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Levels of Acetic and Lactic Acid in RTE Meat and Poultry Products and their Association with Occurrence of *Listeria monocytogenes* at Retail

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

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Dedication

This dissertation is dedicated to my dear husband, Dr. Mohamed Abd-Eldaim, his encouragement and inspiration fuel the fulfillment of my goals, and to my three beautiful daughters, Nada Abd-Edaim, Safa Abd-Eldaim, and Hana Abd-Eldaim. This work is also dedicated to my respected Father Maamoun Ismail and my wonderful mother Ikram Azab who have given me their love and continuous support.

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I thank and honor my dad and mom, Maamoun Attah and Ekram Azab, who had raised me up to be a better person, and who have worked to ensure that I would receive a better education and better life. I respect and appreciate them for the rest of my life for their care and love. I appreciate my sister, Enass Maamoun, for her thoughtfulness and support, and the same for the rest of my family, Amany Maamoun, and Yasser Maamoun.

Abstract

The purposes of this study were (1) to quantify the levels of acetic, and lactic acid occurring in approximately 1800 retail ready-to-eat (RTE) processed deli meat and poultry products to determine the impact of current antimicrobial lethality treatments on occurrence of *Listeria monocytogenes* (LM) at retail, (2) to determine if the intrinsic levels of lactic acid (LA) produced by lactic acid bacteria (LAB) of the processed RTE meat or poultry affect the extrinsic levels of lactic acid added in RTE meat and poultry products, and (3) to evaluate 2% LA for its effect as a post-lethality treatment on the survival of LM on RTE meat and poultry products. Samples were randomly selected and acetic and lactic acids were extracted and analyzed by ion exclusion HPLC. Amount of LA extracted from the samples did not change with increased LAB counts ($P > 0.05$) and with storage time of six weeks ($P>0.05$). Thus, the age of the processed RTE meat or poultry did not affect the levels of lactic acid present in RTE meat and poultry products in six weeks at 4 C. The effect of 2% LA as a post lethality treatment on LM count differed according to meat type and time of storage. However, greater than a 1 log CFU/g reduction was achieved with frankfurters, bologna, and ham after application of 2% LA. Mean concentrations of acetic acid and lactic acid in samples varied by product type and by different manufacturers and ranged from 0.51 to 5.7 mg/g (0.051 – 0.57%), and 12.88 to 23.03 mg/g (1.28% -2.3%). Concentrations of acetic and lactic acids varied among manufacturers ($p<0.0001$) and within products produced by the same manufacturer. Higher levels of AA and LA in RTE meat and poultry products were associated ($p<0.01$) with lower

occurrence of LM. Thus, addition of acetates and lactates as antimicrobials is helpful in formulations as a part of an overall *listeria* control program for processed meat and poultry products; however, even high levels of LA and AA may not prevent contamination of RTE meat and poultry with LM, particularly with post-process contamination.

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I. INTRODUCTION

Listeria monocytogenes

Over the last 20 years, concern over the presence of *Listeria monocytogenes* (LM) on processed foods has greatly increased due to its ability to grow at refrigeration temperatures, its ubiquitous nature, tolerances, severity of disease especially in pregnant and imunocompramised people. This pathogen poses health risks to susceptible consumers through contaminated products such as soft cheeses, deli meats, and other RTE meat and poultry products (Pinner *et al.*, 1992; Wilson, 1995).

Listeria monocytogenes is a small, Gram-positive rod measuring 1-2 µm by 0.5 µm that has been isolated from soil, water, sewage, and the environment. The bacterium is ubiquitous (Mandel *et al.*, 1999). LM resists the deleterious effects of, freezing, drying, and heat (D71.7°C=1 sec) remarkably well for a bacterium that does not form spores (FDA-CFSAN 2007). In general, LM species are able to grow over a pH range from 4.1 to 9.6, but optimum growth occurs from pH 6 to 8 (Jay *et al.*, 2005). Growth is possible at temperatures from 1° C to 45° C. Freezing at -18°C and even repeated freezing/thawing have little effect on survival of LM (Rocourt and Cossart 1997). LM is salt tolerant and can grow in sodium chloride concentrations of up to 6% (Jay *et al.*, 2005). *Listeria* hydrolyzes esculin to 6, 7-dihydroxycomarin, which reacts with iron to form a black pigment. This reaction provides the differential basis of PALCAM, which is used to enumerate *Listeria* spp.

Because LM is ubiquitous, it can be introduced into the food supply in many ways. Crops become contaminated through the use of contaminated irrigation water and from the soil. Animals are infected from silage (Ivanek *et al.*, 2006). Meat is contaminated from feces as domestic farm animals can asymptomatically shed LM in their feces for many months. The bacteria can also be introduced into food from the processing facility itself. Shoes, clothing, transportation equipment, and human carriers are all possible sources (Rocourt & Bille, 1997). In processing plants, LM can be found in drains, conveyer belts, coolers, walls, cleaning tools, and in almost any cool, damp environment (Rocourt & Bille, 1997). At the retail and food service level, Potential sources of the organism in these operations include the environment, food handlers, and incoming raw ingredients (Lianou & Sofos, 2007a). LM was found in both prepackaged and in-store packaged ready to eat luncheon meats with higher prevalence in store-packaged samples (Chen *et al.*, 2003).

Eventually, the bacteria spread from the environment to the processed food and ultimately to the consumer (Tompkin, 2002). Contamination of ready-to-eat products with LM may occur at several stages before consumption. Good manufacturing practices, appropriate cleaning, sanitation and hygiene programs, and temperature control are required for prevention or inhibition of growth of the of LM in the retail and food service sector (Goulet *et al.*, 2001).

