study. A brief description of each strain is available in Table 4.1. Strains studied belonged to four serotypes, with serotypes 1/2a and 4b being the most common (five and six strains, respectively) and three genetic lineages (lineage 1, five strains; lineage 2, five strains; lineage 3, two strains; one strain of unknown lineage) (Table 4.1). Among these strains, three sets (a food and a human isolate linked to the same outbreak or sporadic case of human listeriosis; R2-500 and R2-501, N1-227 and N1-225, and N3-031 and J1-101) originated from the International Life Sciences Institute (ILSI) North America outbreak set (Fugett et al., 2006) and were kindly provided to us by Dr. Martin Wiedmann (Department of Food Science, Cornell University, Ithaca NY). The *L. monocytogenes* strain collection tested in this study also included the wild-type strain 10403S (serotype 1/2a, lineage 2) and its Δ*sigB* derivative, A1-254, gifts from Dr. Kathryn J. Boor (Department of Food Science, Cornell University, Ithaca NY). *L. monocytogenes* A1-254 originated from wild-type strain 10403S by creating a 600-bp *sigB* fragment having an in-frame 297-bp deletion between nucleotides 1490 and 1788 of the *sigB* allele (Wiedmann et al., 1998).

The deleted gene *sigB* encodes for the stress-responsive alternative sigma factor that contributes to the ability of Gram-positive bacteria, including *Bacillus subtilis* and *L. monocytogenes*, to tolerate unfavorable conditions.

Frozen (-70°C) stock cultures of each isolate were maintained in tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks MD) supplemented with 0.6% yeast extract (YE, Acumedia, Baltimore MD) with 20% glycerol added. Bacterial cells were resuscitated by transferring a loopful of stock culture into 10 ml TSBYE and incubating at 30°C for 24 h. The following day, the same procedure was repeated and a loopful of the resulting culture was used to generate tryptic soy agar (TSA, Difco) supplemented with 0.6% YE (TSAYE) slants that were used as working cultures for this experiment. Slants were kept at 4°C and were tested for purity by streaking on TSAYE and PALCAM agar (Difco).

A loopful of bacterial cells from the appropriate slant was transferred into 10 ml TSBYE and grown overnight (22-24 h) at 30°C. From the resulting broth culture, 1 ml was used to inoculate 100 ml of TSB without dextrose (Difco) supplemented with 0.6% YE (TSBYE-G) to yield an initial *L. monocytogenes* cell density of 6.9-7.2 log CFU/ml in TSBYE-G, as determined by serially diluting 1 ml of broth with 10 ml of buffered peptone water (BPW, Difco), immediately upon inoculation, and surface plating onto TSAYE and PALCAM agar (30°C for 48 h). The new TSBYE-G culture was incubated at 30°C for 4 or 16 h. In addition, separate experiments were conducted to monitor the growth of individual *L. monocytogenes* strains during incubation at 30°C for up to 5 or 24 h (1 h intervals for 5 h; 8-10 h intervals for 24 h).

4.2.2. Preparation of simulated gastrointestinal fluids

Artificial saliva was prepared by suspending 6.2 g NaCl, 2.2 g KCl, 0.22 g $CaCl_2$, and 1.2 g $NaHCO_3$ in 1 liter of distilled water (Minekus et al., 1995; Marteau et al., 1997). The solution was sterilized by autoclaving and cooled to ambient temperature (25°C) before use. Simulated gastric fluid contained (Molly et al., 1994; Naim et al., 2004): 0.4 g/liter glucose, 3.0 g/liter yeast extract, 1.0 g/liter Bacto Peptone (Difco, Becton, Dickinson), 4.0 g/liter porcine mucin (Sigma-Aldrich), 0.5 g/liter cysteine, 0.08 g/liter NaCl, 0.4 g/liter $NaHCO_3$, 0.04 g/liter K_2HPO_4, 0.04 g/liter KH_2PO_4, 0.008 g/liter $CaCl_2$-$2H_2O$, 0.008 g/liter $MgSO_4 \cdot 7\ H_2O$, 1.0 g/liter xylan (Sigma-Aldrich, St. Louis, MO), 3.0 g/liter soluble starch (Sigma-Aldrich), 2.0 g/liter pectin (Sigma-Aldrich), and 1 ml/liter Tween 80. The ingredients were mixed thoroughly and the solution was autoclaved and cooled to ambient temperature (25°C), followed by the addition of 3 g/liter pepsin from porcine stomach mucosa (Sigma-Aldrich). Before use in the gastrointestinal challenge, the pH of the gastric fluid was adjusted to 2 using 5N HCl.

Artificial intestinal fluid (Koo et al., 2001) was made by diluting 0.1 g trypsin from porcine pancreas (type IX-S; Sigma-Aldrich) and 3.5 g pancreatin from porcine pancreas (Sigma-Aldrich) with 1 liter distilled water. The solution was filtered through a 0.45-μm pore-diameter filter (cellulose, Millipore Corp., Bedford, MA), for sterilization purposes, and added into a sterile flask. Biliary secretions were simulated by preparing 2% or 4% bile solutions (porcine bile extract, Sigma-Aldrich) in distilled water, which were then filter-sterilized, as described above. Porcine (rather than ox gall) bile was selected due to its similarities to human bile (Marteau et al., 1997).

4.2.3. Dynamic gastrointestinal system

A dynamic model of the stomach and small intestine, previously described by Koo et al. (2001), was used as the basis of the simulated gastrointestinal tract employed in this study. Two 500-ml Erlenmeyer flasks, representing the gastric (GC) and the intestinal (IC) compartments were kept in a water bath (Shaking Water Bath 50, Precision Scientific, Chicago IL), stabilized at 37°C throughout the gastrointestinal challenge. The pH conditions in the GC and IC were monitored continuously using two pH meters (Ultra Basic, Denver Instrument, Arvada, CO) equipped with electrodes (Denver Instrument). Peristaltic pumps (Variable-Speed Pump Low Flow, Fisher Scientific) were used for the delivery of simulated gastric fluid in the GC (at a flow rate of 0.33 ml/min) and simulated intestinal fluid (at a flow rate of 0.33 ml/min) and bile solution (at a flow rate of 0.5 ml/min) in the IC. To simulate physiological conditions prevailing in the human intestine (Northfield and McColl, 1973; Fausa, 1974), a 4% solution of bile was delivered in the IC during the first 30 min of the experiment, followed by the addition of a 2% bile solution for the remaining time (Minekus et al., 1995). The two compartments were connected by a multi-channel peristaltic pump (205U, Watson-Marlow Limited, Cornwall, England) that was used for transferring the gastric contents into the IC (gastric emptying) at a flow rate of 1.1 ml/min, based on results of a study by Doran et al. (1998) concluding that the gastric emptying rate after the consumption of a small meal (217 g) was 1.1 g/min, when subjects were sitting. Introduction of the gastric contents into the IC started 15 min after the beginning of the challenge.

4.2.4. Gastrointestinal passage tolerance assay

Cultures of individual strains, incubated for 4 or 16 h were diluted (1:1, vol/vol) with artificial saliva. The pH of each culture was measured before and after mixing with saliva by inserting a pH electrode (previously sprayed with 70% alcohol, rinsed with sterile distilled water and drained) in the bottle containing the broth. Prior to the beginning of each challenge, 10 ml of simulated gastric fluid were added in the GC to simulate the cephalic phase of acid secretion, whereas the IC contained 7 ml of the 4% bile solution (Minekus et al., 1995). All pumps were started upon addition of the broth-saliva mixture in the GC. The pH of the GC was adjusted manually by adding 5N HCl to reproduce *in vivo* human gastric pH values after ingestion of a standard meal (Dressman et al., 1990): pH 5 at 10 min, pH 4 at 28 min, pH 3 at 58 min, and pH 2 at 88 min. After the final adjustment, the pH in the GC remained constant until the end of the challenge (120 min). The acidified gastric contents were neutralized upon transfer in the IC, as the intestinal pH was maintained at 6.5±0.3 (Minekus et al., 1995; Marteau et al., 1997) via addition of 0.3 M $NaHCO_3$.

Initial (0 min) populations of *L. monocytogenes* were assessed before the introduction of the cell suspension in the GC (before mixing with saliva), by withdrawing 1-ml samples, serially diluting, and plating (PALCAM and TSAYE) as described above. Surviving *L. monocytogenes* populations were enumerated at 15, 30, 60, 90, and 120 min in the GC, and at 30, 60, 90, and 120 min in the IC, by removing 1-ml samples, diluting, and plating in duplicate (PALCAM and TSAYE). Secretion of gastrointestinal fluids in the GC (gastric fluid) and IC (bile and intestinal fluid), as well as gastric emptying continued for 120 min after the beginning of each challenge; however, the IC was

maintained (statically) in the water bath (37°C) for a final microbiological analysis at 240 min. All plates were incubated at 30°C for 48 h and colonies were counted manually.

4.2.5. Statistical analyses

The gastrointestinal challenge was conducted three times for each 4-h or 16-h culture. Cell counts were divided by dilution factors, to account for the continuous addition or removal of gastrointestinal fluids in each compartment (Koo et al., 2001). Dilution factors (DF) were calculated as follows:

$$\text{DF (GC)} = \frac{\text{Remaining sample (ml)}}{\text{Remaining sample (ml)} + \text{HCl (ml)} + \text{Gastric fluid (ml)}}$$

$$\text{DF (IC)} = \frac{\text{Total IC contents (ml)}}{\text{Total IC contents (ml)} + \text{gastric contents (ml)} + \text{bile (ml)} + \text{intestinal fluid (ml)} + \text{NaHCO}_3\text{ (ml)}}$$

The numbers obtained were converted into log CFU/ml and analyzed using the Glimmix Procedure of SAS (SAS, 2002) to determine significant differences ($P < 0.05$) among *L. monocytogenes* strains, with respect to their ability to survive in each compartment of the gastrointestinal system. Independent variables included strain, time and age of the culture and their interactions. Means and standard deviations were calculated and the mean differences were separated at the significance level of 95%.

In addition, *L. monocytogenes* data (log CFU/ml) from each of the three replications were fitted to the model of Baranyi and Roberts (1994) using DMFit software, the in-house program of the Institute of Food Research (Norwich, UK), to determine the shoulder duration (SD) and inactivation rate (IR) of 4- or 16-h cultures of each strain in each gastrointestinal compartment. Inactivation kinetics (SD and IR) were derived from

PALCAM counts, as occasional fitting of the TSAYE data indicated no considerable differences between the kinetic profiles derived from PALCAM or TSAYE counts. Statistical analyses of inactivation kinetics (SD and IR) were performed using the Mixed Procedure of SAS (SAS, 2002), with strain being the independent variable. To determine potential serotype- or lineage-related effects on the gastrointestinal resistance of the pathogen, additional analyses were conducted with the Mixed Procedure of SAS (independent variables were serotype or lineage) after grouping strains according to their serotype or lineage.

4.3. Results

4.3.1. Gastric survival

Viable *L. monocytogenes* populations (PALCAM agar; log CFU/ml) in the GC, graphed together with the gradual gastric pH decline are presented in Figure 4.1. The initial (before mixing with saliva) pH of 4-h *L. monocytogenes* cultures varied from 6.97±0.10 to 7.29±0.21, while 16-h cultures had pH values that ranged from 6.46±0.10 to 6.65±0.03 (Figure 4.1). Although, the pH of 16-h cultures was lower than that of 4-h cultures, it was not sufficiently low to induce considerable resistance to acid (Davis et al., 1996). Moreover, addition of artificial saliva did not result in considerable changes (< 0.2 units; data not shown) of the pH of *L. monocytogenes* cultures. Cell counts of *L. monocytogenes* strains, achieved within 4 and 16 h of incubation (TSBYE-G) at 30°C, ranged from 7.4 to 8.0 log CFU/ml (not including strain A1-254) and from 8.6 to 9.1 log CFU/ml, respectively. Monitoring the growth of individual *L. monocytogenes* during incubation of TSBYE-G suggested that, with the exception of A1-254, all cultures had reached exponential and stationary phase within 4 and 16 h. As suggested by cell

densities, reached by each strain, the Δ*sigB* mutant strain A1-244 exhibited no apparent changes in its populations during the 4-h incubation, suggesting that σ^B may affect the growth potential of the pathogen even at optimal temperatures (30°C).

During the first 60 min of gastric exposure, all *L. monocytogenes* strains exhibited minor ($P \geq 0.05$) reductions (≤ 0.5 log CFU/ml for both 4- and 16-h cultures) in initial (0 min) populations, since the pH of gastric contents during that period was ≥ 3 (Figure 4.1). Regarding 4-h cultures, significant ($P < 0.05$) reductions in populations occurred after the gastric pH was reduced to 2 (90 min) only in strain C1-056. Within 90 min of gastric exposure, reductions in populations were more pronounced for 16-h cultures, as numbers of all *L. monocytogenes* strains (except R2-500 and R2-501) were significantly ($P < 0.05$) reduced. Nevertheless, all strains survived gastric exposure for 90 min with populations remaining as high as 6.4 to 7.6 log CFU/ml and 7.3 to 8.7 log CFU/ml, for 4- and 16-h cultures, respectively (Figure 4.1). Major ($P < 0.05$) reductions in *L. monocytogenes* gastric survival were observed particularly after 120 min of the gastric challenge (30 min at pH 2), and at that point, differences in the acid tolerance among strains became more obvious. In general, the majority of *L. monocytogenes* strains appeared susceptible to the extreme acidity prevailing in the gastric compartment. Stain C1-056 (serotype 1/2a, lineage 2) displayed the highest ($P < 0.05$) acid sensitivity among the strains tested, with initial populations being reduced by approximately 7.5 and 8.0 log CFU/ml after the 120-min gastric exposure of 4- and 16-h cultures, respectively, whereas respective reductions exhibited by the second more acid-sensitive strain, Scott A (serotype 4b, lineage 1) were approximately 6.0 and 6.5 log CFU/ml. Stains R2-500 and 10403S, as 4-h cultures, were the only ones that appeared unaffected by gastric acidity, as minor ($P \geq 0.05$) reductions

in initial numbers were observed during their 120-min exposure in the gastric environment.

Overall, the computed by the Baranyi and Roberts model y_0 values, representing the initial bacterial counts, were similar for most strains, as they ranged from 7.1 to 7.8 log CFU/ml (4-h cultures; except strain A1-254, y_0: 6.5 log CFU/ml) and from 8.2 to 8.8 log CFU/ml (16-h cultures). No y_{end} values were calculated for any of the strains since inactivation curves ceased without reaching a plateau, which in this case, would represent a tailing region (y_0 and y_{end} values are not shown in tabular form).

Since the stressful conditions prevailing in the gastrointestinal system resulted in reductions in microbial populations, calculated IR from the DMFit output were negative. In this paper, however, IR in both gastrointestinal compartments are presented without the minus (-) sign to facilitate interpretation of the findings. The calculated gastric IR (log CFU/ml/min) of *L. monocytogenes* 4-h cultures varied from 0.002±0.003 to 0.262±0.022 (Table 4.2). Clinical isolate C1-056 displayed the highest ($P < 0.05$) IR, as compared to the majority of strains tested, followed ($P \geq 0.05$) by another clinical isolate, Scott A, that had an IR of 0.209 log CFU/ml/min. However, as suggested earlier by plate counts and by the large SD (76.88 and 77.48 min for C1-056 and Scott A, respectively; Table 4.2) displayed by both these strains, reductions in their populations occurred mainly at the later stages of the challenge, at which point the pH of gastric contents had been reduced to < 3. The remaining of the tested *L. monocytogenes* strains possessed IR varying ($P \geq 0.05$) from 0.002 (strains R2-500 and 10403S) to 0.042 (strain J1-158) log CFU/ml/min. Interestingly, A1-254 displayed an IR of 0.032 log CFU/ml/min, which, although higher ($P \geq 0.05$) than that of the wild-type strain, 10403S (0.002 log CFU/ml/min), it was

significantly ($P < 0.05$) lower than that of strains C1-056 and Scott A (Table 4.2). Nevertheless, it should be noted that under the examined conditions, a valid comparison between the gastric resistance of 4-h cultures of the Δ*sigB* mutant strain, A1-254, and that of the wild-type strain 10403S may not be feasible, since, as already mentioned, the slow growth of the mutant strain prevented it from being in the same physiological state (i.e., exponential phase) as the remaining *L. monocytogenes* strains. All food isolates displayed greater IR than the respective human isolates, associated with the same outbreak; however, these differences were slight ($P \geq 0.05$) and thus, they cannot be interpreted as an indication of an origin-related trend in gastric sensitivity, under the conditions of this study.

Regarding 16-h cultures, gastric IR (log CFU/ml/min) varied from 0.009±0.001 (strain R2-500) to 0.221±0.013 (strain C1-056) (Table 4.3). As with 4-h cultures, Scott A followed ($P < 0.05$) C1-056 in gastric sensitivity with an IR of 0.197 log CFU/ml/min. Separate growth experiments suggested that both the Δ*sigB* mutant A1-254 and the parental strain 10403S were at stationary phase within 16 h of incubation, enabling the comparison between the two strains in terms of gastric survival. As a 16-h culture, the Δ*sigB* mutant strain, A1-254, was significantly ($P < 0.05$) more acid-sensitive than the parental strain 10403S and displayed an IR (0.188 log CFU/ml/min) similar ($P \geq 0.05$) to that of the acid-sensitive strain Scott A. No significant ($P \geq 0.05$) differences were observed in IR between 16-h cultures of food and human isolates from the ILSI outbreak set. Inspection of the SAS output indicated that the main effect for the duration of incubation of cultures was statistically significant ($P < 0.05$). With the exception of strains C1-056 and Scott A, IR of 16-h *L. monocytogenes* cultures were greater than those

of respective 4-h cultures (Tables 4.2 and 4.3); however, major differences in IR between 16- and 4-h cultures were displayed only by strains A1-254, J1-158, and N1-227 that appeared more ($P < 0.05$) acid-sensitive as 16-h cultures.

4.3.2. Intestinal survival

Survival of *L. monocytogenes* strains in the IC during the 240-min intestinal exposure is presented in Figure 4.2. Delivering of gastric contents in the IC began while the gastric pH was still high and, thus, viable populations of the pathogen transferred from the GC to the IC within the first 30 min of gastric emptying were correlated to the initial inoculum levels of each *L. monocytogenes* strain achieved in TSBYE-G and ranged from 5.4 to 8.1 log CFU/ml and 7.0 to 9.1 log CFU/ml, for 4- or 16-h cultures, respectively. Thus, populations of the mutant strain, A1-254 delivered in the IC within 30 min of the gastrointestinal challenge were the lowest among *L. monocytogenes* strains, since the strain exhibited the slowest growth during the 4- ($P < 0.05$) or 16-h ($P \geq 0.05$) incubation of TSBYE-G.

The calculated by the Baranyi and Roberts model y_0 values for *L. monocytogenes* strains correspond to the initial bacterial population (30 min) recovered from the IC and ranged from 6.0 (A1-254) to 7.6 (R2-500) log CFU/ml for 4-h cultures and from 7.1 (A1-254) to 8.7 (J1-158) log CFU/ml for 16-h cultures. On the other hand, mean y_{end} values were not calculated, as in many cases the survival curves of individual samples did not form a tailing region within the 240 min of the challenge (y_0 and y_{end} values are not presented in tabular form).

Reductions in populations during the intestinal challenge were not as drastic as those observed in the GC, as demonstrated by the estimated intestinal IR (log CFU/ml/min),

that ranged from 0.004 to 0.016, for 4-h cultures (Table 4.2) and 0.000 to 0.021, for 16-h cultures (Table 4.3). Strain A1-254 exhibited the greatest susceptibility to the intestinal conditions among the strains tested, as it possessed the fastest IR, both as a 4- and a 16-h culture. However, the estimated IR for A1-254 did not differ significantly ($P \geq 0.05$) than the corresponding IR of the wild-type strain (10403S). Once again, estimating the effects of σ^B in the intestinal survival of the pathogen is not possible given that the differences in growth potentials between A1-254 and 10403S during incubation in TSBYE-G led to 4-h cultures being at different physiological states. However, differences in IR ($P \geq 0.05$) observed between the16-h cultures of these strains suggest that, most likely the contribution of σ^B in the intestinal survival of the pathogen was minor. Similar to the observations from the gastric challenge, food isolates had intestinal IR similar ($P \geq 0.05$) to those of human isolates linked to the same outbreak (ILSI outbreak set), suggesting that under the tested intestinal conditions, no origin-related effects existed. Isolates of serotype 4b, as a group possessed significantly ($P < 0.05$) lower intestinal IR than combined serotype 1/2 isolates. However, this serotype-related effect on intestinal survival was present only in 16-h cultures. Similarly, significant lineage-related effects were only identified in 16-h cultures, with combined isolates of lineage 2 exhibiting higher ($P < 0.05$) IR, as compared to those of combined lineage-1 or lineage-3 isolates. Nevertheless, since all 1/2a isolates tested in this study belonged to lineage 2, identifying distinct serotype- and lineage-related effects on the *L. monocytogenes* intestinal survival was not possible.

Generally, microbial count trends on PALCAM agar and those on TSAYE were similar (< 0.3 log CFU/ml difference), throughout the gastric and intestinal challenge,

even when the pH (gastric contents) had been reduced to as low as 2 (TSAYE counts in the GC and IC are shown in Appendix Tables 21-24).

4.4. Discussion

Being able to survive the sequential hostile microenvironments of the human gastrointestinal tract is an important feature affecting the ability of foodborne pathogens to establish infection. Evidence, originating from epidemiological investigations and cell-invasion assays has led to the suggestion that *L. monocytogenes* is a diverse organism in terms of its ability to cause disease (Gellin and Broome, 1989; Rasmussen et al., 1995; Wiedmann et al., 1997; Rocourt et al., 2000; Kathariou, 2002; Gilbreth et al., 2005). In addition, many researchers have observed differences in virulence among *L. monocytogenes* strains in mouse bioassays (Conner et al., 1989; Corral et al., 1990; Lammerding et al., 1992; Brosch et al., 1993; Takeuchi et al., 2003). To our knowledge, no studies have examined strain-to-strain variations specific to the gastrointestinal phase of the *L. monocytogenes* infection. The present study tested differences in survival among 13 *L. monocytogenes* strains under conditions imitating major nonspecific defense mechanisms against foodborne pathogens, the extreme acidity of the stomach, the presence of proteolytic enzymes and the antimicrobial effects of bile. Additional defense mechanisms that were not considered in this study include specific immune responses and the presence of competitive microflora (Duncan and Edberg, 1995).

Gradual decline of gastric pH, similar to that observed *in vivo* (Dressman et al., 1990), demonstrated that *L. monocytogenes* strains may remain unaffected by the conditions in the stomach for a large period of time (60 min), irrespective of their acid susceptibility, which, under the conditions of this study, became evident under conditions of extreme

acid stress (pH $\leq$ 3). Subjecting *L. monocytogenes* to pH values ranging from 5 to 7 was previously shown to have a minor influence on the behavior of eight isolates of the pathogen (Vialette et al., 2003), whereas few publications have assessed differences in the response of *L. monocytogenes* strains under severe acidic conditions (Dykes and Moorhead, 2000; Lianou et al., 2006). Using 25 *L. monocytogenes* strains (including strains tested in the present study), Lianou et al. (2006) found considerable strain-to-strain differences during exposure to pH 3 (lactic acid) for 120 min, with strains C1-056 and Scott A displaying the greatest acid sensitivity. Findings of the present study also demonstrated that clinical isolates C1-056 and Scott A were the most sensitive under lethal acidic conditions, among the 13 *L. monocytogenes* strains tested. However, in this dynamic gastrointestinal model, gradual acidification of the stomach contents in combination with gastric emptying resulted in cells experiencing different levels of acidity, based on their residence time in the GC. Therefore, high cell numbers, even of extremely acid-sensitive strains, survived the gastric transit while the gastric pH was relatively high (> 3), and reached the small intestine in a viable state (intestinal counts at 30 min were 6.2 and 7.1 log CFU/ml for 4-h cultures and 8.7 and 8.9 log CFU/ml for 16-h cultures, for strains C1-056 and Scott A, respectively). The effects of early stages of gastric emptying on the survival of foodborne pathogens during gastric passage are of great importance, particularly after taking into consideration that the peak gastric emptying rates take place at the beginning of the digestion process (Marteau et al., 1997), a factor that was not taken into account in the present study. Dykes and Moorhead (2000) reported increased acid resistance in all clinical *L. monocytogenes* strains tested in their study, a finding that led the authors to remark on the importance of acid tolerance on the

infection process. On the contrary, clinical isolates C1-056 and Scott A were the most acid-susceptible among the 13 strains tested in this study; however, the increased survival of acid-sensitive *L. monocytogenes* isolates during the initial stages of the gastric challenge, may help elucidate their implication in human disease, particularly if high contamination levels were involved. Overall, all *L. monocytogenes* strains examined in this study (even the extremely acid-sensitive C1-056 and Scott A) displayed small reductions in populations within the IC, indicating that once crossing the gastric barrier, the pathogen should be capable of withstanding *in vivo* bile concentrations and induce intestinal colonization. Indeed, the ability of *L. monocytogenes* to withstand bile is well documented. Begley et al. (2002) observed that the pathogen tolerated levels of human, porcine, or bovine bile higher than those encountered *in vivo*. Similarly, 50 *L. monocytogenes* isolates were able to tolerate high concentrations of bile, as they grew on agar and in broth supplemented with 2 and 5%, respectively, of porcine bile salts (Olier et al., 2004).

Unlike the findings of other researchers (Davis et al., 1996; King et al., 2003) estimation of IR in this study suggested that the acid resistance of 4-h cultures (exponential phase) of *L. monocytogenes* strains was generally higher than that of 16-h cultures (stationary phase) of respective strains. Higher tolerance of *L. monocytogenes* cells at exponential phase as compared to cells at stationary phase to a specific combination of sublethal stresses (pH 5, 4% NaCl, 10°C) has been previously reported (Vialette et al., 2003). Davis et al. (1996) studied the effects of growth phase on the acid resistance of *L. monocytogenes* and concluded that mid-exponential *L. monocytogenes* cells were the most sensitive to pH 3, whereas the highest level of acid tolerance was

obtained during the transition between exponential and stationary phase. A possible explanation for the findings of the present study could be that *L. monocytogenes* cultures after 4 h of incubation were still at early stages of exponential phase and thus, maintained, to some degree, the high acid resistance of the 24-h (stationary phase) inoculum used to initiate the culture. Exposure of *L. monocytogenes* cells under conditions of constantly increasing acidity during the gastric challenge might have induced an acid tolerance response (ATR). Indeed, according to Davis et al. (1996), ATR is gradually induced in exponentially grown *L. monocytogenes* by subjecting cells to pH between 4.8 and 5.2 over time, reaching a peak within 60 min. In the present study, however, *L. monocytogenes* cells encountered such a pH range for only 30 min. The age of the tested *L. monocytogenes* strains examined may have also contributed to the low acid resistance displayed by 16-h cultures as strains of *Streptococcus mutans* kept in laboratory media for long periods of time were unable of inducing stationary-phase ATR (Svensäter et al., 2001).

Bacterial cells at stationary phase possess a pH-independent acid resistance mechanism relying primarily on the stationary-phase-specific alternative sigma factor (Lee et al., 1994; Davis et al., 1996). An alternative sigma factor (σ^B), contributing to survival under a variety of stressful conditions, has been described in low-GC content, Gram-positive bacteria, including *L. monocytogenes* (Becker et al., 1998; Wiedmann et al., 1998; Gahan and Hill, 1999; Becker et al., 2000; Ferreira et al., 2001; 2003; Hecker and Volker, 2001; Kazmierczak et al., 2003; Moorhead and Dykes, 2003; Chaturongakul and Boor, 2004; Begley et al., 2005b; 2006). In this study, we examined the role of σ^B function in the gastrointestinal survival of *L. monocytogenes*, using an in-frame *sigB*

deletion mutant strain, generated from *L. monocytogenes* strain 10403S (Wiedmann et al., 1998). Overall, results indicated that the wild-type strain (10403S) was more acid-resistant than the *sigB* mutant strain (A1-254), whereas as previously stated, the contribution of σ^B in gastric-survival could be demonstrated only in cultures had been grown for 16 h. The growth phase-dependent contribution of σ^B in stress-resistance has been previously shown for *L. monocytogenes* and *B. subtilis* (Gaidenko and Price, 1998; Becker et al., 2000). According to Ferreira et al. (2003), σ^B may play a critical role in the ability of *L. monocytogenes* to survive in the human stomach, as a deletion mutant strain displayed lower survival in simulated gastric fluid (pH 2.5) than the parental strain throughout growth. Findings of the latter study also demonstrated that the magnitude of the σ^B effect in cellular survival during exposure to gastric fluid differed depending on the growth phase (mid-exponential vs. stationary phase) of *L. monocytogenes*, with different effects being observed, however, in acid-adapted or non-acid-adapted cells.

Limited information is available on the contribution of σ^B to the bile-tolerance of *L. monocytogenes*. Sue et al. (2003) and Begley et al. (2005b) showed that σ^B, at least partially, regulated the expression of the *bsh* (bile salt hydrolase) gene. In this study, it was demonstrated that loss of a functional *sigB* slightly sensitized *L. monocytogenes* cells to the simulated intestinal conditions, whereas the contribution of σ^B to intestinal survival of 4- and 16-h cultures was similar. Nevertheless, the estimated IR indicated that the strain A1-245 lacking *sigB* displayed the greatest reductions during the intestinal challenge among the *L. monocytogenes* strains examined in this study.

Overall, the differences in the gastrointestinal IR of the *L. monocytogenes* strains observed in this study did not indicate any clear origin-related trends. Although clinical

isolates C1-056 and Scott A were the most acid-susceptible among strains, no major differences were shown in survival when comparing food and clinical isolates, linked to the same outbreak, suggesting that, under these conditions, clinical isolates did not possess any advantages from human carriage. Human and food isolates belonging to two of the outbreak-associated sets (R2-500 and R2-501, J1-101 and N3-031) shared identical serotypes, ribotypes and PFGE types, whereas isolates within the third set (N1-225 and N1-227) had different, but related PFGE patterns (Fugett et al., 2006). The high similarity between the genetic profiles of food and clinical isolates, found in the study by Fugett et al. (2006), may justify the absence of significant differences in the physiological responses between strains linked to the same outbreak in this study. No significant serotype- or lineage-related effects in gastric survival were identified under the conditions of this study. Lianou et al. (2006) also reported the lack of trends among serotypes in a study investigating the acid resistance of 25 *L. monocytogenes* strains, whereas to our knowledge, no published studies have been conducted to identify lineage-related effects on the ability of *L. monocytogenes* to tolerate acidic conditions. Significant variation among serotypes was identified in the intestinal tolerance of 16-h cultures since combined 4b isolates displayed slower inactivation than isolates of serotype 1/2. Significant variation was also observed among lineages, in terms of intestinal survival of 16-h cultures, with isolates of lineage 2 displaying faster IR as compared to those of lineages 1 or 3.

Findings of this study may help elucidate, to some degree, certain differences in epidemiology and environmental distribution of *L. monocytogenes* strains. The increased intestinal-resistance, observed in serotype 4b isolates, could be a contributing factor to

their frequent association with outbreak-related cases of listeriosis (Schuchat et al., 1991). On the other hand, acidity is a common environmental stress, and unlike bile, it is not exclusively linked to intra-host conditions. Thus, the absence of trends in gastric resistance between serotypes 4b and 1/2 could be explained after taking into consideration that both clinical isolates (mainly 4b) and food isolates (mainly 1/2) may encounter some form of acidic conditions within the human body or during food processing, respectively. Additionally, as previously discussed, although considerable strain-to-strain variation was observed in terms of gastric survival, differences in acid resistance had slight effects in the overall gastrointestinal survival of the pathogen, considering that large levels of pathogenic cells were transferred to the intestine during the first stages of gastric emptying. Similar conclusions can be drawn by observing the lineage-related differences identified in this study, since lineages 1 and 3, that were characterized as more bile-tolerant than lineage 2 in this study, are associated primarily with foodborne disease (epidemic or sporadic cases) isolates and animal isolates, respectively (Rasmussen et al., 1995; Wiedmann et al., 1997; Jeffers et al., 2001; Ward et al., 2004), and thus, are required to encounter intestinal hurdles in order to colonize the human or animal intestinal tract. Nevertheless, it should be noted that the indicated serotype- and lineage-related trends were not present when 4-h cultures were studied. Moreover, differences among serotypes or lineages were found only when the combined (based on serotype or lineage) observations of *L. monocytogenes* strains were compared, while the behavior of strains within serotypes or lineages was not identical during the intestinal challenge.

Results of the present study identified differences among *L. monocytogenes* strains in terms of their ability to tolerate passage through a simulated gastrointestinal tract. Certain correlations between *L. monocytogenes* survival and serotype or lineage were observed; however, they were related only to intestinal survival. Overall, loss of a functional *sigB* appeared to sensitize the pathogen to the defense barriers of the gastrointestinal tract; however, the contribution of *sigB* to the survival of *L. monocytogenes* in the simulated gastrointestinal tract was more pronounced in the GC, while its effects during the intestinal challenge were minor. Nevertheless, under the conditions of this study, all *L. monocytogenes* strains, even the extremely acid-sensitive ones, survived the *in vitro* gastrointestinal passage, suggesting that although acid resistance may be an important element in terms of intrahost survival, assumptions regarding the gastrointestinal survival of the pathogen, as a virulence-related factor, may be more accurate when other gastrointestinal-related aspects (e.g., gastric emptying and bile-resistance) have also been taken into consideration.

Table 4.1. *Listeria monocytogenes* strains used in this study.

Strain	Serotype	Lineage	Origin	Reference
558	1/2	NK	Pork meat	-
R2-500	4b	1	Food, epidemic, North Carolina (2000)	Fugett et al. (2006)[1]
R2-501	4b	1	Human, epidemic, North Carolina (2000)	Fugett et al. (2006)[1]
Scott A	4b	1	Human	-
N1-225	4b	1	Human, epidemic US (1998-99)	Fugett et al. (2006)[1]
N1-227	4b	1	Food, epidemic US (1998-99)	Fugett et al. (2006)[1]
C1-056	1/2a	2	Human, sporadic case	Fugett et al. (2006)[1]
N3-031	1/2a	2	Food (hot dog), sporadic case (1989)	Fugett et al. (2006)[1]
J1-101	1/2a	2	Human, sporadic case (1989)	Fugett et al. (2006)[1]
10403S	1/2a	2	NK	Bishop and Hinrichs (1987)[2]
A1-254	Δ*sigB* mutant of 10403S			Wiedmann et al. (1998)[2]
J1-158	4b	3	Goat	Fugett et al. (2006)[1]
J1-168	4a	3	Human, sporadic case	Fugett et al. (2006)[1]

NK: not known

Kindly provided by [1]Dr. Martin Wiedmann and [2]Dr. Kathryn J. Boor (Department of Food Science, Cornell University, Ithaca NY)

Serotype and lineage designation is as provided by donor/reference (except for strains 558 and Scott A)

Table 4.2. Mean kinetics (± standard deviation) of *Listeria monocytogenes* in a simulated gastrointestinal system (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 4 h at 30°C.

Strain	Gastrointestinal compartment			
	Gastric		Intestinal	
	Shoulder duration (min)	Maximum inactivation rate (log CFU/ml/min)	Shoulder duration (min)	Maximum inactivation rate (log CFU/ml/min)
558	$0.00\pm0.00_{A}$	$0.003\pm0.001_{A}$	-[1]	$0.008\pm0.006_{A}$
R2-500	-[1]	$0.003\pm0.003_{A}$	-[1]	$0.010\pm0.009_{A}$
R2-501	$68.66\pm42.85_{BC}$	$0.002\pm0.003_{A}$	-[1]	$0.004\pm0.001_{A}$
Scott A	$77.48\pm0.74_{B}$	$0.209\pm0.019_{B}$	-[1]	$0.006\pm0.002_{A}$
N1-225	$40.77\pm34.76_{C}$	$0.010\pm0.007_{A}$	-[1]	$0.009\pm0.007_{A}$
N1-227	$71.49\pm3.31_{B}$	$0.019\pm0.006_{A}$	-[1]	$0.011\pm0.012_{A}$
C1-056	$76.88\pm2.37_{B}$	$0.262\pm0.022_{B}$	-[1]	$0.004\pm0.005_{A}$
N3-031	$29.64\pm51.33_{D}$	$0.029\pm0.047_{A}$	-[1]	$0.009\pm0.011_{A}$
J1-101	-[1]	$0.005\pm0.002_{A}$	-[1]	$0.013\pm0.011_{A}$
10403S	-[1]	$0.002\pm0.001_{A}$	-[1]	$0.008\pm0.007_{A}$
A1-254	$39.10\pm32.04_{CD}$	$0.032\pm0.018_{A}$	-[1]	$0.016\pm0.007_{A}$
J1-158	$51.94\pm19.76_{BC}$	$0.042\pm0.029_{A}$	-[1]	$0.009\pm0.003_{A}$
J1-168	$40.90\pm31.77_{CD}$	$0.013\pm0.009_{A}$	-[1]	$0.006\pm0.009_{A}$

[1] No shoulder was observed (inactivation was immediate)

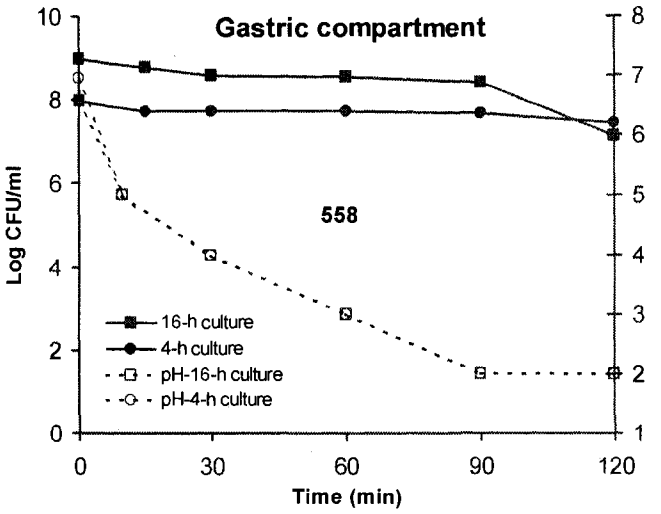
ABC…: means within a column lacking a common letter are significantly different ($P < 0.05$)

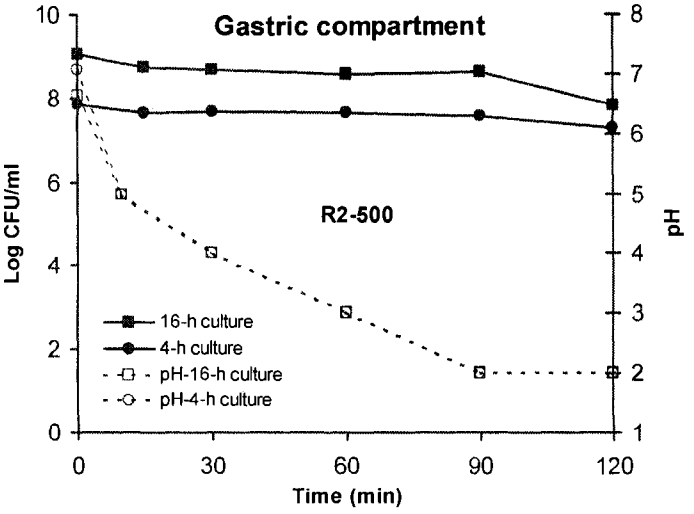
Table 4.3. Mean kinetics (± standard deviation) of *Listeria monocytogenes* in a simulated gastrointestinal system (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 16 h at 30°C.