Listeriosis

Listeriosis is a life-threatening, primarily foodborne illness caused by *Listeria monocytogenes.* Listeriosis is the name of the general group of disorders caused by LM. The manifestations of listeriosis include septicemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. The onset of the disorders is usually preceded by influenza-like symptoms including persistent fever. It is reported that gastrointestinal symptoms such as nausea, vomiting, and diarrhea may proceed more serious forms of listeriosis or may be the only symptoms expressed (FDA-CFSAN 2007). Listeriosis may appear mild in healthy adults and more severe in neonates, the elderly, and the immunocompromised. Epidemiologic surveillance data show that the case-fatality rate varies by age, with a higher case-fatality rate among newborns and the elderly (Mead *et al.*, 1999b).

According to the Centers for Disease Control and Prevention (CDC), the rate of listeriosis has fallen by 35 percent from 1996-2002. Still, each year, LM causes an estimated 2,493 cases of listeriosis and 499 deaths (CDC, 2008). The case-fatality rate is high across the whole population – 20 deaths per 100 cases of illness. Preliminary FoodNet data on the incidence of foodborne illnesses for the United States in 2001 indicated that the incidence of infection from LM decreased between 1996 and 2001 from 0.5 to 0.3 cases per 100,000 people per year (CDC, 2008). Although significant declines in the incidence have occurred since 1996, these declines all occurred before 2004.The level then reached a plateau

(FDA *et al.*, 2003). In 2007, the number of laboratory-confirmed cases of listeriosis infection in FoodNet surveillance areas, and incidence per 100,000 populations were decreased (122; 0.27, respectively). Comparing 2007 with 2004–2006, the estimated incidence of infections caused by *Listeria* decline only slightly. The incidence of listerial infections in 2007 (0.27 cases per 100,000) was 00,000) was close to the national target for 2010 (0.25) (CDC, 2008)(figure I.1).

Listeria in ready-to-eat meat and poultry products

LM has two unique characteristics that influence its transmission to humans through ready-to-eat foods. First, it is a colonizer that favors moist, cool environments, such as food processing plants; to produce resistant biofilms thus, eradication is difficult (Gravani, 1999). Second, although it is easily killed by cooking, LM multiplies at refrigeration temperatures, whereas most other competing microflora do not (Lou & Yousef, 1999).

Ready-to-eat meat and poultry products provide a particularly favorable environment for growth of LM (Glass & Doyle, 1989). These products are usually fully cooked during manufacture and are usually consumed without further heating or after just warming. They present high risks to the consumer if these RTE products are contaminated with LM. If the pathogen is already present in product ingredients, a processing error, such as incorrect formulation (lower concentrations of antimicrobials) or inadequate processing time or temperature, can result in the production of products containing live organisms (USDA/FSIS, 2007). A product that has undergone a successful lethality treatment can be

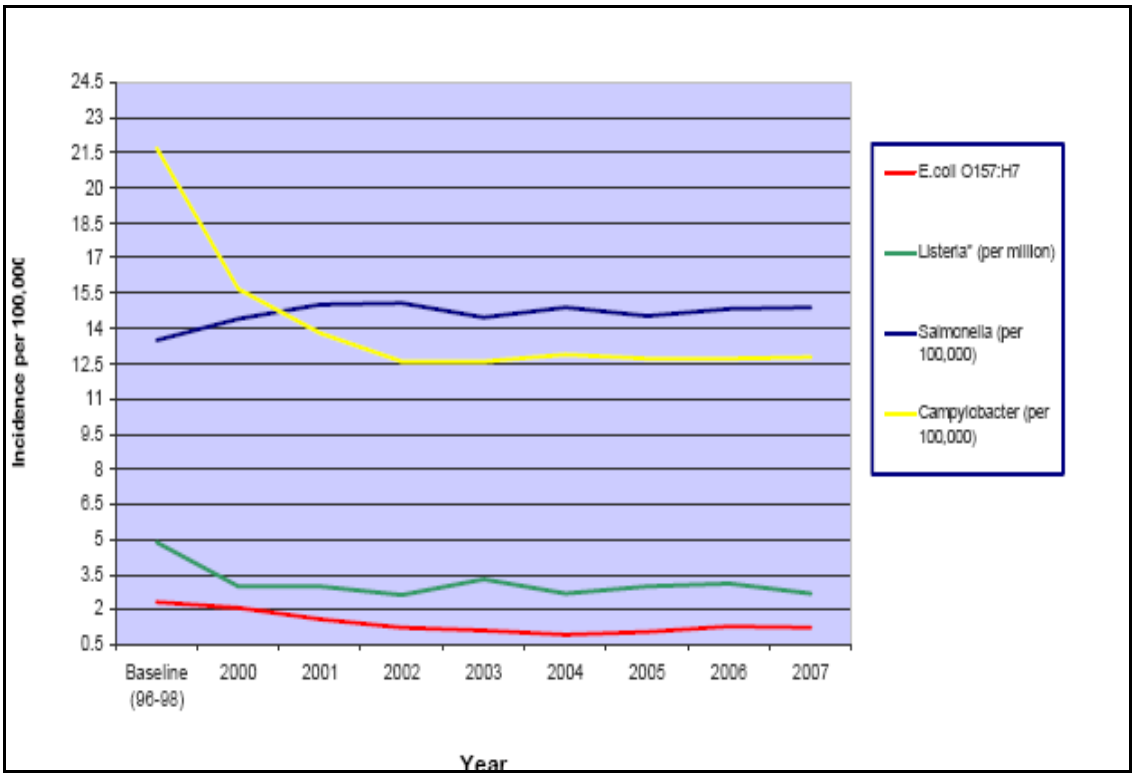


Figure I.1 Incidence of foodborne illness for 4 pathogens, 1996-89 to 2007(CDC, 2008)

contaminated by biofilms on food-contact surfaces of equipment used for processing, handling, or packaging the product (Gibbons *et al.*, 2006; USDA/FSIS, 2007).