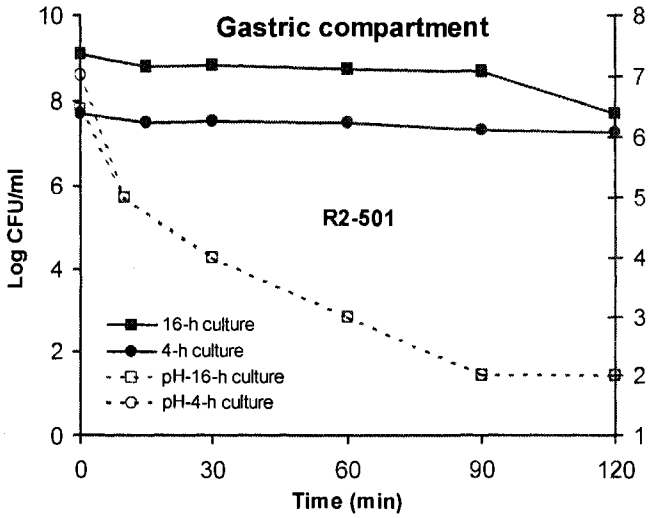
Strain	Gastrointestinal compartment			
	Gastric		Intestinal	
	Shoulder duration (min)	Maximum inactivation rate (log CFU/ml/min)	Shoulder duration (min)	Maximum inactivation rate (log CFU/ml/min)
558	63.73±16.28 $_{A}$	0.043±0.028 $_{AD}$	-[1]	0.002±0.002 $_{AB}$
R2-500	-[1]	0.009±0.001 $_{A}$	-[1]	0.000±0.003 $_{A}$
R2-501	71.17±1.00 $_{A}$	0.023±0.001 $_{A}$	-[1]	0.003±0.005 $_{AB}$
Scott A	74.71±1.59 $_{A}$	0.197±0.010 $_{B}$	-[1]	0.008±0.005 $_{ABD}$
N1-225	70.45±3.30 $_{A}$	0.039±0.009 $_{AD}$	-[1]	0.003±0.001 $_{AB}$
N1-227	70.35±2.94 $_{A}$	0.092±0.001 $_{BD}$	-[1]	0.004±0.001 $_{AB}$
C1-056	70.74±4.99 $_{A}$	0.221±0.013 $_{C}$	-[1]	0.014±0.010 $_{BC}$
N3-031	86.48±2.32 $_{A}$	0.047±0.007 $_{AD}$	1.32±2.29	0.016±0.004 $_{BC}$
J1-101	68.15±1.55 $_{A}$	0.062±0.010 $_{AD}$	-[1]	0.018±0.007 $_{CD}$
10403S	67.35±2.77 $_{A}$	0.048±0.003 $_{AD}$	-[1]	0.010±0.005 $_{BC}$
A1-254	83.68±22.73 $_{A}$	0.188±0.113 $_{B}$	-[1]	0.021±0.013 $_{C}$
J1-158	76.66±1.30 $_{A}$	0.103±0.019 $_{BD}$	-[1]	0.008±0.006 $_{ABD}$
J1-168	65.32±7.12 $_{A}$	0.049±0.008 $_{AD}$	-[1]	0.009±0.006 $_{BC}$

[1] No shoulder was observed (inactivation was immediate)

ABC…: means within a column lacking a common letter are significantly different ($P < 0.05$)


Gastric compartment
558
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
R2-500
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
R2-501
Log CFU/ml
pH
Time (min)
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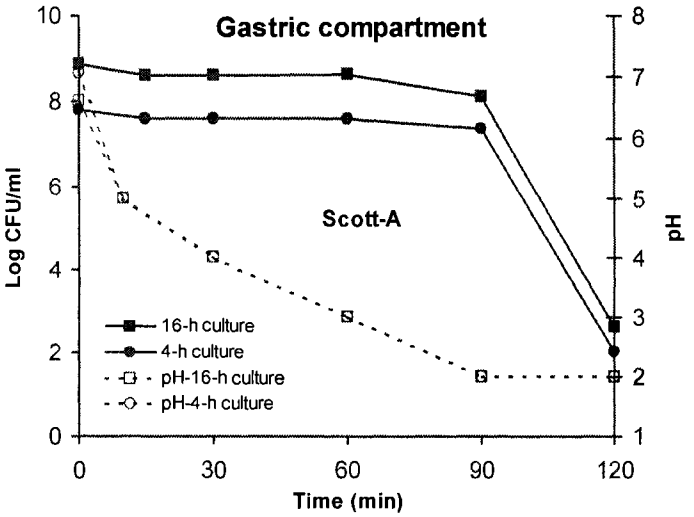
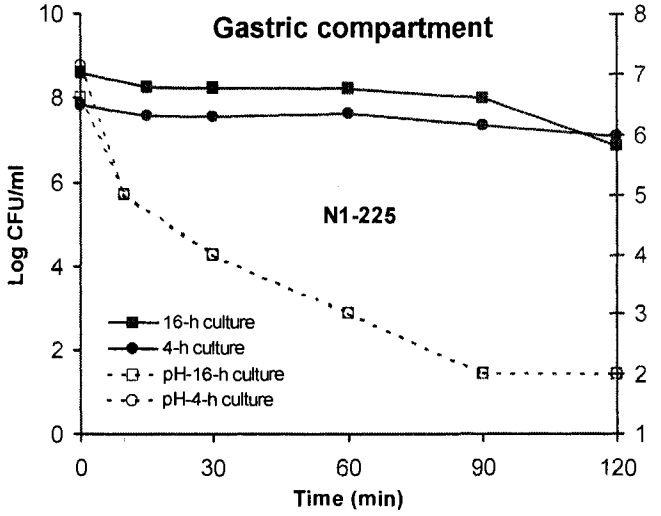
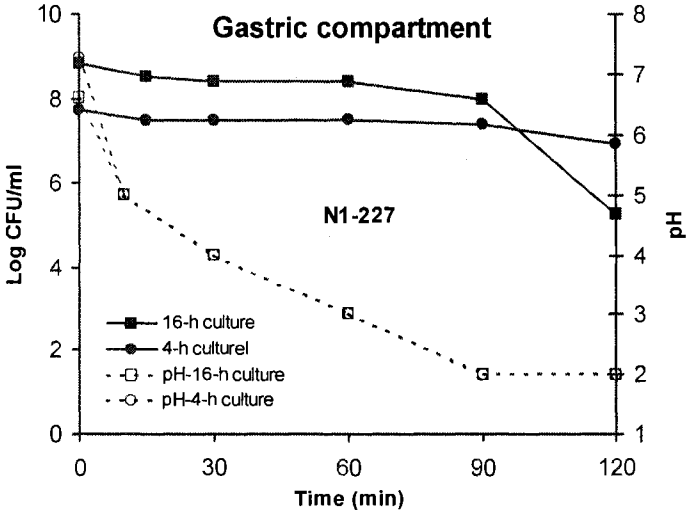
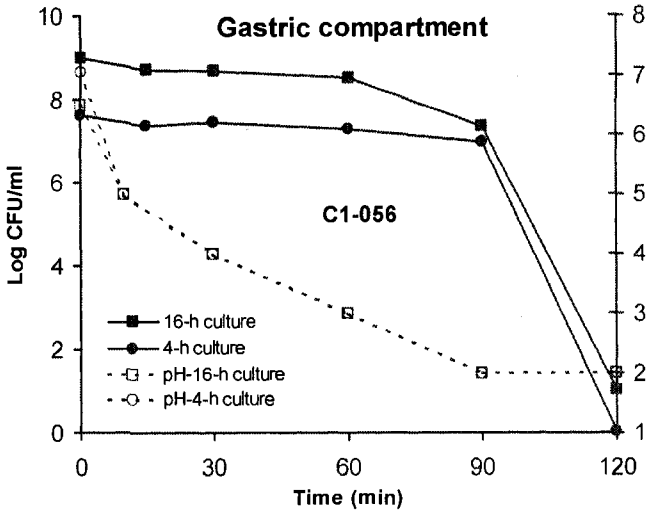
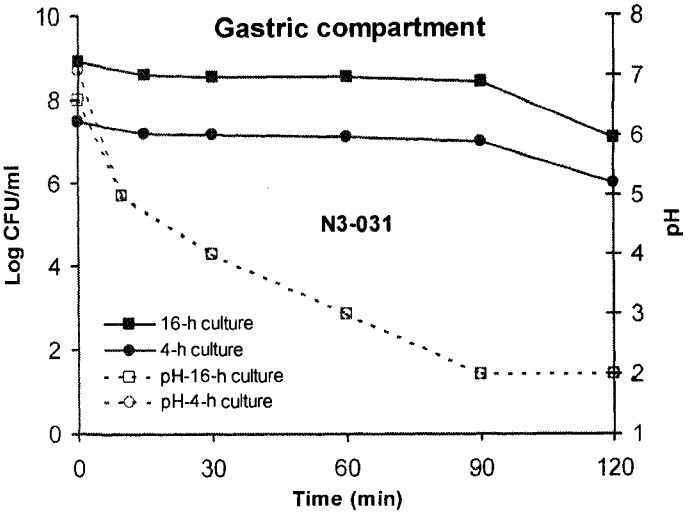
4-h culture
pH-16-h culture
pH-4-h culture

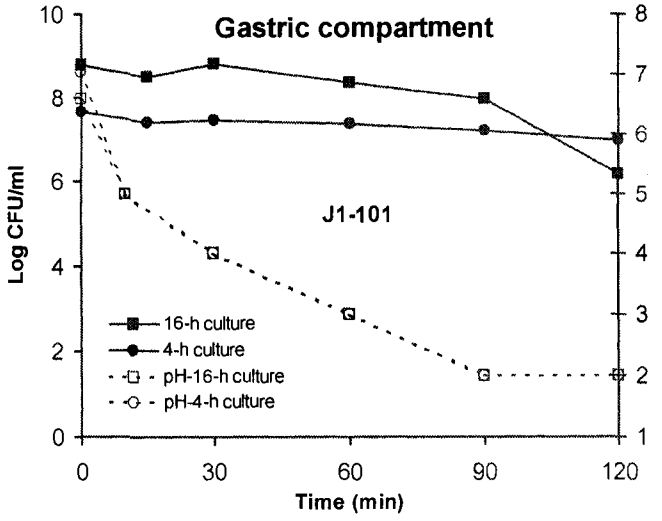

Gastric compartment
Scott-A
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture

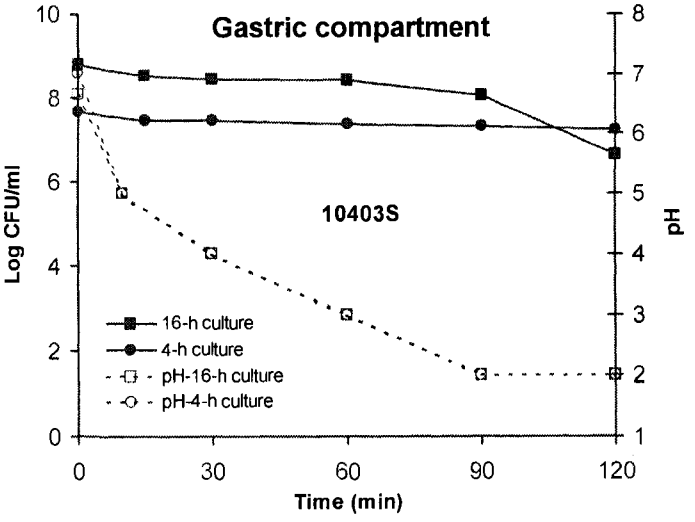

Gastric compartment
N1-225
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture

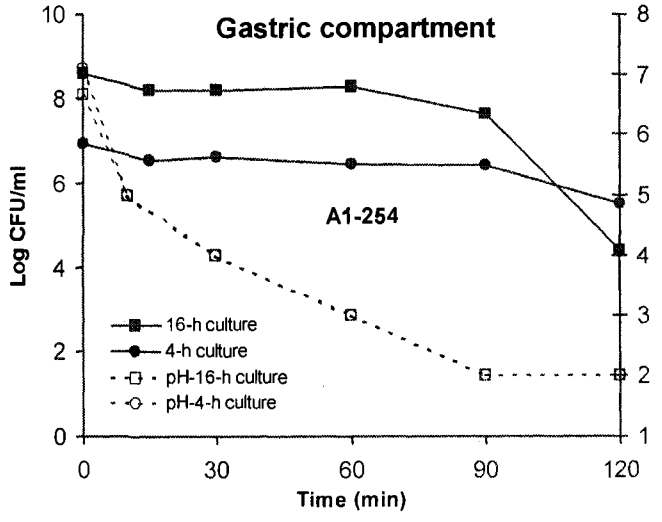

Gastric compartment
N1-227
Log CFU/ml
pH
Time (min)
16-h culture
4-h culturel
pH-16-h culture
pH-4-h culture

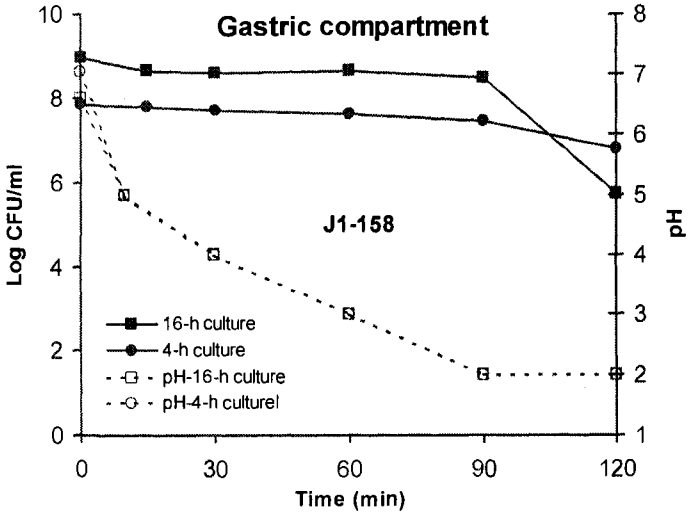

Gastric compartment
C1-056
Log CFU/ml
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
N3-031
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
J1-101
Log CFU/ml
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
10403S
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
A1-254
Log CFU/ml
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culture


Gastric compartment
J1-158
Log CFU/ml
pH
Time (min)
16-h culture
4-h culture
pH-16-h culture
pH-4-h culturel

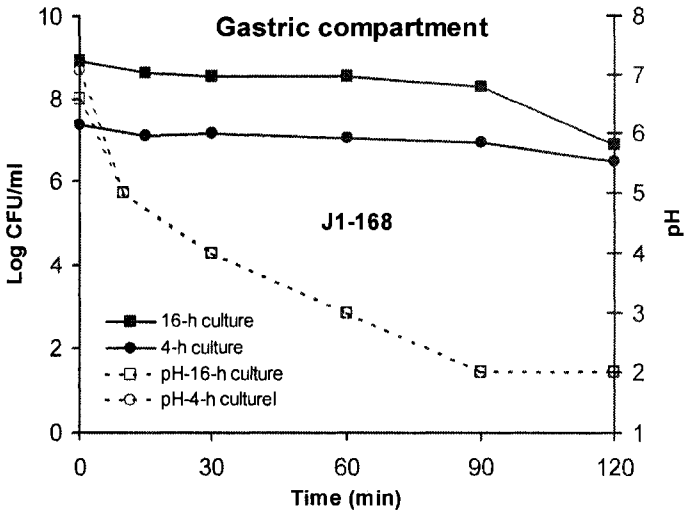
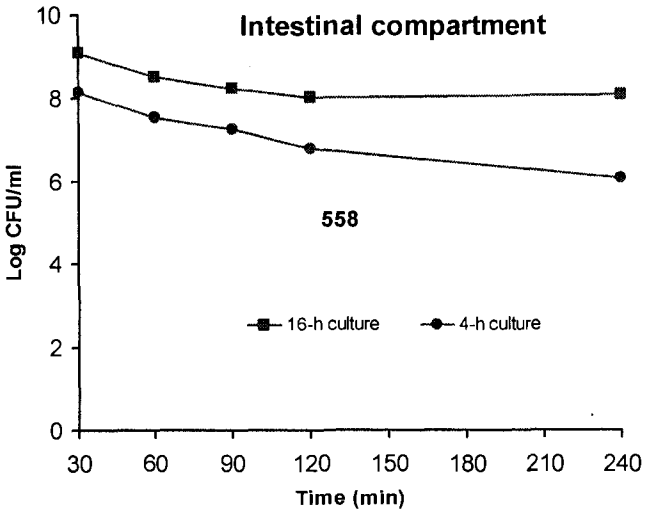
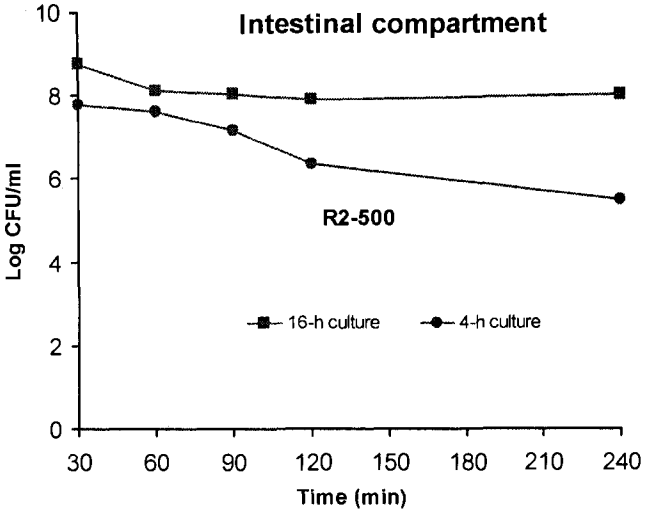
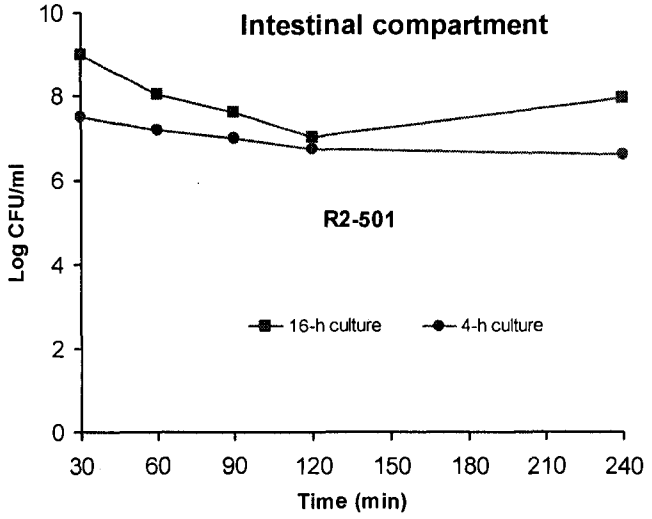
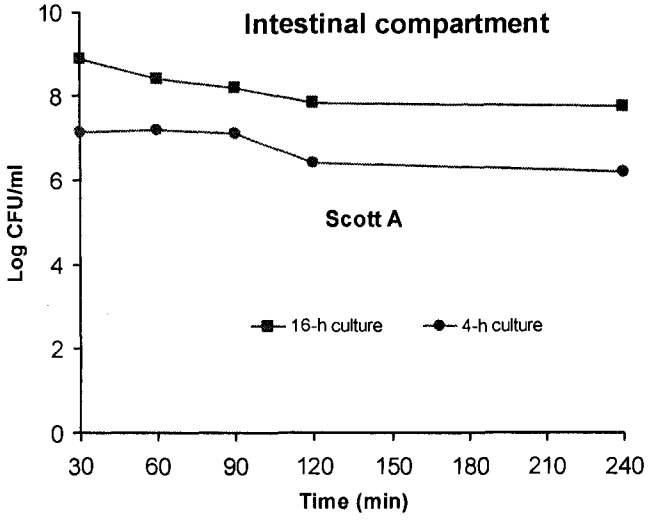


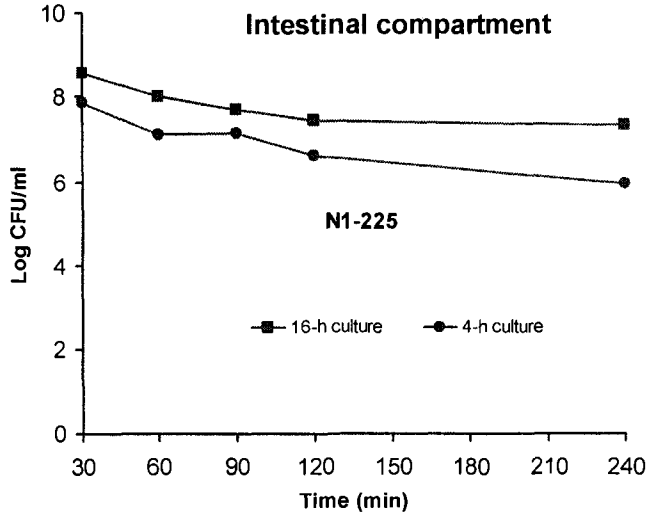
Figure 4.1 (Appendix Tables 17 and 18). Survival (log CFU/ml; PALCAM agar counts) of individual *L. monocytogenes* strains and pH values within the gastric compartment (37°C) during a simulated gastrointestinal challenge, conducted after inoculation into 100 ml TSBYE without dextrose and incubation for 4 or 16 h at 30°C

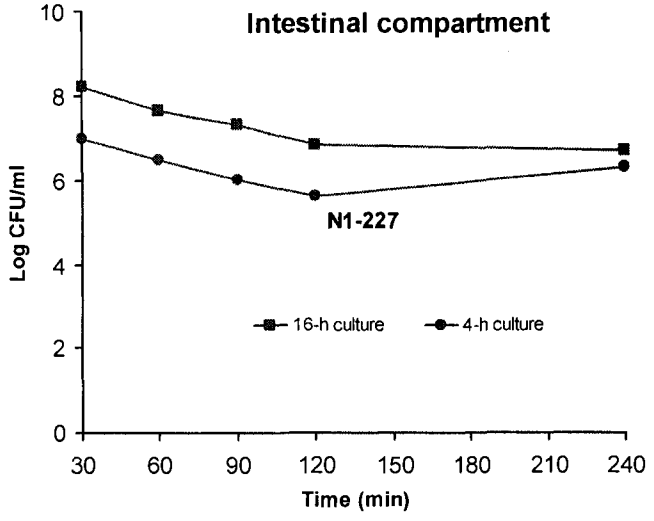

Intestinal compartment
Log CFU/ml
558
16-h culture
4-h culture
Time (min)

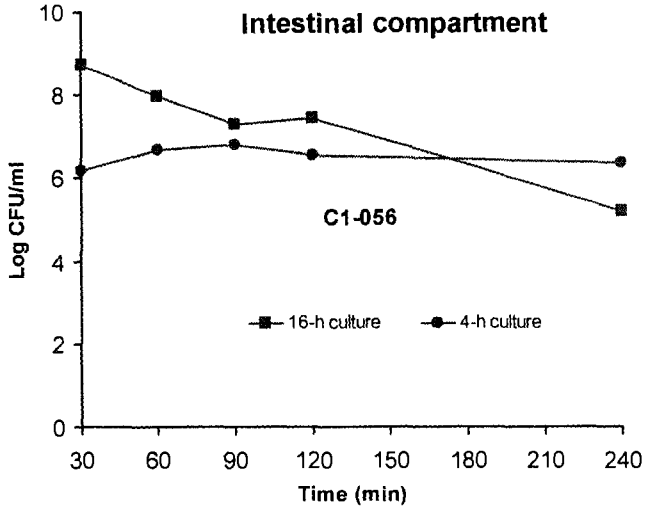

Intestinal compartment
Log CFU/ml
R2-500
16-h culture
4-h culture
Time (min)

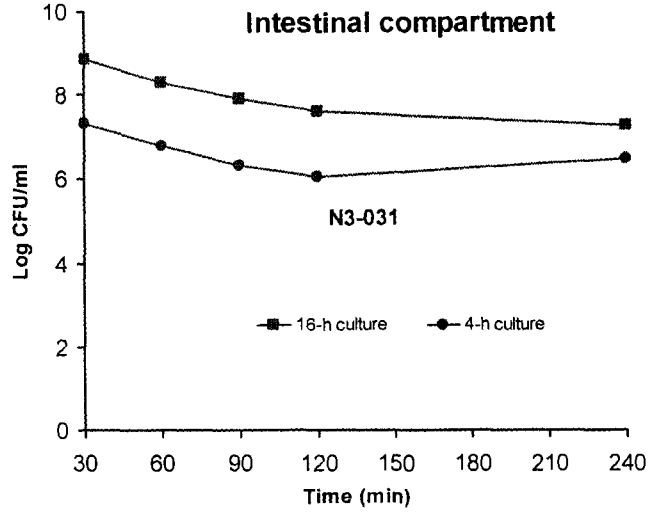

Intestinal compartment
Log CFU/ml
R2-501
16-h culture
4-h culture
Time (min)

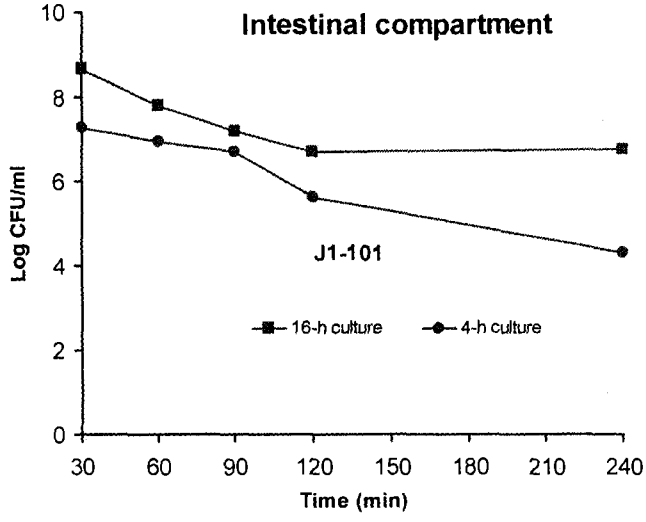

Intestinal compartment
Log CFU/ml
Scott A
16-h culture
4-h culture
Time (min)

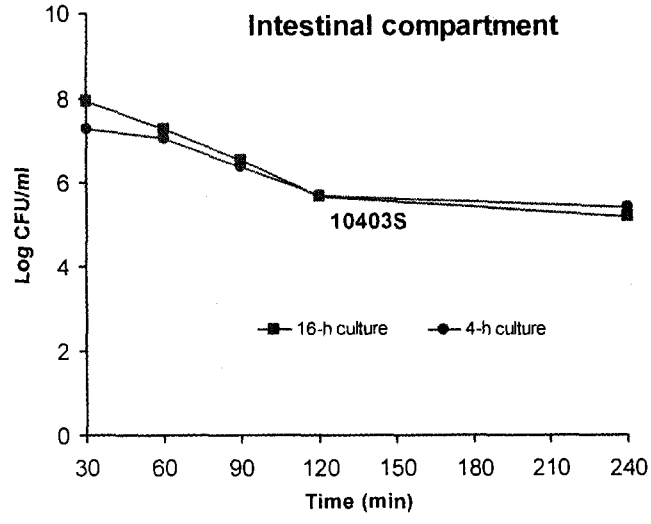

Intestinal compartment
Log CFU/ml
N1-225
16-h culture
4-h culture
Time (min)

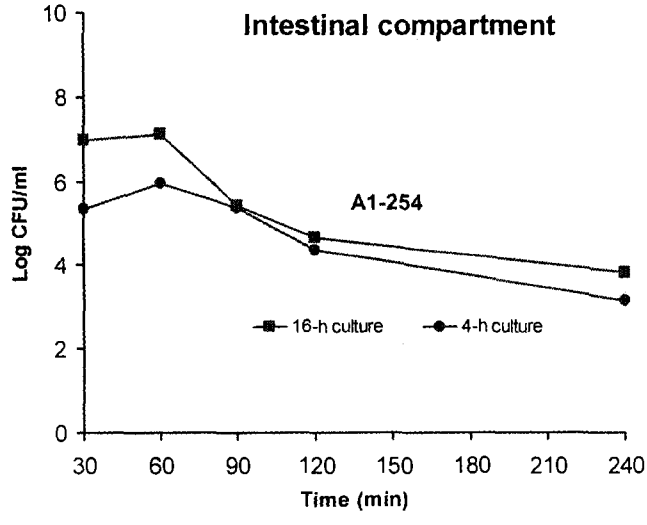

Intestinal compartment
Log CFU/ml
N1-227
16-h culture
4-h culture
Time (min)

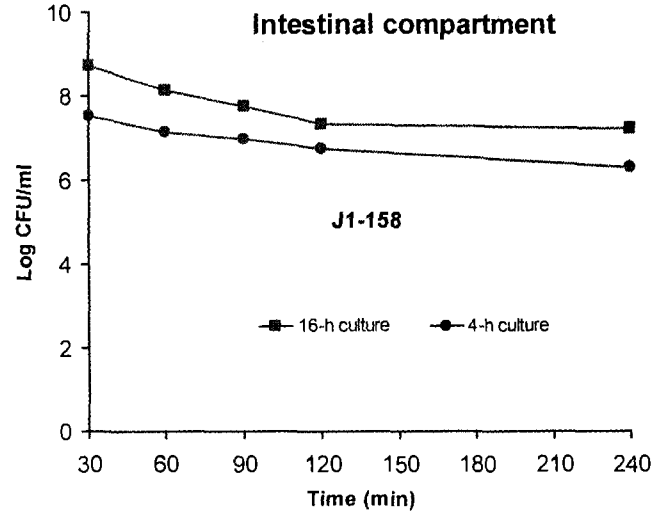

Intestinal compartment
C1-056
16-h culture
4-h culture
Log CFU/ml
Time (min)


Intestinal compartment
N3-031
16-h culture
4-h culture
Log CFU/ml
Time (min)


Intestinal compartment
J1-101
16-h culture
4-h culture
Log CFU/ml
Time (min)


Intestinal compartment
10403S
16-h culture
4-h culture
Log CFU/ml
Time (min)


Intestinal compartment
A1-254
16-h culture
4-h culture
Log CFU/ml
Time (min)


Intestinal compartment
J1-158
16-h culture
4-h culture
Log CFU/ml
Time (min)

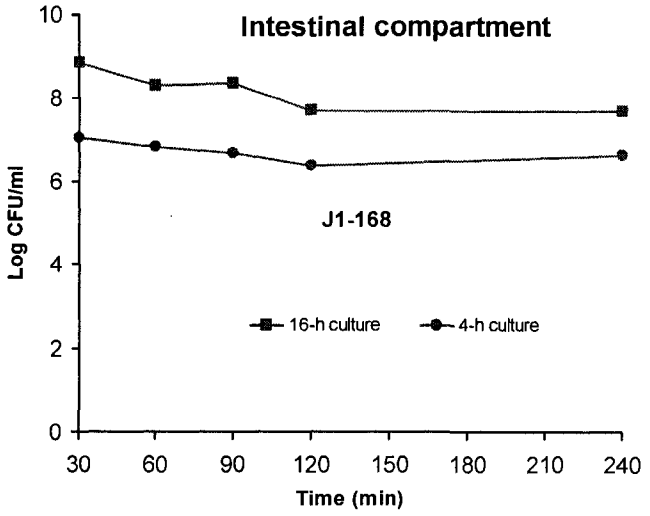


Figure 4.2 (Appendix Tables 19 and 20). Survival (log CFU/ml; PALCAM agar counts) of individual *L. monocytogenes* strains within the intestinal compartment (pH 6.5±0.3; 37°C) during a simulated gastrointestinal challenge, conducted after inoculation into 100 ml TSBYE without dextrose and incubation for 4 or 16 h at 30°C

CHAPTER 5

Changes of *Listeria monocytogenes* counts in a dynamic gastrointestinal model following inoculation onto bologna or salami slices and storage at 4°C in vacuum packages

ABSTRACT

Listeria monocytogenes counts were determined during storage (82 days, 4°C) in vacuum packages of inoculated (4.0-5.0 log CFU/g) bologna or salami slices, following exposure to a dynamic gastrointestinal model (37°C). Simulated variables included gastric emptying and gastrointestinal fluid secretion rates, gradual gastric acidification (pH reduction to 2.0 within 88 min), and intestinal pH maintenance (6.5±0.3). Inactivation curves in each gastrointestinal compartment (gastric, GC and intestinal, IC) were modeled using the Baranyi and Roberts model. As expected, *L. monocytogenes* populations increased on bologna and decreased on salami during storage, reaching 8.7 and 1.4 log CFU/g, respectively, on day-82. Throughout storage, inactivation rates (IR) of the pathogen inoculated onto bologna or salami during exposure in the GC ranged from 0.085 (day-1) to 0.158 (day-57) log CFU/g/min and 0.013 (day-42) to 0.051 (day-1) log CFU/g/min, respectively. Gastric IR for populations present on salami were lower than those for populations on bologna on corresponding days, indicating potential protective effects of the former product. However, it is also possible that the increased

acid tolerance of the pathogen observed during gastric exposure of salami samples was not solely due to product-related effects, as the low initial *L. monocytogenes* levels reached with storage of this product (≤ 2.5 log CFU/g after day-27) may have experienced slower decreases during the acid challenge than the high levels on bologna. Since delivery of gastric contents in the IC began while the gastric pH was still high (> 4.41), populations of the pathogen transferred from the GC to the IC within the first 30 min of gastric emptying depended on the initial contamination levels on each product, and overall initial *L. monocytogenes* populations in the IC (30 min) were ≤ 1 log CFU/g lower than initial levels on each product. Subsequently, reductions resulting from the 240-min intestinal challenge were smaller than those displayed in the GC. Intestinal IR were similar for both products, ranging from 0.003 to 0.048 (bologna) and 0.002 to 0.056 (salami) log CFU/g/min, throughout storage. Results indicated potential protective effects of salami against acid destruction of *L. monocytogenes*. However, any effects of the food matrix *per se* on the gastrointestinal survival of the pathogen were overwhelmed by the high and low contamination levels reached on bologna and salami, respectively, during storage.

5.1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a rare but potentially fatal disease. The pathogen is frequently isolated from a variety of foods (Farber and Peterkin, 1991; Jay, 1996), including meat and various meat products (Farber et al., 1988; Farber and Daley, 1994; Jay, 2000; Levine et al., 2001; Wallace et al., 2003; Farber et al., 2007). The occurrence of *L. monocytogenes* in processed meat products, intended to be consumed without further heating poses a major public health concern and such products

have been linked to fatal listeriosis outbreaks and numerous recalls in the United States (Bernard and Scott, 1999; CDC, 1998; 2000; 2002). Among 23 categories of ready-to-eat (RTE) foods (including seafood, produce, dairy, meat, and combination products), the highest relative risk for listeriosis on both per serving and per annum basis was attributed to deli meats (HHS-FDA/USDA-FSIS, 2003). Even though presence of the pathogen in dry and semidry fermented sausages has been widely reported (Brackett, 1988; Farber et al., 1988; 1989; Trussel, 1989; Levine et al., 2001; USDA-FSIS, 2001), a listeriosis risk assessment (HHS-FDA/USDA-FSIS, 2003) identified such products as posing a low relative risk for listeriosis, as they possess intrinsic properties (e.g., acidity, low water activity, starter cultures, and preservative ingredients) that are traditionally known to suppress growth of *L. monocytogenes*. While several researchers have shown that manufacturing processes (i.e., fermentation, drying) applied to these foods may not necessarily eliminate *L. monocytogenes* presence in the finished product (Johnson et al., 1988; Glass and Doyle, 1989b; Berry et al., 1990; Farber et al., 1993; Nightingale et al., 2006), levels of the pathogen found in naturally-contaminated dry, fermented products are generally low (Gianfranceschi et al., 2006). To date, no epidemiological evidence linking dry fermented sausages with cases of listeriosis is available (USDA-FSIS, 2001), however, such products have been implicated in cases of foodborne illness caused by other pathogens (Tilden et al., 1996). In response to an *Escherichia coli* O157:H7 outbreak associated with dry fermented salami (CDC, 1995), the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) required meat processors to apply validated manufacturing processes for dry and semi-dry fermented sausages that achieve a 5-log or greater reduction in *E. coli* O157:H7 numbers (Reed,

1995). Moreover, as dry and semidry fermented meats are considered as RTE by the USDA-FSIS (USDA-FSIS, 2001), current food safety regulations require absence of *L. monocytogenes* from 25-g samples of product ('zero tolerance' policy; Shank et al., 1996). The negative impact of the 'zero tolerance' requirement in RTE meat/poultry products has gained attention in recent years (Tompkin, 2002; Chen et al., 2003), while requests for modifying the zero tolerance policy in RTE foods that do not allow *L. monocytogenes* growth may be supported by scientific data, including a dose-response study by Chen et al. (2003), indicating that consumption of foods contaminated with low *L. monocytogenes* levels ($< 10^2$ CFU/g) pose very little risk to public health. In addition, the risk assessment conducted by the Food and Drug Administration (FDA) of the US Department of Health and Human Services (HHS) and the USDA-FSIS (HHS-FDA/USDA-FSIS, 2003) demonstrated that most listeriosis cases arise from heavily contaminated foods, although exposure to low *L. monocytogenes* numbers may cause disease to extremely susceptible individuals (Lyytikäinen et al., 2000; Maijala et al., 2001). Clearly, contamination of RTE meat products that permit multiplication of *L. monocytogenes* cannot be tolerated, even at low levels, particularly since such foods have an extended shelf life, while the phychrotrophic nature of the pathogen renders refrigerated storage inadequate as a control measure (Junttila et al., 1988; Walker et al., 1990; Barbosa et al., 1994; Hudson et al., 1994).

L. monocytogenes contamination on dry and semidry fermented sausages may occur due to either survival of raw material contaminants during product manufacture or post-processing contamination in the food-processing environment. Pathogen levels on these products are generally expected to be low; however, consumption of contaminated

sausages may still pose a food safety risk if surviving *L. monocytogenes* cells have been 'stress hardened' (Lou and Yousef, 1997; Samelis and Sofos, 2003) as a result of their exposure to the stress conditions prevailing on the surface of these foods. Numerous studies have shown that the survival properties *L. monocytogenes* cells under a specific stress may be enhanced by their pre-exposure to similar or different sublethal stresses (Farber and Pagotto, 1992; Davis et al., 1996; Gahan et al., 1996; Lou and Yousef, 1996; O' Driscoll et al., 1996; 1997; Mazzota, 2001; Samelis et al., 2003a). Strains of *L. monocytogenes* originating from sausages or sausage-processing environments were more resistant to processes associated with sausage manufacturing than clinical strains (Thévenot et al., 2005b). Consequently, stresses imposed to *L. monocytogenes* present on a fermented dried product (i.e., acidity, low water activity, microbial competition) during its commercial life may lead to greater survival under subsequent host-related stresses (e.g., gastric acid and bile) and, thus, affect the likelihood of infection.

To establish successful infection, *L. monocytogenes* must survive various hostile microenvironments encountered during its passage through the stomach and small intestine. The high acidity of the stomach is the first barrier that the pathogen encounters following consumption of contaminated food (Smith, 2003). Increased survival of microorganisms during gastric passage may be observed due to protective effects of food (Conway et al., 1987; Peterson et al., 1989; Tamplin, 2005) and gastric emptying, (Takumi et al., 2000), factors that may allow cells to reach the small intestine before gastric acidity reaches lethal levels. Moreover, similarly to other foodborne pathogens, *L. monocytogenes* has the ability to develop mechanisms that may enable increased survival under acidic conditions (Davis et al., 1996; O' Driscoll et al., 1996; Cotter et al., 2001a;

Cotter and Hill, 2003; Smith, 2003; Gahan and Hill, 2005). Subsequently, *L. monocytogenes* cells that reach the small intestine in a viable state encounter unfavorable conditions, resulting from the presence of volatile fatty acids and bile salts in conjunction with low oxygen and high osmolarity conditions (Chowdhury et al., 1996; Phan-Thanh et al., 2000; Begley et al., 2002; 2005a; Gahan and Hill, 2005). Examination of factors that may influence survival of *L. monocytogenes* in the gastrointestinal tract has received significant consideration (Roering et al., 1999; Phan-Thanh et al., 2000; Cotter et al., 2001a; Begley et al., 2002; King et al., 2003; Olier et al., 2004; Wonderling and Bayles, 2004; Stopforth et al., 2005; Formato et al., 2007); however, the majority of studies in this area have used artificial gastrointestinal fluids as broth systems, and thus overlooked important gastrointestinal parameters such as the sequential stresses and changing conditions to which pathogens are subjected during gastrointestinal transit.

Currently, not enough scientific information exists for consideration of the food matrix as a variable in the hazard characterization component of the *L. monocytogenes* risk assessment (Rocourt et al., 2003). In this respect, characteristics of the food vehicle that could affect survival of pathogens in the gastrointestinal tract, such as structural or compositional attributes and conditions that can induce stress responses may be of great importance (FAO/WHO, 2003; HHS-FDA/USDA-FSIS, 2003). For instance, the fat content of a food has been long suspected as a factor affecting the dose-response relationship (Buchanan et al., 2000), since high-fat foods have been frequently implicated in outbreaks of listeriosis (Linnan et al., 1988; McLauchlin et al., 1991; CDC, 1998; Lyytikäinen et al., 2000). Foods with high salt content have also been epidemiologically linked to human listeriosis (Junttila and Brander, 1989), suggesting that exposure of *L.*

monocytogenes cells to osmotic stress could enhance their ability to tolerate the acidic conditions associated with defense mechanisms of the human host (O'Driscoll et al., 1996; HHS-FDA/USDA-FSIS, 2003). Nevertheless, information regarding the contribution of food matrix characteristics on survival of the pathogen during the gastrointestinal phase of infection is very limited. *In vitro* data have indicated that glutamate, a commonly used food ingredient, may protect the pathogen in gastric fluid (Cotter et al., 2001a).

Currently, factors such as the amount of food consumption, the frequency and extent of contamination, and the potential for *L. monocytogenes* growth are considered critical on the risk per serving classification of these RTE products (HHS-FDA/USDA-FSIS, 2003); however, it is not clear whether exposure of the organism on the surface a specific product may alter its survival properties in the gastrointestinal tract. As mentioned above, there are data supporting that subjecting *L. monocytogenes* to an acidic environment (e.g., on a fermented product) could induce protective responses against subsequent stresses. The study presented in this chapter examined the survival of *L. monocytogenes* during simulated digestion of inoculated bologna and salami samples tested at intervals during refrigerated storage. The objective was to identify potential effects of characteristics associated with these RTE meat products on the gastrointestinal survival of this pathogen at different stages of storage.

5.2. Materials and methods

5.2.1. Meat products

Bologna was manufactured at the Department of Animal Sciences Meat Science Laboratory at Colorado State University. Fresh pork (approximately 30% fat) and beef

(approximately 25% fat) trimmings were obtained from Swift Co. (Greeley, CO). The basic bologna formulation (Samelis et al., 2001a) consisted of (%, wt/wt): meat (40% pork and 60% beef) trimmings (82.2), ice (10), sodium chloride (2), dextrose (2), dry mustard (0.9), corn syrup solids (2), polyphosphate (0.4; sodium tripolyphosphate and sodium hexameta-phosphate; Heller Inc., Bedford Park, IL), sodium nitrite (0.0156), sodium erythorbate (0.05), paprika (0.25), onion powder (0.05), garlic powder (0.05), coriander (0.05), and white pepper (0.05). Spices and seasonings were purchased from AC Legg Co. (Birmingham, AL). Ingredients were emulsified in a 35-L bowl chopper (RMF, Kansas City, MO) for 3-5 min to a final temperature of 15.5°C. The mixture was extruded (Handtmann Inc., Buffalo Grove, IL) into 65 mm diameter fibrous cellulose casings (Koch, Kansas City, MO) and the bologna was cooked in a smokehouse (Alkar, DEC International Inc., Lodi, WI). Specifically, bologna was cooked in dry air for 1 hour (smokehouse temperature 60°C), followed by hot smoking (60°C; Zesti liquid smoke, Hickory Specialties Inc., Crossville, TN) for 38 min. After smoking, the bologna was cooked with steam for 1 hour (smokehouse temperature 71°C, relative humidity 50%). Then the smokehouse temperature was increased to 88°C and the bologna was cooked until its internal temperature reached 70°C. After cooking, the bologna was showered with cool tap water for 5 min and cooled overnight at 4°C. The casings were then removed and the bologna sticks were sliced into approximately 4 mm thick slices with a delicatessen slicer (Globe slicer, Mozley Manufacturing, Stamford, CT).

Salami sticks (61 mm diameter) consisted of pork, beef, nonfat dry milk, salt, sugar, corn syrup solids, spices, wine, garlic powder, sodium ascorbate, lactic acid starter culture, sodium nitrite, butylated hydroxyanisole, butylated hydroxytoluene and citric

acid, and were obtained from a commercial source. Salami sticks were sliced into approximately 4 mm slices as described above.

Fat and moisture contents of the products were determined in triplicate for two lots of each product using AOAC International Official methods 960.39 and 950.46.B (AOAC, 1998), respectively. The fat content (%) of bologna and salami was 18.8±0.7 and 29.1±2.1, respectively and the moisture content (%) was 61.8±0.5 and 35.6±1.0, respectively.

5.2.2. Preparation of bacterial cultures

A 10-*L. monocytogenes* strain mixture was used to inoculate the surface of bologna and salami slices. Strains included NA-1 (serotype 3b, pork sausage isolate), N-7150 (serotype 3a, meat isolate), 558 (serotype 1/2, pork meat isolate), N1-225 and N1-227 (both serotype 4b, human and food isolate, respectively; associated with the same epidemic), R2-500 and R2-501 (both serotype 4b, food and human isolate, respectively; associated with the same epidemic), R2-763, R2-764, and R2-765 (all serotype 4b, human, food, and environmental isolate, respectively; associated with the same epidemic) (Fugett et al., 2006; Lianou et al., 2007). Strains N1-225, N1-225, R2-500, R2-501, R2-763, R2-764, and R2-765 originated from the International Life Sciences Institute (ILSI) North America outbreak set (Fugett et al., 2006) and were kindly provided by Dr. Martin Wiedmann (Department of Food Science, Cornell University, Ithaca NY). Frozen (-70°C) stock cultures were maintained separately in tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks MD) supplemented with 0.6% yeast extract (Acumedia, Baltimore MD) (TSBYE) and 20% glycerol. Working cultures were kept on tryptic soy agar (Difco), supplemented with 0.6% yeast extract (TSAYE) slants at 4°C. Each strain was

activated by transferring a loopful of bacterial cells from the appropriate slant into 10 ml TSBYE and incubating at 30°C for 24 h. The resultant cultures were then subcultured (0.1 ml), in duplicate, into 10 ml of fresh TSBYE and incubated at 30°C for 24 h.

5.2.3. Inocula preparation and product inoculation

For inocula preparation, TSBYE cultures were individually centrifuged (Eppendorf, model 5810 R, Brinkmann Instruments, Inc., Westbury, NY) at 4,629 x *g* for 15 min (4°C). The cell pellets were washed with 10 ml of phosphate-buffered saline (prepared by suspending 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7\ H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 liter of distilled water, pH 7.4), and centrifuged (4629 x *g* for 15 min at 4°C). The procedure was performed twice for the two sets of *L. monocytogenes* cultures. The harvested cells of each strain were resuspended in 10 ml of homogenate prepared from products that were either prepared in the Colorado State University Meat Laboratory, as described above (bologna) or commercially obtained (salami) (Lianou et al., 2007). The purpose of suspending *L. monocytogenes* cells in product homogenate instead of culture media was to 'habituate' the pathogen to the bologna or salami environment prior to inoculation. Indeed, a previous study by Geornaras et al. (2006) showed that *L. monocytogenes* cells previously grown in sausage extract exhibited shorter lag phases than cells grown in TSBYE after inoculation onto frankfurters containing antimicrobials. Product homogenates were prepared by homogenizing (2 min, Masticator, IUL Instruments, Barcelona, Spain) a 10% (wt/wt) suspension of each product with distilled water. Product slurries were then filtered twice through two layers of cheesecloth, autoclaved (15 min), cooled to ambient temperature (25°C), and stored overnight at 4°C. *L. monocytogenes* strain cultures, suspended in product homogenates, were kept at 4°C for approximately 72

h. Subsequently, cultures of each strain in bologna or salami homogenate were mixed, serially diluted with freshly prepared product homogenate and used to inoculate the surface of bologna or salami slices (approximately 9 g each), respectively, to obtain an inoculum level of 4.0-5.0 log CFU/g when 0.1 ml of inoculum was applied on the surface of bologna or salami slices.

Slices of each product were inoculated under a biological safety cabinet by spreading 0.1 ml of the appropriate inoculum on their flat surface; after 15 min at 4°C, slices were turned over and the procedure was repeated for the other side. Six inoculated slices of each product were placed on top of each other, transferred into vacuum bags (15 by 20 cm, 3 mil std barrier, Nylon/PE vacuum pouch, Koch, Kansas City, MO), vacuum packaged (Hollymatic Corp., Countryside, IL) and stored at 4°C for 82 days. Total microbial and *L. monocytogenes* populations (log CFU/g) were determined on the day of the inoculation by adding 50 g of maximum recovery diluent (prepared from ingredients in the laboratory; 0.85% NaCl and 0.1% peptone) to two slices of each product, followed by blending for 2 min at high speed, serially diluting in 0.1% buffered peptone water (Difco) and surface plating on TSAYE and PALCAM agar (Difco). Colonies on agar plates were counted manually after incubation at 25°C for 72 h (TSAYE) and 30°C for 48 h (PALCAM agar).

5.2.4. Preparation of simulated gastrointestinal fluids

Artificial saliva was prepared by suspending 6.2 g NaCl, 2.2 g KCl, 0.22 g $CaCl_2$, and 1.2 g $NaHCO_3$ in 1 liter of distilled water (Minekus et al., 1995; Marteau et al., 1997). The solution was sterilized by autoclaving, and cooled to approximately 25°C before use.

Gastric fluid contained (Molly et al., 1994; Naim et al., 2004): 0.4 g/liter glucose, 3.0 g/liter yeast extract, 1.0 g/liter Bacto Peptone (Difco, Becton, Dickinson), 4.0 g/liter porcine mucin (Sigma-Aldrich), 0.5 g/liter cysteine, 0.08 g/liter NaCl, 0.4 g/liter $NaHCO_3$, 0.04 g/liter K_2HPO_4, 0.04 g/liter KH_2PO_4, 0.008 g/liter $CaCl_2$-$2H_2O$, 0.008 g/liter $MgSO_4 \cdot 7\ H_2O$, 1.0 g/liter xylan (Sigma-Aldrich, St. Louis, MO), 3.0 g/liter soluble starch (Sigma-Aldrich), 2.0 g/liter pectin (Sigma-Aldrich), and 1 ml/liter Tween 80. Finally, pepsin from porcine stomach mucosa (Sigma-Aldrich) was added to achieve a concentration of 3 g/liter after sterilization by autoclaving and equilibration to ambient temperature (approximately 25°C). Before use in the gastrointestinal challenge, the pH of the gastric fluid was adjusted to 2 with 5N HCl.

Artificial intestinal fluid (Koo et al., 2001) was made by diluting 0.1 g trypsin from porcine pancreas (type IX-S; Sigma-Aldrich) and 3.5 g pancreatin from porcine pancreas (Sigma-Aldrich) with 1 liter distilled water. The solution was filtered through a 0.45-μm pore-diameter filter (cellulose, Millipore Corp., Bedford, MA), for sterilization purposes, and added into a sterile flask. Biliary secretions were simulated by preparing 2% or 4% bile solutions (porcine bile extract, Sigma-Aldrich) in distilled water, which were then filter-sterilized, as described above. Porcine (rather than ox gall) bile was selected due to its similarities to human bile (Marteau et al., 1997).

5.2.5. Dynamic gastrointestinal system

A dynamic model of the stomach and small intestine, previously described by Koo et al. (2001), was used as the basis of the simulated gastrointestinal tract employed in this study. Two 250-ml Erlenmeyer flasks, representing the gastric (GC) and the intestinal (IC) compartments, were kept in a water bath (Shaking Water Bath 50, Precision

Scientific, Chicago IL), stabilized at 37°C throughout the gastrointestinal challenge. The pH conditions in the GC and IC were monitored continuously using two pH meters (Ultra Basic, Denver Instrument, Arvada, CO) equipped with electrodes (Denver Instrument). Three peristaltic pumps (Variable-Speed Pump Low Flow, Fisher Scientific) were used for the delivery of simulated gastric fluid in the GC (at a flow rate of 0.33 ml/min) and simulated intestinal fluid (at a flow rate of 0.33 ml/min) and bile solution (at a flow rate of 0.5 ml/min) in the IC. To simulate physiological conditions prevailing in the human intestine (Northfield and McColl, 1973; Fausa, 1974), a 4% solution of bile was delivered in the IC during the first 30 min of the experiment, followed by the addition of a 2% bile solution for the remaining time (Minekus et al., 1995). The two compartments were connected through a multi-channel peristaltic pump (205U, Watson-Marlow Limited, Cornwall, England) that was used for transferring the gastric contents into the IC (gastric emptying) at a flow rate of 1.1 ml/min, based on results of a study (Doran et al., 1998) concluding that the gastric emptying rate after the consumption of a small meal (217 g) was 1.1 g/min, when subjects were sitting. Preliminary tests suggested that 1 ml of chyme weighted approximately 1 g (throughout the challenge). Introduction of the gastric contents into the IC started within 15 min of the simulated ingestion.

5.2.6. Gastrointestinal passage tolerance assay

The gastrointestinal challenge was performed at 1, 6, 14, 27, 42, 57, and 82 days after inoculation of bologna and salami slices. On each of these storage days, artificial saliva (70 g) was added to six slices (all contained within a single vacuum bag) of each product (50-55 g total) and blended for 3 min at high speed. The pH of the product-saliva mixture was measured by inserting a pH electrode, previously sprayed with 70% alcohol, rinsed

with sterile distilled water and drained. Overall, the pH of the product-saliva suspension was very similar to that of the product itself, as determined by measuring the pH of blended bologna or salami before addition of artificial saliva. Prior to the beginning of each challenge, 10 ml of simulated gastric fluid were added in the GC to simulate the cephalic phase of acid secretion, whereas the IC contained 12.5 ml of the 4% bile solution (Marteau et al., 1997). Before the introduction of the mixture in the GC, both flasks were inserted in the shaking water bath. All pumps were started immediately upon the addition of the product-saliva mixture in the GC. The pH of the GC was adjusted manually (5N HCl) to reproduce *in vivo* human gastric pH values after ingestion of a standard meal (Dressman et al., 1990): pH 5 at 10 min (not for salami, since its pH was < 5 throughout the storage period), pH 4 at 28 min, pH 3 at 58 min, and pH 2 at 88 min. After the final adjustment, the gastric pH remained constant until the end of the challenge (120 min). The acidified gastric contents were neutralized upon transfer to the IC, as the intestinal pH was maintained at 6.5±0.3 (Minekus et al., 1995) using 0.3 M $NaHCO_3$.

Initial *L. monocytogenes* populations were assessed by withdrawing a 1-ml aliquot from the blended sample, prior to introduction in the GC, serially diluting, and plating on PALCAM agar TSAYE. Subsequently, during the gastrointestinal challenge, surviving *L. monocytogenes* populations were enumerated at 30, 60, 90, and 120 min in the GC, and at 30, 90, and 120 min in the IC, by removing 1-ml samples of gastric or intestinal contents and plating in duplicate on PALCAM agar and TSAYE. Secretion of gastrointestinal fluids in the gastrointestinal compartments and gastric emptying continued for 120 min after the beginning of each challenge; however, the IC was maintained (statically) at 37°C

in the water bath for a final microbiological analysis at 240 min. Plates were incubated as previously described and colonies were counted manually.

5.2.7. Statistical analyses

Two replicate experiments were conducted using two different product lots and bacterial cultures. In each replicate, two samples (six slices each) per product were tested on each storage day. Cell counts were divided by dilution factors, to account for the continuous addition or removal of gastrointestinal fluids in each compartment (Koo et al., 2001). Dilution factors (DF) were calculated as follows:

$$DF\ (GC) = \frac{\text{Remaining sample (ml)}}{\text{Remaining sample (ml)} + \text{HCl (ml)} + \text{Gastric fluid (ml)}}$$

$$DF\ (IC) = \frac{\text{Total IC contents (ml)}}{\text{Total IC contents (ml)} + \text{gastric contents (ml)} + \text{bile (ml)} + \text{intestinal fluid (ml)} + NaHCO_3\ \text{(ml)}}$$

The obtained numbers were converted to log CFU/g and analyzed using the Mixed Procedure of SAS (SAS, 2002) to identify potential effects ($P < 0.05$) of the product and the storage day on the ability of *L. monocytogenes* to survive in each compartment of the gastrointestinal system. Means and standard deviations were calculated and the mean differences were separated at the significance level of 95%.

Shoulder durations (SD; min) and inactivation rates (IR; log CFU/g/min) for each product and storage day in each gastrointestinal compartment were estimated by fitting *L. monocytogenes* data (log CFU/g) from each of the two replicate experiments to the model of Baranyi and Roberts (1994) using DMFit software, the in-house program of the

Institute of Food Research (Norwich, UK). Statistical analyses of the computed inactivation kinetics were performed using the Mixed Procedure of SAS (SAS, 2002).

5.3. Results

5.3.1. Chemical and physical properties of products

Changes in pH values of inoculated and stored bologna and salami are presented in Figure 5.1. The pH values of uninoculated products on the day of inoculation (day-0) were 6.16±0.04 (bologna) and 4.47±0.13 (salami) (not shown in figures). On day-1, the pH values of bologna and salami were 6.13±0.09 and 4.49±0.09, respectively. Reductions in pH, observed during storage of bologna, were suggestive of microbial growth and the pH value of the product had declined to 5.24±0.11 within 82 days. On the other hand, the pH of salami remained virtually unchanged during product storage, ranging between 4.41±0.29 (day-82) and 4.61±0.34 (day-42). On day-0, the water activity of inoculated bologna and salami was 0.968±0.006 and 0.892±0.013, respectively.

5.3.2. Changes in microbial populations during storage

Initial (0 min) levels of *L. monocytogenes* represented those that had grown or survived on each product at 1, 6, 14, 27, 42, 57 or 82 days and were enumerated prior to introduction of the product-saliva mixture in the gastric compartment. Initial *L. monocytogenes* populations on both products were at comparable ($P \geq 0.05$) levels (4.0-5.0 log CFU/g) only on day-1, as subsequently during storage at 4°C, populations of the pathogen exhibited increases on bologna and reductions on salami (3.5 and 3.0 log CFU/g, respectively, within 82 days). Changes in *L. monocytogenes* levels during storage of each product are shown in Figure 5.1 (0 min populations). Overall, total microbial populations on bologna followed similar trends with *L. monocytogenes* populations

during storage, as suggested by comparing counts obtained on PALCAM agar (Figure 5.1) and TSAYE (Figure 5.2). On the contrary, colonies that grew on TSAYE after plating of salami samples were predominately small, resembling colonies of lactic acid bacteria, and tested negative for catalase. Lactic acid bacteria have been shown to be the predominant component of microflora recovered from fermented sausages on non-selective media (Holley et al., 1988). Differences in counts between *L. monocytogenes* (PALCAM agar) and suspected lactic acid bacteria (TSAYE) became more pronounced as storage of the product progressed (Figures 5.1 and 5.2). This observation was likely due to the resistance of lactic acid starter cultures to the intrinsic properties of the salami. Thus, while *L. monocytogenes* populations displayed reductions, levels of presumed lactic acid bacteria remained high (approximately 7 log CFU/g) throughout storage of salami samples.

5.3.3. Gastric survival

Surviving *L. monocytogenes* populations in the GC of the dynamic gastrointestinal model on storage days 1, 6, 14, 27, 42, 57, and 82 are presented in Figure 5.1. Overall, reductions in initial *L. monocytogenes* populations were relatively small (0.2 to 1.5 log CFU/g) during the first 60 min of gastric challenge, as the pH of the gastric contents during that period was ≥ 3. After the pH in the GC had been reduced to 2 (90 min), reductions in *L. monocytogenes* levels in the GC became greater, ranging from 1.3 to 3 log CFU/g, depending on the type and age of product used for the delivery of the pathogen in the simulated digestive tract (Figure 5.1). Effects of product type and storage duration on the gastric survival of the pathogen became more obvious within 120 min of gastric exposure (30 min at pH 2), as reductions of *L. monocytogenes* populations present

on bologna and salami, respectively, were 4.9 (day-1) to 7.6 (day-27) log CFU/g and 1.2 (day-82) to 3.0 (day-1) log CFU/g, from the initial levels of 5.0 (day-1) and 7.9 (day-27) log CFU/g (bologna) and 1.4 (day-82) and 4.4 (day-1) log CFU/g (salami) (Figure 5.1).

Computed inactivation parameters are presented in Tables 5.1 and 5.2. The calculated gastric IR of *L. monocytogenes* inoculated onto bologna or salami varied from 0.085 (day-1) to 0.158 (day-57) log CFU/g/min and 0.013 (day-42) to 0.051 (day-1) log CFU/g/min, respectively (Table 5.1). It should be noted that the decline of initial *L. monocytogenes* cell populations to < 2.5 log CFU/g during storage of salami resulted in almost linear inactivation curves during the gastric challenge. Moreover, tailing of the survival curve was observed in one of the replicates on days 6 and 27 of storage, while on day-82 the tailing effect was reproducible (Table 5.2). Tailing of the inactivation curve suggests presence of a subpopulation with very high acid resistance. Prolonged incubation at pH 3.5 led to isolation of acid-tolerant spontaneous mutants, as demonstrated in a study by O'Driscoll et al. (1996). Consequently, exposure on the salami surface (pH 4.41-4.61) could have led to similar effects. Nevertheless, it is not known whether and to what degree exposure of each individual strain to conditions prevailing on salami for 1 to 82 days could have influenced their subsequent behavior during acid exposure, particularly since the *L. monocytogenes* strains comprising the inoculum possess various levels of acid tolerance (chapter 4; Lianou et al., 2006). Perhaps, the acid-resistant cells, whose presence led to the tailing effects, may have belonged to a different strain on each day this phenomenon was observed.

During gastric exposure of bologna samples on day-1 of storage, *L. monocytogenes* levels present on bologna were reduced faster than those on salami (IR: 0.085 and 0.051

log CFU/g/min, respectively); however, the difference was not significant ($P \geq 0.05$). Nevertheless, although initial *L. monocytogenes* populations on both products were similar (4.0-5.0 log CFU/g), after 120 min of gastric challenge, counts on salami samples were 1.4 log CFU/g, whereas no survivors were detected on bologna samples. On corresponding days during subsequent storage, gastric IR of populations on bologna were significantly ($P < 0.05$) higher than those of populations on salami. It appears that the surface of salami may have rendered bacterial cells more resistant to the extreme acidity of the stomach. Prolonged exposure to low pH (approximately 4.5) may have resulted in selection of resistant cells that were capable of withstanding the subsequent gastric challenge. Nevertheless, the low initial population densities on salami (Figure 5.1) could also have contributed to the low inactivation rates observed during digestion of this product. For instance, results by Yoon et al. (2004) showed that low *E. coli* O157:H7 levels, previously exposed to acetic acid, displayed lower rates of inactivation during drying of inoculated jerky than high levels of the pathogen.

In addition to the type, the age of the inoculated product also appeared to influence the gastric survival of the pathogen. Previous studies (Stopforth et al., 2005; Formato et al., 2007) have also investigated the survival properties of *L. monocytogenes* during simulated digestion of inoculated and stored RTE products (frankfurters or bologna), with results indicating that the resistance of *L. monocytogenes* to gastric fluid (pH 1) increased at later stages of product storage. Results of the present study showed that on day-27 of bologna storage, *L. monocytogenes* populations present on the product had a gastric IR of 0.132 log CFU/g/min, which was significantly ($P < 0.05$) greater than the IR displayed by the pathogen at earlier stages of the storage period (days-1 to 14; IR: 0.079 to 0.094 log

CFU/g/min) (Table 5.1). Subsequent IR of *L. monocytogenes* populations observed during storage of bologna remained high, reaching a peak on day-57 (0.158 log CFU/g/min). On day-82, however, the calculated gastric IR of pathogenic populations was 0.100 log CFU/g/min, which was significantly lower than that observed on day-57. It should also be mentioned that reductions in initial *L. monocytogenes* counts observed during gastric exposure of bologna stored for 42, 57, or 82 days were lower than that observed on day-27. Findings from other studies performed in our laboratory (Stopforth et al., 2005; Formato et al., 2007) have also suggested that the resistance of *L. monocytogenes* to artificial gastric fluid may vary based on the duration of the storage period. More specifically, results of all studies agree in that survival of the pathogen during the gastric challenge increased, after *L. monocytogenes* populations on the stored product reached a plateau, probably due to activation of the stationary phase-dependant acid resistance system (Samelis et al., 2003a; Samelis and Sofos, 2003). Therefore, on days-42 to 82 of storage, the increased acid tolerance of *L. monocytogenes* cells in combination with the high initial populations (> 8.5 log CFU/g) led to high numbers of surviving cells being present in the GC, especially during the first 90 min of the gastric challenge. *L. monocytogenes* populations present on salami displayed the highest gastric IR (0.051 log CFU/g/min) on day-1 storage. On the other hand, the slow gastric reductions displayed by the pathogen during storage of inoculated salami (particularly after day-14) may have been resulted from acid adaptation of cells exposed to the dry and acidic (pH 4.41 to 4.61) surface of this product.

In general, reductions in total microbial populations present on bologna followed similar trends to those exhibited by *L. monocytogenes* (Figures 5.1 and 5.2). However,

higher counts observed on the non-selective medium (as compared to those on PALCAM), particularly at later stages of the gastric challenge were indicative of acid injury (Hurst, 1977). Throughout storage, total counts present on salami (initial populations: 6.6-7.4 log CFU/g) exhibited reductions during the 120-min gastric challenge that ranged from 3.3 to 4.4 log CFU/g, except for day-27, on which a 6.1 log CFU/g reduction was observed. On the other hand, as already mentioned, corresponding reductions in *L. monocytogenes* levels varied between 1.2 (day-82) and 3.0 (day-1) log CFU/g, from initial populations of 1.4 and 4.4 log CFU/g, respectively. Overall, the acid susceptibility of total microbial populations on salami appeared unaffected by the storage duration, probably because populations, unlike those on bologna, remained relatively constant during storage.

5.3.4. Intestinal survival

L. monocytogenes populations in the IC during the 240-min exposure are presented in Figure 5.3. Interestingly, on day-1 of storage, although contamination levels on both products were similar ($P \geq 0.05$), initial intestinal populations for bologna were higher ($P \geq 0.05$) than those for salami, while surviving populations on bologna after 120 min of exposure were significantly ($P < 0.05$) higher than those on salami (Figure 5.3). Transferring of gastric contents in the IC began while the gastric pH was still high and, thus, viable populations of the pathogen transferred from the GC to the IC within the first 30 min of gastric emptying were related to the initial contamination levels on each product. As a result, the duration of the storage period in combination with the type of product (supportive vs. not supportive of growth) had a major effect on the number of pathogenic cells being present in the IC. More specifically, during storage of bologna,

initial (30 min) *L. monocytogenes* populations in the IC increased from 4.5 (day-1) to 7.9 (day-82) log CFU/g, while initial pathogenic populations during the 120-day storage period of salami decreased from 3.8 to 1.0 log CFU/g. Subsequently, reductions resulting from the 240-min intestinal exposure were less dramatic than those achieved within the GC. Intestinal IR were similar for both products, ranging from 0.003 to 0.048 log CFU/g/min and 0.002 to 0.056 log CFU/g/min throughout storage of bologna and salami, respectively (Table 5.1). The age of the inoculated products did not appear to have any marked effects on the ability of *L. monocytogenes* to tolerate intestinal stresses, as suggested by the calculated intestinal IR.

Overall, total microbial populations present on bologna exhibited reductions during the 240-min of intestinal challenge (Figure 5.4). Differences in counts recovered by the two media (TSAYE and PALCAM agar) were observed in some cases (e.g., day-14; Figures 5.3 and 5.4) and may have been due to the acid or bile exposure of cells, that prevented them from growing on the selective medium. An apparent discrepancy was also observed on day-1, as initial (30 min) total microbial counts were higher than *L. monocytogenes* counts by 1 log cycle and could have been due to technical error. Declines in initial levels were also observed for total microbial populations present on salami during the 240-min intestinal challenge; however, in most cases, reductions in initial (30 min) levels were slight ($P \geq 0.05$), suggesting that the microflora of salami samples had the ability to overcome intestinal stresses. Although other studies (Gänzle et al., 1999; Jacobsen et al., 1999; Kimoto et al., 2002) have shown that lactic acid bacteria are susceptible to the toxic effects of bile, heterogeneity in bile resistance among strains

(Jacobsen et al., 1999) and protective effects of food components (Gänzle et al., 1999; Kos et al., 2000) may account for the findings of the present study.

5.4. Discussion

The 2003 listeriosis risk assessment conducted by the Food and Drug Administration (FDA) of the United States (US) Department of Health and Human Services (HHS) and the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) stated that factors that contribute to the dose-response relationship for *L. monocytogenes* include the virulence properties of the specific strain, the susceptibility of the host, and the nature of the food matrix (HHS-FDA/USDA-FSIS, 2003). Although, the role of the *L. monocytogenes*-contaminated food vehicle in the dose-response relationship is not well understood, it is suspected that certain physico-chemical food properties that may promote survival of the pathogen in the human gastrointestinal tract may be of great importance in this respect. For example, foods with high buffering capacity may protect *L. monocytogenes* cells in the gastric environment by neutralizing the acid. Other conditions in the matrix (e.g., low pH, high salt content) may alter physiological responses of the cells and influence their ability to overcome natural defense barriers, such as gastric acidity (O'Driscoll et al., 1996).

In this study, the type of product (bologna vs. salami) used for *L. monocytogenes* delivery in the simulated gastrointestinal tract appeared to affect the ability of the pathogen to tolerate stomach acidity. More specifically, pathogenic populations on salami exhibited lower IR than those on bologna throughout the storage period. Enhanced survival of *L. monocytogenes* populations present on salami may have been due to either adaptation of pathogenic cells to the acidic conditions prevailing in the product, the high

fat content of the product, or the combination of both factors. Nevertheless, as already mentioned, the low initial levels, reached with prolonged storage of salami, may have also contributed to the slow death rates. Although *L. monocytogenes* populations on bologna decreased faster than those on salami, the high initial contamination levels, reached during storage of this product resulted in higher ($P < 0.05$) numbers of survivors being detected in the GC: (i) for the first 90 min of the gastric challenge, on days-6 to 27 of storage, and (ii) throughout the gastric challenge, on days-42 to 82, as compared to those detected during the simulated digestion of salami. Overall, gradual decline of gastric pH, similar to that observed *in vivo* (Dressman et al., 1990), resulted in a large fraction of the initial population being delivered into the IC. In most cases, initial *L. monocytogenes* populations in the IC at 30 min were ≤ 1 log CFU/g lower than initial numbers (0 min counts) on each product throughout storage. Therefore, populations transferred to the IC greatly depended on the initial levels of the pathogen on each product, and thus increased or decreased with storage of bologna or salami, respectively. These results agree with those by Marteau et al. (1997) demonstrating the protective effects of gastric emptying against the gastric destruction of lactic acid bacteria in a dynamic gastrointestinal system. Considering that the peak gastric emptying rates occur at the beginning of the digestion process (Marteau et al., 1997), early stages of gastric emptying may allow bacteria to escape to the small intestine before gastric acidity becomes lethal. Subsequently, *L. monocytogenes* populations transferred in the IC of he dynamic model underwent relatively small reductions in both products. This observation came as no surprise, as the main antimicrobial factor in the simulated intestine used in

this study was bile and the bile-tolerance of *L. monocytogenes* is well documented (Allerberger et al., 1989; Briones et al., 1992; Begley et al., 2002; Olier et al., 2004).

Under the conditions of this study, levels of *L. monocytogenes* in the compartments of the simulated gastrointestinal tract depended on the type of product and the duration of storage. Although salami appeared to a have protective effect against acid destruction of the pathogen, the high pathogenic levels reached on bologna during storage overshadowed any effects of the food matrix in gastric survival. Thus, the potential for *L. monocytogenes* growth, as affected by the intrinsic characteristics of each product and the length of the storage period were the most influential factors, in terms of *L. monocytogenes* levels being present in the compartments of the gastrointestinal system. According to the risk assessments of *L. monocytogenes* in RTE foods conducted by HHS-FDA/USDA-FSIS and the Food and Agriculture Organization of the United Nations (FAO) and the World Heath Organization (WHO), the vast majority of listeriosis cases result from foods contaminated with high levels of *L. monocytogenes* (HHS-FDA/USDA-FSIS, 2003; Buchanan et al., 2004). Results of this study also suggest that the odds of contracting *L. monocytogenes* infection may increase with the contamination levels ingested and thus, they highlight the importance of implementing control measures in RTE products allowing growth of *L. monocytogenes* to high levels. In addition, findings also demonstrated the protective effects of gastric emptying against *L. monocytogenes* acid destruction, and, thus, highlight the importance of simulating gastrointestinal-related features (e.g., gastric emptying and bile-resistance) other than static acid challenging in studies designed to examine the gastrointestinal survival of pathogen.