A small amount of LM contamination at a processing plant that occurs in post-lethality environment, after cooking but before packaging, may lead to a large infectious dose being delivered to a susceptible consumer, because of multiplication of bacteria during storage (Glass & Doyle, 1989).

Outbreaks of listeriosis related to RTE meat and poultry products have been reported in North America, Europe and Japan (Swaminathan & Gerner-Smidt, 2007). A listeriosis outbreak in France in 1992, involved 279 cases and pork RTE deli products was implicated. Deli products were contaminated secondarily during handling in food stores which helped the spread of the outbreak (Jacquet *et al.*, 1995). Another outbreak in France involving 38 persons was related to RTE pork product (Goulet *et al.*, 1998). .In Japan, epidemiological data were collected from 1996 to 2002. It was estimated that there is an average of 83 cases of listeriosis per year and an incidence of 0.65 cases per million of the population in Japan (Okutani *et al.*, 2004).

RTE meats have been the focus of several risk assessments and have been specifically targeted for *Listeria* control by food regulatory agencies, and food processors in the United States. Despite a United States Department of Agriculture (USDA) policy of zero tolerance (0 CFU/25g sample), LM has been isolated from retail turkey, chicken, pork, and beef frankfurters. *Listeria* species (5%) were isolated from 14 products out of 8000 ready to eat meat and poultry

products (Wilson, 1995). Found in 20% (22/110) of vacuumed sealed RTE products from the retail market, originating from different producers. (Johansson *et al.*, 1999). Turkey deli meat was the source of a large multi-state (9 states) outbreak of listeriosis in 2000 (Gottlieb *et al.*, 2006), and meat frankfurters was implicated in an outbreak involving residents of 24 US states (Mead *et al.*, 2006). In 2001, FSIS conducted microbiological testing programs for ready-to-eat (RTE) meat and poultry products produced at approximately 1,800 federally inspected establishments. All samples were collected at production facilities and not at retail. The cumulative 10-year (1990-1999) LM prevalence was as follows: jerky, 0.52%; cooked, uncured poultry products, 2.12%; large-diameter cooked sausages, 1.31%; small-diameter cooked sausages, 3.56%; cooked beef, roast beef, and cooked corned beef, and sliced ham and luncheon meat, 5.16% (Levine *et al.*, 2001). In a Belgian market, a variety of 252 ready-to-eat food products were analyzed. Overall, LM was detected in 23.4% of the samples. The highest prevalence of LM was found in prepared minced meat (42.1%) (Van Coillie *et al.*, 2004).

Regulatory background of LM in RTE meat and poultry products

The U.S. government required the absence of LM in any RTE meat and poultry product in late 1980's. USDA-FSIS and FDA enforce a zero tolerance policy for LM in RTE foods. Zero tolerance means the absence of the organism in a 25 g samples, thus, any RTE meats that contain this organism are considered adulterated and subjected to recall (Jay *et al.*, 2005).

FSIS's Hazard Analysis and Critical Control Point (HACCP) final rule was released in July 1996 to enhance the safety of meat and poultry. Under HACCP, all meat and poultry slaughtering and processing plants must examine their operations and identify hazards (physical, biological, or chemical) and the specific points that pose the greatest food safety risks. In1998, an especially virulent strain of LM emerged and associated with a major LM outbreak in hotdogs and deli meats, in response FSIS advised manufacturers of RTE meat and poultry products of the need to reassess their HACCP plans to ensure the plans were adequately addressing LM (USDA/FSIS, 2007). Both the plants and FSIS are responsible for verifying the effectiveness of HACCP.

Recent risk assessment models have estimated that RTE deli meats and non-reheated hot dogs have the highest risk of listeriosis per serving due to contamination through post lethality processes (FDA *et al.*, 2003). That led to *Listeria* Interim final rule in 2003.It included three alternatives to address post-lethality contamination in RTEmeat and poultry products only exposed to processing environment after lethality procedures. Establishments must use one of three alternative controls for LM in the post-lethality environment: Alternative 1: Use of post-lethality treatment AND antimicrobial agent/process. Alternative 2: Use of post-lethality treatment OR antimicrobial agent/process. Alternative 3: Use of sanitation procedures (FSIS, 2003).

FDA, FSIS, and CDC efforts to reduce foodborne listeriosis were reaffirmed as a national public health goal in the Healthy People 2010 by the Department of Health and Human Services (HHS).The Healthy People 2010 objective for

listeriosis was to achieve a 50% reduction in listeriosis incidence, from 5 cases per million population in 1997 to 2.5 cases per million population in 2010 (HHS, 2000). In response to another highly publicized listeriosis outbreak in 2000 was caused by RTE turkey deli meat (Gottlieb *et al.*, 2006; Goulet *et al.*, 2001), the government pledged to achieve this goal by 2005. In 2007, still the incidence of listerial infections (2.7 cases per million) , but was close to the national target to the national target (CDC, 2008).

Current programs can provide effective control of LM in meat processing environments. However, competent delivery of food safety education and training to retail and food service managers and food handlers at retail must be in place for successful implementation of such a system (Lianou & Sofos, 2007a). Further decreases in listeriosis incidence will require continued efforts of industry and government to reduce contamination of food. Prevention of persistent LM contamination in food processing plants still presents a critical challenge to food safety professionals (Olsen *et al.*, 2005).