Table 5.1. Mean inactivation parameters (shoulder duration or maximum inactivation rate ± standard deviation; determined from counts on PALCAM agar using the model of Baranyi and Roberts) of *Listeria monocytogenes* exposed to a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

Product		Gastrointestinal compartment			
		Gastric		Intestinal	
	Day of storage	Shoulder duration (min)	Maximum inactivation rate (log CFU/g/min)	Shoulder duration (min)	Maximum inactivation rate (log CFU/g/min)
Bologna	1	$46.85\pm3.80_{AD}$	$0.085\pm0.023_{AD}$	-[1]	$0.004\pm0.000_{A}$
	6	$68.53\pm9.56_{BC}$	$0.094\pm0.020_{A}$	$27.85\pm39.39_{A}$	$0.018\pm0.024_{AB}$
	14	$52.13\pm0.11_{ABD}$	$0.079\pm0.000_{AD}$	$80.71\pm3.09_{B}$	$0.034\pm0.012_{AC}$
	27	$64.68\pm7.14_{ABC}$	$0.132\pm0.016_{BC}$	-[1]	$0.004\pm0.002_{A}$
	42	$70.77\pm11.57_{BC}$	$0.104\pm0.037_{AB}$	$64.01\pm23.70_{C}$	$0.043\pm0.016_{BC}$
	57	$83.26\pm3.66_{C}$	$0.158\pm0.010_{C}$	$60.04\pm30.88_{C}$	$0.048\pm0.051_{BC}$
	82	$63.77\pm1.84_{ABC}$	$0.100\pm0.011_{AB}$	-[1]	$0.003\pm0.000_{A}$
Salami	1	$62.61\pm11.60_{ABC}$	$0.051\pm0.010_{DE}$	-[1]	$0.007\pm0.004_{A}$
	6	$35.78\pm13.48_{D}$	$0.039\pm0.006_{EF}$	-[1]	$0.002\pm0.002_{A}$
	14	-[1]	$0.016\pm0.003_{F}$	-[1]	$0.005\pm0.002_{A}$
	27	-[1]	$0.021\pm0.009_{F}$	-[1]	$0.014\pm0.012_{A}$
	42	-[1]	$0.013\pm0.007_{F}$	$53.62\pm3.03_{C}$	$0.056\pm0.028_{C}$
	57	-[1]	$0.014\pm0.007_{F}$	-[1]	$0.007\pm0.006_{A}$
	82	-[1]	$0.024\pm0.023_{F}$	$24.49\pm34.63_{A}$	$0.021\pm0.010_{AC}$

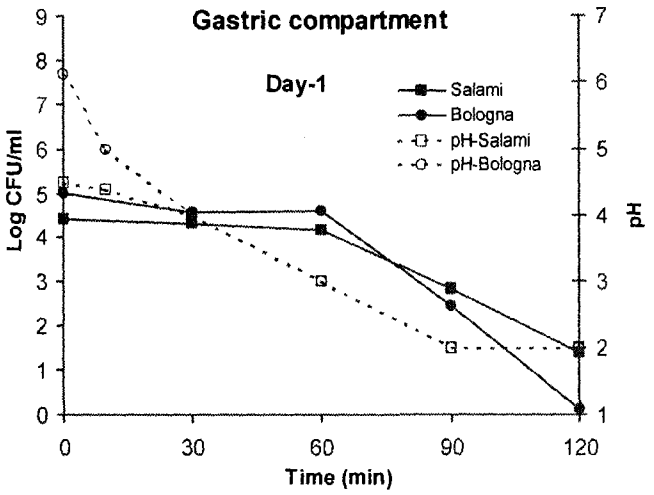
[1] No shoulder was observed (inactivation was immediate)

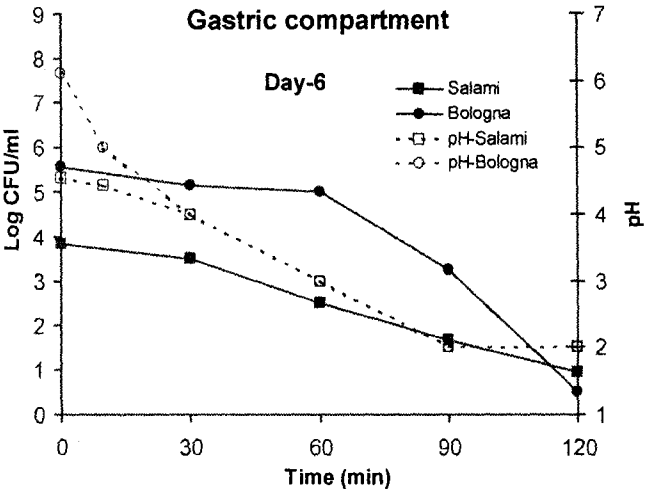
Means within a column lacking a common letter are significantly different ($P < 0.05$)

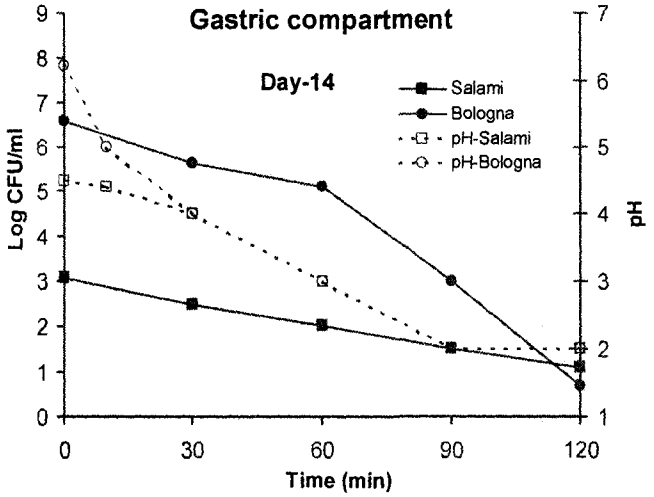
Table 5.2. Mean inactivation parameters (y_0 or y_{end} ± standard deviation; determined from counts on PALCAM agar using the model of Baranyi and Roberts) of *Listeria monocytogenes* exposed to a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

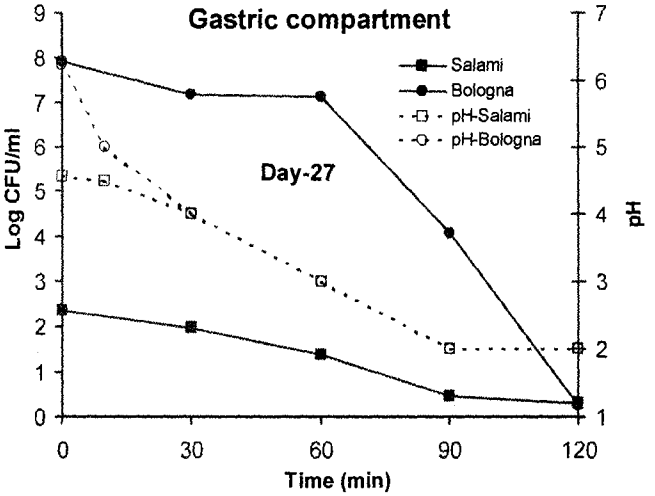
Product		Gastrointestinal compartment			
		Gastric		Intestinal	
	Day of storage	Y_0 (log CFU/g)	Y_{end} (log CFU/g)	Y_0 (log CFU/g)	Y_{end} (log CFU/g)
Bologna	1	4.8±0.3	-[1]	4.6±0.1	-[1]
	6	5.5±0.9	-[1]	5.1±1.0	4.8±0.6
	14	6.6±0.6	-[1]	5.9±0.6	5.0-[2]
	27	8.0±0.7	-[1]	6.3±1.2	-[1]
	42	8.6±0.2	-[1]	8.0±0.2	7.3±0.2
	57	9.2±0.6	-[1]	8.1±0.1	7.3±0.2
	82	8.7±0.1	-[1]	7.7±0.0	-[1]
Salami	1	4.2±0.0	-[1]	3.6±0.1	-[1]
	6	3.6±0.3	0.2-[2]	2.3±0.3	-[1]
	14	3.0±0.6	-[1]	0.9±0.3	-[1]
	27	2.3±1.3	0.1-[2]	1.3±0.7	0.5-[2]
	42	1.9±0.5	-[1]	1.5±1.1	0.4±0.4
	57	2.2±0.8	-[1]	1.6±1.2	-[1]
	82	0.1±0.0	0.1±0.0	1.2±0.6	0.2±0.1

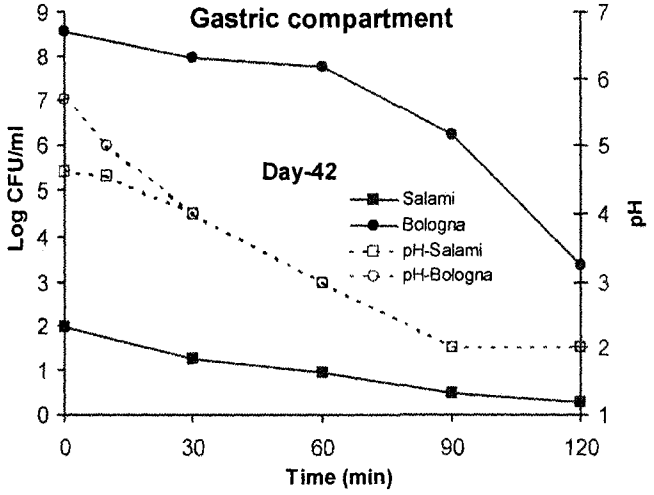
Y_0: upper asymptote corresponding to the initial bacterial counts, Y_{end}: lower asymptote corresponding to the final bacterial counts; [1] No y_{end} value could be estimated when survival curve ceased without forming a tailing region; [2] y_{end} values were estimated for one replicate of the experiment only

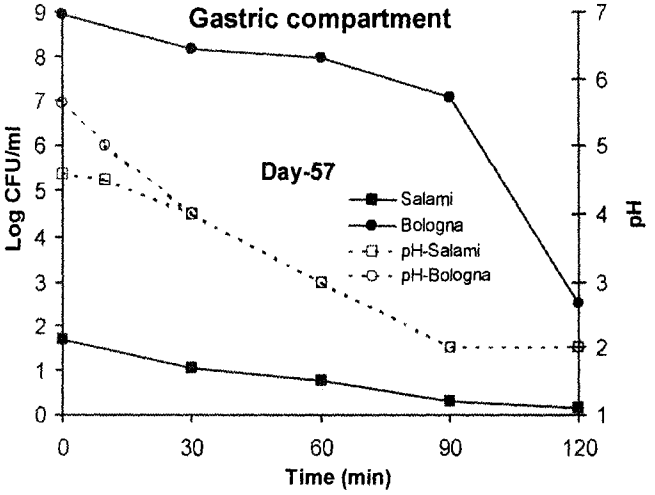

Gastric compartment
Day-1
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)


Gastric compartment
Day-6
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)


Gastric compartment
Day-14
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)


Gastric compartment
Day-27
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)


Gastric compartment
Day-42
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)


Gastric compartment
Day-57
Salami
Bologna
pH-Salami
pH-Bologna
Log CFU/ml
pH
Time (min)

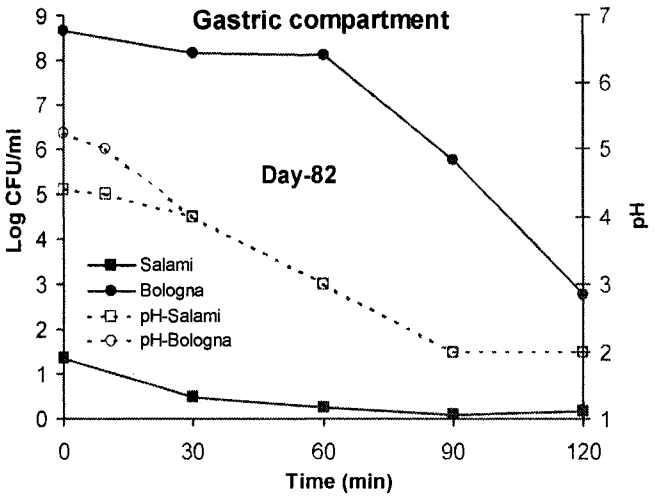
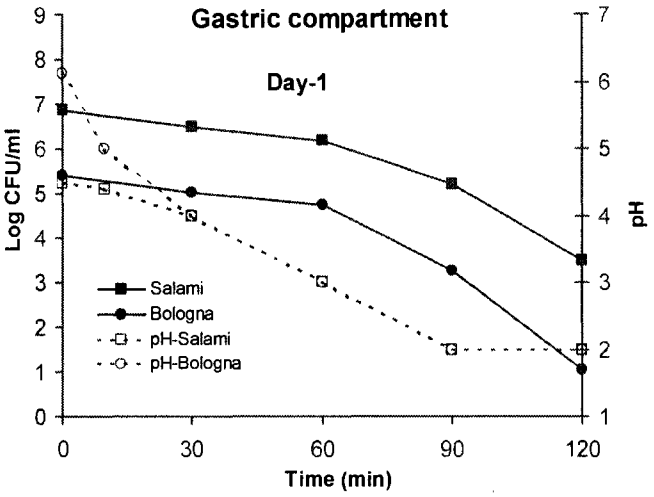
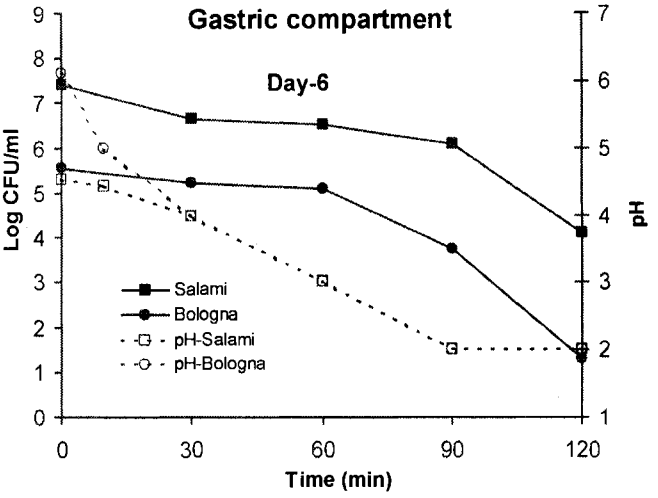
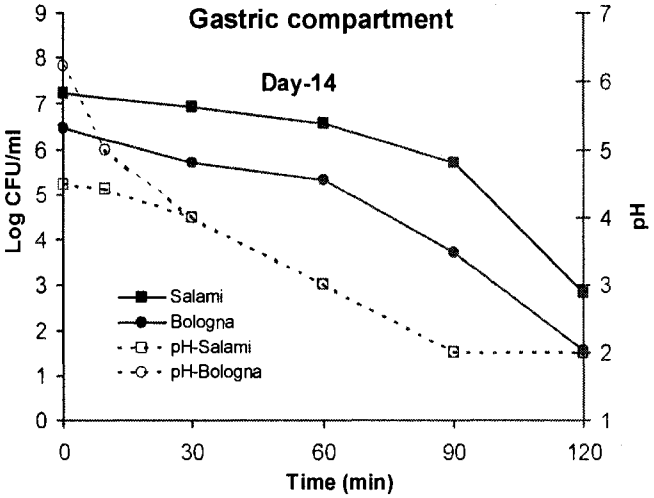
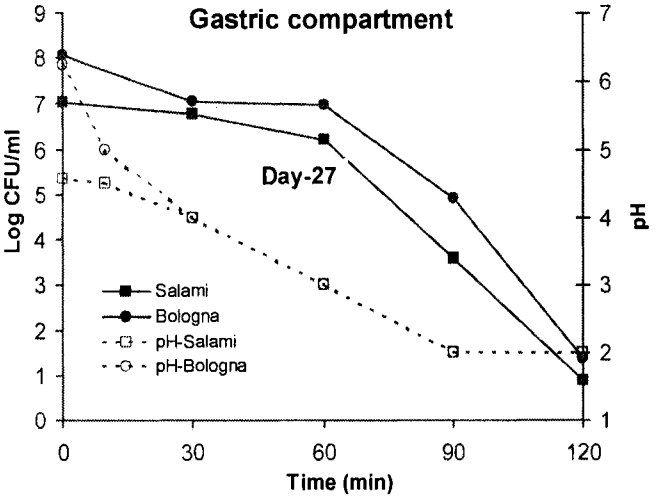


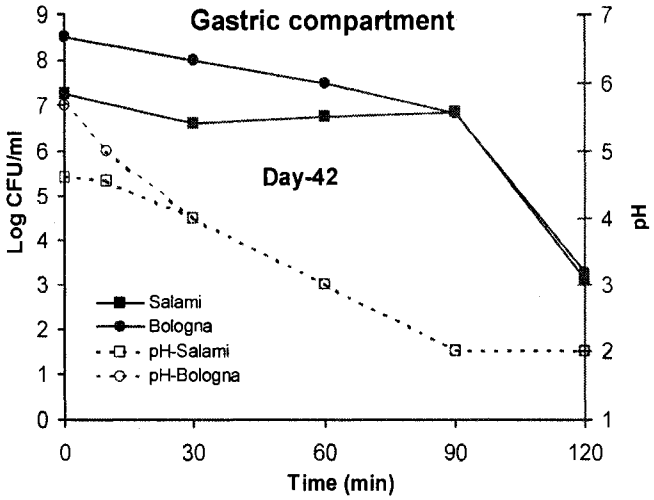
Figure 5.1 (Appendix Table 25). *Listeria monocytogenes* populations (log CFU/g; PALCAM agar) and pH values in the gastric compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation (4.0-5.0 log CFU/g) onto bologna or salami slices and storage at 4°C in vacuum packages for 82 days

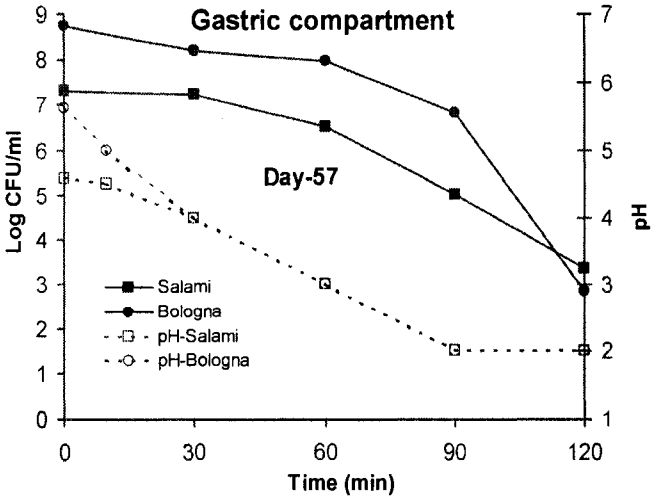

Gastric compartment
Day-1
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna


Gastric compartment
Day-6
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna


Gastric compartment
Day-14
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna


Gastric compartment
Day-27
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna


Gastric compartment
Day-42
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna


Gastric compartment
Day-57
Log CFU/ml
pH
Time (min)
Salami
Bologna
pH-Salami
pH-Bologna

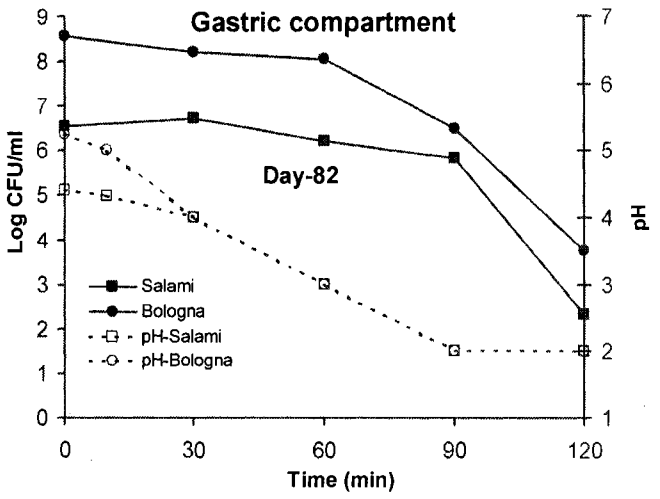
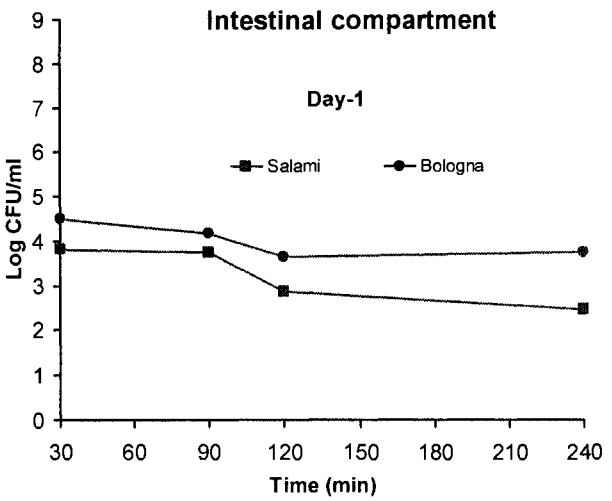
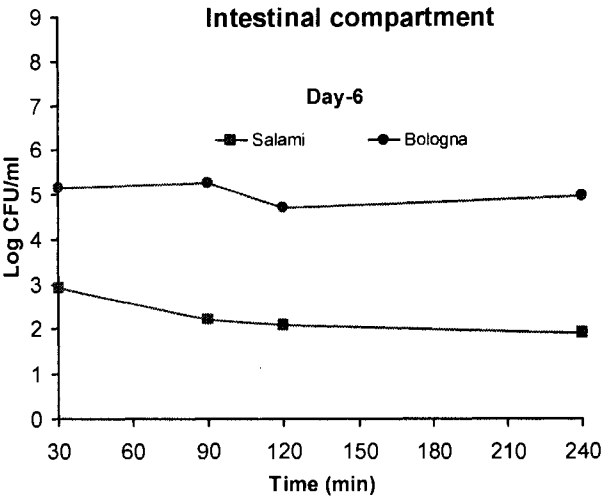
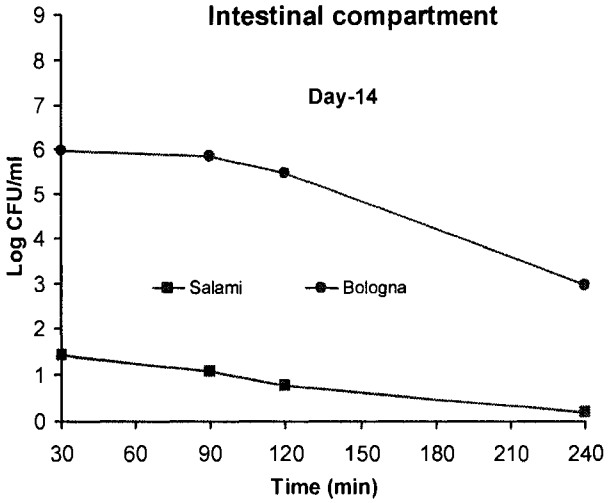
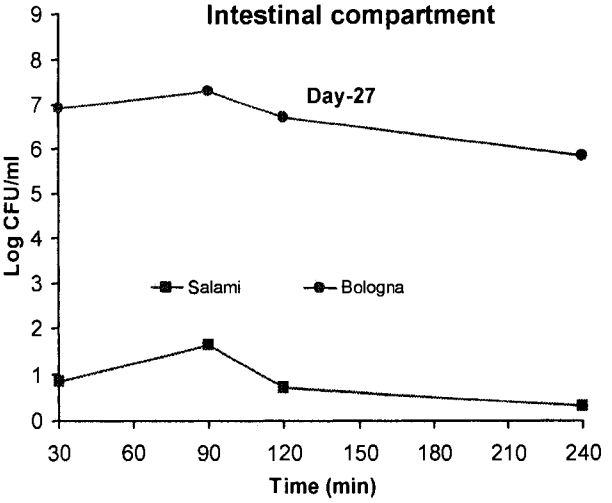


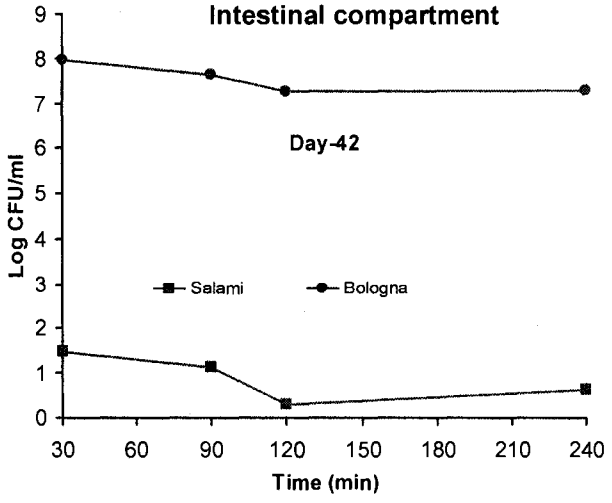
Figure 5.2 (Appendix Table 26). Total microbial populations (log CFU/g; TSAYE) and pH values in the gastric compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation of *L. monocytogenes* (4.0-5.0 log CFU/g) onto bologna or salami slices and storage at 4°C in vacuum packages for 82 days

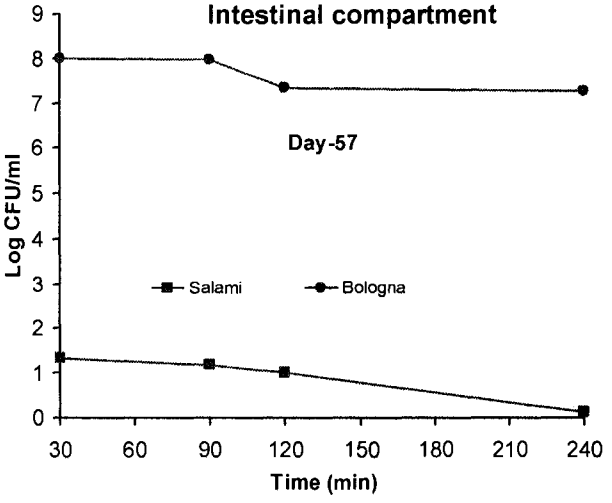

Intestinal compartment
Day-1
Salami
Bologna
Log CFU/ml
Time (min)


Intestinal compartment
Day-6
Salami
Bologna
Log CFU/ml
Time (min)


Intestinal compartment
Day-14
Salami
Bologna
Log CFU/ml
Time (min)


Intestinal compartment
Day-27
Salami
Bologna
Log CFU/ml
Time (min)


Intestinal compartment
Day-42
Salami
Bologna
Log CFU/ml
Time (min)


Intestinal compartment
Day-57
Salami
Bologna
Log CFU/ml
Time (min)

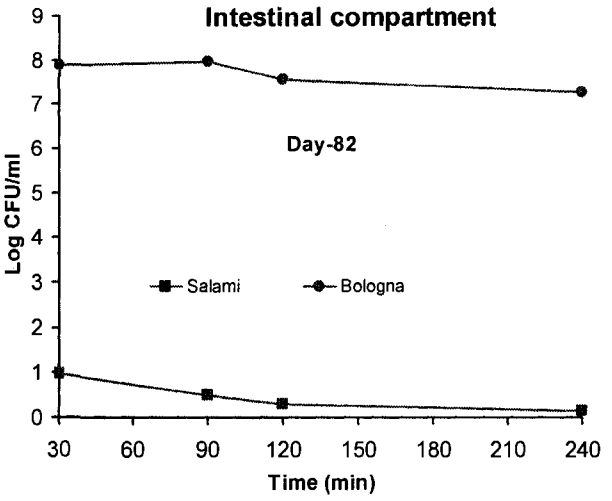
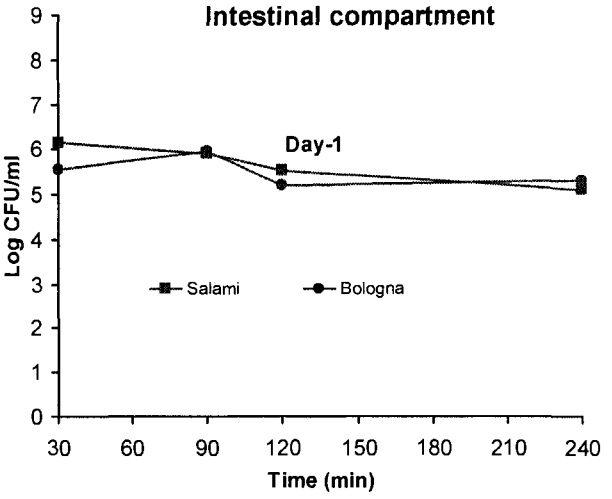
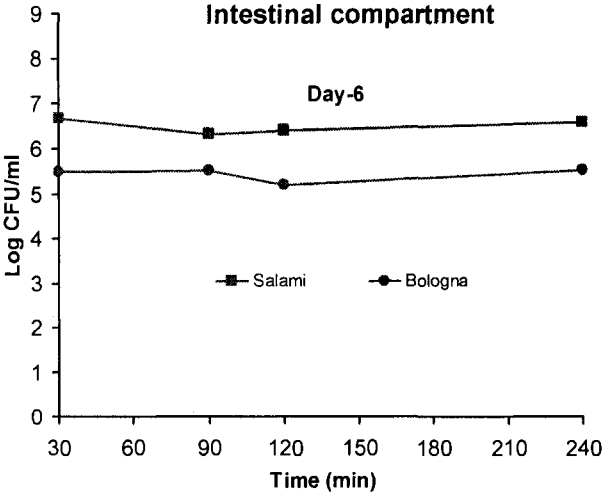
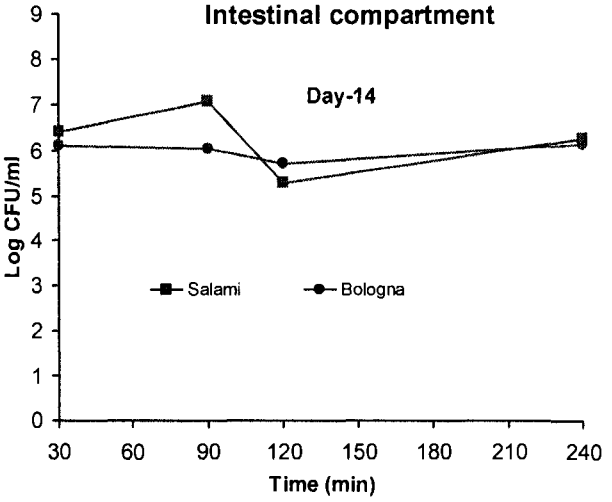
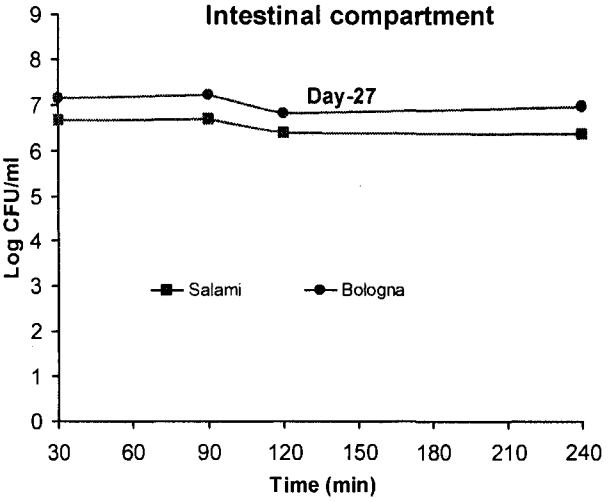


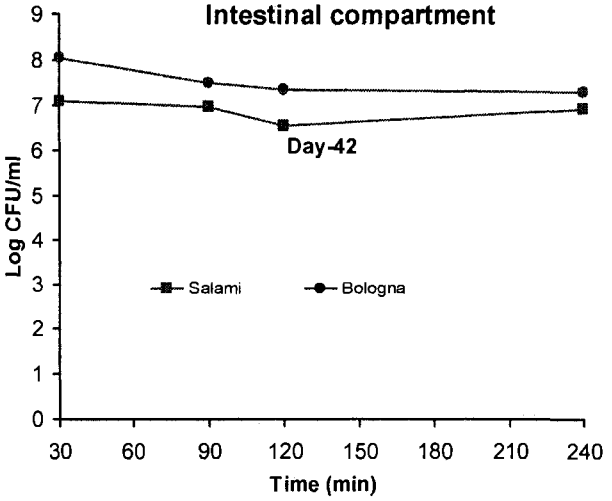
Figure 5.3 (Appendix Table 27). *Listeria monocytogenes* populations (log CFU/g; PALCAM agar) in the intestinal compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation (4.0-5.0 log CFU/g) onto bologna or salami slices and storage at 4°C in vacuum packages for 82 days

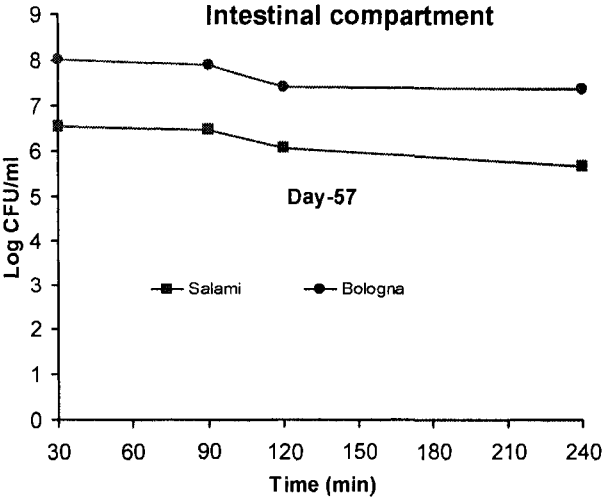

Intestinal compartment
Day-1
Log CFU/ml
Time (min)
Salami
Bologna


Intestinal compartment
Day-6
Log CFU/ml
Time (min)
Salami
Bologna


Intestinal compartment
Day-14
Log CFU/ml
Time (min)
Salami
Bologna


Intestinal compartment
Day-27
Log CFU/ml
Time (min)
Salami
Bologna


Intestinal compartment
Day-42
Log CFU/ml
Time (min)
Salami
Bologna


Intestinal compartment
Day-57
Log CFU/ml
Time (min)
Salami
Bologna

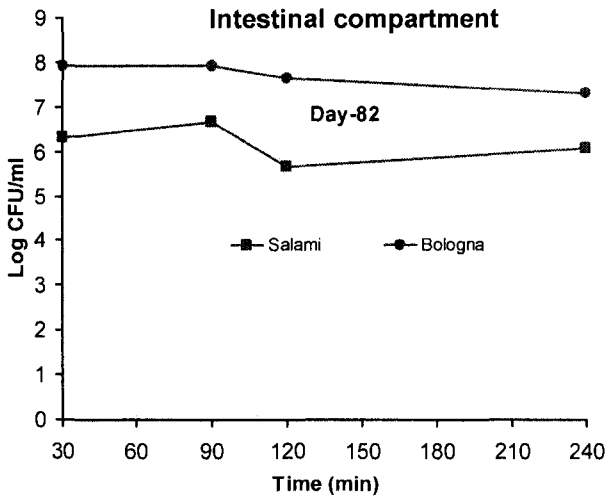


Figure 5.4 (Appendix Table 28). Total microbial populations (log CFU/g; TSAYE) in the intestinal compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation of *L. monocytogenes* (4.0-5.0 log CFU/g) onto bologna or salami slices and storage at 4°C in vacuum packages for 82 days

CHAPTER 6

Effect of fat content on survival of *Listeria monocytogenes* during simulated digestion of inoculated beef frankfurters during storage at 7°C in vacuum packages

ABSTRACT

Potential effects of the fat content and storage of frankfurters on the ability of *L. monocytogenes* to survive transit through a simulated gastrointestinal tract were investigated. *L. monocytogenes* counts were determined during storage (7°C for 55 days) of inoculated (2.0-3.0 log CFU/g) and vacuum-packaged frankfurters of low (~4.5%) or high (~32.5%) fat content, following exposure to a dynamic gastrointestinal model (37°C). Controlled parameters included gastric emptying and gastrointestinal fluid secretion rates, gradual gastric acidification (pH reduction to 2.0 within 88 min) and intestinal pH maintenance (6.5±0.3). Survival curves in each gastrointestinal compartment (gastric, GC; intestinal, IC) were fitted with the Baranyi and Roberts model. Growth of *L. monocytogenes* was observed during storage of low- and high-fat frankfurters with populations reaching 8.3 (day-39) and 8.0 (day-55) log CFU/g, respectively. In the GC, major reductions in *L. monocytogenes* populations were observed mainly after 60 min of exposure (pH ≤ 3), with reductions at 120 min (30 min at pH 2) ranging from 2.6 (day-1) to > 7.2 (day-39) log CFU/g, in low-fat frankfurters and 1.6 (day-1) to 5.2 (day-55) log CFU/g, in high-fat frankfurters. Inactivation rates indicated that on most storage days, *L. monocytogenes* populations present on low-fat frankfurters

declined faster than those on high-fat frankfurters; however, differences in *L. monocytogenes* IR during gastric exposure of low- or high-fat frankfurters were significant ($P < 0.05$) only on days-1 and 6. Since delivery of gastric contents in the IC began while the gastric pH was still high (> 5), *L. monocytogenes* cell numbers transferred from the GC to the IC within the first 30 min of gastric emptying depended on the initial contamination levels of frankfurters, with populations recovered from the IC at 30 min being generally < 1.3 log CFU/g lower than initial populations on each product. As a result, storage duration affected the number of pathogenic cells being present during the intestinal challenge, particularly since subsequent reductions in *L. monocytogenes* populations during the 240-min intestinal exposure of both products were relatively small ranging from 0.1 to 1.4 log CFU/g. Findings indicated that the fat content of frankfurters may enhance survival of *L. monocytogenes* during gastric exposure. However, the effects of fat were observed mainly at later stages of the gastric challenge and, thus did not affect the numbers of pathogenic cells reaching the IC.

6.1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a foodborne illness that may be manifested as mild febrile gastroenteritis or life-threatening systemic disease (Farber and Peterkin, 1991; Painter and Slutsker, 2007). Occurrence of this pathogen in foods, mainly processed ready-to eat (RTE) meat products, poses a major concern to the meat industry, as such foods have been linked to fatal listeriosis outbreaks and numerous recalls in the United States (Bernard and Scott, 1999; CDC, 1998; 2000; 2002). According to the 2003 risk assessment conducted by the Food and Drug Administration (FDA) of the United States Department of Health and Human Services (HHS) and the

Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA), consumption of delicatessen meats and non-reheated frankfurters are major risk factors for contracting listeriosis (HHS-FDA/USDA-FSIS, 2003). The high risk for listeriosis associated with these products is partly attributed to characteristics of these products (e.g., high pH and water activity) that create the potential for *L. monocytogenes* growth to high numbers during storage. Indeed, the listeriosis risk assessment also concluded that the majority of listeriosis cases arise from heavily contaminated foods (HHS-FDA/USDA-FSIS, 2003). Estimating a definite infectious dose for this pathogen, however, is a complicated task due to variables, such as the amount and characteristics of the food ingested and host susceptibility (King et al., 2003).

Food matrix-related characteristics that that could affect *L. monocytogenes* survival during transit through the human gastrointestinal tract are thought to contribute to the likelihood of *L. monocytogenes* infection (HHS-FDA/USDA-FSIS, 2003). In this respect, structural and compositional elements of the ingested food, as well as conditions that may enhance the ability of the pathogen to tolerate host defense barriers by inducing stress responses (e.g., acidity) may be of great importance (FAO/WHO, 2003). In addition, the residence time of bacteria within the human stomach, which depends on the physical form, the energy density, the volume, and the composition of the ingested meal (Hunt and McDonald, 1954; Hunt and Knox, 1968; Malagelada et al., 1979; Hunt, 1983; Notivol et al., 1984; Fisher et al., 1987) is also a factor influencing the antimicrobial efficacy of the gastric barrier (Takumi et al., 2000). Among compositional characteristics of foods, the fat content has been long believed to be a factor influencing the dose-response relationship (Buchanan et al., 2000), since high-fat vehicles have been frequently

implicated in outbreaks of listeriosis (Linnan et al., 1988; McLauchlin et al., 1991; CDC, 1998; Lyytikäinen et al., 2000). However, studies with animal models, undertaken to investigate potential effects of the fat content on the likelihood of developing illness, have provided conflicting results. Gastric inoculation of Sprague-Dawley rats resulted in lower rates of *L. monocytogenes* infection when milk, rather than brain heart infusion broth, was used as a delivery vehicle (Schlech, 1993). Similarly, Sprong et al. (1999) observed that feeding rats with high-fat milk content prevented intestinal colonization of *L. monocytogenes* and diarrhea as compared to milk with low-fat milk. On the other hand, Smith et al. (2003) showed that the oral infectivity of *L. monocytogenes* in pregnant primates increased when whipping cream (30% milk fat) was used as the delivery vehicle as compared to skim milk (0.25% milk fat) or half-and-half (11% milk fat). Findings by Mytle et al. (2006) illustrated that the fat content of the food matrix (skim milk, half-and-half, or whipping cream) did not affect *L. monocytogenes* colonization in the murine gastrointestinal tract. The authors of the latter study, however, reported that any effects of the food matrix were probably overwhelmed the high numbers of the pathogen in the food. Nevertheless, discrepancies among studies employing different animal hosts highlight that results relative to oral-infection information obtained from animal models might be difficult to interpret with respect to humans, as physiological differences between animal species may influence susceptibility to *L. monocytogenes* infection (Kathariou, 2002; Mytle et al., 2006). In addition, investigating the direct effects of fat on the gastrointestinal survival of *L. monocytogenes* and other foodborne pathogens via animal models might be challenging due to possible interactions of dietary lipids with immune responses of the host (Puertollano et al., 2004).

To overcome limitations associated with animal studies, including interspecies extrapolation of data, animal welfare issues and high cost, several researchers have examined various aspects of foodborne infection by employing simulated gastrointestinal fluids. Indeed, *in vitro* gastrointestinal challenge studies have provided useful information regarding conditions and factors contributing to the gastrointestinal survival of various foodborne microorganisms, including *L. monocytogenes* (Roering et al., 1999; Cotter et al., 2001a; King et al., 2003; Corcoran et al., 2005; Tamplin, 2005; Stopforth et al., 2005; Formato et al., 2007). However, to our knowledge, only a few studies have evaluated the effects of food matrix-related characteristics on the ability of *L. monocytogenes* to tolerate gastrointestinal stresses. More specifically, findings by Cotter et al. (2001b) suggested that glutamate might protect *L. monocytogenes* during exposure to artificial gastric fluid. In addition, Stopforth et al. (2005) showed that immersion of frankfurters, formulated with sodium diacetate (0.25%), into a 2.5% solution of lactic acid may have resulted in increased resistance of surviving cells to artificial gastric fluid as storage of the product progressed. However, it was not clear whether the increased resistance of the pathogen in gastric fluid was exclusively due to the antimicrobial treatments applied on the product. Nevertheless, conventional procedures (i.e., static models) used in *in vitro* studies discussed above (Cotter et al., 2001a; Stopforth et al., 2005) might have compromised the predictive value of the findings, as they overlooked the sequential stresses and the constantly shifting conditions to which ingested pathogens are subjected during gastrointestinal passage. Simulation of the successive hurdles, encountered by pathogens during passage through the gastrointestinal transit, may be important, since prior exposure to one form of sublethal stress may impart tolerance against subsequent

stresses (Farber and Pagotto, 1992; Lou and Yousef, 1996; Mazzota, 2001). Consequently, exposure of pathogens in the acidic gastric environment may affect their ability to withstand intestinal stresses. For instance, findings by Begley et al. (2002) demonstrated that exposing *L. monocytogenes* to sublethal levels of bile acids, acid, heat, salt, or sodium dodecyl sulfate increased its ability to tolerate bile.

As mentioned earlier, the effect of the fat content of delivery vehicles on the ability of *L. monocytogenes* to establish infection has been assessed in studies using animal surrogates (Schlech, 1993; Sprong et al., 1999; Mytle et al., 2006). However, to our knowledge, no information is available regarding potential effects of the fat level of foods on the *L. monocytogenes* survival during the gastrointestinal stages of infection. Thus, this study was conducted to assess the survival patterns of *L. monocytogenes* during *in vitro* digestion of low (approximately 4.5% fat) or high-fat (approximately 32.5% fat) beef frankfurters, in order to determine whether the fat content and the storage time of the contaminated food can influence the pathogen's ability to tolerate stresses similar to those encountered in the human gastrointestinal tract.

6.2. Materials and methods

6.2.1. Preparation of beef frankfurters

Beef frankfurters were manufactured at the Department of Animal Sciences Meat Laboratory at Colorado State University. Fresh beef knuckles (approximately 5% fat) and frozen beef trimmings (approximately 50% fat), purchased from Swift Co. (Greeley, CO), were coarse ground (Model 4341, Hobart Corp., Troy, OH) separately through a 0.25-in plate, mixed thoroughly, and reground through a 0.125-in plate. The Pearson-square was then used to determine appropriate amounts of ground knuckles and beef

trimmings needed to generate a ground beef mixture that contained approximately 36% fat. Ground knuckles or the 36% ground beef mixture were used for the preparation of frankfurters having a target fat level of approximately of 5 (low-fat product) or 30% (high-fat product), respectively. It should be noted that since the contribution of fat to the sensory properties of meat products is very important, commercial production of reduced-fat processed meat products involves taking additional steps, such as raising the moisture content and using ingredients that supply water-holding capacity. For the purposes of the present study, however, the formulations of low- and high-fat frankfurters were exactly the same.