Regulatory-Approved Food Antimicrobials Used in Meat Products against LM

Meat processors rely on many different methods to eliminate or reduce contamination by LM and add a margin of safety for the consumers. For RTE meat products, the most frequently applied hurdles include thermal processing, vacuum packaging, refrigerated storage, and nitrite. However, because LM is ubiquitous (Beresford *et al.*, 2001), has an ability to grow at refrigerated

temperatures under anaerobic condition and is resistant to salt and nitrite (Lou & Yousef, 1999), thus, other hurdles are often necessary. Formulating meat products with antimicrobial additives is a common practice to control the growth of LM after processing (Lou & Yousef, 1999; Glass & Doyle, 1989; Mbandi & Shelef, 2001).

Some antimicrobials are approved by U.S. regulatory agencies to be added directly to foods to retard growth or kill microorganisms. Food antimicrobials do not preserve food indefinitely as most of them are bacteriostatic or fungistatic at permitted use concentrations. Therefore, antimicrobials are often used in combination with other preservation procedures. Food preservation by antimicrobials is best achieved when the microorganism to be inhibited are low in number. Antimicrobial type and concentration, storage time and temperature, and food pH and buffering capacity must be taken into consideration. These factors could be classified as microbial (resistance, initial number, growth rate, interaction with other microorganisms, and gram reaction), intrinsic (food nutrients, pH, oxidation reduction potential, and water activity), extrinsic (temperature and time of storage, atmosphere, and relative humidity), and processing (heat, high pressure, and low pH inhibition processes) (Davidson & Taylor, 2007).

Regulatory approved antimicrobials in the US are classified as traditional and naturally occurring (Davidson & Taylor, 2007). Traditional antimicrobials include organic acids, phenolics, and inorganic acids. Organic acids such as lactic acid, acetic acid, citric acid, sorbic acid, benzoic acid, propionic acid, and their salts

have all been shown to be effective at various concentrations, combination, and storage temperature against LM in processed meat (Glass *et al.*, 2007; Barmpalia *et al.*, 2004b; Blom *et al.*, 1997; Islam *et al.*, 2002). Sodium lactate and sodium diacetate are used as an antimicrobial barrier against LM in RTE meat formulations.

Organic acids and their salts

Organic acids are approved and listed in FDA regulations for a variety of technical purposes in addition to preservation, such as acidulants, antioxidants, flavoring agent, pH adjusters, and even nutrients (9 CFR 424.21).In such applications they are considered to be ingredients of the product.

Acetic acid

Acetic acid (AA) and its sodium, potassium, and calcium salts are some of the oldest food antimicrobials. Acetic acid is produced naturally by the bacterium *Acetobacter* which derives its energy from the oxidation of ethanol to acetic acid during respiration. *Acetobacter* is also used in the production of vinegar (Theron & Lues, 2007). Acetic acid (pK_a: 4.75) is the primary component of vinegar, and as such is primarily used for its flavoring abilities. Acetic acid is generally regarded as safe (GRAS) for general-purpose usage (21 CFR 184.1005). Sodium diacetate (SDA) is approved for use in processed meat and poultry products by the USDA (9 CFR 424.21) not to exceed 0.25% of the product formulation (Figure I.1).

Bacteria inhibited by acetic acid include *Bacillus* spp., *Campylobacter jejuni*, *Clostridium* spp., *E- coli*, LM, *Salmonella*, and *Staphylococcus aureus* Only *Acetobacter* species (microorganisms involved in vinegar production), lactic acid bacteria, and butyric bacteria are tolerant to acetic acid (Davidson & Taylor, 2007).

Acetic acid or its salts is most often used in combination with sodium or potassium lactate to inhibit LM in meat and poultry products. Sodium diacetate is effective at 0.2% in decreasing the growth rate of LM, and has been shown to cause a greater than a 1 log CFU/g decline in LM in meat during storage for 25 days at 10°C (Mbandi & Shelef, 2001). Samelis *et al.* (2001b) evaluated aqueous dipping solutions of organic acids (2.5 or 5% acetic acid) or its salts (2.5% sodium acetate or 5% sodium diacetate) to control LM on sliced, vacuum-packaged bologna stored at 4°C for up to 120 days. There was no significant ($P > 0.05$) increase in LM population on bologna slices treated with 2.5 or 5% acetic acid, 5% sodium diacetate from day 0 to 120. Post-process control of LM by antimicrobial treatments of acetic acid was successful in increasing the safety of post-process antimicrobial treatments on commercially manufactured frankfurters formulated with and without a 1.5% potassium lactate-0.05% sodium diacetate combination (Geornaras *et al.*, 2006a). Inoculated frankfurters were dipped in acetic acid (AA; 2.5%), lactic acid (LA; 2.5%), potassium benzoate (PB; 5%). Initial LM populations were reduced by 1.0 to 1.8 logs CFU/cm^2 following treatment with AA, LA, or PB solutions. The dipping of products formulated with

potassium lactate-sodium diacetate in AA or LA alone increased lag-phase duration of the pathogen.

Lactic acid

Lactic acid (pK_a = 3.79) is produced naturally during fermentation of food by lactic acid bacteria. Lactic acid (LA) and lactate salts act as antimicrobials, pH control agents, and flavorings in food products (Davidson & Taylor, 2007). Lactic acid is used in the manufacture of jams, jellies, and beverages, adjusting the acidity in brines for pickles, as a firming agent for apple slices, and to prevent discoloration in fruit (Doores, 1993). Lactic acid is approved as a GRAS substance for general purpose usage (21 CFR 184.1061). Potassium (21 CFR 184.1639), sodium (21 CFR 184.1768), and calcium lactates (21 CFR 184.1207) are also approved as GRAS compounds. Sodium and potassium lactate are approved for use as antimicrobial agent in processed meat and poultry products by the USDA (9 CFR 424.21) not to exceed 4.8 % of the product formulation (Figure I.1).

In the meat industry, lactic acid has been shown to be efficacious as a sanitizer on meat and poultry carcasses to reduce or eliminate pathogens (Castillo *et al.*, 1999; Russell, 1998)) . A 2% lactic acid spray at 55°C was effective in reducing aerobic plate counts (APC) and counts of *Enterobacteriaceae*, total coliforms, thermotolerant coliforms, and *Escherichia coli* on beef carcass surfaces (Castillo *et al.*, 1999). At levels of 5% or above, LA eliminated or inhibited all spoilage bacteria on fresh poultry broiler carcasses (Russell, 1998).

Sodium lactate (SL) (2.5 to 5.0%) inhibits LM in various meat products (Gonzalez-Fandos & Dominguez, 2006a; Houtsma *et al.*, 1993a).Sodium or potassium lactate (4%) is listeriostatic that incease the lag phase but did not kill bacteria at refrigeration temperature (Chen & Shelef, 1992). Sodium, potassium, and calcium lactates were equally effective in inhibiting growth of LM in cooked strained beef stored at 20°C (Chen & Shelef, 1992).

Mixtures of sodium or calcium lactate and sodium diacetate have been demonstrated to be effective in inhibiting growth and causing reduction in LM in various meat products. Enhanced inhibition of LM was achieved by combinations of sodium lactate (2.5%) and sodium diacetate (0.2%) at 5°C and 10°C in beef bologna for up to 60 days (Mbandi & Shelef, 2002b). Similarly, a mixture of sodium lactate (2.5%) and sodium acetate (0.25%) inhibited the growth of LM in sliced cooked ham and sausage product at 4°C for 5 weeks (Blom *et al.*, 1997). The antilisterial activity of sodium lactate and sodium diacetate was evaluated by Barmpalia *et al.* (2004b) in a frankfurter formulation and in combination with a dipping treatment (solutions of lactic acid or acetic acid) after processing and inoculation. The combination of 1.8% SL with 0.25% SDA provided complete inhibition of LM growth throughout storage at 10° C for 40 days.

Synergistic combination of lactic acid and/or acetic acid with other antimicrobials was proven effective against LM. A combination of lactate (4%) and nisin (400 IU/ml) was listericidal at pH 5.5 and 4°C (Buncic *et al.*, 1995). When no nitrite was included in the formulation, and 0.2% propionate used alone, a combination of 0.1% propionate with 0.1% sorbate, or a combination of 3.2% lactate with

0.2% diacetate was required to prevent listerial growth on the product stored at 4 ° C for 12 weeks (Glass *et al.*, 2007).

Mechanisms of Action of Organic acids

The antimicrobial effectiveness of organic acids is related to pH, and the undissociated form of the acid. Therefore, in selecting an organic acid for use as an antimicrobial food additive, both the product pH and the acid pK_a must be taken into account. LM optimally grows at neutral or slightly alkaline pH, but can grow at much lower pH (Lou and Yousef 1999). Glass and Doyle (1989) observed that LM grew well on meats with a pH above 6.0, but did not grow well on meats below pH 5.0.

Organic acids affect bacteria by interfering with the permeability of the cell membrane, which causes a disruption in the electron transport system. This leads to acidification of the inside of the cell and inhibition or death of bacteria (Ahamad and Marth 1989).

The undissociated form of the organic acid can penetrate the cell membrane lipid bilayer. Once inside the cell, the acid dissociates because of the cell interior has a higher pH than the exterior. Proton generated intracellularly acidifies the cytoplasm, inhibiting many metabolic processes. In response, Bacteria extrude protons to the exterior of the cell to maintain neutral interior pH. According to the chemiosmotic theory, the cytoplasmic membrane is impermeable to protons and they must be transported to the exterior. They can only pass through a specific proton channel, which is ATPase enzyme mediated. This proton extrusion

creates an electrochemical potential across the membrane called the proton motive force. ATPase pumps protons out of the cell utilizing energy in the form of ATP. The resultant energy depletion is a major factor in the inhibition caused by organic acids. In summary, inhibition and/or inactivation of bacterial cell by organic acids may be due to loss of cellular energy or inactivation of critical cellular functions due to low intracellular pH (Davidson & Taylor, 2007)(figure I.2). Eventually, the intracellular pH is raised to a point that the cell may resume growth. The time it takes to accomplish that depends on the extra cellular pH and inhibitor concentration and is termed lag time.

Accumulation of inhibitory concentrations of anions in the cytoplasm in the cell may also affect cellular functions. High concentrations of anions could lead to an increased osmolarity and to interference with metabolic process. One problem with extruding anions and protons is the potential for recombination in the extra cellular and reentry into the cell. To prevent this exhausting cycle, adapted cells may react by altering cell membrane structure.

Adaptation

Bacteria may be innately resistant to certain food antimicrobials either by preventing entry of the antimicrobial through cellular barriers, or by pumping compounds out of the cell through cellular efflux. Considering the long time that some antimicrobials (benzoic, sorbic) have been applied to food products, some microorganisms have innate resistance to these antimicrobials as they can metabolize these compounds (Chipley, 1993) .On the other hand, sensitive