The basic frankfurter formulation (Bedie et al., 2001) consisted of (%, wt/wt): ground beef (5% fat ground knuckles or 36% fat ground beef mixture) (82.2), ice (10), sodium chloride (2), dextrose (2), dry mustard (0.9), corn syrup solids (2), polyphosphate (0.4; sodium tripolyphosphate and sodium hexameta-phosphate; Heller Inc., Bedford Park, IL), sodium nitrite (0.0156), sodium erythorbate (0.05), paprika (0.25), onion powder (0.05), garlic powder (0.05), coriander (0.05), and white pepper (0.05). All spices and seasonings were purchased from AC Legg Co. (Birmingham, AL). Meat and non-meat ingredients of each batch were emulsified in a 35-L bowl chopper (RMF, Kansas City, MO) for 3-5 min to a final temperature of 15.5°C. The mixture was extruded (Handtmann Inc., Buffalo Grove, IL) into 24 mm diameter fibrous cellulose casings (Koch, Kansas City, MO) and linked at 10 to 11 cm in length. Stuffed frankfurters were cooked and smoked in a smokehouse (Alkar, DEC International Inc., Lodi, WI), first in dry air (relative humidity 0%, smokehouse temperature 60°C for 15 min and 66°C for 15 min), followed by hot smoking (Zesti liquid smoke, Hickory Specialties Inc., Crossville, TN) for 30 min

(relative humidity 48%, smokehouse temperature 66°C), steam cooking for 40 min (100% relative humidity, smokehouse temperature 66°C), dry cooking for 10 min (relative humidity 0%, 74°C), and hot steam cooking (relative humidity 100%, 74°C) until the internal temperature of the product reached 71.1°C. After cooking and smoking, frankfurters were showered with cool tap water for 5 min and cooled overnight at 4°C. Frankfurters were then transferred to the microbiology lab, where the casings were manually peeled, before inoculation of the links.

Fat and moisture contents of finished products were determined in triplicate for two lots of each product using AOAC International Official methods 960.39 and 950.46.B (AOAC, 1998), respectively. Results indicated that the fat and moisture content of frankfurters were 4.6%±1.6 (low-fat product) or 32.5%±2.0 (high-fat product) and 49.9±3.0 (low-fat product) and 40.2±1.8 (high-fat product), respectively.

6.2.2. Preparation of bacterial cultures

The ten-strain composite of *L. monocytogenes* used in this study included NA-1 (serotype 3b, pork sausage isolate), N-7150 (serotype 3a, meat isolate), 558 (serotype 1/2, pork meat isolate), N1-225 and N1-227 (both serotype 4b, human and food isolate, respectively; associated with the same epidemic), R2-500 and R2-501 (both serotype 4b, food and human isolate, respectively; associated with the same epidemic), R2-763, R2-764, and R2-765 (all serotype 4b, human, food, and environmental isolate, respectively; associated with the same epidemic) (Fugett et al., 2006; Lianou et al., 2007). Strains N1-225, N1-225, R2-500, R2-501, R2-763, R2-764, and R2-765 originated from the International Life Sciences Institute (ILSI) North America outbreak set (Fugett et al., 2006) and were kindly provided by Dr. Martin Wiedmann (Department of Food Science,

Cornell University, Ithaca NY). Frozen (-70°C) stock cultures were maintained separately in tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks MD) supplemented with 0.6% yeast extract (Acumedia, Baltimore MD) (TSBYE) and 20% glycerol. Working cultures were kept on tryptic soy agar (Difco), supplemented with 0.6% yeast extract (TSAYE) slants at 4°C. Each strain was activated by transferring a loopful of bacterial cells from the appropriate slant into 10 ml TSBYE and incubating at 30°C for 24 h. The resultant cultures were then subcultured (0.1 ml) into 10 ml of fresh TSBYE and further incubated at 30°C for 24 h.

6.2.3. Product inoculation

For inoculum preparation, individual cultures (TSBYE; 30°C for 24 h) were individually centrifuged (Eppendorf, model 5810 R, Brinkmann Instruments, Inc., Westbury, NY) at 4,629 x *g* for 15 min (4°C). Resultant cell pellets were washed with 10 ml of phosphate-buffered saline (prepared by suspending 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7\ H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 liter of distilled water, pH 7.4), and centrifuged (4629 x *g* for 15 min at 4°C). The harvested cells of each strain were resuspended in 10 ml of homogenate prepared from frankfurters (low fat content), prepared as described above (Lianou et al., 2007). Suspension of *L. monocytogenes* cells in product homogenate instead of culture media aimed to 'habituate' the pathogen to the frankfurter environment prior to inoculation. Product homogenates were prepared by homogenizing (2 min, Masticator, IUL Instruments, Barcelona, Spain) a 10% (wt/wt) suspension of product with distilled water. Slurries were then filtered twice through two layers of cheesecloth, autoclaved, cooled to ambient temperature (approximately 25°C), and stored overnight at 7°C. Each *L. monocytogenes* strain culture, suspended in product homogenate, was

maintained at 7°C for approximately 72 h. To prepare the ten-strain composite, the 10-ml cultures of each strain were mixed, serially diluted with freshly prepared product homogenate and used to inoculate the surface of frankfurters (approximately 25 g each), respectively, to obtain an inoculum level of 2.0-3.0 log CFU/g when 0.25 ml of inoculum was applied on the surface of each frankfurter link under a biological safety cabinet. Inoculated links were kept at 4°C for 30 min to allow attachment of *L. monocytogenes* on the surface of the product. Subsequently, two inoculated frankfurter links were placed on top of each other into a vacuum bag (15 by 20 cm, 3 mil std barrier, Nylon/PE vacuum pouch, Koch), vacuum packaged (Hollymatic Corp., Countryside, IL) and stored at 7°C for 55 days. Enumeration of *L. monocytogenes* and total microbial populations on the day of inoculation (day-0) was performed by adding 50 g of maximum recovery diluent (0.85% NaCl and 0.1% peptone) to two frankfurter links, blending for 2 min at high speed, withdrawing 1 ml from the blended sample, serially diluting in 0.1% buffered peptone water (Difco) and surface plating on PALCAM agar (Difco) and TSAYE. Colonies were counted manually after incubation at 30°C for 48 h (PALCAM agar) and 25°C for 72 h (TSAYE). In addition, the initial (day-0) water activity of inoculated low- and high-fat frankfurters was determined with an AquaLab (model series 3, Decagon Devices Inc., Pullman, Wash.) water activity meter.

6.2.4. Preparation of simulated gastrointestinal fluids

Artificial saliva was prepared by suspending 6.2 g NaCl, 2.2 g KCl, 0.22 g $CaCl_2$, and 1.2 g $NaHCO_3$ in 1 liter of distilled water (Minekus et al., 1995; Marteau et al., 1997). The solution was sterilized by autoclaving and cooled to room temperature (approximately 25°C) before use. Artificial gastric fluid contained (Molly et al., 1994;

Naim et al., 2004): 0.4 g/liter glucose, 3.0 g/liter yeast extract, 1.0 g/liter Bacto Peptone (Difco, Becton, Dickinson), 4.0 g/liter porcine mucin (Sigma-Aldrich), 0.5 g/liter cysteine, 0.08 g/liter NaCl, 0.4 g/liter $NaHCO_3$, 0.04 g/liter K_2HPO_4, 0.04 g/liter KH_2PO_4, 0.008 g/liter $CaCl_2$-$2H_2O$, 0.008 g/liter $MgSO_4 \cdot 7\ H_2O$, 1.0 g/liter xylan (Sigma-Aldrich, St. Louis, MO), 3.0 g/liter soluble starch (Sigma-Aldrich), 2.0 g/liter pectin (Sigma-Aldrich), and 1 ml/liter Tween 80. The ingredients were mixed thoroughly and the solution was autoclaved and cooled to room temperature, followed by the addition of 3 g/liter pepsin from porcine stomach mucosa (Sigma-Aldrich). Before use in the gastrointestinal challenge, the pH of the gastric fluid was adjusted to 2 using 5N HCl.

Artificial intestinal fluid (Koo et al., 2001) was made by diluting 0.1 g trypsin from porcine pancreas (type IX-S; Sigma-Aldrich) and 3.5 g pancreatin from porcine pancreas (Sigma-Aldrich) with 1 liter distilled water. The solution was filtered through a 0.45-μm pore-diameter filter (cellulose, Millipore Corp., Bedford, MA), for sterilization purposes, and added into a sterile flask. Biliary secretions were simulated by preparing 2% or 4% bile solutions (porcine bile extract, Sigma-Aldrich) in distilled water, which were then filter-sterilized. Porcine (rather than ox gall) bile was selected due to its similarities to human bile (Marteau et al., 1997).

6.2.5. Dynamic gastrointestinal system

A dynamic model of the stomach and small intestine, previously described by Koo et al. (2001), was used as the basis of the simulated gastrointestinal tract employed in this study. Two 250-ml Erlenmeyer flasks, representing the gastric (GC) and the intestinal (IC) compartments and were kept in a water bath (Shaking Water Bath 50, Precision Scientific, Chicago IL), stabilized at 37°C throughout the gastrointestinal challenge. The

pH conditions in the GC and IC were monitored continuously using two pH meters (Ultra Basic, Denver Instrument, Arvada, CO) equipped with electrodes (Denver Instrument). Three peristaltic pumps (Variable-Speed Pump Low Flow, Fisher Scientific) were used for the delivery of simulated gastric fluid in the GC (at a flow rate of 0.33 ml/min) and simulated intestinal fluid (at a flow rate of 0.33 ml/min) and bile solution (at a flow rate of 0.5 ml/min) in the IC. To simulate physiological conditions prevailing in the human intestine (Northfield and McColl, 1973; Fausa, 1974), a 4% solution of bile was delivered in the IC during the first 30 min of the experiment, followed by the addition of a 2% bile solution for the remaining time (Minekus et al., 1995). The two compartments were connected by a multi-channel peristaltic pump (205U, Watson-Marlow Limited, Cornwall, England) that was used for transferring the gastric contents into the IC (gastric emptying) at a flow rate of 1.1 ml/min, based on results of a study (Doran et al., 1998) concluding that the gastric emptying rate after the consumption of a small meal (217 g) was 1.1 g/min, when subjects were sitting. Preliminary tests suggested that 1 ml of chyme weighted approximately 1 g (throughout the challenge). Introduction of the gastric contents into the IC started within 15 min of the simulated ingestion.

6.2.6. Gastrointestinal passage tolerance assay

The gastrointestinal challenge of low-and high-fat frankfurters was performed on days 1, 6, 20, 39, and 55 of product storage. On each storage day, 60 g of artificial saliva were added to two frankfurter links (50 g total) followed by blending at high speed for 3 min. The pH of the product-saliva mixture was measured by inserting a pH electrode, previously sprayed with 70% alcohol, rinsed with sterile distilled water and drained. Overall, the pH of the product-saliva suspension was very similar to that of the product

itself, as determined by measuring the pH of blended product before addition of artificial saliva. Prior to the beginning of each challenge, 10 ml of simulated gastric fluid were added in the GC to simulate the cephalic phase of acid secretion, whereas, the IC contained 12.5 ml of the 4% bile solution (Marteau et al., 1997). Before the introduction of the mixture in the GC, both flasks were inserted in the shaking water bath. All pumps were started immediately upon the addition of the product-saliva mixture in the GC. The pH of the GC was adjusted manually by adding 5N HCl appropriately to reproduce *in vivo* human gastric pH values after ingestion of a standard meal (Dressman et al., 1990): pH 5 at 10 min, pH 4 at 28 min, pH 3 at 58 min, and pH 2 at 88 min. After the final adjustment, the pH in the GC remained constant until the end of the challenge (120 min). The acidified gastric contents were neutralized upon addition in the IC, as the intestinal pH was maintained at 6.5±0.3 (Minekus et al., 1995; Marteau et al., 1997) with 0.3 M $NaHCO_3$.

Initial *L. monocytogenes* populations were assessed by withdrawing a 1-ml aliquot from the blended sample, prior to introduction in the GC, serially diluting, and plating on PALCAM agar TSAYE. Subsequently, during the gastrointestinal challenge, surviving *L. monocytogenes* populations were enumerated at 30, 60, 90, and 120 min in the GC, and at 30, 90, and 120 min in the IC, by removing 1-ml samples of gastric or intestinal contents and plating in duplicate on PALCAM agar and TSAYE. Secretion of gastrointestinal fluids in the GC (gastric fluid) and IC (bile and intestinal fluid) and gastric emptying continued for 120 min after the beginning of each challenge; however, the IC was maintained (statically) at 37°C in the water bath for a final microbiological analysis at

240 min. Plates were incubated as previously described and colonies were counted manually.

6.2.7. Statistical analyses

Two replicate experiments were conducted with three samples tested per product (low- or high-fat frankfurters) on each storage day in each replicate. Cell counts on PALCAM agar and TSAYE were divided by dilution factors, to account for the addition or removal of gastrointestinal fluids in each gastrointestinal compartment (Koo et al., 2001). Dilution factors (DF) were calculated as follows:

$$DF\ (GC) = \frac{\text{Remaining sample (ml)}}{\text{Remaining sample (ml)} + \text{HCl (ml)} + \text{Gastric fluid (ml)}}$$

$$DF\ (IC) = \frac{\text{Total IC contents (ml)}}{\text{Total IC contents (ml)} + \text{gastric contents (ml)} + \text{bile (ml)} + \text{intestinal fluid (ml)} + NaHCO_3\ \text{(ml)}}$$

The numbers obtained were converted into log CFU/g and analyzed using the Mixed Procedure of SAS (SAS, 2002) to identify potential effects ($P < 0.05$) of the product (low- or high-fat frankfurters) and the storage day on the ability of *L. monocytogenes* to survive in each compartment of the gastrointestinal system. Means and standard deviations were calculated and the mean differences were separated at the significance level of 95%.

In addition, the shoulder duration (SD; min) and inactivation rate (IR; log CFU/g/min) in each gastrointestinal compartment for each product on each challenge day were determined with the Baranyi and Roberts model (Baranyi and Roberts, 1994) using the DMFit Microsoft Excel program (Institute of Food Research, Norwich, UK). Inactivation

kinetics were calculated using data (log CFU/g) on PALCAM agar. Statistical analyses of inactivation kinetics were performed using the Mixed Procedure of SAS (SAS, 2002).

6.3. Results

6.3.1. Chemical and physical properties of products

Changes in pH values of inoculated and stored low- and high-fat frankfurters are shown in Figures 6.1 and 6.2, whereas, the pH values obtained for uninoculated products (day-0) were 5.87±0.04 (low-fat) and 5.78±0.04 (high-fat) (not shown in figures). On day-1 of storage, the pH values of low- and high-fat frankfurters were 6.00±0.24 and 6.06±0.18, respectively. Reductions in pH, which were suggestive of microbial growth, were observed during storage, as the pH of products reached 5.24±0.35 (low-fat) and 5.47±0.46 (high-fat) within 55 days. On day-0, the water activity of inoculated low- and high-fat frankfurters was 0.967±0.001 and 0.960±0.006, respectively.

6.3.2. Changes in microbial populations during storage

Initial (0 min) populations of *L. monocytogenes* (Figure 6.1) reflected those that had grown on frankfurters at 1, 6, 20, 39, or 55 days of storage and were enumerated prior to introduction of the product-saliva mixture in the GC. Extensive growth of the pathogen was observed on both low- and high-fat frankfurters during storage, with populations reaching or exceeding 8.0 log CFU/g on day-39 on low-fat frankfurters and on day-55 on high-fat frankfurters. Throughout the storage period, initial *L. monocytogenes* populations were similar ($P \geq 0.05$) on both products, except for day-39, on which populations on low-fat frankfurters were significantly higher ($P < 0.05$) than those on the high-fat product. Considering that stress-susceptibility may vary with the growth phase or age of bacterial cells (Stopforth et al., 2005; Formato et al., 2007), observing the *L.*

monocytogenes growth patterns during storage may be of great importance as they may help elucidate differences in stress responses. Growth of total microbial populations followed similar patterns with that of *L. monocytogenes* on both products (Figure 6.2). Overall, initial counts on TSAYE were < 0.5 log CFU/g higher than those on PALCAM agar throughout the storage period, suggesting that presence of spoilage flora on frankfurters was limited. Total counts reached by the end of storage on low- and high-fat frankfurters were 8.2 log CFU/g (Figure 6.2).

6.3.3. Gastric survival

Surviving *L. monocytogenes* populations in the GC of the dynamic gastrointestinal model are shown in Figure 6.1. Overall, reductions in initial populations during the first 30 min of the gastric challenge (gastric pH ≥ 4) were relatively small and similar for both products throughout storage, ranging from 0.3 to 1.1 log CFU/g (low-fat frankfurters) and 0.3 to 0.6 log CFU/g (high-fat frankfurters) during storage. Potential food matrix effects on the *L. monocytogenes* survival were observed mainly after 60 min of gastric exposure (pH ≤ 3). Specifically, at 90 min, populations declined by 2.6 (day-1) to 3.8 (day-39) log CFU/g in low-fat frankfurters and 1.4 (days-1 and 39) to 2.3 (day-55) log CFU/g in high-fat frankfurters. At 120 min of gastric challenge (30 min at pH 2), *L. monocytogenes* populations present on low-fat frankfurters had declined by 2.6 (day-1) to > 7.2 (day-39) log CFU/g. *L. monocytogenes* present on high-fat frankfurters exhibited enhanced survival, as suggested by reductions in populations that ranged between 1.6 (day-1) and 5.2 (day-55) log CFU/g. The 120-min gastric challenge also resulted in reductions in total microbial populations present on both products; however, the lower reductions observed on TSAYE (2 to > 6 log CFU/g, for low-fat frankfurters and 1.3 to 4.7 log CFU/g, for

high-fat frankfurters), as opposed to respective reductions on PALCAM agar (shown above) were indicative of the inability of acid-injured cells to form colonies on the selective agar (Figure 6.2).

Inactivation parameters are presented in Tables 6.1 and 6.2. On day-1 and 6 of storage, *L. monocytogenes* populations present on low-fat frankfurters declined faster ($P < 0.05$) than those on high-fat frankfurters, as suggested by calculated IR (Table 6.1). Since the initial counts on day-1 were similar on both products (2.8 and 2.9 log CFU/g on low- and high-fat frankfurters, respectively), the increased acid sensitivity of populations on the low-fat frankfurters cannot be attributed to the growth phase of cells being present on each product. It should be mentioned, however, that on day-1, the high standard deviation of IR assessed for populations on low-fat frankfurters (Table 6.1) suggests that there was a great degree of variability between replicate experiments. Thus, the significant difference in IR of *L. monocytogenes* present on low- and high-fat frankfurters on day-1 could have been a function of the experimental procedures followed, rather than the fat level of the product. Subsequently during storage (days-20 to 55), differences in *L. monocytogenes* IR during gastric exposure of low- or high-fat frankfurters were not significant ($P \geq 0.05$). The storage duration of products did not have significant ($P \geq 0.05$) effects on the gastric IR. However, populations present on the high-fat product exhibited faster reductions as storage progressed, possibly because of the reduced acid resistance of exponentially growing *L. monocytogenes* cells. These results are in accordance with those of other studies (Stopforth et al., 2005; Formato et al., 2007), indicating that *L. monocytogenes* cells exhibited greater resistance to artificial gastric fluid when at stationary phase, probably due to activation of the stationary phase-dependent acid

resistance system (Samelis and Sofos, 2003). Different effects of storage duration on the *L. monocytogenes* gastric survival were observed on low-fat frankfurters, as the pathogen displayed the fastest and slowest ($P \geq 0.05$) decline on day-1 (IR 0.194 log CFU/g/min) and 55 (IR 0.080 log CFU/g/min) of storage, respectively. The slow IR displayed by the pathogen on day-55 was no surprise considering that the fast growth of the pathogen on the low-fat product resulted in cells reaching stationary phase by day-55. As already mentioned, the reason for the high IR observed on day-1 was not necessarily a function of the tested conditions (as assessed by the high standard deviation of the IR value on day-1). In addition, comparing the findings of the present study with those by Stopforth et al. (2005) and Formato et al. (2007) may not be feasible, due to differences in methodology (i.e., dynamic model vs. static model) and experimental conditions (i.e., different products, storage temperature). For instance, reproducing the *in vivo* gradual gastric acidification in this study allowed assessment of the acid resistance of the pathogen even at early stages of product storage. On the other hand, exposure of low initial populations (e.g., on day-0) to gastric fluid of pH 1 resulted in no detectable numbers of the pathogen at the next sampling interval (20 min; Stopforth et al., 2005; Formato et al., 2007). Nevertheless, under the conditions of the present study, overall differences in IR observed with product storage in were not significant ($P \geq 0.05$). Overall, calculated SD were similar ($P \geq 0.05$) and varied from 53.33 (day-55) to 66.48 (day-1) min (low fat frankfurters) and from 27.54 (day-6) to 78.32 (day-39) min (high-fat frankfurters), indicating that, in most cases, gastric inactivation occurred after the pH of gastric contents had been reduced to 3 (Table 6.1).

6.3.4. Intestinal survival

Surviving *L. monocytogenes* and total microbial populations during the 240-min intestinal exposure are shown in Figures 6.3 and 6.4, respectively. Gradual acidification of the gastric environment in combination with the emptying of gastric contents in the IC (pH 6.5) resulted in *L. monocytogenes* cells experiencing acid challenges of different intensity, according to their residence time in the GC. As delivery of gastric contents in the IC began while the gastric pH was still high (> 5), *L. monocytogenes* cell numbers transferred from the GC to the IC within the first 30 min of gastric emptying depended on the initial contamination levels on each product, with counts recovered from the IC at 30 min being generally < 1.3 log CFU/g lower than counts on each product prior to the beginning of each gastrointestinal challenge. Therefore, the duration of storage had a major effect on the number of pathogenic cells being present in the IC, particularly since subsequent reductions in *L. monocytogenes* populations during the 240-min intestinal exposure of both products were relatively small ranging from 0.1 to 1.4 log CFU/g. However, slightly higher (≤ 0.9 log CFU/g) reductions were observed in *L. monocytogenes* populations present on high-fat frankfurters, as compared to those of populations on low-fat frankfurters on corresponding days. Reductions were observed in total microbial populations in the IC were similar to those of *L. monocytogenes*, as they varied between 0.1 and 1.4 log CFU/g, suggesting that exposure of cells to intestinal stresses did not lead to sublethal injury that could have prevented growth on the selective agar (Figure 6.4).

Calculated IR of *L. monocytogenes* in the IC were similar ($P \geq 0.05$) throughout the storage period, irrespective of product, as they varied between 0.002 (day-39) and 0.062

(day-1) log CFU/g/min, for low-fat frankfurters and 0.002 (day-39) and 0.056 (day-1) log CFU/g/min, for high-fat frankfurters, except for day-55, on which the IR of populations present on the high-fat product was 0.119 log CFU/g/min (Table 6.1). Although no clear reason exists for the high IR of *L. monocytogenes* populations grown on the high-fat product for 55 days, an explanation may be provided by the emulsifying activity of bile, resulting in dispersion of fat globules and exposure of *L. monocytogenes* cells to intestinal hurdles. However, since the intestinal IR of *L. monocytogenes* on high-fat frankfurters was low on days-1, 6, 20 and 39, another factor, possibly the high stress-susceptibility of cells resulting from prolonged storage, may be involved as well. Indeed, it has been shown that prolonged exposure of *L. monocytogenes* cells at moderately acidic pH may sensitize cells to subsequent stresses, such as lethal pH (Phan-Thanh and Montagne, 1998). Observing the initial contamination levels on each product during storage may help explain the reason *L. monocytogenes* populations present on low-fat frankfurters had a low IR (0.004 log CFU/g/min) during the intestinal challenge. Nevertheless, even though the IR of *L. monocytogenes* was fast during the intestinal exposure of high-fat frankfurters on day-55, levels of the pathogen in the IC high (> 6 log CFU/g) throughout the 240-min challenge.

6.4. Discussion

Although, there is great uncertainty about the contribution of the ingested food vehicle on the *L. monocytogenes* dose-response relationship, it is believed that certain physico-chemical food characteristics may enhance the ability of the pathogen to overcome gastrointestinal stresses and, thus increase the likelihood of intestinal colonization and infection. For instance, it has been long thought that the reason fat-rich foods are

commonly associated with listeriosis (Linnan et al., 1988; McLauchlin et al., 1991; CDC, 1998; Lyytikäinen et al., 2000) is that the high fat content serves as a barrier against gastric acidity. Waterman and Small (1998) hypothesized that pathogenic cells that become entrapped into hydrophobic lipid moieties are more likely to evade gastric killing and reach the small intestine.

As already noted, the protective role of ingested fat against foodborne listeriosis has been speculated due to findings of epidemiological investigations and studies with animal surrogates. The study presented in this chapter was conducted to investigate potential effects of the fat content and the storage duration of a high-risk RTE product on the survival of *L. monocytogenes* during a simulated digestion challenge. Under the tested conditions, the fat content of frankfurters appeared to protect *L. monocytogenes* against gastric fluid throughout the storage period, as suggested by comparing gastric reductions in bacterial populations achieved during gastric exposure of both products. The protective role of fat against gastric killing was also suggested by calculated gastric IR, although, in this case, obvious effects were observed only during the early stages (days-1 and 6) of the storage period, with IR of *L. monocytogenes* populations on low-fat frankfurters being significantly ($P < 0.05$) faster than those of populations on the high-fat product. Nevertheless, product-related differences, possibly due to protective effects of fat contained in high-fat frankfurters, were observed mostly at later stages of gastric exposure (> 60 min). In the meantime, *L. monocytogenes* cells that had been exposed to low acidity during their gastric residence time had already been transferred to IC.

Findings of previous studies (Stopforth et al., 2005; Formato et al., 2007) have shown that prolonged storage of inoculated products (frankfurters or bologna) may result in

increased survival of *L. monocytogenes* in artificial gastric fluid. Potential effects of storage duration on the gastric survival of the pathogen were also identified in this study, as cells exhibited higher acid resistance on day-55 of storage of the low-fat product. It is possible that the lower moisture content of the high-fat product decreased the growth potential of the pathogen, preventing cells from reaching stationary phase and acquiring stationary phase-dependent growth resistance during the 55-day storage period. The lower pH reached with storage of low-fat samples, as compared to that of high-fat samples (5.25 and 5.47, respectively, on day-55) could have also resulted in increased gastric survival, due to their habituation to acidic conditions adequate of inducing adaptive acid tolerance response (ATR). According to Davis et al. (1996), maximal pH-dependent ATR in *L. monocytogenes* is achieved by subjecting the pathogen to pH values that range between 4.8 and 5.2. Accordingly, based on our data, ATR may have been activated in cells present on low-fat frankfurters during storage between 39 (pH 3.32) and 55 (pH 5.24) days.

It has already been mentioned that, in consequence of the gradual gastric acidification and emptying of gastric contents, *L. monocytogenes* numbers reaching the IC depended mostly on the initial contamination levels on each product on each storage day. Hence, *L. monocytogenes* levels passing to the IC were similar for low- and high-fat frankfurters because overall growth patterns (in terms of initial numbers) were similar on both products during storage. With some exceptions, subsequent reductions in the IC were relatively low. For both products, faster ($P \geq 0.05$) intestinal IR were observed on day-1, probably because of the high stress susceptibility of cells, trying to adjust their metabolism to the new environment. It should be noted that on day-1 of storage, the

gastric IR of the pathogen was also high, however, this effect was only observed during the digestion of the low-fat product only. The highest IR during intestinal exposure was exhibited by *L. monocytogenes* populations present on high-fat frankfurters on day-55. Although the reason for this observation is not clear, the prolonged exposure the mildly acidic (pH < 6) environment of the product, in conjunction with the loss of the protective 'coating' provided by fat due to emulsification by bile salts may have rendered cells susceptible to the intestinal conditions. On the other hand, since growth of the pathogen was faster on low-fat frankfurters than that on high-fat frankfurters, the high bile resistance of populations on the low-fat product may have been due to their physiological state (i.e., stationary phase). In addition, the low-fat product may have had a protective effect against bile destruction of cells, as already suggested by the lower reductions of *L. monocytogenes* populations present on the low-fat product as compared to those of populations on high-fat frankfurters on corresponding days.

Results of the present study indicate that, under the conditions of the present study, the fat content of frankfurters may enhance survival of *L. monocytogenes* during gastric passage. However, the effects of fat were relatively small and occurred at later stages of the gastric challenge and, thus did not affect the numbers of pathogenic cells being transferred to the IC. It should be mentioned, that since foods with high protein content have also been shown to protect foodborne pathogens against gastric acid (Waterman and Small, 1998), effects of fat might have been more obvious if a low-fat, low-protein product had been also been used for comparison. Findings of the study also highlight the importance of simulating the dynamics of gastric emptying and gradual acidification in studies designed to investigate gastrointestinal aspects of the *L. monocytogenes* infection.

Table 6.1. Mean inactivation parameters (shoulder duration or maximum inactivation rate ± standard deviation; determined from counts on PALCAM agar using the model of Baranyi and Roberts) of *Listeria monocytogenes* exposed to a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation onto frankfurters of low (~4.5%) or high- (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat content of frankfurters		Gastrointestinal compartment			
		Gastric		Intestinal	
	Day of storage	Shoulder duration (min)	Maximum inactivation rate (log CFU/g/min)	Shoulder duration (min)	Maximum inactivation rate (log CFU/g/min)
Low	1	$66.48\pm13.82_{A}$	$0.194\pm0.190_{B}$	$41.68\pm58.94_{A}$	$0.062\pm0.010_{AB}$
	6	$57.29\pm2.88_{AB}$	$0.121\pm0.034_{B}$	-[1]	$0.001\pm0.004_{A}$
	20	$62.05\pm2.06_{A}$	$0.086\pm0.010_{AB}$	-[1]	$0.004\pm0.005_{A}$
	39	$62.96\pm3.88_{A}$	$0.116\pm0.012_{AB}$	-[1]	$0.002\pm0.002_{A}$
	55	$53.33\pm1.68_{AB}$	$0.080\pm0.002_{AB}$	-[1]	$0.004\pm0.002_{A}$
High	1	$55.17\pm0.64_{AB}$	$0.037\pm0.015_{A}$	$41.98\pm59.36_{A}$	$0.056\pm0.069_{AB}$
	6	$27.54\pm38.94_{B}$	$0.030\pm0.015_{A}$	-[1]	$0.003\pm0.001_{A}$
	20	$71.09\pm18.72_{A}$	$0.092\pm0.056_{AB}$	$135.72\pm57.11_{A}$	$0.013\pm0.000_{A}$
	39	$78.32\pm1.61_{A}$	$0.093\pm0.011_{AB}$	-[1]	$0.002\pm0.001_{A}$
	55	$65.86\pm17.64_{A}$	$0.097\pm0.022_{AB}$	$78.80\pm4.18_{A}$	$0.119\pm0.076_{B}$

[1] No shoulder was observed (inactivation was immediate)

ABC…: means within a column lacking a common letter are significantly different ($P < 0.05$)

Table 6.2. Mean inactivation parameters (y_0 or y_{end} ± standard deviation; determined from counts on PALCAM agar using the model of Baranyi and Roberts) of *Listeria monocytogenes* exposed to a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation onto frankfurters of low (~4.5%) or high- (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat content of frankfurters		Gastrointestinal compartment			
		Gastric		Intestinal	
	Day of storage	Y_0 (log CFU/g)	Y_{end} (log CFU/g)	Y_0 (log CFU/g)	Y_{end} (log CFU/g)
Low	1	2.7±0.4	-[1]	2.4±0.4	1.8±0.5
	6	3.7±0.2	0.2±0.1	2.0±0.4	-[1]
	20	6.6±1.6	-[1]	5.4±1.1	-[1]
	39	8.3±0.5	-[1]	7.3±1.3	-[1]
	55	7.9±1.1	-[1]	7.3±1.1	-[1]
High	1	2.9±0.1	1.3±0.1	2.5±0.1	-[1]
	6	3.8±0.3	-[1]	2.0±0.6	-[1]
	20	6.2±1.8	-[1]	4.7±1.5	-[1]
	39	7.2±2.0	-[1]	6.0±2.8	-[1]
	55	8.0±0.6	-[1]	7.1±1.1	5.7±2.4

Y_0: upper asymptote corresponding to the initial bacterial counts, Y_{end}: lower asymptote corresponding to the final bacterial counts; [1] No y_{end} value could be estimated when survival curve ceased without forming a tailing region

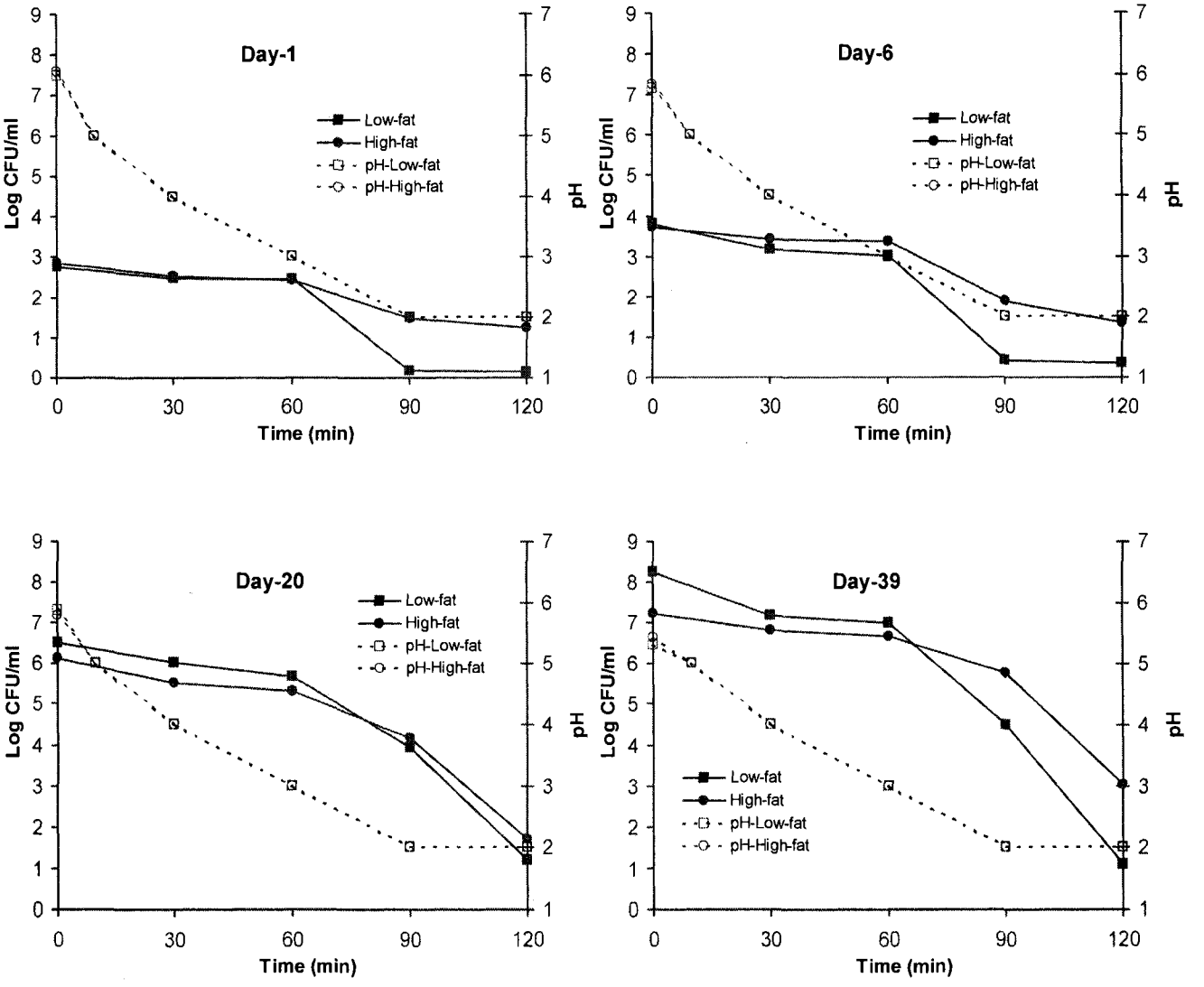


Figure 6.1 (Appendix Table 29). *Listeria monocytogenes* populations (log CFU/g; PALCAM agar) and pH values in the gastric compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation (2.0-3.0 log CFU/g) onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages

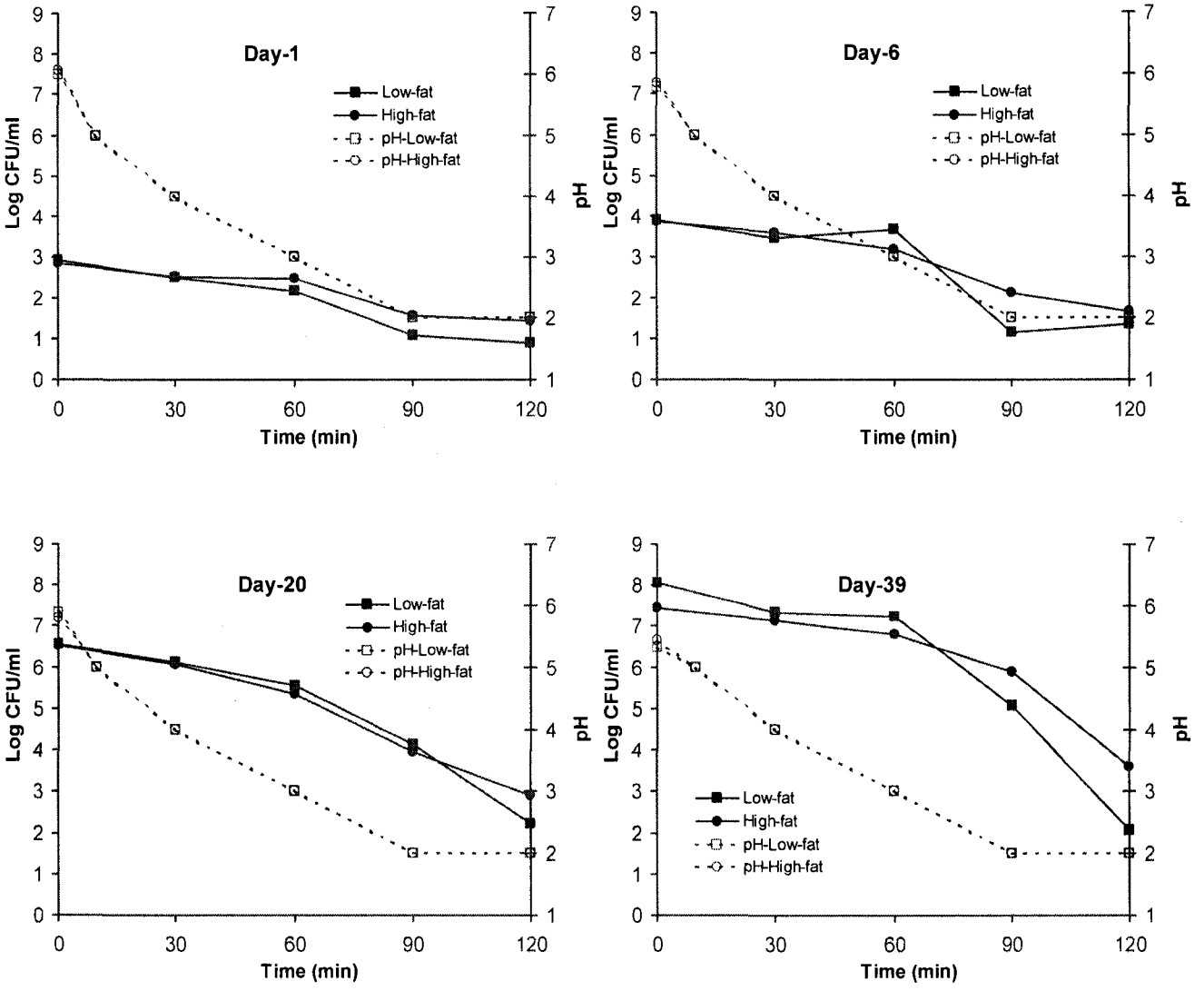


Figure 6.2 (Appendix Table 30). Total microbial populations (log CFU/g; PALCAM agar) and pH values in the gastric compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation of *Listeria monocytogenes* (2.0-3.0 log CFU/g) onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages

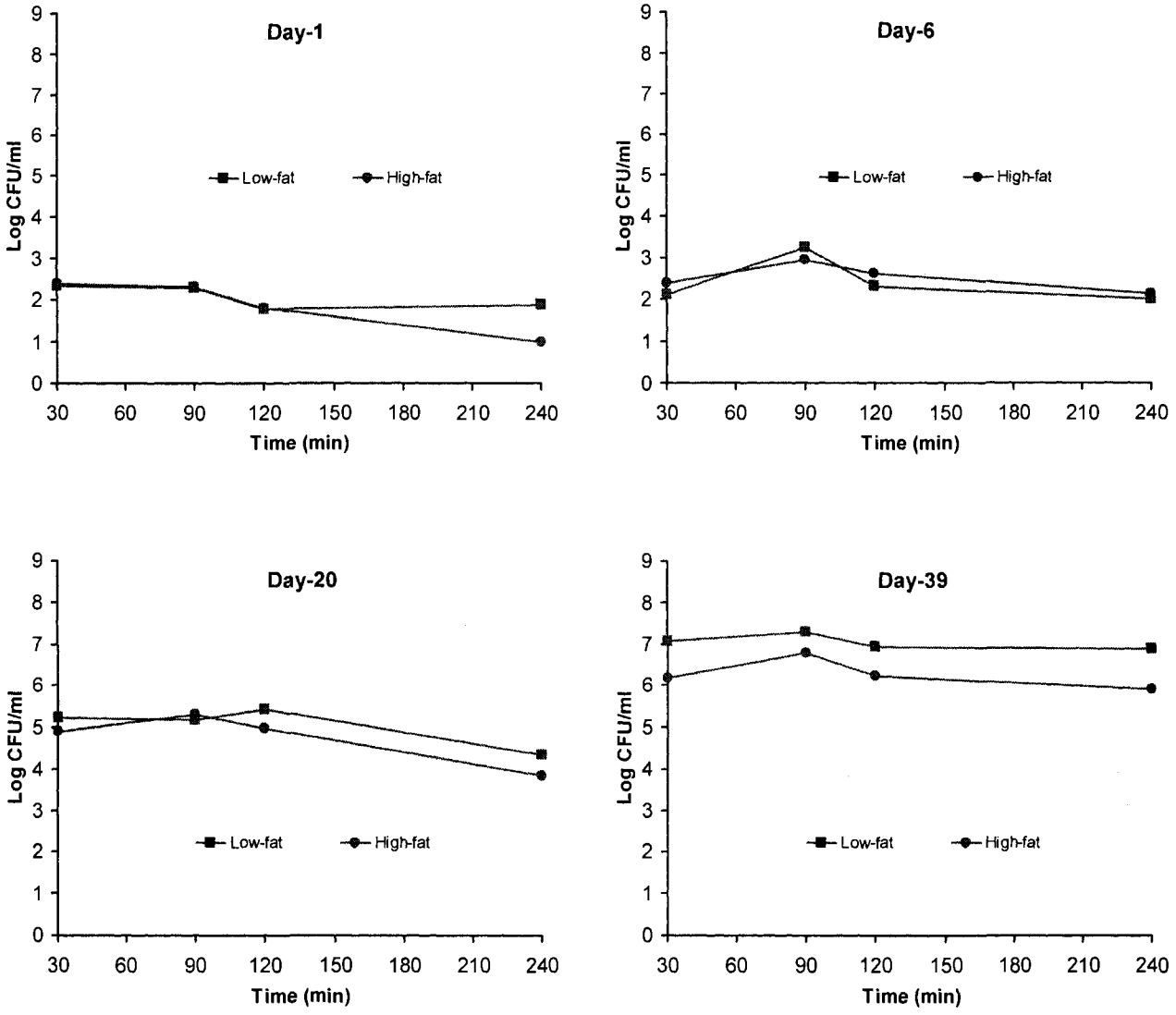


Figure 6.3 (Appendix Table 31). *Listeria monocytogenes* populations (log CFU/g; PALCAM agar) and pH values in the intestinal compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation (2.0-3.0 log CFU/g) onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages

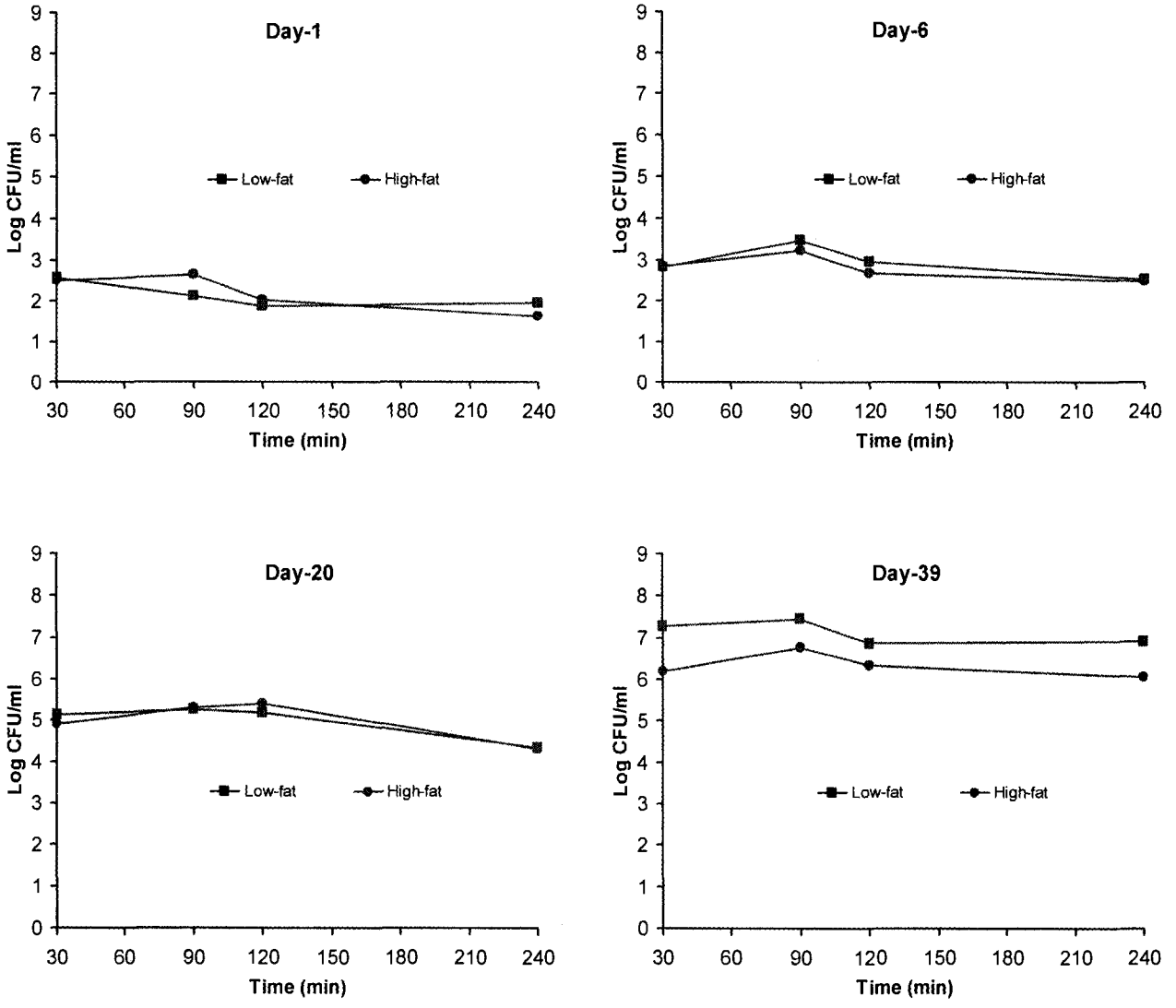


Figure 6.4 (Appendix Table 32). Total microbial populations (log CFU/g; PALCAM agar) and pH values in the intestinal compartment of a dynamic gastrointestinal system during a simulated gastrointestinal challenge, conducted after inoculation of *Listeria monocytogenes* (2.0-3.0 log CFU/g) onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages

CHAPTER 7

SUMMARY OF DISSERTATION

Listeria monocytogenes is the causative agent of listeriosis, a rare foodborne infection that may induce serious or even fatal clinical manifestations in susceptible individuals (i.e., the immunocompromised, neonates, and the elderly). The establishment of successful foodborne infection is closely related to the ability of this pathogen to tolerate inhospitable conditions encountered both outside and inside the host. Specifically, to cause disease, *L. monocytogenes* must overcome stresses associated with food processing, storage, and preparation (e.g., heat, high acidity, antimicrobials), as well as various elements of the host defense system. The studies presented in this dissertation investigated the survival properties of *L. monocytogenes* in various ready-to-eat (RTE) meat and poultry products treated with antimicrobial agents and under conditions simulating digestion.

The fate of *L. monocytogenes* was examined during storage of inoculated RTE products formulated with lactoferrin, surface treated with the activated form of the protein (ALF), or both, in combination or in comparison with organic acids and salts (i.e., lactic acid, acetic acid, potassium lactate, and sodium diacetate). Findings of these studies indicated that although the ALF surface treatments products caused significant ($P < 0.05$) reductions (0.4-1.5 log CFU/cm^2) in initial *L. monocytogenes* contamination levels, the long-term antilisterial effects of the compound were very limited as populations of the pathogen

reached high levels (7.0-8.0 log CFU/cm^2) eventually. Lactoferrin incorporated in product formulations caused slight inhibition of growth during initial storage, but did not sustain its antilisterial effects as *L. monocytogenes* populations reached high numbers (> 7.0 log CFU/cm^2) by the end of storage in samples that contained the compound. No substantial enhancement in the antilisterial activity of organic acids/salts was observed when they were applied as surface treatments or ingredients in combination with ALF or lactoferrin, respectively. Nevertheless, use of ALF as a surface treatment of frankfurters appeared to enhance the activity of antimicrobial additives (plus 1.8% potassium lactate and 0.125% sodium diacetate; 1.8% potassium lactate plus 0.5% lactoferrin) against the pathogen. Moreover, inclusion of lactoferrin in the formulation of frankfurters apperarred to increase the antilisterial effectiveness of acetic acid applied as a surface solution. Under the conditions of these studies, non-activated or activated lactoferrin applied individually as formulation or surface treatments, respectively, were not as effective as organic acids and salts. However, under certain conditions, use of ALF and lactoferrin enhanced the antilisterial effects of other additives or surface treatments, respectively, suggesting that both forms of the protein could be regarded as antilisterial treatments for RTE products when appropriately combined with other antimicrobial compounds.

Three studies were conducted to investigate strain and food matrix-related aspects that may affect the fate of *L. monocytogenes* during simulated digestion (gastric exposure: 120 min; intestinal exposure: 240 min) using a dynamic model of the stomach and small intestine maintained at 37°C. Simulated parameters included gastric emptying and gastrointestinal fluid secretion rates (gastric fluid, bile, pancreatic secretions), gradual

gastric acidification (pH reduction to 2.0 within 88 min), and intestinal pH maintenance (6.5±0.3).

Strain variations in gastric resistance were observed in a study using thirteen *Listeria monocytogenes* strains (cultures in tryptic soy broth without dextrose plus 0.6% yeast extract incubated for 4 or 16 h at 30°C) that represented different serotypes (1/2, 1/2a, 4a, 4b) and three genotypic lineages, with clinical strains Scott A and C1-056 displaying the greatest gastric-susceptibility among the strains tested; however, under the tested conditions, serotype and lineage did not appear to have any significant effects in gastric survival. All strains tested underwent small reductions in populations during exposure in the intestinal compartment. However, significant ($P < 0.05$) serotype- and lineage-related effects in intestinal survival were identified (16-h cultures only), with serotype 4b and lineage 2 isolates exhibiting greater survival in the intestinal compartment than isolates belonging to serotype 1/2 and lineage 1 or 3, respectively.

In two other studies, *L. monocytogenes* counts were determined during exposure of inoculated: (i) bologna or salami and, (ii) beef frankfurters of low (~4.5%) or high (~32.5%) fat level, to the dynamic gastrointestinal model throughout storage in order to identify potential effects of the food matrix properties (e.g., acidity, fat content) and the storage time on the gastrointestinal survival of the pathogen. In general, findings indicated potential effects of the product type on gastric survival of the pathogen, as gastric inactivation of *L. monocytogenes* populations present on salami and high-fat frankfurters was slower than that of populations on bologna and low-fat frankfurters, respectively. It should be noted, however, that it is not clear whether the increased acid tolerance of the organism observed during gastric exposure of salami was exclusively due to product

properties, as the low initial contamination levels reached with prolonged storage of this product may have undergone slower reductions than the high populations on bologna. Effects of storage duration on gastric survival were observed only in populations present on products that supported growth (i.e., bologna and frankfurters) and in most cases could be explained by examining the growth patterns of *L. monocytogenes* on each product during storage. For instance, the increased acid resistance observed after populations had reached a plateau was probably because of the activation of the stationary phase-dependent acid resistance system. As a result of gastric emptying and gradual gastric acidification, cells transferred from the gastric to the intestinal compartment during the early stages of the gastrointestinal challenge (30 min) were unaffected by gastric acidity (pH > 4); thus, populations of the pathogen transferred to the intestinal compartment within the first 30 min of gastric emptying depended on the initial contamination levels on each product. Subsequently, population reductions due to intestinal stresses (i.e., bile) were low for all products. Overall, the potential for *L. monocytogenes* growth, as affected by attributes of each product and the length of the storage period were the most influential factors, in terms of *L. monocytogenes* levels being present in the compartments of the gastrointestinal model. Thus, any effects of the food matrix on the survival of the pathogen were probably overshadowed by the high and low contamination levels reached on bologna and salami, respectively. On the other hand, since *L. monocytogenes* growth patterns on low- and high-fat frankfurters were generally similar ($P \geq 0.05$) on corresponding days, pathogen levels being present in the intestinal compartment were not affected by the fat level of the product.

In summary, examination of factors that may affect survival of the pathogen in the gastrointestinal tract using a dynamic model of the stomach and the small intestine indicated that gastric survival may be affected by strain differences and features of the food matrix. However, due to effects of gastric emptying and gradual gastric pH reduction, pathogen levels transferred to the small intestine depended on the initial contamination levels, as affected by the length of storage or incubation period, the growth potential of the specific strain and the type of product (supportive vs. not supportive of growth). Therefore, results also highlight the importance of simulating the dynamics of gastric emptying and gradual acidification in studies designed to investigate gastrointestinal aspects of the *L. monocytogenes* infection.

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APPENDIX

Appendix Table 1 (Figure 3.1). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on the surface of commercial ham slices inoculated with the pathogen before or after dipping into water (120 s), 1% acetic acid (30 s), or 2% activated lactoferrin (120 s) vacuum packaged and stored at 7°C.

		Day of storage					
Application	Treatment	0	5	10	15	22	28
None	Control	$2.4\pm0.0_{Aa}$	$3.2\pm0.2_{Ab}$	$4.4\pm0.1_{Ac}$	$6.2\pm0.2_{ADd}$	$7.2\pm0.1_{ACe}$	$7.3\pm0.1_{Ae}$
Pre-inoculation	Water	$2.3\pm0.0_{ABa}$	$3.8\pm0.2_{Bb}$	$5.4\pm0.3_{Bc}$	$7.0\pm0.4_{Bd}$	$7.5\pm0.0_{Ad}$	$7.4\pm0.1_{Ad}$
	AA (1%)	$2.4\pm0.1_{Aa}$	$2.3\pm0.1_{Ca}$	$2.5\pm0.0_{Ca}$	$2.9\pm0.2_{Cab}$	$3.1\pm0.1_{Bb}$	$4.4\pm0.7_{Bc}$
	ALF (2%)	$2.3\pm0.1_{ABa}$	$2.6\pm0.2_{CDa}$	$4.5\pm0.1_{Ab}$	$6.0\pm0.2_{Ac}$	$7.1\pm0.2_{ACd}$	$7.5\pm0.1_{Ad}$
Post-inoculation	Water	$1.8\pm0.0_{Ba}$	$2.9\pm0.3_{ADb}$	$5.9\pm0.4_{Bc}$	$6.7\pm0.4_{BDd}$	$7.6\pm0.0_{Ae}$	$7.4\pm0.2_{Ae}$
	AA (1%)	$2.2\pm0.1_{ABa}$	$2.2\pm0.1_{Ca}$	$2.3\pm0.2_{Ca}$	$2.7\pm0.3_{Cab}$	$3.3\pm0.6_{Bbc}$	$3.5\pm0.1_{Cc}$
	ALF (2%)	$1.8\pm0.1_{Ba}$	$2.4\pm0.1_{CDb}$	$4.2\pm0.3_{Ac}$	$5.9\pm0.3_{Ac}$	$6.9\pm0.3_{Cd}$	$7.4\pm0.5_{Ae}$

AA: acetic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 2 (Figure 3.2). Mean populations of *Listeria monocytogenes* (log CFU/cm^2 ± standard deviation) on the surface of frankfurters, inoculated with the pathogen, dipped into antimicrobial solutions for 30, 60, 90 or 120 s, vacuum packaged and stored at 7°C.

	Day of storage							
Treatment	0	4	8	12	16	20	24	32
Control	$2.9\pm0.1_{Aa}$	$3.2\pm0.0_{Ab}$	$4.6\pm0.1_{Ac}$	$5.9\pm0.5_{Ad}$	$6.5\pm0.3_{Ae}$	$7.1\pm0.3_{Ae}$	$7.8\pm0.1_{Af}$	$7.9\pm0.1_{Af}$
Water (30 s)	$2.2\pm0.1_{Ba}$	$3.1\pm0.2_{ADb}$	$5.1\pm0.3_{Bc}$	$6.4\pm0.2_{Bd}$	$7.2\pm0.2_{Be}$	$8.0\pm0.1_{Bf}$	$8.1\pm0.1_{Bf}$	$8.3\pm0.1_{Af}$
LA (2 %; 30 s)	$2.2\pm0.0_{Ba}$	$2.0\pm0.2_{Ba}$	$2.7\pm0.1_{Cb}$	$4.2\pm0.3_{Cc}$	$5.4\pm0.4_{Ce}$	$5.9\pm0.3_{Cf}$	$7.5\pm0.0_{Bg}$	$8.1\pm0.1_{Ah}$
SD (3%; 30 s)	$2.4\pm0.0_{Ba}$	$2.5\pm0.1_{Ca}$	$3.1\pm0.5_{Db}$	$4.6\pm0.0_{Dc}$	$5.5\pm0.2_{Cd}$	$6.3\pm0.1_{De}$	$6.9\pm0.1_{Cf}$	$7.9\pm0.1_{Ag}$
ALF (2%; 30 s)	$2.1\pm0.1_{Ba}$	$3.4\pm0.4_{Ab}$	$5.0\pm0.1_{Bc}$	$5.9\pm0.2_{Ad}$	$7.4\pm0.3_{Be}$	$7.6\pm0.1_{Eef}$	$7.9\pm0.1_{Af}$	$8.3\pm0.1_{Ag}$
ALF (2%; 60 s)	$1.9\pm0.0_{Ca}$	$3.3\pm0.1_{Ab}$	$4.5\pm0.2_{Ac}$	$5.7\pm0.1_{Ad}$	$6.6\pm0.0_{Ae}$	$7.4\pm0.3_{AEf}$	$8.1\pm0.0_{Bg}$	$8.0\pm0.1_{Ag}$
ALF (2%; 90 s)	$1.9\pm0.1_{Ca}$	$2.9\pm0.1_{Db}$	$4.4\pm0.2_{Ac}$	$5.7\pm0.1_{Ad}$	$6.4\pm0.2_{Ae}$	$7.4\pm0.1_{AEf}$	$7.9\pm0.4_{Ag}$	$8.2\pm0.0_{Ag}$
ALF (2%; 120 s)	$2.0\pm0.2_{BCa}$	$3.1\pm0.1_{Ab}$	$4.3\pm0.1_{Ac}$	$5.9\pm0.2_{Ad}$	$6.6\pm0.1_{Ae}$	$7.2\pm0.1_{Af}$	$7.6\pm0.2_{Bg}$	$7.5\pm0.3_{Bh}$

LA: lactic acid, SD: sodium diacetate, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 3 (Figure 3.3). Mean *Listeria monocytogenes* populations (log CFU/cm^2 ± standard deviation) on the surface of beef or beef and pork bologna slices, inoculated with the pathogen, and dipped into water (120 s), 2% acetic acid (AA; 60 s), or 2% activated lactoferrin (ALF; 60, 120, or 180 s), vacuum packaged and stored at 10°C.

Meat used in bologna formulation	Treatment	Day of storage					
		0	4	8	12	16	26
Beef	Control	3.5±0.1 $_{Aa}$	5.8±0.3 $_{Ab}$	7.3±0.3 $_{Ac}$	8.2±0.1 $_{Ad}$	8.1±0.1 $_{Ad}$	8.0±0.1 $_{ACd}$
	Water	3.1±0.1 $_{Ba}$	6.5±0.1 $_{Bb}$	8.2±0.0 $_{Bc}$	8.2±0.1 $_{Ac}$	8.2±0.2 $_{ADc}$	8.0±0.4 $_{ACc}$
	AA (2%)	2.8±0.2 $_{BCa}$	2.4±0.3 $_{Cab}$	2.3±0.2 $_{Cbc}$	2.0±0.5 $_{Bcd}$	1.8±0.5 $_{Bd}$	1.3±0.5 $_{Be}$
	ALF (2%; 60 s)	3.0±0.2 $_{Ba}$	5.8±0.4 $_{Ab}$	7.9±0.3 $_{BDcd}$	8.3±0.1 $_{Ac}$	8.3±0.1 $_{ADc}$	7.7±0.2 $_{Ad}$
	ALF (2%; 120 s)	2.8±0.1 $_{BCa}$	5.3±0.1 $_{Cb}$	7.9±0.0 $_{BDc}$	8.2±0.1 $_{Ac}$	8.2±0.1 $_{ADc}$	7.9±0.2 $_{Ac}$
	ALF (2%; 180 s)	2.8±0.1 $_{BCa}$	5.6±0.4 $_{ACb}$	7.7±0.3 $_{Dc}$	8.3±0.1 $_{Ad}$	8.2±0.2 $_{ADd}$	7.9±0.1 $_{Acd}$
Beef and pork	Control	3.5±0.1 $_{Aa}$	6.0±0.3 $_{ADb}$	7.9±0.2 $_{BDc}$	8.2±0.2 $_{Ac}$	8.1±0.2 $_{Ac}$	8.3±0.2 $_{CDc}$
	Water	3.1±0.5 $_{Ba}$	6.3±0.4 $_{BDb}$	8.1±0.2 $_{BDc}$	8.2±0.1 $_{Ac}$	7.9±0.4 $_{Ac}$	8.1±0.1 $_{ACc}$
	AA (2%)	2.6±0.5 $_{Ca}$	2.1±0.2 $_{Cb}$	2.2±0.4 $_{Cab}$	1.8±0.5 $_{Bb}$	2.1±0.2 $_{Bb}$	1.1±0.7 $_{Bc}$
	ALF (2%; 60 s)	3.0±0.2 $_{Ba}$	6.0±0.5 $_{ADb}$	7.9±0.2 $_{BDc}$	8.3±0.1 $_{Ac}$	8.2±0.1 $_{ADc}$	8.1±0.1 $_{ACc}$
	ALF (2%; 120 s)	3.0±0.1 $_{Ba}$	6.4±0.3 $_{Bb}$	8.2±0.1 $_{Bc}$	8.3±0.2 $_{Ac}$	8.9±0.9 $_{Cd}$	8.2±0.1 $_{ADc}$
	ALF (2%; 180 s)	2.7±0.1 $_{Ca}$	6.0±0.3 $_{ADb}$	8.2±0.1 $_{Bc}$	8.2±0.2 $_{Ac}$	8.6±0.5 $_{CDc}$	8.2±0.1 $_{ADc}$

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 4 (Figure 3.4). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on bologna slices inoculated with the pathogen and dipped in (30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control), vacuum packaged and stored at 7°C.

		Day of storage							
Application	Treatment	0	4	8	12	16	22	28	43
None	Control	$3.1\pm0.0_{Aa}$	$5.3\pm0.0_{Ab}$	$5.9\pm0.0_{Ac}$	$6.6\pm0.3_{ACd}$	$7.2\pm0.1_{Ae}$	$7.4\pm0.0_{Ae}$	$7.6\pm0.4_{Ae}$	$7.4\pm0.0_{ABe}$
Dipping	Water	$2.5\pm0.0_{Ba}$	$4.9\pm0.2_{Bb}$	$6.8\pm0.1_{Bc}$	$6.8\pm0.0_{Ac}$	$7.7\pm0.0_{Bd}$	$7.6\pm0.1_{Ad}$	$7.8\pm0.1_{Ad}$	$7.7\pm0.1_{Ad}$
	LA (2%)	$2.7\pm0.1_{Ba}$	$2.7\pm0.1_{Ca}$	$3.5\pm0.5_{Cb}$	$3.5\pm0.2_{Bb}$	$4.8\pm0.2_{Cc}$	$6.1\pm0.9_{Bd}$	$7.0\pm0.0_{Be}$	$7.6\pm0.1_{ABf}$
	ALF (2%)	$2.7\pm0.0_{Ba}$	$4.9\pm0.1_{Bb}$	$6.4\pm0.0_{Dc}$	$6.3\pm0.1_{Cc}$	$7.5\pm0.1_{ABd}$	$7.6\pm0.1_{Ad}$	$7.5\pm0.0_{Ad}$	$7.7\pm0.0_{Ad}$
Spraying	Water	$2.4\pm0.1_{Ba}$	$4.3\pm0.0_{Db}$	$6.1\pm0.1_{ADc}$	$6.5\pm0.0_{ACc}$	$7.5\pm0.1_{ABd}$	$7.7\pm0.0_{Ad}$	$7.5\pm0.0_{Ad}$	$7.7\pm0.1_{Ad}$
	LA (2%)	$2.6\pm0.0_{Ba}$	$3.1\pm0.1_{Eb}$	$3.7\pm0.1_{Cc}$	$4.0\pm0.3_{Dc}$	$5.2\pm0.5_{Dd}$	$5.8\pm0.1_{Be}$	$6.8\pm0.2_{Bf}$	$7.3\pm0.1_{Bg}$
	ALF (2%)	$2.8\pm0.1_{ABa}$	$4.0\pm0.2_{Db}$	$5.5\pm0.0_{Ec}$	$5.9\pm0.1_{Ed}$	$6.7\pm0.3_{Ee}$	$7.3\pm0.1_{Cf}$	$7.8\pm0.4_{Ag}$	$7.6\pm0.2_{ABg}$

LA: lactic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 5 (Figure 3.5). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on the surface of commercial cured turkey breast slices, inoculated with the pathogen, dipped into antimicrobial solutions or water for 60 s (except control), vacuum packaged and stored at 7°C.

	Day of storage							
Treatment (dipping)	0	4	8	12	16	22	28	43
Control (undipped)	3.2±0.1$_{Aa}$	3.1±0.1$_{ABa}$	4.3±0.2$_{Ab}$	4.8±0.2$_{Ac}$	5.9±0.3$_{ADd}$	6.6±0.1$_{Ae}$	7.0±0.1$_{Aef}$	7.3±0.2$_{Af}$
Water	2.5±0.1$_{Ba}$	3.2±0.2$_{Ab}$	4.0±0.3$_{Ac}$	4.8±0.2$_{Ad}$	6.4±0.0$_{Ae}$	7.2±0.3$_{Bf}$	6.7±0.1$_{Af}$	7.0±0.2$_{Aef}$
PL (3%)	2.6±0.1$_{Ba}$	3.2±0.2$_{Ab}$	4.0±0.5$_{Ac}$	5.5±0.1$_{Bd}$	6.4±0.4$_{Ae}$	7.2±0.1$_{Bf}$	7.2±0.2$_{Af}$	6.4±0.0$_{Be}$
SD (3%)	2.7±0.1$_{ABab}$	2.7±0.0$_{Bab}$	2.5±0.2$_{BDa}$	2.6±0.0$_{CGab}$	2.6±0.1$_{Bab}$	3.2±0.2$_{Cb}$	3.0±0.6$_{Bab}$	2.6±0.1$_{Cab}$
LA (1%)	2.7±0.1$_{ABa}$	2.7±0.1$_{Ba}$	3.2±0.1$_{Cab}$	3.4±0.2$_{Db}$	4.8±1.1$_{Cc}$	5.7±0.3$_{Dd}$	6.7±0.0$_{Ae}$	7.1±0.1$_{Af}$
AA (1%)	2.8±0.0$_{ABa}$	2.8±0.1$_{ABa}$	2.9±0.2$_{Ba}$	2.8±0.2$_{Ca}$	2.8±0.3$_{Ba}$	3.2±0.7$_{Cab}$	3.4±0.5$_{Bb}$	3.6±0.6$_{Db}$
ALF (2%)	2.5±0.1$_{Ba}$	2.9±0.1$_{ABab}$	3.5±0.1$_{Cb}$	4.3±0.2$_{Ec}$	4.9±1.0$_{Cd}$	6.6±1.0$_{Ae}$	7.2±0.1$_{Af}$	6.9±0.2$_{Aef}$
ALF (1%)	2.6±0.0$_{Ba}$	3.4±0.1$_{Ab}$	5.1±0.2$_{Dc}$	6.4±0.4$_{Fd}$	6.5±0.3$_{Ad}$	7.3±0.1$_{Be}$	7.3±0.2$_{Ae}$	7.2±0.5$_{Ae}$
ALF (1%) + PL (3%)	2.4±0.1$_{Ba}$	3.0±0.1$_{ABb}$	4.3±0.2$_{Ac}$	5.0±0.5$_{Ad}$	5.6±0.5$_{De}$	6.7±0.3$_{Afb}$	7.1±0.1$_{Af}$	6.9±0.2$_{Af}$
ALF (1%) + SD (3%)	2.5±0.0$_{Ba}$	2.3±0.1$_{BCa}$	2.2±0.2$_{Da}$	2.3±0.2$_{CGa}$	2.4±0.0$_{Ba}$	3.4±0.5$_{Cb}$	2.3±0.2$_{Ca}$	2.2±0.3$_{Ca}$
ALF (1%) + LA (1%)	2.4±0.1$_{Ba}$	2.5±0.1$_{BCa}$	2.3±0.1$_{Da}$	2.7±0.0$_{CGa}$	3.7±0.4$_{Eb}$	5.1±0.4$_{Ec}$	5.5±0.3$_{Dc}$	6.9±0.6$_{Ad}$
ALF (1%) + AA (1%)	2.4±0.1$_{Ba}$	2.1±0.3$_{Ca}$	2.3±0.1$_{Da}$	2.2±0.1$_{Ga}$	2.4±0.1$_{Ba}$	4.1±0.2$_{Fb}$	3.7±1.2$_{Bbc}$	3.5±1.0$_{Dc}$

PL: potassium lactate, SD: sodium diacetate, LA: lactic acid, AA: acetic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 6 (Figure 3.6). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on the surface of bologna slices formulated with or without antimicrobials, inoculated with the pathogen after slicing, vacuum packaged and stored at 4°C.

	Day of storage					
Treatment (formulation)	0	10	20	43	75	95
Control	2.0±0.0 $_{Aa}$	3.5±0.1 $_{Ab}$	4.9±0.2 $_{Ac}$	7.0±0.0 $_{Ad}$	6.9±0.1 $_{Ad}$	6.6±0.1 $_{Ad}$
PL (1.8%)	2.1±0.0 $_{Aa}$	2.1±0.0 $_{Ba}$	2.2±0.0 $_{Ba}$	3.6±0.2 $_{Bb}$	4.5±0.8 $_{Bc}$	6.1±0.2 $_{Bd}$
LF (0.5%)	2.0±0.1 $_{Aa}$	2.6±0.2 $_{Cb}$	3.2±0.0 $_{Cc}$	5.1±0.1 $_{Cd}$	6.9±0.1 $_{Ae}$	7.2±0.3 $_{De}$
LF (1%)	2.1±0.0 $_{Aa}$	2.9±0.0 $_{Cb}$	4.2±0.1 $_{Dc}$	7.3±0.1 $_{Ad}$	6.8±0.3 $_{Ae}$	7.2±1.2 $_{Dde}$
PL (1.8%) + SD (0.125%)	2.1±0.1 $_{Aab}$	2.0±0.1 $_{Ba}$	2.0±0.0 $_{Ba}$	1.8±0.1 $_{Da}$	1.7±0.0 $_{Ca}$	2.5±0.5 $_{Eb}$
PL (1.8%) + LF (0.5%)	2.1±0.0 $_{Aab}$	2.0±0.1 $_{Bab}$	1.9±0.1 $_{Ba}$	2.4±0.3 $_{Eb}$	5.8±0.5 $_{Dc}$	6.8±0.2 $_{Ad}$
SD (0.125%) + LF (0.5%)	2.0±0.1 $_{Aa}$	3.5±0.1 $_{Ab}$	5.4±0.2 $_{Ec}$	8.0±0.1 $_{Fd}$	8.0±0.1 $_{Ed}$	8.0±0.1 $_{Fd}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	2.0±0.1 $_{Aa}$	2.0±0.0 $_{Ba}$	2.2±0.2 $_{Ba}$	2.4±0.2 $_{Eab}$	2.8±0.1 $_{Fb}$	4.5±0.2 $_{Gc}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	2.0±0.1 $_{Aa}$	2.0±0.1 $_{Ba}$	2.7±0.3 $_{Fb}$	4.9±0.2 $_{Cc}$	4.5±0.5 $_{Bc}$	6.1±0.1 $_{Bd}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 7 (Figure 3.6). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on the surface of bologna slices formulated with or without antimicrobials, inoculated with the pathogen after slicing, vacuum packaged and stored at 7°C.

Treatment (formulation)	Day of storage								
	0	4	8	12	16	20	28	43	57
Control	$2.0\pm0.0_{Aa}$	$3.3\pm0.0_{Ab}$	$4.5\pm0.1_{Ac}$	$5.3\pm0.1_{Ad}$	$6.4\pm0.2_{Ae}$	$6.4\pm0.4_{Ae}$	$7.1\pm0.1_{Af}$	$6.7\pm0.3_{Aef}$	$6.8\pm0.5_{Aef}$
PL (1.8%)	$2.1\pm0.0_{Aa}$	$2.1\pm0.1_{Ba}$	$2.3\pm0.0_{Bab}$	$2.3\pm0.2_{Bab}$	$2.6\pm0.0_{Bb}$	$3.1\pm0.1_{Bc}$	$3.6\pm0.1_{Bd}$	$6.0\pm0.0_{Be}$	$6.0\pm0.0_{Be}$
LF (0.5%)	$2.0\pm0.1_{Aa}$	$2.7\pm0.1_{Cb}$	$3.4\pm0.1_{Cc}$	$3.9\pm0.1_{Cd}$	$4.5\pm0.1_{Ce}$	$5.1\pm0.0_{Cf}$	$5.8\pm0.0_{Cg}$	$7.0\pm0.1_{Ch}$	$7.2\pm0.2_{ACh}$
LF (1%)	$2.1\pm0.0_{Aa}$	$2.7\pm0.0_{Cb}$	$3.5\pm0.1_{Cc}$	$4.1\pm0.1_{Cd}$	$5.2\pm0.2_{De}$	$5.6\pm0.1_{Df}$	$6.1\pm0.2_{Cg}$	$7.1\pm0.2_{Ch}$	$7.4\pm0.1_{Ch}$
PL (1.8%) + SD (0.125%)	$2.1\pm0.1_{Aa}$	$2.0\pm0.1_{Ba}$	$1.9\pm0.0_{Ba}$	$1.9\pm0.0_{BDa}$	$2.1\pm0.1_{Ea}$	$1.9\pm0.0_{Ea}$	$2.0\pm0.1_{Da}$	$1.9\pm0.0_{Da}$	$2.5\pm0.0_{Da}$
PL (1.8%) + LF (0.5%)	$2.1\pm0.0_{Aa}$	$2.1\pm0.1_{Ba}$	$2.2\pm0.2_{Ba}$	$2.5\pm0.1_{Ca}$	$2.9\pm0.0_{Bb}$	$3.1\pm0.1_{Bb}$	$3.8\pm0.1_{Ec}$	$4.6\pm0.2_{Ed}$	$5.8\pm0.3_{Be}$
SD (0.125%) + LF (0.5%)	$2.0\pm0.1_{Aa}$	$3.0\pm0.1_{Ab}$	$4.5\pm0.1_{Ac}$	$5.0\pm0.0_{Ad}$	$5.3\pm0.1_{Cd}$	$6.2\pm0.0_{Ae}$	$7.4\pm0.6_{Ff}$	$7.9\pm0.1_{Fg}$	$8.2\pm0.6_{Eg}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	$2.0\pm0.1_{Aa}$	$1.9\pm0.1_{Ba}$	$2.1\pm0.2_{Ba}$	$1.5\pm0.4_{Db}$	$1.9\pm0.0_{Ea}$	$2.2\pm0.0_{Ea}$	$2.6\pm0.3_{Gc}$	$3.4\pm0.3_{Gd}$	$5.3\pm0.9_{Fe}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	$2.0\pm0.1_{Aa}$	$2.0\pm0.0_{Ba}$	$2.8\pm0.3_{Db}$	$2.5\pm0.0_{Cb}$	$2.7\pm0.0_{Bb}$	$4.0\pm0.2_{Fc}$	$5.7\pm0.1_{Cd}$	$7.2\pm0.1_{Ce}$	$7.4\pm0.3_{Ce}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 8 (Figure 3.7). Mean populations (log CFU/cm^2 ± standard deviation) of *Listeria monocytogenes* on the surface of frankfurters formulated with or without antimicrobials, inoculated with the pathogen, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Treatment (formulation)	Day of storage							
		0	5	10	15	20	25	35	50
None	Control	$2.0\pm0.1_{Aac}$	$1.8\pm0.2_{Aa}$	$2.9\pm0.4_{Abc}$	$2.5\pm0.1_{Ac}$	$3.0\pm0.5_{Ab}$	$4.5\pm0.1_{Ad}$	$5.6\pm0.7_{Ae}$	$7.1\pm0.9_{Af}$
	PL (1.8%) + SD (0.125%)	$2.1\pm0.1_{Aa}$	$1.8\pm0.1_{Aa}$	$1.9\pm0.0_{Ba}$	$1.8\pm0.1_{Ba}$	$1.7\pm0.1_{Ba}$	$1.8\pm0.0_{Ba}$	$1.9\pm0.3_{Ba}$	$2.1\pm0.5_{Ba}$
	LF (0.5%)	$2.2\pm0.1_{Aab}$	$1.9\pm0.0_{Aa}$	$2.3\pm0.3_{Bab}$	$2.6\pm0.1_{Ab}$	$2.8\pm0.2_{Abc}$	$3.3\pm0.1_{Cc}$	$5.7\pm0.5_{Ad}$	$6.6\pm0.7_{Ae}$
	PL (1.8%) + LF (0.5%)	$2.0\pm0.0_{Aa}$	$1.9\pm0.1_{Aa}$	$1.9\pm0.1_{Ba}$	$1.7\pm0.1_{Ba}$	$1.9\pm0.0_{Ba}$	$1.8\pm0.3_{Ba}$	$1.7\pm0.1_{Bs}$	$1.8\pm0.3_{Ba}$
AA (2%)	Control	$1.2\pm0.1_{BDa}$	$0.9\pm0.1_{Ba}$	$0.8\pm0.2_{Ca}$	$0.2\pm0.3_{CEb}$	$0.8\pm0.1_{CFa}$	$0.9\pm0.3_{Da}$	$1.9\pm0.2_{Bc}$	$1.3\pm0.4_{Ba}$
	PL (1.8%) + SD (0.125%)	$1.0\pm0.2_{BCa}$	$0.5\pm0.1_{Ba}$	$0.3\pm0.0_{Cb}$	$0.5\pm0.1_{CEb}$	$0.2\pm0.1_{Db}$	$0.3\pm0.1_{Eb}$	$0.4\pm0.2_{Cb}$	$0.3\pm0.3_{CEb}$
	LF (0.5%)	$1.0\pm0.2_{BCa}$	$0.9\pm0.1_{BCa}$	$0.9\pm0.2_{Ca}$	$0.8\pm0.0_{Ca}$	$0.9\pm0.0_{CFa}$	$0.8\pm0.1_{Da}$	$1.1\pm0.3_{Da}$	$1.0\pm0.6_{BDa}$
	PL (1.8%) + LF (0.5%)	$1.1\pm0.1_{BCa}$	$0.8\pm0.1_{BCab}$	$0.6\pm0.3_{Cab}$	$0.6\pm0.3_{Cab}$	$0.9\pm0.0_{CFab}$	$0.4\pm0.1_{DEb}$	$0.6\pm0.4_{CDab}$	$0.7\pm0.1_{DEa}$
ALF (2%)	Control	$0.5\pm0.1_{Ca}$	$1.2\pm0.3_{Cb}$	$1.9\pm0.1_{Bc}$	$3.3\pm0.5_{Dd}$	$4.8\pm0.1_{Ee}$	$5.2\pm0.5_{Fef}$	$5.6\pm0.5_{Af}$	$7.2\pm0.4_{Ag}$
	PL (1.8%) + SD (0.125%)	$0.6\pm0.3_{Ca}$	$0.5\pm0.1_{Ba}$	$0.4\pm0.2_{Cab}$	$0.1\pm0.2_{Eb}$	$0.2\pm0.1_{Dab}$	$0.4\pm0.1_{DEab}$	$0.3\pm0.2_{CFa}$	$1.3\pm0.8_{Bc}$
	LF (0.5%)	$0.9\pm0.2_{CDa}$	$1.2\pm0.6_{Ca}$	$1.2\pm0.1_{Ca}$	$3.1\pm0.7_{Db}$	$4.5\pm0.2_{Ec}$	$4.1\pm0.5_{Ac}$	$6.1\pm0.5_{Ed}$	$6.7\pm0.4_{Ad}$
	PL (1.8%) + LF (0.5%)	$0.7\pm0.2_{Ca}$	$0.3\pm0.1_{Bab}$	$0.6\pm0.2_{Cab}$	$0.5\pm0.3_{CEab}$	$0.4\pm0.1_{DFab}$	$0.2\pm0.3_{Ebc}$	$-0.1\pm0.3_{Fc}$	$0.0\pm0.2_{Cc}$

AA: acetic acid, ALF: activated lactoferrin, PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 9. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of ham slices inoculated with *Listeria monocytogenes* before or after dipping into water (120 s), 1% acetic acid (30 s), or 2% activated lactoferrin (120 s) vacuum packaged and stored at 7°C.