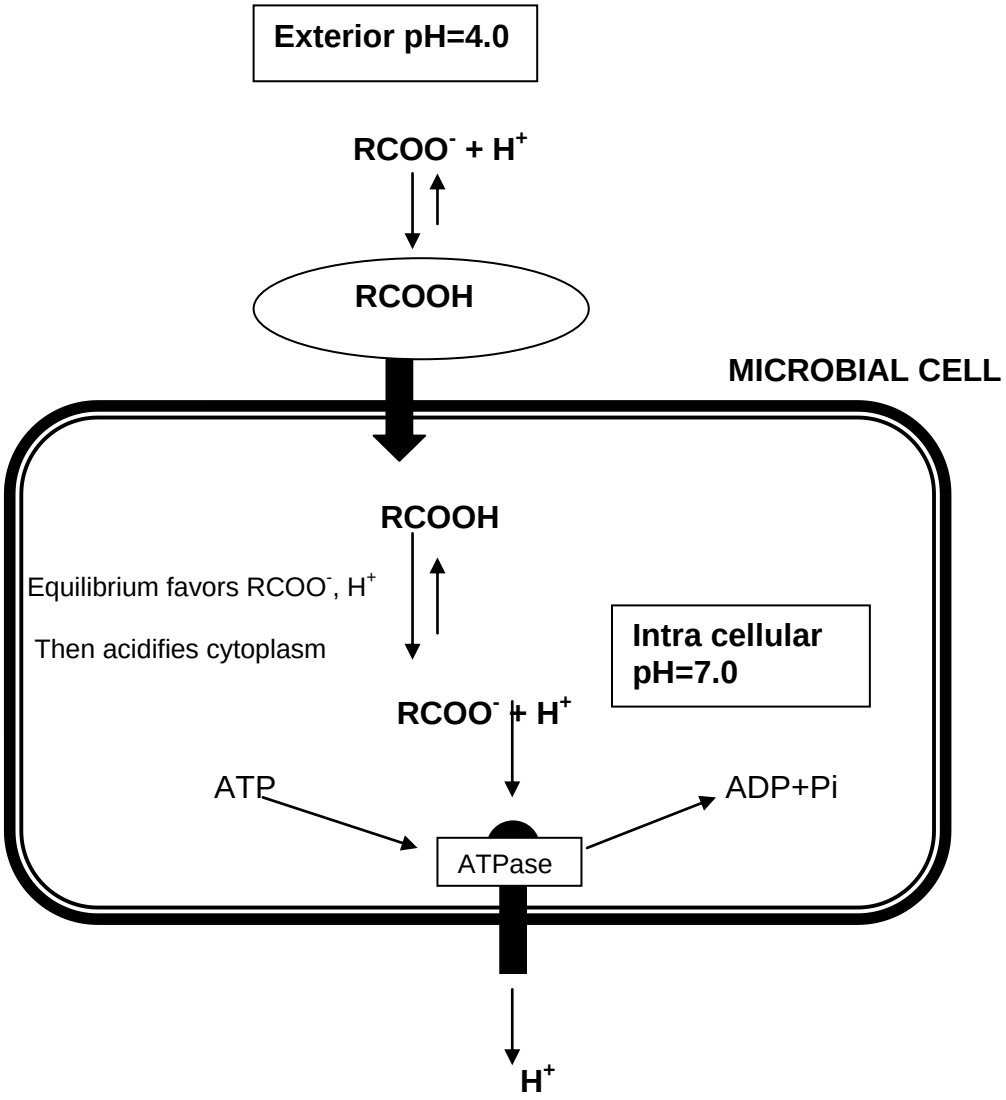


Figure I.2 Fate of an organic acid (RCOOH) in a low pH environment in the presence of a microbial cell (Davidson & Taylor, 2007)

microorganisms may not mutate or acquire resistance because antimicrobials are generally non-specific (have no specific target sites in microbial cell).

However, exposure of sensitive organisms to sub-inhibitory antimicrobial levels may cause a temporary adaptation, so subsequent exposure to lethal levels is less effective (McEntire & Montville, 2007). There is no standard definition, or threshold, to characterize a microbe as resistant to a specific food antimicrobial. In many cases, resistance is manifested as a temporary adaptation that is not displayed by subsequent generations. Bacterial adaptation is the term used to describe temporary phenotypic changes in response to stress. New genetic material is not required for bacteria to adapt, as stress factor activates certain existing pathways mechanisms to produce a physiological response that helps the microbe withstand the stress (McEntire & Montville, 2007).

When a microorganism is adapted to a stress, it may also resist a similar or different stress that was previously lethal or injurious to the cell. For example, LM became more acid resistant and possibly more resistant to other stresses (heat, osmotic pressure) if subjected to relatively mild acidity or multiple sublethal stresses before exposure to more acidic conditions (Skandamis *et al.*, 2008). LM was also shown to exhibit a rapid and significant adaptive acid tolerance response following a 1-h exposure to mild acid (pH 5.5), which was capable of protecting cells from severe acid stress (pH 3.5) exposure (ODriscoll *et al.*, 1996). Some mechanisms of adaptation are known, such as stress proteins. The synthesis of stress response proteins is triggered by low levels of stress (heat, cold, acid, osmotic stress). These proteins protect the cell from subsequent

related or unrelated stresses. Common genetic regulatory factors called sigma factors (σ) produced in response to stress, bind to microbial RNA polymerase, and leading to the production of stress proteins which protect the cell from the stress (Davidson & Harrison, 2002).

One of the changes in response to stress is a major alteration of the fatty acid composition of lipids in the bacterial membrane. To increase fluidity in response to cold temperatures, bacteria increase unsaturation or decrease the chain length (Russell *et al.*, 1995).

Analytical Methods for Determination of Organic Acids

Organic acids play an important role in maintains the quality, flavor, and nutritional value of a variety of foods. Because of their importance, they are considered one of the most commonly analyzed components of food systems. Many methods have been used to determine organic acids in foods, including volumetric, electrochemical, enzymatic, and chromatographic (paper, thin-layer, gas-liquid, or high-performance liquid chromatography (HPLC)) methods. Of the methods listed, HPLC has long been used as the industry standard for the analysis of organic acids in a food sample and requires the least sample pretreatment (Friedrich *et al*. 2001). HPLC found many applications allowing fast, sensitive, and highly specific analysis of organic acids in food and entailing relatively uncomplicated sample treatment (Gomis, 2000; Nassos *et al.*, 1984). For example, one of the advantages of HPLC over gas chromatography is that

derivatization is not required and non-volatile inorganic matter does not have to be removed (Nassos *et al.*, 1984).