		Day of storage					
Application	Treatment	0	5	10	15	22	28
None	Control	$2.4\pm0.0_{Aa}$	$3.4\pm0.5_{Ab}$	$4.4\pm0.1_{Ac}$	$6.3\pm0.2_{Ad}$	$7.7\pm0.5_{Ae}$	$7.4\pm0.1_{Ae}$
Pre-inoculation	Water	$2.4\pm0.0_{Aa}$	$3.8\pm0.2_{Ab}$	$5.4\pm0.2_{Bc}$	$7.1\pm0.3_{Bd}$	$7.3\pm0.6_{Ad}$	$7.6\pm0.0_{Ad}$
	AA (1%)	$2.4\pm0.0_{Aa}$	$2.4\pm0.2_{Ba}$	$2.5\pm0.0_{Ca}$	$3.4\pm0.5_{Cb}$	$3.4\pm0.7_{Bb}$	$4.8\pm0.7_{Bc}$
	ALF (2%)	$2.4\pm0.1_{Aa}$	$2.7\pm0.1_{Ba}$	$4.6\pm0.1_{Ab}$	$6.1\pm0.2_{Ac}$	$7.3\pm0.2_{Ad}$	$7.7\pm0.1_{Ad}$
Post-inoculation	Water	$2.0\pm0.1_{Aa}$	$3.4\pm0.4_{Ab}$	$5.8\pm0.3_{Bc}$	$7.2\pm0.4_{Bd}$	$7.5\pm0.0_{Ad}$	$7.6\pm0.1_{Ad}$
	AA (1%)	$2.3\pm0.0_{Aa}$	$2.3\pm0.2_{Ba}$	$2.4\pm0.1_{Ca}$	$3.3\pm1.3_{Cb}$	$5.7\pm0.3_{Cc}$	$5.0\pm0.6_{Bd}$
	ALF (2%)	$2.1\pm0.1_{Aa}$	$2.4\pm0.1_{Ba}$	$3.3\pm1.2_{Db}$	$4.9\pm2.0_{Dc}$	$7.7\pm1.1_{Ad}$	$7.6\pm0.3_{Ad}$

AA: acetic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 10. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of frankfurters, inoculated with *Listeria monocytogenes*, dipped into antimicrobial solutions for 30, 60, 90 or 120 s, vacuum packaged and stored at 7°C.

	Day of storage							
Treatment	0	4	8	12	16	20	24	32
Control	$3.1\pm0.0_{Aa}$	$3.5\pm0.1_{ACa}$	$5.4\pm0.4_{Ab}$	$6.5\pm0.2_{Ac}$	$7.0\pm0.2_{Ad}$	$7.3\pm0.2_{Ad}$	$8.0\pm0.0_{ACe}$	$7.9\pm0.1_{ABe}$
Water (30 s)	$2.2\pm0.1_{BCa}$	$3.1\pm0.3_{ADb}$	$5.0\pm0.3_{Bc}$	$6.4\pm0.2_{Ad}$	$7.3\pm0.2_{Ae}$	$7.9\pm0.1_{Bfg}$	$7.8\pm0.7_{ACf}$	$8.3\pm0.1_{Ag}$
LA (2 %; 30 s)	$2.6\pm0.6_{Bab}$	$2.3\pm0.2_{Ba}$	$3.0\pm0.2_{Cb}$	$4.2\pm0.3_{Bc}$	$5.4\pm0.4_{Bd}$	$6.1\pm0.3_{Ce}$	$7.6\pm0.0_{Af}$	$8.1\pm0.0_{ABg}$
SD (3%; 30 s)	$2.4\pm0.2_{BCDa}$	$2.5\pm0.1_{Ba}$	$3.1\pm0.5_{Cb}$	$4.5\pm0.0_{Bc}$	$6.2\pm0.1_{Cd}$	$6.4\pm0.1_{Cd}$	$6.8\pm0.1_{Bd}$	$7.8\pm0.1_{Be}$
ALF (2%; 30 s)	$2.1\pm0.1_{Ca}$	$3.7\pm0.2_{Cb}$	$5.0\pm0.0_{Bc}$	$5.9\pm0.2_{Cd}$	$7.4\pm0.2_{Ae}$	$7.6\pm0.1_{ABf}$	$8.0\pm0.0_{ACf}$	$8.2\pm0.0_{ABf}$
ALF (2%; 60 s)	$2.0\pm0.1_{BCDa}$	$3.3\pm0.2_{ACb}$	$4.4\pm0.2_{Dc}$	$5.7\pm0.1_{CDd}$	$6.4\pm0.5_{Ce}$	$7.4\pm0.3_{ABf}$	$8.5\pm0.7_{Cg}$	$8.1\pm0.1_{ABg}$
ALF (2%; 90 s)	$1.9\pm0.1_{Da}$	$2.9\pm0.0_{Db}$	$4.4\pm0.3_{Dc}$	$5.6\pm0.2_{CDd}$	$6.6\pm0.3_{Ce}$	$7.5\pm0.0_{ABf}$	$8.2\pm0.5_{ACg}$	$8.2\pm0.1_{ABg}$
ALF (2%; 120 s)	$2.1\pm0.4_{Ca}$	$3.3\pm0.1_{ACDb}$	$5.4\pm0.3_{Ac}$	$5.1\pm0.0_{Dc}$	$7.2\pm0.1_{Ad}$	$7.8\pm0.0_{Be}$	$8.1\pm0.5_{Ce}$	$7.8\pm0.4_{Be}$

LA: lactic acid, SD: sodium diacetate, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 11. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of beef or beef and pork bologna slices, inoculated with *Listeria monocytogenes*, and dipped into water (120 s), 2% acetic acid (AA; 60 s), or 2% activated lactoferrin (ALF; 60, 120, or 180 s), vacuum packaged and stored at 10°C.

Meat used in bologna formulation	Treatment	Day of storage					
		0	4	8	12	16	26
Beef	Control	3.5±0.1 $_{Aa}$	5.9±0.3 $_{Ab}$	7.3±0.3 $_{Ac}$	8.2±0.2 $_{Ad}$	8.1±0.2 $_{Ad}$	8.2±0.2 $_{Ad}$
	Water	3.1±0.1 $_{Ba}$	6.7±0.1 $_{Bb}$	8.0±0.6 $_{BDcd}$	8.2±0.1 $_{Ac}$	8.1±0.3 $_{Ac}$	7.7±0.6 $_{Bd}$
	AA (2%)	2.9±0.2 $_{Ba}$	2.9±0.2 $_{Cab}$	2.7±0.2 $_{Ca}$	2.8±0.2 $_{Ba}$	3.4±0.8 $_{Bb}$	2.7±0.8 $_{Ca}$
	ALF (2%; 60 s)	3.0±0.1 $_{Ba}$	5.9±0.4 $_{Ab}$	8.0 ±0.3 $_{Bcd}$	8.3±0.1 $_{Ac}$	8.2±0.2 $_{ACc}$	7.7±0.3 $_{Bd}$
	ALF (2%; 120 s)	3.0±0.2 $_{Ba}$	5.4±0.1 $_{Db}$	7.9±0.1 $_{Bc}$	8.2±0.1 $_{Ac}$	8.0±0.3 $_{Ac}$	8.1±0.1 $_{ACc}$
	ALF (2%; 180 s)	2.9±0.1 $_{Ba}$	5.8±0.1 $_{Ab}$	7.8±0.3 $_{Bc}$	8.3±0.1 $_{Ad}$	8.3±0.2 $_{Ad}$	7.9±0.2 $_{BCc}$
Beef and pork	Control	3.5±0.1 $_{Ba}$	6.1±0.2 $_{Ab}$	8.0±0.2 $_{BDc}$	8.2±0.1 $_{Ac}$	8.0±0.2 $_{Ac}$	8.3±0.1 $_{Ac}$
	Water	2.8±0.2 $_{Ba}$	6.5±0.5 $_{Bb}$	8.2±0.3 $_{BDcd}$	8.3±0.2 $_{Ac}$	7.9±0.5 $_{Ad}$	8.1±0.1 $_{ACcd}$
	AA (2%)	3.2±0.2 $_{ABa}$	2.6±0.1 $_{Cb}$	2.7±0.2 $_{Cb}$	2.7±0.4 $_{Bb}$	2.6±0.3 $_{Ab}$	1.9±0.4 $_{Dc}$
	ALF (2%; 60 s)	2.9±0.2 $_{Ba}$	6.2±0.6 $_{ABb}$	8.1±0.1 $_{BDc}$	8.3±0.1 $_{Ac}$	8.2±0.2 $_{ACc}$	8.2±0.1 $_{Ac}$
	ALF (2%; 120 s)	2.8±0.2 $_{Ba}$	6.5±0.4 $_{Bb}$	8.3±0.1 $_{Dc}$	8.3±0.1 $_{Ac}$	9.0±1.0 $_{Dd}$	8.3±0.1 $_{Ac}$
	ALF (2%; 180 s)	2.8±0.1 $_{Ba}$	6.0±0.4 $_{Ab}$	8.4±0.1 $_{Dc}$	8.2±0.1 $_{Ac}$	8.5±0.3 $_{Cc}$	8.2±0.1 $_{Ac}$

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 12. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on bologna slices inoculated with *Listeria monocytogenes* and dipped in (30 s) or sprayed with (0.69 bar, 2 s each side) antimicrobial solutions or water (except control), vacuum packaged and stored at 7°C.

		Day of storage							
Application	Treatment	0	4	8	12	16	22	28	43
None	Control	$3.2\pm0.0_{Aa}$	$5.2\pm0.7_{Ab}$	$6.0\pm0.1_{Ac}$	$6.6\pm0.1_{Ad}$	$7.3\pm0.1_{Ae}$	$6.5\pm1.8_{Ad}$	$7.7\pm0.4_{Ae}$	$7.4\pm0.0_{ABe}$
Dipping	Water	$2.6\pm0.1_{Ba}$	$4.9\pm0.2_{Bb}$	$6.5\pm0.5_{Bc}$	$6.9\pm0.0_{Ac}$	$7.7\pm0.0_{Bd}$	$7.7\pm0.1_{Bd}$	$7.8\pm0.0_{Ad}$	$7.6\pm0.1_{Ad}$
	LA (2%)	$2.8\pm0.0_{Ba}$	$3.0\pm0.3_{Cab}$	$3.2\pm0.1_{Cb}$	$3.2\pm0.7_{Bb}$	$5.2\pm0.5_{Cc}$	$5.7\pm0.1_{Cd}$	$7.0\pm0.1_{Be}$	$7.5\pm0.1_{ABf}$
	ALF (2%)	$2.8\pm0.1_{Ba}$	$4.8\pm0.1_{Bb}$	$6.4\pm0.2_{Bc}$	$6.8\pm0.2_{Ac}$	$7.6\pm0.1_{Bd}$	$7.6\pm0.0_{Bd}$	$7.5\pm0.0_{Ad}$	$7.5\pm0.0_{ABd}$
Spraying	Water	$2.5\pm0.1_{Ba}$	$4.2\pm0.1_{Db}$	$6.0\pm0.0_{Ac}$	$6.6\pm0.0_{Ad}$	$7.5\pm0.2_{Be}$	$7.8\pm0.1_{Be}$	$7.6\pm0.0_{Ae}$	$7.5\pm0.0_{ABe}$
	LA (2%)	$2.8\pm0.3_{Ba}$	$3.1\pm0.1_{Ca}$	$3.8\pm0.2_{Cb}$	$4.1\pm0.3_{Cb}$	$4.9\pm0.0_{Cc}$	$5.8\pm0.0_{Ce}$	$6.9\pm0.2_{Bf}$	$7.2\pm0.1_{Bf}$
	ALF (2%)	$2.9\pm0.0_{ABa}$	$4.1\pm0.1_{Db}$	$5.8\pm0.3_{Ac}$	$5.9\pm0.1_{Dc}$	$6.8\pm0.3_{Ad}$	$7.3\pm0.1_{Be}$	$7.7\pm0.4_{Ae}$	$7.6\pm0.2_{Ae}$

LA: lactic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 13. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of cured turkey breast slices, inoculated with *Listeria monocytogenes*, dipped into antimicrobial solutions or water for 60 s (except control), vacuum packaged and stored at 7°C.

	Day of storage							
Treatment (dipping)	0	4	8	12	16	22	28	43
Control (undipped)	$3.1\pm0.1_{Aa}$	$3.2\pm0.2_{ABa}$	$4.7\pm0.4_{AEb}$	$4.8\pm0.3_{Ab}$	$5.9\pm0.3_{ADc}$	$6.7\pm0.1_{Ad}$	$7.1\pm0.1_{Aa}$	$7.3\pm0.1_{Ae}$
Water	$2.5\pm0.1_{ABa}$	$3.3\pm0.2_{ABb}$	$4.1\pm0.3_{ACc}$	$4.8\pm0.2_{Ac}$	$6.4\pm0.0_{AEd}$	$7.2\pm0.3_{Ae}$	$6.8\pm0.1_{Ae}$	$7.0\pm0.3_{ABe}$
PL (3%)	$2.7\pm0.1_{ABa}$	$3.3\pm0.2_{ABa}$	$4.7\pm0.7_{Ab}$	$5.6\pm0.1_{Bc}$	$6.5\pm0.4_{Ad}$	$7.2\pm0.0_{Ae}$	$7.2\pm0.3_{Ae}$	$6.5\pm0.2_{Bd}$
SD (3%)	$2.7\pm0.1_{ABa}$	$2.6\pm0.1_{ACDa}$	$2.6\pm0.4_{Ba}$	$2.7\pm0.1_{Ca}$	$2.7\pm0.0_{Ba}$	$4.3\pm0.2_{Bb}$	$4.1\pm1.0_{Bb}$	$2.5\pm0.2_{Ca}$
LA (1%)	$2.7\pm0.1_{ABa}$	$2.7\pm0.1_{ACDa}$	$3.3\pm0.1_{BDb}$	$3.4\pm0.2_{Db}$	$4.9\pm1.1_{Cc}$	$5.7\pm0.4_{Cd}$	$6.7\pm0.0_{Aa}$	$7.2\pm0.2_{ABe}$
AA (1%)	$2.8\pm0.0_{ABa}$	$2.8\pm0.1_{ABCa}$	$2.8\pm0.1_{Ba}$	$2.9\pm0.2_{Cab}$	$2.9\pm0.2_{Bab}$	$3.3\pm0.6_{Dab}$	$3.4\pm0.5_{Cab}$	$3.5\pm0.8_{Db}$
ALF (2%)	$2.6\pm0.1_{ABa}$	$2.9\pm0.1_{ABCa}$	$3.8\pm0.6_{CDb}$	$4.3\pm0.2_{Ab}$	$5.6\pm0.6_{Dc}$	$7.2\pm1.0_{Ad}$	$7.2\pm0.0_{Ad}$	$7.1\pm0.4_{ABd}$
ALF (1%)	$2.6\pm0.0_{ABa}$	$3.5\pm0.1_{Bb}$	$5.2\pm0.0_{Ec}$	$5.9\pm0.0_{Bcd}$	$6.5\pm0.2_{Adf}$	$7.3\pm0.1_{Ae}$	$7.3\pm0.2_{Ae}$	$7.2\pm0.6_{Aef}$
ALF (1%) + PL (3%)	$2.3\pm0.1_{Ba}$	$3.1\pm0.0_{ABb}$	$4.0\pm0.5_{Cc}$	$5.0\pm0.2_{Ad}$	$5.7\pm0.4_{DEd}$	$6.7\pm0.3_{Ae}$	$7.1\pm0.1_{Ae}$	$6.9\pm0.2_{ABe}$
ALF (1%) + SD (3%)	$2.5\pm0.0_{ABa}$	$2.4\pm0.0_{CDa}$	$2.5\pm0.1_{Ba}$	$2.5\pm0.2_{Ca}$	$2.4\pm0.0_{Ba}$	$3.9\pm0.5_{BDb}$	$2.7\pm0.3_{Da}$	$3.4\pm1.7_{Db}$
ALF (1%) + LA (1%)	$2.4\pm0.1_{Ba}$	$2.4\pm0.1_{CDa}$	$2.4\pm0.1_{Ba}$	$2.5\pm0.2_{Ca}$	$3.7\pm0.3_{Fb}$	$5.0\pm0.3_{Bc}$	$5.6\pm0.3_{Ec}$	$6.9\pm0.7_{ABd}$
ALF (1%) + AA (1%)	$2.5\pm0.0_{ABa}$	$2.1\pm0.2_{Da}$	$2.4\pm0.0_{Ba}$	$2.3\pm0.2_{Ca}$	$2.4\pm0.1_{Ba}$	$4.2\pm0.0_{Bb}$	$3.6\pm1.5_{Cb}$	$3.0\pm1.1_{Dc}$

PL: potassium lactate, SD: sodium diacetate, LA: lactic acid, AA: acetic acid, ALF: activated lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 14. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of bologna slices formulated with or without antimicrobials, inoculated with *Listeria monocytogenes* after slicing, vacuum packaged and stored at 4°C.

	Day of storage					
Treatment (formulation)	0	10	20	43	75	95
Control	2.4±0.7$_{Aa}$	4.9±0.2$_{Ab}$	6.3±0.4$_{Ac}$	7.3±0.0$_{Ad}$	7.2±0.2$_{Ad}$	7.7±1.5$_{ACDd}$
PL (1.8%)	2.2±0.0$_{Aa}$	2.2±0.2$_{Bb}$	2.5±0.1$_{Ba}$	5.2±0.4$_{Bb}$	5.8±0.7$_{Bb}$	7.3±1.4$_{ACDc}$
LF (0.5%)	2.3±0.1$_{Aa}$	4.0±1.1$_{ACb}$	3.8±0.3$_{Cb}$	6.3±0.4$_{Cc}$	7.2±0.1$_{Ad}$	7.8±0.8$_{ADd}$
LF (1%)	2.3±0.2$_{Aa}$	3.4±0.1$_{Cb}$	5.0±0.6$_{Dc}$	7.5±0.2$_{Ad}$	7.2±0.0$_{Ad}$	7.8±1.0$_{ADd}$
PL (1.8%) + SD (0.125%)	2.5±0.4$_{Aab}$	2.0±0.0$_{Ba}$	2.1±0.1$_{Ba}$	2.0±0.1$_{Da}$	2.4±1.2$_{Cab}$	2.9±0.6$_{Bb}$
PL (1.8%) + LF (0.5%)	2.3±0.1$_{Aa}$	2.2±0.1$_{Ba}$	1.9±0.1$_{Ba}$	2.7±0.5$_{Da}$	5.8±0.4$_{Bb}$	6.9±0.2$_{CFc}$
SD (0.125%) + LF (0.5%)	2.2±0.1$_{Aa}$	3.8±0.4$_{Cb}$	5.5±0.1$_{Dc}$	8.0±0.1$_{Ad}$	8.0±0.1$_{Ad}$	8.1±0.1$_{Dd}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	2.2±0.2$_{Aa}$	2.1±0.0$_{Ba}$	3.2±0.5$_{Cb}$	3.6±0.6$_{Eb}$	4.6±0.8$_{Dc}$	4.9±0.4$_{Ec}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	3.0±1.1$_{Aab}$	2.1±0.2$_{Ba}$	3.7±0.5$_{Cb}$	5.5±0.3$_{Bcd}$	5.4±0.3$_{Bc}$	6.4±0.5$_{Fd}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 15. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of bologna slices formulated with or without antimicrobials, inoculated with *Listeria monocytogenes* after slicing, vacuum packaged and stored at 7°C.

	Day of storage								
Treatment (formulation)	0	4	8	12	16	20	28	43	57
Control	$2.4\pm0.7_{ABa}$	$4.5\pm0.5_{Ab}$	$4.5\pm0.1_{Ab}$	$5.8\pm0.2_{Ac}$	$6.8\pm0.1_{Ad}$	$6.8\pm0.1_{Ad}$	$7.4\pm0.0_{Ad}$	$7.1\pm0.0_{Ad}$	$7.3\pm0.2_{Ad}$
PL (1.8%)	$2.2\pm0.0_{Aa}$	$2.2\pm0.0_{Ba}$	$2.3\pm0.0_{Ba}$	$2.4\pm0.3_{BEa}$	$2.9\pm0.2_{Bb}$	$3.4\pm0.5_{Bb}$	$5.3\pm0.2_{Bc}$	$5.9\pm0.1_{Bd}$	$6.0\pm0.4_{Bd}$
LF (0.5%)	$2.3\pm0.1_{Aa}$	$2.9\pm0.1_{Cb}$	$3.8\pm0.6_{Cc}$	$4.4\pm0.2_{Cd}$	$5.4\pm0.2_{Ce}$	$5.7\pm0.1_{Aef}$	$6.3\pm0.1_{Cfh}$	$7.1\pm0.1_{Ag}$	$7.0\pm0.3_{Agh}$
LF (1%)	$2.3\pm0.2_{Aa}$	$3.1\pm0.2_{Cb}$	$3.5\pm0.1_{Cb}$	$4.2\pm0.8_{Cc}$	$5.1\pm0.6_{Cd}$	$5.6\pm0.1_{Ce}$	$6.0\pm0.6_{Ce}$	$7.2\pm0.2_{Af}$	$7.5\pm0.1_{Af}$
PL (1.8%) + SD (0.125%)	$2.5\pm0.4_{ABa}$	$2.1\pm0.0_{Bab}$	$1.9\pm0.1_{Bb}$	$1.9\pm0.0_{BDb}$	$2.2\pm0.2_{Dab}$	$1.8\pm0.4_{Db}$	$2.1\pm0.1_{Dab}$	$2.3\pm0.4_{Cab}$	$3.1\pm1.0_{Cc}$
PL (1.8%) + LF (0.5%)	$2.2\pm0.1_{Aa}$	$2.3\pm0.1_{Ba}$	$2.3\pm0.1_{Ba}$	$2.5\pm0.1_{BEa}$	$3.3\pm0.1_{Bb}$	$3.1\pm0.1_{Bb}$	$4.1\pm0.6_{Ec}$	$4.6\pm0.3_{Dc}$	$5.8\pm0.4_{Bd}$
SD (0.125%) + LF (0.5%)	$2.2\pm0.1_{Aab}$	$3.0\pm0.2_{Cb}$	$4.5\pm0.1_{Ac}$	$5.1\pm0.2_{Acd}$	$5.5\pm0.2_{Cd}$	$6.1\pm0.2_{Ce}$	$7.2\pm0.1_{Af}$	$7.9\pm0.0_{Eg}$	$8.2\pm0.6_{Dg}$
PL (1.8%) + SD (0.125%) + LF (0.5%)	$2.2\pm0.2_{Aa}$	$1.9\pm0.0_{Bab}$	$2.0\pm0.0_{Bab}$	$1.7\pm0.0_{Da}$	$2.3\pm0.3_{Db}$	$2.2\pm0.0_{Dab}$	$3.0\pm0.8_{Fc}$	$3.4\pm0.2_{Fc}$	$5.3\pm0.9_{Ed}$
PL (1.8%) + SD (0.0625%) + LF (0.25%)	$3.0\pm1.1_{Ba}$	$2.1\pm0.1_{Bb}$	$2.8\pm0.3_{Ba}$	$2.8\pm0.1_{Ea}$	$4.0\pm0.1_{Ec}$	$4.6\pm0.2_{Ec}$	$5.9\pm0.2_{BCc}$	$7.2\pm0.3_{Ae}$	$7.3\pm0.1_{Ae}$

PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 16. Mean total microbial populations (log CFU/cm^2 ± standard deviation) on the surface of frankfurters formulated with or without antimicrobials, inoculated with *Listeria monocytogenes*, left undipped or dipped into solutions of 2% acetic acid or 2% activated lactoferrin for 120 s, vacuum packaged and stored at 7°C.

Treatment (dipping)	Treatment (formulation)	Day of storage							
		0	5	10	15	20	25	35	50
None	Control	$2.1\pm0.1_{Aa}$	$2.3\pm0.1_{Aa}$	$3.0\pm0.2_{Ac}$	$3.5\pm1.4_{Ac}$	$2.9\pm0.1_{Ac}$	$5.0\pm0.1_{Ad}$	$5.5\pm0.8_{Ad}$	$7.3\pm0.4_{Ae}$
	PL (1.8%) + SD (0.125%)	$2.1\pm0.1_{Aa}$	$2.0\pm0.1_{ABa}$	$2.0\pm0.1_{Ba}$	$1.8\pm0.3_{Ba}$	$1.8\pm0.1_{Ba}$	$1.8\pm0.3_{BEa}$	$2.0\pm0.1_{Ba}$	$2.9\pm2.0_{Bb}$
	LF (0.5%)	$2.2\pm0.1_{Aa}$	$2.1\pm0.1_{ABa}$	$2.5\pm0.4_{ABab}$	$3.0\pm1.0_{Ab}$	$3.1\pm0.6_{Ab}$	$4.0\pm1.0_{Cc}$	$5.3\pm0.1_{Ad}$	$6.7\pm0.7_{Ae}$
	PL (1.8%) + LF (0.5%)	$2.1\pm0.1_{Aa}$	$1.9\pm0.1_{ABa}$	$2.0\pm0.0_{Ba}$	$1.8\pm0.1_{Ba}$	$1.9\pm0.0_{Ba}$	$2.0\pm0.3_{Ba}$	$2.0\pm0.1_{Ba}$	$2.1\pm0.6_{BCa}$
AA (2%)	Control	$1.1\pm0.1_{BCa}$	$1.5\pm1.0_{BDab}$	$0.9\pm0.2_{CDa}$	$1.8\pm0.8_{Bab}$	$0.9\pm0.1_{CEa}$	$1.5\pm0.2_{BEab}$	$1.9\pm0.3_{BDb}$	$1.5\pm0.4_{Cab}$
	PL (1.8%) + SD (0.125%)	$1.1\pm0.2_{BCa}$	$0.6\pm0.1_{Ca}$	$0.6\pm0.1_{CDa}$	$0.7\pm0.1_{Ca}$	$0.7\pm0.1_{CEa}$	$0.6\pm0.2_{Da}$	$0.5\pm0.5_{CEa}$	$0.6\pm0.5_{Ds}$
	LF (0.5%)	$1.4\pm0.0_{ABab}$	$1.2\pm0.2_{Da}$	$1.2\pm0.4_{Da}$	$1.4\pm0.4_{BDab}$	$1.3\pm0.4_{BCa}$	$1.2\pm0.1_{DEa}$	$1.2\pm0.3_{CDa}$	$2.0\pm0.2_{BCb}$
	PL (1.8%) + LF (0.5%)	$1.4\pm0.1_{ABa}$	$0.8\pm0.0_{Ca}$	$0.8\pm0.1_{CDa}$	$0.9\pm0.1_{CDa}$	$0.8\pm0.2_{CEa}$	$0.7\pm0.2_{Da}$	$0.7\pm0.3_{CEa}$	$1.1\pm0.3_{CEa}$
ALF (2%)	Control	$0.7\pm0.1_{Ca}$	$1.0\pm0.5_{CDa}$	$2.0\pm0.2_{Bb}$	$3.4\pm0.3_{Ac}$	$4.8\pm1.1_{Dd}$	$5.2\pm0.4_{Ade}$	$5.8\pm0.4_{Ae}$	$7.2\pm0.4_{Af}$
	PL (1.8%) + SD (0.125%)	$0.7\pm0.0_{Ca}$	$0.5\pm0.1_{CDa}$	$0.4\pm0.3_{Ca}$	$0.5\pm0.0_{Ca}$	$0.2\pm0.3_{Ea}$	$0.4\pm0.3_{Da}$	$0.1\pm0.2_{Ea}$	$1.5\pm0.9_{CEs}$
	LF (0.5%)	$1.0\pm0.2_{BCab}$	$1.3\pm0.5_{Da}$	$0.5\pm0.0_{Ca}$	$3.2\pm0.7_{Ac}$	$4.6\pm0.3_{Fd}$	$4.3\pm0.2_{Cd}$	$6.1\pm0.5_{Ae}$	$7.0\pm0.7_{Af}$
	PL (1.8%) + LF (0.5%)	$1.8\pm0.1_{Aa}$	$0.5\pm0.1_{CDb}$	$2.0\pm1.0_{Ba}$	$0.7\pm0.4_{Cb}$	$0.3\pm0.1_{Eb}$	$0.4\pm0.0_{Db}$	$0.6\pm0.6_{Cb}$	$0.9\pm0.3_{Db}$

AA: acetic acid, ALF: activated lactoferrin, PL: potassium lactate, SD: sodium diacetate, LF: lactoferrin

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 17 (Figure 4.1). *Listeria monocytogenes* counts (log CFU/ml ± standard deviation; 13 strains; PALCAM agar) in the GC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 4 h at 30°C.

Strain	Time of exposure					
	0	15	30	60	90	120
558	8.0±0.1 $_{Aa}$	7.7±0.1 $_{Aab}$	7.7±0.1 $_{Aab}$	7.7±0.1 $_{Aab}$	7.7±0.2 $_{Aab}$	7.5±0.0 $_{Ab}$
R2-500	7.9±0.1 $_{Aa}$	7.6±0.0 $_{ACab}$	7.7±0.0 $_{Aab}$	7.7±0.2 $_{ADab}$	7.6±0.2 $_{Aab}$	7.3±0.3 $_{Ab}$
R2-501	7.7±0.0 $_{Aa}$	7.5±0.2 $_{ACa}$	7.5±0.1 $_{ACa}$	7.5±0.1 $_{ADa}$	7.3±0.3 $_{Aa}$	7.3±0.1 $_{Aa}$
Scott A	7.8±0.1 $_{Aa}$	7.6±0.1 $_{ACa}$	7.6±0.2 $_{ACa}$	7.6±0.1 $_{ACa}$	7.4±0.1 $_{Aa}$	2.0±0.5 $_{Cb}$
N1-225	7.8±0.1 $_{Aa}$	7.6±0.1 $_{ACab}$	7.6±0.1 $_{ACab}$	7.6±0.0 $_{ACa}$	7.4±0.0 $_{Aab}$	7.1±0.3 $_{Ab}$
N1-227	7.7±0.2 $_{Aa}$	7.5±0.2 $_{ACab}$	7.5±0.1 $_{ACa}$	7.5±0.1 $_{ADa}$	7.4±0.2 $_{Aa}$	7.0±0.4 $_{ADb}$
C1-056	7.6±0.3 $_{Aa}$	7.4±0.2 $_{ACab}$	7.5±0.1 $_{ACa}$	7.3±0.1 $_{ADab}$	7.0±0.3 $_{Ab}$	0.0±0.0 $_{Bc}$
N3-031	7.5±0.4 $_{ACa}$	7.2±0.3 $_{Ca}$	7.2±0.2 $_{Ca}$	7.1±0.3 $_{CDa}$	7.0±0.2 $_{Aa}$	6.0±1.2 $_{Db}$
J1-101	7.7±0.1 $_{Aa}$	7.4±0.0 $_{ACab}$	7.5±0.1 $_{ACab}$	7.4±0.1 $_{ADab}$	7.2±0.2 $_{Aab}$	7.0±0.2 $_{ADb}$
10403S	7.7±0.0 $_{Aa}$	7.5±0.1 $_{ACa}$	7.5±0.0 $_{ACa}$	7.4±0.0 $_{ADa}$	7.3±0.0 $_{Aa}$	7.3±0.1 $_{Aa}$
A1-254	6.9±0.1 $_{Ba}$	6.5±0.1 $_{Ba}$	6.6±0.1 $_{Ba}$	6.4±0.1 $_{Ba}$	6.4±0.1 $_{Ba}$	5.5±0.6 $_{Db}$
J1-158	7.8±0.2 $_{Aa}$	7.8±0.2 $_{Aa}$	7.7±0.1 $_{Aa}$	7.6±0.1 $_{ACa}$	7.4±0.1 $_{Aa}$	6.8±0.5 $_{ADb}$
J1-168	7.4±0.4 $_{Ca}$	7.1±0.3 $_{Ca}$	7.2±0.4 $_{Ca}$	7.0±0.2 $_{Da}$	6.9±0.3 $_{Aa}$	6.5±0.4 $_{Db}$

GC: gastric compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 18 (Figure 4.1). *Listeria monocytogenes* counts (log CFU/ml ± standard deviation; 13 strains; PALCAM agar) in the GC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 16 h at 30°C.

Strain	Time of exposure					
	0	15	30	60	90	120
558	9.0±0.1 $_{Aa}$	8.8±0.2 $_{ABab}$	8.6±0.1 $_{ABab}$	8.6±0.0 $_{Aab}$	8.4±0.1 $_{ACb}$	7.1±0.6 $_{Ac}$
R2-500	9.1±0.0 $_{Aa}$	8.8±0.1 $_{ABa}$	8.7±0.1 $_{ABa}$	8.6±0.1 $_{Aa}$	8.6±0.1 $_{Aab}$	7.8±0.3 $_{Bb}$
R2-501	9.1±0.1 $_{Aa}$	8.8±0.1 $_{Aa}$	8.8±0.0 $_{Aa}$	8.7±0.0 $_{Aa}$	8.7±0.0 $_{Aa}$	7.7±0.5 $_{Bb}$
Scott A	8.9±0.2 $_{Aa}$	8.6±0.0 $_{ABCab}$	8.6±0.0 $_{ABab}$	8.6±0.0 $_{Aab}$	8.1±0.2 $_{CDb}$	2.6±0.6 $_{Cc}$
N1-225	8.6±0.1 $_{Aa}$	8.3±0.2 $_{BCab}$	8.2±0.2 $_{Bab}$	8.2±0.1 $_{Aab}$	8.0±0.3 $_{CDb}$	6.9±0.4 $_{ADc}$
N1-227	8.8±0.0 $_{Aa}$	8.5±0.1 $_{ABCa}$	8.4±0.0 $_{ABa}$	8.4±0.1 $_{Aa}$	8.0±0.2 $_{CDb}$	5.3±0.2 $_{Ec}$
C1-056	9.0±0.1 $_{Aa}$	8.7±0.2 $_{ABCab}$	8.7±0.0 $_{ABab}$	8.5±0.1 $_{Ab}$	7.3±0.8 $_{Bc}$	1.0±1.4 $_{Fd}$
N3-031	8.9±0.0 $_{Aa}$	8.6±0.0 $_{ABCab}$	8.5±0.1 $_{ABab}$	8.6±0.1 $_{Aab}$	8.4±0.1 $_{ACb}$	7.1±0.1 $_{Ac}$
J1-101	8.8±0.1 $_{Aa}$	8.5±0.1 $_{ABCa}$	8.8±0.5 $_{Aa}$	8.4±0.0 $_{Aa}$	8.0±0.1 $_{CDb}$	6.2±0.4 $_{Dc}$
10403S	8.8±0.0 $_{Aa}$	8.5±0.1 $_{ABCab}$	8.4±0.1 $_{ABab}$	8.4±0.1 $_{Aab}$	8.1±0.1 $_{Cb}$	6.7±0.1 $_{ADc}$
A1-254	8.6±0.1 $_{Aa}$	8.2±0.1 $_{Cab}$	8.2±0.0 $_{Bab}$	8.3±0.1 $_{Aab}$	7.6±0.1 $_{BDb}$	4.4±1.0 $_{Gc}$
J1-158	9.0±0.1 $_{Aa}$	8.7±0.2 $_{ABCa}$	8.6±0.0 $_{ABab}$	8.7±0.1 $_{Aa}$	8.5±0.1 $_{ACb}$	5.7±0.4 $_{Dc}$
J1-168	8.9±0.1 $_{Aa}$	8.6±0.1 $_{ABCab}$	8.6±0.1 $_{ABab}$	8.5±0.1 $_{Aab}$	8.3±0.2 $_{ACb}$	6.9±0.1 $_{ADc}$

GC: gastric compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 19 (Figure 4.2). *Listeria monocytogenes* counts (log CFU/ml ± standard deviation; 13 strains; PALCAM agar) in the IC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 4 h at 30°C.