There were no much available information for organic acid extraction and analysis from meat, but according to (Gomis, 2000) organic acids could generally be extracted from solid and semi-solid samples by HPLC. Because of high water solubility of organic acids, they could be extracted from samples by cutting up and grinding an adequate portion, followed by blending in water, and acidified water.

In most methods applied in organic acid extraction from dairy products such as cheeses, an ion exchange or ion exclusion column was used (Lues *et al.*, 1998; Bouzas *et al.*, 1991), while the use of Reverse phase (RP)-HPLC has also been reported by others as well suited method for the quantitative analysis of a broad spectrum of organic acids (Dinkci *et al.*, 2007; Tormo & Izco, 2004). According to (Gomis, 2000), RP-chromatography with C_{18} bonded phase column is used more often for the separation of organic acids because of the existing disadvantages of very expensive ion exchange columns. However, ion exchange HPLC has become more prominent among current analytical methods for organic acids. Lues *et al* compared reverse-phase to ion-exclusion HPLC and concluded in favor of the latter. The ion-exchange method yielded best results for the concentration of compounds analyzed, resolution, ease of analysis, and short duration of separation compared to a longer run time by RP-HPLC, and resolution was not as good as with the ion-exchange method (Lues *et al.*, 1998).

Research Objectives

This research focused on the use of sodium lactate and sodium diacetate (acetate and lactate) as antimicrobial food preservatives against LM in processed meats. The specific objectives of the research were to:

1. Quantify lactate and diacetate in RTE processed deli meat and poultry products that were analyzed in an earlier study for the presence of LM to determine the association with the presence of the compounds and presence or absence of LM.
2. Determine if there was a relationship between lactic acid bacteria and the presence of lactic acid in RTE meat and poultry products throughout the shelf-life of the product.
3. Evaluate the effectiveness of the use of a 2% lactic acid spray as a post-processing lethality treatment for LM on RTE meat and poultry product.

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II. ACETATE AND LACTATE CONCENTRATIONS IN READY-TO-EAT MEAT AND POULTRY PRODUCTS AND ASSOCIATION WITH DETECTION OF *LISTERIA MONOCYTOGENES*

Abstract

Listeria monocytogenes (LM) is a psychrotrophic foodborne pathogen that has been isolated from ready-to-eat (RTE) processed deli meat and poultry products. Contaminated food products are responsible for approximately 2000 cases of listeriosis in the US each year. The purpose of this study was to quantify lactic and acetic acids occurring in a group of retail RTE processed deli meat and poultry products that had been tested in a previous study for the presence of LM. The data were used to determine the potential association of antimicrobial lethality treatments of acetic and lactic acids on occurrence of LM at retail. Pre-packaged and deli meat and poultry luncheon meats samples (~1800 samples) were randomly selected from 8000 samples collected from four FoodNet states (TN, GA, CA, and MN) that had been analyzed for the presence of LM. Products were extracted after blending 50 g from each sample with de-ionized water. Extracts were analyzed for lactic acid and acetic acid using an ion exclusion column on an HPLC system with photodiode array (PDA) detector. In general, the mean concentrations of acetic acid in samples varied with product type and with different manufacturers and ranged from 0.51 to 5.7 mg/g (0.051 – 0.57%). Lactic acid in RTE meat and poultry products ranged from 12.88 mg/g (1.28%) to 23.03 mg/g (2.3%). Concentrations of acetic and lactic acids varied among manufactures ($p<0.0001$), among products and even within the same manufacturer's product. Lactic acid detected in beef products was higher than pork, poultry, and mixed products. Concentrations of lactic and acetic acid in

samples that had been positive for *L. monocytogenes* ranged from 0.13 – 2.41 mg/ g and 0.055 to 5.75 mg/g, respectively. Effects of acetic acid and lactic acid were additive and interacted significantly ($p<0.01$) and were associated with lower occurrence of *L. monocytogenes* in RTE meat and poultry products. Based on these results, addition of acetates and lactates as antimicrobials is helpful as a part of an overall *listeria* control program for ensuring Listeria-safe RTE processed meat and poultry products; however, a rigorous sanitation and an effective HACCP program are also essential for control of listeria.

Introduction

Listeria monocytogenes (LM) is a foodborne pathogenic bacterium that causes listeriosis, a severe disease for individuals with compromised immune systems, the elderly, pregnant women, and newborns. Listeriosis is a rare disease, with an annual estimated incidence rate between 0.1 and 11.3 cases per million of population (Notermans *et al.*, 1998) but it is implicated with 28% of all confirmed infant deaths associated with foodborne illnesses in the United States annually (Mead *et al.*, 1999). The pathogen is psychrotrophic and can survive and grow in adverse conditions such as refrigeration temperature, low pH, and high salt concentrations at which other microorganisms could not grow or survive (Norrung, 2000; Rocourt et al., 2003). Ready-to-eat (RTE) foods that are commonly consumed without further cooking are of particular concern. Because of the significant public health concern, U.S. regulatory agencies established a “zero” tolerance policy of *L. monocytogenes* for ready-to eat (RTE)

foods in the 1980s (Gombas *et al.*, 2003a). Also, the “Listeria rules” issued by the United States Department of Agriculture Food Safety and Inspection Services (USDA_FSIS) encourages the use of antimicrobial agents for control of *L. monocytogenes* in RTE meat or poultry products (CFR, 2003).