Strain	Time of exposure				
	30	60	90	120	240
558	8.1±0.09 $_{Aa}$	7.5±0.1 $_{Aab}$	7.3±0.1 $_{Aab}$	6.8±0.7 $_{ACbc}$	6.0±1.0 $_{Ac}$
R2-500	7.8±0.32 $_{Aa}$	7.6±0.4 $_{Aa}$	7.2±0.2 $_{Aab}$	6.4±1.1 $_{ACbC}$	5.5±1.7 $_{ACc}$
R2-501	7.5±0.33 $_{Aa}$	7.2±0.2 $_{ABa}$	7.0±0.2 $_{Aa}$	6.8±0.1 $_{ACa}$	6.6±0.0 $_{Aa}$
Scott A	7.1±0.27 $_{ABa}$	7.2±0.2 $_{ABa}$	7.1±0.3 $_{Aa}$	6.4±1.1 $_{ACa}$	6.2±0.6 $_{Aa}$
N1-225	7.9±0.27 $_{Aa}$	7.1±0.5 $_{ABab}$	7.1±0.3 $_{Aab}$	6.6±0.5 $_{ACb}$	6.0±2.1 $_{ACb}$
N1-227	7.0±0.09 $_{ABa}$	6.5±0.8 $_{ABab}$	6.0±1.4 $_{ABb}$	5.6±1.3 $_{BCb}$	6.3±0.2 $_{Aab}$
C1-056	6.2±0.83 $_{BCa}$	6.7±0.6 $_{ABa}$	6.8±0.4 $_{ABa}$	6.5±0.4 $_{ACa}$	6.3±0.0 $_{Aa}$
N3-031	7.3±0.33 $_{ABa}$	6.8±0.4 $_{ABab}$	6.3±0.8 $_{ABab}$	6.1±0.8 $_{ACb}$	6.4±0.0 $_{Aab}$
J1-101	7.3±0.32 $_{ABa}$	7.0±0.0 $_{ABa}$	6.7±0.6 $_{ABa}$	5.6±2.0 $_{BCb}$	4.3±2.8 $_{BCc}$
10403S	7.3±0.49 $_{ABa}$	7.1±0.2 $_{ABa}$	6.4±1.1 $_{ABab}$	5.7±1.1 $_{Cb}$	5.4±0.1 $_{ACb}$
A1-254	5.4±0.39 $_{Cab}$	6.0±0.1 $_{Ba}$	5.4±0.2 $_{Ba}$	4.4±1.1 $_{Bb}$	3.2±0.0 $_{Bb}$
J1-158	7.5±0.18 $_{Aa}$	7.1±0.1 $_{ABa}$	7.0±0.3 $_{Aa}$	6.8±0.3 $_{ACa}$	6.3±0.1 $_{Aa}$
J1-168	7.0±0.82 $_{ABa}$	6.9±0.2 $_{ABa}$	6.7±0.3 $_{ABa}$	6.4±0.6 $_{ACa}$	6.6±0.0 $_{Aa}$

IC: intestinal compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 20 (Figure 4.2). *Listeria monocytogenes* counts (log CFU/ml ± standard deviation; 13 strains; PALCAM agar) in the IC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation (1 ml) into 100 ml TSBYE without dextrose and incubation for 16 h at 30°C.

Strain	Time of exposure				
	30	60	90	120	240
558	9.1±0.1 $_{Aa}$	8.5±0.2 $_{Aab}$	8.2±0.3 $_{Ab}$	8.0±0.4 $_{Ab}$	8.1±0.1 $_{Ab}$
R2-500	8.8±0.2 $_{ACa}$	8.1±0.5 $_{ACa}$	8.0±0.5 $_{Aa}$	7.9±0.4 $_{Aa}$	8.0±0.2 $_{Aa}$
R2-501	9.0±0.1 $_{Aa}$	8.0±0.5 $_{ACb}$	7.6±0.3 $_{ACbc}$	7.0±0.1 $_{Ac}$	8.0±0.0 $_{Ab}$
Scott A	8.9±0.2 $_{Aa}$	8.4±0.0 $_{Aa}$	8.2±0.2 $_{Aab}$	7.9±0.2 $_{Ab}$	7.8±0.3 $_{Ab}$
N1-225	8.6±0.2 $_{ACa}$	8.0±0.3 $_{ABab}$	7.7±0.3 $_{ACab}$	7.5±0.3 $_{Ab}$	7.4±0.2 $_{Ab}$
N1-227	8.2±0.1 $_{ACa}$	7.7±0.1 $_{ABab}$	7.3±0.3 $_{ACab}$	6.9±0.4 $_{Ab}$	6.7±0.4 $_{Cb}$
C1-056	8.7±0.6 $_{ACa}$	8.0±0.2 $_{ABb}$	7.3±1.1 $_{ACb}$	7.4±0.3 $_{Ab}$	5.2±1.9 $_{Bc}$
N3-031	8.8±0.1 $_{Aa}$	8.3±0.1 $_{ACab}$	7.9±0.4 $_{Aab}$	7.6±0.3 $_{Ab}$	7.3±0.1 $_{ACb}$
J1-101	8.7±0.1 $_{ACa}$	7.8±0.2 $_{ABb}$	7.2±0.3 $_{ACbc}$	6.7±0.1 $_{Ac}$	6.8±0.2 $_{Cc}$
10403S	7.9±0.3 $_{BCa}$	7.3±0.3 $_{BCb}$	6.5±0.2 $_{Cc}$	5.7±0.4 $_{Bd}$	5.2±1.1 $_{Bd}$
A1-254	7.0±0.5 $_{Ba}$	7.1±0.2 $_{Ba}$	5.4±1.4 $_{BCb}$	4.7±1.7 $_{Bc}$	3.8±2.4 $_{Dd}$
J1-158	8.7±0.1 $_{ACa}$	8.1±0.2 $_{ACab}$	7.8±0.3 $_{ACbc}$	7.4±0.3 $_{Ac}$	7.2±0.4 $_{ACc}$
J1-168	8.9±0.1 $_{Aa}$	8.3±0.1 $_{ACab}$	8.4±0.4 $_{Aab}$	7.7±0.2 $_{Ab}$	7.7±0.0 $_{Ab}$

IC: intestinal compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 21. Total microbial counts (log CFU/ml ± standard deviation; 13 strains; TSAYE) in the GC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation of *Listeria monocytogenes* (1 ml) into 100 ml TSBYE without dextrose and incubation for 4 h at 30°C.

Strain	Time of exposure					
	0	15	30	60	90	120
558	8.0±0.1 $_{Aa}$	7.8±0.1 $_{Aab}$	7.8±0.0 $_{Aab}$	7.8±0.1 $_{ACab}$	7.7±0.1 $_{Aab}$	7.5±0.0 $_{Ab}$
R2-500	7.9±0.1 $_{ACa}$	7.7±0.1 $_{ACab}$	7.7±0.1 $_{Aab}$	7.7±0.0 $_{Aab}$	7.5±0.1 $_{AEab}$	7.3±0.3 $_{Ab}$
R2-501	7.7±0.1 $_{ACa}$	7.6±0.1 $_{ACa}$	7.6±0.1 $_{ACa}$	7.6±0.0 $_{ACa}$	7.5±0.1 $_{AEa}$	7.3±0.2 $_{Aa}$
Scott A	7.9±0.0 $_{ACa}$	7.5±0.1 $_{ACa}$	7.6±0.2 $_{ACa}$	7.6±0.1 $_{ACa}$	7.4±0.0 $_{AEa}$	2.1±0.2 $_{Cb}$
N1-225	7.9±0.0 $_{ACa}$	7.6±0.1 $_{ACa}$	7.7±0.1 $_{ACa}$	7.7±0.1 $_{Aa}$	7.4±0.0 $_{AEab}$	7.1±0.2 $_{Ab}$
N1-227	7.8±0.2 $_{ACa}$	7.6±0.2 $_{ACa}$	7.5±0.2 $_{ACa}$	7.5±0.1 $_{ACa}$	7.4±0.1 $_{AEab}$	7.0±0.3 $_{AFb}$
C1-056	7.6±0.2 $_{ACa}$	7.5±0.1 $_{ACa}$	7.5±0.1 $_{ACa}$	7.4±0.2 $_{ACa}$	6.8±0.5 $_{BCb}$	0.3±0.4 $_{Bc}$
N3-031	7.5±0.2 $_{ABa}$	7.2±0.3 $_{BCa}$	7.2±0.3 $_{ACa}$	7.2±0.3 $_{BCa}$	7.1±0.3 $_{CEa}$	6.0±1.2 $_{DEb}$
J1-101	7.7±0.1 $_{ACa}$	7.4±0.1 $_{ACab}$	7.5±0.1 $_{ACab}$	7.5±0.0 $_{ACab}$	7.1±0.3 $_{CEab}$	7.2±0.0 $_{Ab}$
10403S	7.6±0.1 $_{ACa}$	7.3±0.3 $_{ACa}$	7.5±0.1 $_{ACa}$	7.5±0.0 $_{ACa}$	7.4±0.0 $_{AEa}$	7.3±0.0 $_{Aa}$
A1-254	7.0±0.1 $_{Ba}$	6.7±0.1 $_{Ba}$	6.7±0.1 $_{Ba}$	6.7±0.1 $_{Ba}$	6.6±0.0 $_{BDa}$	5.8±0.3 $_{Db}$
J1-158	7.8±0.1 $_{ACa}$	7.8±0.1 $_{Aa}$	7.7±0.2 $_{Aa}$	7.6±0.1 $_{ACa}$	7.5±0.2 $_{AEab}$	7.1±0.3 $_{Ab}$
J1-168	7.3±0.4 $_{BCa}$	7.2±0.2 $_{BCa}$	7.2±0.4 $_{BCa}$	7.1±0.3 $_{BCa}$	7.0±0.3 $_{CDa}$	6.5±0.5 $_{EFb}$

GC: gastric compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 22. Total microbial counts (log CFU/ml ± standard deviation; 13 strains; TSAYE) in the GC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation of *Listeria monocytogenes* (1 ml) into 100 ml TSBYE without dextrose and incubation for 16 h at 30°C.

Strain	Time of exposure					
	0	15	30	60	90	120
558	9.1±0.3 $_{Aa}$	8.7±0.1 $_{ABb}$	8.6±0.1 $_{ABb}$	8.6±0.0 $_{Ab}$	8.5±0.1 $_{ACb}$	7.2±0.6 $_{AFc}$
R2-500	9.0±0.2 $_{Aa}$	8.8±0.0 $_{Aa}$	8.8±0.0 $_{Aa}$	8.6±0.1 $_{Aa}$	8.7±0.1 $_{ACa}$	7.6±0.6 $_{Ab}$
R2-501	9.1±0.1 $_{Aa}$	8.8±0.1 $_{Aa}$	8.8±0.0 $_{ABab}$	8.3±0.8 $_{Abc}$	8.7±0.0 $_{Aab}$	8.2±0.1 $_{Bc}$
Scott A	9.0±0.1 $_{Aa}$	8.5±0.2 $_{ABab}$	8.6±0.0 $_{ABab}$	8.6±0.0 $_{Aab}$	8.2±0.2 $_{CDb}$	2.7±0.6 $_{Dc}$
N1-225	8.7±0.2 $_{Aa}$	8.2±0.2 $_{Bab}$	8.3±0.1 $_{Bab}$	8.2±0.1 $_{Aab}$	8.0±0.2 $_{CDb}$	7.0±0.3 $_{Fc}$
N1-227	8.9±0.0 $_{Aa}$	8.5±0.1 $_{ABab}$	8.5±0.1 $_{ABab}$	8.4±0.1 $_{Aab}$	8.1±0.2 $_{CDb}$	5.4±0.2 $_{Ec}$
C1-056	9.1±0.1 $_{Aa}$	8.8±0.1 $_{Aa}$	8.7±0.0 $_{ABa}$	8.7±0.0 $_{Aa}$	7.5±0.8 $_{Bb}$	0.9±1.5 $_{Cc}$
N3-031	8.9±0.1 $_{Aa}$	8.5±0.0 $_{ABab}$	8.6±0.1 $_{ABab}$	8.6±0.1 $_{Aab}$	8.4±0.0 $_{ACb}$	7.1±0.1 $_{Fc}$
J1-101	8.9±0.1 $_{Aa}$	8.5±0.1 $_{ABab}$	8.5±0.1 $_{ABab}$	8.5±0.1 $_{Aab}$	8.2±0.1 $_{CDb}$	6.2±0.7 $_{HJc}$
10403S	8.8±0.0 $_{Aa}$	8.6±0.0 $_{ABab}$	8.6±0.1 $_{ABab}$	8.5±0.1 $_{Aab}$	8.2±0.1 $_{CDb}$	6.5±0.3 $_{Gc}$
A1-254	8.7±0.2 $_{Aa}$	8.4±0.1 $_{ABa}$	8.3±0.0 $_{ABa}$	8.4±0.1 $_{Aa}$	7.8±0.2 $_{BDb}$	4.4±1.5 $_{Fc}$
J1-158	9.0±0.1 $_{Aa}$	8.7±0.1 $_{ABab}$	8.6±0.1 $_{ABab}$	8.6±0.1 $_{Aab}$	8.5±0.1 $_{ACb}$	5.8±0.5 $_{Elc}$
J1-168	9.0±0.0 $_{Aa}$	8.7±0.1 $_{ABab}$	8.6±0.1 $_{ABab}$	8.6±0.0 $_{Aab}$	8.4±0.2 $_{ACb}$	6.9±0.2 $_{Jc}$

GC: gastric compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 23. Total microbial counts (log CFU/ml ± standard deviation; 13 strains; TSAYE) in the IC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation of *Listeria monocytogenes* (1 ml) into 100 ml TSBYE without dextrose and incubation for 4 h at 30°C.

Strain	Time of exposure				
	30	60	90	120	240
558	8.1±0.1 $_{Aa}$	7.5±0.1 $_{Aab}$	7.3±0.1 $_{Aab}$	6.8±0.6 $_{ABb}$	6.4±1.1 $_{Ab}$
R2-500	7.8±0.3 $_{Aa}$	7.6±0.4 $_{Aa}$	7.1±0.2 $_{Aa}$	7.5±0.3 $_{Aa}$	5.7±1.6 $_{Ab}$
R2-501	7.6±0.3 $_{Aa}$	7.2±0.2 $_{ABa}$	7.0±0.2 $_{Aa}$	6.8±0.2 $_{ABa}$	6.8±0.1 $_{Aa}$
Scott A	7.2±0.3 $_{Aa}$	7.2±0.1 $_{Aa}$	7.2±0.3 $_{Aa}$	6.5±0.9 $_{ABa}$	7.0±0.0 $_{Aa}$
N1-225	7.9±0.3 $_{Aa}$	7.2±0.4 $_{ABab}$	7.1±0.4 $_{Aab}$	6.6±0.5 $_{ABb}$	6.0±2.1 $_{Ab}$
N1-227	7.2±0.1 $_{ABa}$	6.7±0.7 $_{ABab}$	6.2±1.2 $_{ABab}$	5.9±1.3 $_{BCb}$	6.4±0.1 $_{Aab}$
C1-056	6.1±1.0 $_{BCa}$	7.0±0.2 $_{ABa}$	6.9±0.3 $_{Aa}$	6.6±0.4 $_{ABa}$	6.6±0.0 $_{Aa}$
N3-031	7.5±0.3 $_{Aa}$	6.8±0.3 $_{ABab}$	6.4±0.7 $_{ABab}$	6.1±0.7 $_{Bb}$	6.5±0.0 $_{Aab}$
J1-101	7.4±0.3 $_{Aa}$	7.0±0.1 $_{ABa}$	6.4±1.5 $_{ABab}$	5.8±1.9 $_{BCb}$	4.3±3.1 $_{Bc}$
10403S	7.5±0.4 $_{Aa}$	7.5±0.4 $_{Aa}$	6.5±0.9 $_{Aab}$	5.9±1.3 $_{BCb}$	5.6±0.1 $_{Ab}$
A1-254	5.7±0.5 $_{Cab}$	6.1±0.1 $_{Ba}$	5.4±0.3 $_{Bab}$	4.8±0.9 $_{Cb}$	3.5±0.5 $_{Bc}$
J1-158	7.6±0.2 $_{Aa}$	7.2±0.1 $_{ABa}$	7.1±0.3 $_{Aa}$	6.8±0.3 $_{ABa}$	6.9±0.5 $_{Aa}$
J1-168	7.1±0.8 $_{ABa}$	6.9±0.2 $_{ABa}$	6.8±0.4 $_{Aa}$	6.2±0.5 $_{Ba}$	6.7±0.2 $_{Aa}$

IC: intestinal compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 24. Total microbial counts (log CFU/ml ± standard deviation; 13 strains; TSAYE) in the IC of a dynamic gastrointestinal model (gastric compartment: pH 2.0 within 88 min, intestinal compartment: pH 6.5; 37°C) after inoculation of *Listeria monocytogenes* (1 ml) into 100 ml TSBYE without dextrose and incubation for 16 h at 30°C.

Strain	Time of exposure				
	30	60	90	120	240
558	9.1±0.0 $_{Aa}$	8.5±0.1 $_{Aab}$	8.3±0.3 $_{ABab}$	8.1±0.3 $_{Aab}$	7.6±0.4 $_{Ab}$
R2-500	8.9±0.1 $_{ACa}$	8.2±0.5 $_{Aa}$	8.1±0.5 $_{ABa}$	8.0±0.5 $_{Aa}$	8.1±0.2 $_{Aa}$
R2-501	9.0±0.1 $_{ACa}$	8.1±0.4 $_{Aab}$	7.9±0.2 $_{ABab}$	7.6±0.6 $_{Ab}$	7.8±0.3 $_{Aab}$
Scott A	8.8±0.2 $_{ACa}$	8.4±0.1 $_{Aa}$	8.2±0.3 $_{ABa}$	7.9±0.1 $_{Aa}$	7.8±0.2 $_{Aa}$
N1-225	8.6±0.1 $_{ACa}$	8.0±0.2 $_{Aa}$	7.7±0.2 $_{ABa}$	7.5±0.3 $_{Aa}$	7.4±0.1 $_{Aa}$
N1-227	8.4±0.0 $_{ACa}$	7.7±0.0 $_{Aab}$	7.4±0.2 $_{Bab}$	6.9±0.4 $_{ACb}$	6.8±0.4 $_{ABb}$
C1-056	9.0±0.7 $_{ACa}$	8.0±0.2 $_{Aab}$	7.4±1.1 $_{ABb}$	7.7±0.2 $_{Ab}$	5.9±2.5 $_{Bc}$
N3-031	8.9±0.0 $_{ACa}$	8.3±0.1 $_{Aab}$	8.0±0.3 $_{ABab}$	7.7±0.1 $_{Ab}$	7.6±0.0 $_{ABb}$
J1-101	8.8±0.1 $_{ACa}$	7.9±0.1 $_{Aab}$	7.3±0.3 $_{Bb}$	6.9±0.1 $_{ACb}$	6.9±0.1 $_{ABb}$
10403S	8.0±0.5 $_{BCa}$	7.5±0.3 $_{Aa}$	6.3±0.4 $_{BCb}$	5.7±0.5 $_{BCbc}$	5.0±0.7 $_{Cc}$
A1-254	7.4±0.7 $_{Ba}$	7.4±0.2 $_{Aa}$	5.7±1.2 $_{Cb}$	4.8±1.7 $_{Bbc}$	4.1±2.2 $_{Cc}$
J1-158	8.9±0.1 $_{ACa}$	8.2±0.2 $_{Aab}$	7.9±0.3 $_{ABab}$	7.5±0.3 $_{Ab}$	7.3±0.4 $_{Ab}$
J1-168	8.9±0.1 $_{ACa}$	8.4±0.1 $_{Aab}$	8.3±0.6 $_{ABab}$	7.8±0.1 $_{Ab}$	7.4±0.0 $_{ABb}$

IC: intestinal compartment

ABC…: means within a column; and, abc…: means within a row lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 25 (Figure 5.1). Mean populations (log CFU/g ± standard deviation) of *Listeria monocytogenes* (PALCAM agar) in the GC of a dynamic gastrointestinal model after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

Product	Exposure time (min)	Day of storage						
		1	6	14	27	42	57	82
Bologna	0	5.0±0.1 $_{A}$	5.6±0.7 $_{A}$	6.6±0.5 $_{A}$	7.9±0.5 $_{A}$	8.6±0.2 $_{A}$	8.9±0.4 $_{A}$	8.7±0.1 $_{A}$
	30	4.6±0.2 $_{AD}$	5.2±0.6 $_{AB}$	5.6±0.3 $_{B}$	7.2±0.2 $_{B}$	8.0±0.1 $_{B}$	8.2±0.1 $_{B}$	8.2±0.1 $_{A}$
	60	4.6±0.5 $_{AD}$	5.0±0.5 $_{B}$	5.1±0.1 $_{B}$	7.1±0.3 $_{B}$	7.8±0.3 $_{B}$	8.0±0.1 $_{B}$	8.1±0.2 $_{A}$
	90	2.4±0.9 $_{B}$	3.3±1.0 $_{C}$	3.0±0.5 $_{CE}$	4.1±0.9 $_{C}$	6.2±0.7 $_{C}$	7.1±1.0 $_{C}$	5.7±0.6 $_{B}$
	120	<0.1 $_{C}$	<0.5 $_{D}$	0.7±0.6 $_{D}$	<0.3 $_{D}$	3.4±0.7 $_{D}$	2.5±0.4 $_{D}$	2.8±0.7 $_{C}$
Salami	0	4.4±0.4 $_{AD}$	3.8±0.4 $_{C}$	3.1±0.6 $_{C}$	2.4±1.0 $_{E}$	2.0±0.3 $_{E}$	1.7±0.7 $_{E}$	1.4±0.4 $_{D}$
	30	4.3±0.3 $_{AD}$	3.5±0.1 $_{C}$	2.5±0.3 $_{EF}$	2.0±1.4 $_{E}$	1.3±0.4 $_{F}$	1.1±0.5 $_{F}$	0.5±0.3 $_{E}$
	60	4.2±0.3 $_{D}$	2.5±0.7 $_{E}$	2.0±0.6 $_{FG}$	1.4±1.0 $_{F}$	1.0±0.5 $_{FG}$	0.8±0.6 $_{F}$	0.3±0.2 $_{E}$
	90	2.8±0.4 $_{B}$	1.7±0.6 $_{F}$	1.5±0.2 $_{GH}$	0.5±0.5 $_{D}$	0.5±0.3 $_{GH}$	<0.3 $_{F}$	<0.1 $_{E}$
	120	1.4±0.6 $_{E}$	0.9±0.5 $_{D}$	1.1±0.2 $_{H}$	<0.3 $_{D}$	<0.3 $_{H}$	<0.2 $_{F}$	<0.2 $_{E}$

GC: gastric compartment; Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 26 (Figure 5.2). Total microbial populations (log CFU/g ± standard deviation; tryptic soy agar supplemented with 0.6% yeast extract) in the GC of a dynamic gastrointestinal model after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

Product	Exposure time (min)	Day of storage						
		1	6	14	27	42	57	82
Bologna	0	5.4±0.9 $_{A}$	5.6±0.7 $_{AE}$	6.5±0.7 $_{A}$	8.0±0.4 $_{A}$	8.5±0.3 $_{A}$	8.8±0.5 $_{A}$	8.6±0.1 $_{A}$
	30	5.0±0.5 $_{A}$	5.2±0.6 $_{A}$	5.7±0.2 $_{B}$	7.1±0.3 $_{B}$	8.0±0.1 $_{B}$	8.2±0.1 $_{AB}$	8.2±0.1 $_{A}$
	60	4.7±0.6 $_{A}$	5.1±0.5 $_{A}$	5.3±0.1 $_{B}$	7.0±0.5 $_{B}$	7.5±0.7 $_{BC}$	8.0±0.1 $_{B}$	8.0±0.1 $_{A}$
	90	3.3±1.0 $_{B}$	3.7±0.7 $_{B}$	3.7±0.1 $_{C}$	4.9±0.5 $_{C}$	6.8±0.5 $_{C}$	6.8±0.1 $_{C}$	6.5±0.6 $_{BD}$
	120	1.1±0.8 $_{C}$	1.3±1.0 $_{C}$	1.6±0.4 $_{D}$	1.4±0.4 $_{D}$	3.3±0.8 $_{D}$	2.8±0.3 $_{D}$	3.8±0.7 $_{C}$
Salami	0	6.9±0.4 $_{D}$	7.4±1.2 $_{D}$	7.2±1.0 $_{A}$	7.0±0.6 $_{B}$	7.3±0.8 $_{BC}$	7.3±0.6 $_{B}$	6.6±1.2 $_{BD}$
	30	6.5±0.5 $_{D}$	6.7±1.1 $_{DE}$	6.9±0.9 $_{A}$	6.8±0.8 $_{BE}$	6.6±0.9 $_{C}$	7.2±1.3 $_{B}$	6.7±0.3 $_{B}$
	60	6.2±0.5 $_{D}$	6.5±1.0 $_{DE}$	6.6±1.1 $_{A}$	6.2±0.5 $_{E}$	6.8±0.6 $_{C}$	6.5±0.5 $_{C}$	6.2±0.1 $_{BD}$
	90	5.2±1.0 $_{E}$	6.1±1.1 $_{E}$	5.7±0.7 $_{B}$	3.6±0.7 $_{F}$	6.9±1.2 $_{C}$	5.0±1.4 $_{E}$	5.8±0.4 $_{D}$
	120	3.5±0.2 $_{F}$	4.1±0.1 $_{B}$	2.8±0.6 $_{D}$	0.9±0.2 $_{D}$	3.1±0.0 $_{D}$	3.4±2.1 $_{D}$	2.3±0.8 $_{E}$

GC: gastric compartment; Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 27 (Figure 5.3). Mean populations (log CFU/g ± standard deviation) of *Listeria monocytogenes* (PALCAM agar) in the IC of a dynamic gastrointestinal model after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

Product	Exposure time (min)	Day of storage						
		1	6	14	27	42	57	82
Bologna	30	$4.5\pm0.2_{A}$	$5.2\pm0.8_{A}$	$6.0\pm0.6_{A}$	$6.9\pm1.0_{A}$	$8.0\pm0.2_{A}$	$8.0\pm0.1_{A}$	$7.9\pm0.2_{AB}$
	90	$4.2\pm0.1_{AB}$	$5.3\pm0.5_{A}$	$5.8\pm0.5_{A}$	$7.3\pm0.4_{A}$	$7.7\pm0.6_{AB}$	$8.0\pm0.1_{A}$	$8.0\pm0.1_{A}$
	120	$3.6\pm0.5_{B}$	$4.7\pm0.5_{A}$	$5.5\pm0.5_{A}$	$6.7\pm0.3_{A}$	$7.3\pm0.2_{B}$	$7.3\pm0.3_{B}$	$7.6\pm0.2_{AB}$
	240	$3.7\pm0.3_{B}$	$4.9\pm0.5_{A}$	$3.0\pm2.8_{B}$	$5.9\pm1.3_{B}$	$7.3\pm0.1_{B}$	$7.3\pm0.2_{B}$	$7.3\pm0.0_{B}$
Salami	30	$3.8\pm0.3_{AB}$	$2.9\pm0.5_{B}$	$1.4\pm0.3_{C}$	$0.9\pm0.2_{C}$	$1.5\pm0.7_{C}$	$1.3\pm0.8_{C}$	$1.0\pm0.5_{C}$
	90	$3.7\pm0.2_{B}$	$2.2\pm0.6_{BC}$	$1.1\pm0.2_{C}$	$1.6\pm0.7_{D}$	$1.1\pm1.0_{C}$	$1.2\pm0.9_{C}$	$<0.5_{D}$
	120	$2.9\pm0.0_{C}$	$2.1\pm0.5_{C}$	$0.8\pm0.5_{C}$	$<0.7_{C}$	$0.3\pm0.2_{D}$	$1.0\pm0.8_{C}$	$<0.3_{D}$
	240	$2.5\pm0.5_{C}$	$1.9\pm0.3_{C}$	$<0.2_{D}$	$<0.3_{C}$	$0.6\pm0.7_{D}$	$<0.1_{D}$	$<0.1_{D}$

IC: intestinal compartment; Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 28 (Figure 5.4). Total microbial populations (log CFU/g ± standard deviation; tryptic soy agar supplemented with 0.6% yeast extract) in the IC of a dynamic gastrointestinal model after inoculation onto bologna or salami slices and storage at 4°C in vacuum packages.

Product	Exposure time (min)	Day of storage						
		1	6	14	27	42	57	82
Bologna	30	$5.5\pm0.4_{AB}$	$5.5\pm0.5_{AC}$	$6.1\pm0.5_{AC}$	$7.1\pm0.6_{A}$	$8.0\pm0.2_{A}$	$8.0\pm0.2_{A}$	$7.9\pm0.3_{A}$
	90	$6.0\pm1.1_{AB}$	$5.5\pm0.3_{AC}$	$6.0\pm0.2_{AC}$	$7.2\pm0.3_{A}$	$7.5\pm0.6_{A}$	$7.9\pm0.1_{A}$	$7.9\pm0.1_{A}$
	120	$5.2\pm0.6_{AB}$	$5.2\pm0.5_{A}$	$5.7\pm0.2_{AC}$	$6.8\pm0.2_{A}$	$7.3\pm0.4_{AB}$	$7.4\pm0.2_{AB}$	$7.6\pm0.2_{A}$
	240	$5.3\pm0.9_{AB}$	$5.5\pm0.4_{AC}$	$6.1\pm0.5_{AC}$	$7.0\pm0.2_{A}$	$7.3\pm0.3_{AB}$	$7.4\pm0.2_{AB}$	$7.3\pm0.1_{AC}$
Salami	30	$6.2\pm0.4_{A}$	$6.7\pm1.1_{B}$	$6.4\pm1.8_{A}$	$6.7\pm1.1_{A}$	$7.1\pm0.7_{AB}$	$6.5\pm0.5_{BC}$	$6.3\pm0.8_{BC}$
	90	$5.9\pm0.2_{AB}$	$6.3\pm1.1_{BC}$	$7.1\pm1.2_{B}$	$6.7\pm0.7_{A}$	$7.0\pm0.6_{AB}$	$6.5\pm0.6_{BC}$	$6.7\pm1.7_{BC}$
	120	$5.5\pm0.6_{AB}$	$6.4\pm1.2_{BC}$	$5.3\pm2.9_{C}$	$6.4\pm0.9_{A}$	$6.5\pm0.7_{B}$	$6.1\pm0.5_{C}$	$5.7\pm1.1_{D}$
	240	$5.1\pm1.5_{B}$	$6.6\pm1.1_{BC}$	$6.3\pm1.1_{A}$	$6.4\pm0.5_{A}$	$6.9\pm0.2_{AB}$	$5.7\pm0.7_{C}$	$6.1\pm0.1_{BD}$

IC: intestinal compartment; Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 29 (Figure 6.1). Mean populations (log CFU/g ± standard deviation) of *Listeria monocytogenes* (PALCAM agar) in the GC of a dynamic gastrointestinal model after inoculation onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat level	Exposure time	Day of storage				
	(min)	1	6	20	39	55
Low	0	2.8±0.4 $_{A}$	3.8±0.5 $_{A}$	6.5±1.3 $_{A}$	8.3±0.4 $_{E}$	8.0±0.7 $_{A}$
	30	2.5±0.4 $_{AB}$	3.2±0.6 $_{D}$	6.0±1.3 $_{B}$	7.2±1.0 $_{A}$	7.7±0.7 $_{AB}$
	60	2.5±0.5 $_{AB}$	3.0±0.4 $_{D}$	5.7±1.0 $_{B}$	7.0±0.8 $_{A}$	7.3±0.8 $_{AB}$
	90	0.2±0.1 $_{D}$	0.4±0.4 $_{E}$	3.9±1.1 $_{C}$	4.5±0.7 $_{F}$	4.8±0.7 $_{C}$
	120	<0.2	0.4±0.1 $_{E}$	1.2±0.7 $_{D}$	<1.1	2.8±0.8 $_{D}$
High	0	2.9±0.3 $_{A}$	3.7±0.2 $_{AD}$	6.1±1.4 $_{A}$	7.2±1.4 $_{A}$	8.0±0.5 $_{A}$
	30	2.5±0.2 $_{AB}$	3.4±0.6 $_{AD}$	5.5±1.2 $_{B}$	6.8±1.5 $_{B}$	7.7±0.4 $_{AB}$
	60	2.4±0.2 $_{B}$	3.4±0.7 $_{AD}$	5.3±1.3 $_{B}$	6.7±1.4 $_{B}$	7.4±0.5 $_{B}$
	90	1.5±0.2 $_{C}$	1.9±0.5 $_{B}$	4.2±1.7 $_{C}$	5.8±1.4 $_{C}$	5.7±1.4 $_{C}$
	120	1.3±0.2 $_{C}$	1.4±0.1 $_{C}$	1.7±0.5 $_{D}$	3.0±1.7 $_{D}$	2.8±0.8 $_{D}$

GC: gastric compartment
Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 30 (Figure 6.2). Total microbial populations (log CFU/g ± standard deviation) (TSAYE) in the GC of a dynamic gastrointestinal model after inoculation of *Listeria monocytogenes* onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat level	Exposure time	Day of storage				
	(min)	1	6	20	39	55
Low	0	2.9±0.3 $_{A}$	3.9±0.5 $_{A}$	6.6±1.3 $_{A}$	8.1±0.7 $_{A}$	8.2±0.7 $_{A}$
	30	2.5±0.3 $_{A}$	3.5±0.5 $_{AB}$	6.1±1.3 $_{A}$	7.3±0.8 $_{AB}$	7.6±0.7 $_{AB}$
	60	2.2±0.4 $_{A}$	3.7±0.8 $_{AB}$	5.6±1.1 $_{B}$	7.2±0.8 $_{AB}$	7.6±0.8 $_{AB}$
	90	1.1±0.4 $_{B}$	1.1±0.7 $_{C}$	4.1±0.9 $_{C}$	5.1±0.6 $_{C}$	5.5±0.7 $_{C}$
	120	0.9±0.5 $_{B}$	1.3±0.6 $_{C}$	2.2±0.4 $_{D}$	<2.1	3.1±1.7 $_{D}$
High	0	2.9±0.2 $_{A}$	3.9±0.6 $_{A}$	6.5±1.2 $_{A}$	7.4±1.3 $_{A}$	8.2±0.5 $_{A}$
	30	2.5±0.3 $_{A}$	3.6±0.8 $_{AB}$	6.1±1.2 $_{A}$	7.1±1.1 $_{AB}$	7.8±0.5 $_{AB}$
	60	2.5±0.4 $_{A}$	3.2±0.8 $_{B}$	5.4±1.3 $_{B}$	6.8±1.2 $_{B}$	7.5±0.4 $_{B}$
	90	1.4±0.4 $_{B}$	2.1±0.3 $_{C}$	4.0±1.2 $_{C}$	5.9±1.5 $_{C}$	6.0±0.9 $_{C}$
	120	1.6±0.3 $_{B}$	1.7±0.3 $_{C}$	2.9±1.0 $_{D}$	3.6±1.9 $_{D}$	3.5±1.3 $_{D}$

GC: gastric compartment
Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 31 (Figure 6.3). Mean populations (log CFU/g ± standard deviation) of *Listeria monocytogenes* (PALCAM agar) in the IC of a dynamic gastrointestinal model after inoculation onto frankfurters of low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat level	Exposure time	Day of storage				
	(min)	1	6	20	39	55
Low	30	2.4±0.4 $_{A}$	2.1±0.3 $_{B}$	5.2±1.0 $_{B}$	7.1±0.8 $_{B}$	7.4±0.7 $_{AC}$
	60	2.3±0.3 $_{A}$	3.2±0.6 $_{C}$	5.2±0.8 $_{B}$	7.3±1.0 $_{B}$	7.8±0.7 $_{C}$
	90	1.8±0.3 $_{B}$	2.3±0.5 $_{AB}$	5.4±1.2 $_{B}$	7.0±0.6 $_{B}$	7.1±0.6 $_{A}$
	240	1.9±0.6 $_{B}$	2.0±1.1 $_{B}$	4.4±1.6 $_{C}$	6.9±0.6 $_{B}$	7.0±1.0 $_{A}$
High	30	2.4±0.2 $_{A}$	2.4±0.7 $_{AB}$	4.9±0.9 $_{A}$	6.2±1.7 $_{A}$	7.1±1.0 $_{A}$
	60	2.3±0.1 $_{A}$	2.9±0.7 $_{AC}$	5.3±0.8 $_{B}$	6.8±1.6 $_{B}$	7.5±1.1 $_{AC}$
	90	1.8±0.3 $_{B}$	2.6±0.5 $_{AB}$	5.0±1.0 $_{B}$	6.2±1.5 $_{A}$	6.1±1.6 $_{B}$
	240	1.0±0.4 $_{C}$	2.1±0.5 $_{B}$	3.8±1.5 $_{C}$	5.9±1.7 $_{A}$	6.0±1.9 $_{B}$

IC: intestinal compartment
Means within a column lacking a common letter are significantly different ($P < 0.05$)

Appendix Table 32 (Figure 6.4). Total microbial populations (log CFU/g ± standard deviation) (TSAYE) in the IC of a dynamic gastrointestinal model after inoculation of *Listeria monocytogenes* onto frankfurters of high low (~4.5%) or high (~32.5%) fat content and storage at 7°C in vacuum packages.

Fat level	Exposure time	Day of storage				
	(min)	1	6	20	39	55
Low	30	2.6±0.4 $_{A}$	2.8±0.5 $_{AB}$	5.1±1.0 $_{A}$	7.3±0.6 $_{B}$	7.3±0.8 $_{AB}$
	60	2.1±0.6 $_{AB}$	3.5±0.7 $_{A}$	5.3±0.7 $_{A}$	7.5±0.9 $_{B}$	7.9±0.8 $_{A}$
	90	1.9±0.3 $_{B}$	3.0±0.4 $_{AB}$	5.2±1.6 $_{A}$	6.9±0.8 $_{AB}$	7.2±0.7 $_{AB}$
	240	2.0±0.4 $_{B}$	2.5±0.8 $_{B}$	4.3±1.3 $_{B}$	6.9±0.7 $_{AB}$	6.5±1.2 $_{C}$
High	30	2.5±0.2 $_{AB}$	2.9±0.7 $_{AB}$	4.9±0.9 $_{A}$	6.2±1.9 $_{A}$	7.3±0.5 $_{AB}$
	60	2.7±0.2 $_{A}$	3.2±0.5 $_{A}$	5.3±0.7 $_{A}$	6.8±1.5 $_{AB}$	7.8±0.6 $_{A}$
	90	2.0±0.2 $_{B}$	2.7±0.8 $_{AB}$	5.4±1.2 $_{A}$	6.4±1.4 $_{AB}$	7.0±0.6 $_{B}$
	240	1.6±0.4 $_{C}$	2.5±0.4 $_{B}$	4.3±1.1 $_{B}$	6.1±1.6 $_{A}$	6.0±1.5 $_{C}$

IC: gastric compartment
Means within a column lacking a common letter are significantly different ($P < 0.05$)