Acetic acid (AA) and lactic acid (LA) are considered the most widely used antimicrobial chemical compounds in the meat industry, individually or in combination and often in the form of salts. There has been an increased interest in the anti-listerial activity of these generally recognized as safe (GRAS) organic acids in processed meat, since their commercial application is simple and cost-effective. Acetic and lactic acid could be added to products during formulation, to finished meat products by spraying or dipping (Samelis *et al.*, 2001) to packaging material (Ouattara *et al.*, 2000; Quintavalla & Vicini, 2002), or by the use of edible antimicrobial film (Cagri *et al*., 2004).

Salts of organic acids are approved for use as food ingredients and have been utilized traditionally to enhance the quality of cooked or cured meat products. They have been employed as color and flavor enhancers, and to control pH (Houtsma *et al.*, 1993). They are also a normal component of muscle tissue, and can improve palatability of products. The limit acceptability of sodium lactate would appear to be 4% since panelists noted a mild throat irritation at this concentration (Papadopoulos *et al.*, 1991).

Studies examining the effects of lactate and diacetate on LM have been conducted mainly at different application levels and conditions of use of these additives in different product types and under various conditions (Abou-Zeid *et*

al., 2007; Gonzalez-Fandos & Dominguez, 2006; Samelis *et al.*, 2001). However, data on the effectiveness of these organic acids applied in actual commercial practice are limited and the effectiveness of organic acids and their applicability in the food industry have been questioned.

The goal of our study was to provide much needed information regarding AA, and LA occurrence, distribution and levels in over 1800 RTE meat and poultry products from a large cross-section of meat manufacturers. RTE meat and poultry products have a shelflife of four or more weeks in unopened packages stored at 4 C or lower. If lactic acid bacterial levels change during storage of RTE meat at 4 C, they may be associated with increased levels of lactic acid in samples. Prior to initiating this study, a preliminary study was conducted to determine if this is a confounding factor in evaluating lactic acid levels in a large collection of samples collected at different points in their shelflife.

The objective of this study was to quantify lactate and acetate in RTE processed deli meat and poultry products that were analyzed in an earlier study for the presence of LM to determine the association of LA and AA with the presence or absence of LM. A preliminary study was conducted to evaluate changes in lactic acid bacteria and lactic acid in bologna and RTE beef, pork and poultry samples stored at 4 C for 6 weeks.

Materials and methods

Sample selection

RTE meat and poultry samples used in this study were obtained from 8000 samples that were collected, refrigerated for a maximum of 24 hrs and frozen at -70°C until analysis for lactic and acetic acid. All samples had been tested for *Listeria monocytogenes* by the USDA method (Draughon, 2006). At the time of sample collection, Draughon et al (2006) also obtained information on each sample that identified type of meat or poultry, curing, location of sample collection, manufacturing information, and sell-by date. All products were collected at least 7 days before the sell-by date and frozen before sell-by date.

All samples positive for LM (and available) were selected (39) and 1883 samples were selected randomly using a random number generator from the remaining 8000 samples. Samples represented different categories including uncured and cured poultry products, pork, and beef which were sliced at retail deli supermarkets or packaged in USDA or state inspected plants. Some samples were categorized as mixed products since they were prepared from the mixture of beef, pork and/or poultry. Samples were obtained from 4 states (California, Georgia, Minnesota, and Tennessee) representing geographic diversity in the US. All four states participate in FoodNet and PulseNet.

Sample extraction

Lactic and acetic acid contents in various RTE meat and poultry samples were analyzed according to the procedures of Nassos et al (1984) and Friedrich

(2006) with modification. The analysis of the acids consisted of sample extraction and separation of acids using high performance liquid chromatography (HPLC).

Samples (50 g) were added with 450 ml of de-ionized, distilled water and homogenized in a blender at high speed for 2 minutes. The homogenized samples were filtered with Whatman No. 113 filter paper under vacuum. An aliquot (filtrate) of 50 ml of each sample was added and mixed with 100 ml of 0.5N perchloric acid in a 200 ml flask and allowed to stand for 5 minutes at room temperature to precipitate protein. The sample was filtered once again with Whatman No. 4 filter paper under vacuum to remove the protein. The extracted samples (about 20 ml) were stored in closed vials at refrigeration temperature (4ºC) until HPLC analysis. A final filtration through 0.45 μm Millipore membrane filter was done prior to injection into the HPLC system.

Average recovery percentage of AA, and LA was 91.81% ± 5.5 and 96.64% ± 6.8, respectively. Percentage recovery was determined by adding known concentrations (1000 ppm) of AA, and LA organic acid standards to the samples and extracting using the method described above. The concentration of LA and AA added to the samples was then determined by running a separation as for the sample analysis. Non-spiked samples were analyzed also to quantify the background analyte amount. Recovery % is calculated by (100* amount of analyte recovered)/ (amount of analyte added +background analyte amount).

Preliminary Study

To determine if lactic acid levels change during storage of RTE meat and poultry at 4 C, RTE pork and bologna samples were collected from a

manufacturer. Poultry and beef were freshly sliced and collected from a retail grocery. All the samples were sliced and vacuumed packed then stored at 4°C for six weeks. Each week RTE meat and poultry samples were randomly selected and analyzed for lactic acid bacteria (LAB) and lactic acid level using HPLC.

For LAB enumeration, twenty-five gram portions of sample were aseptically removed from the package and mixed with 225 ml of sterile 0.1% Buffered Peptone-Water (BPW) (Merck, Darmstadt, Germany) in sterile stomacher bags with filter inlay. Samples were mixed in a Stomacher for 120 sec. After ten-fold serial dilution, samples were pour-plated in duplicates in deMan Rogosa Sharpe (MRS, pH 5.5) agar (Difco, Becton Dickinson, Sparks, Md.). All plates were left for 2 h at room temperature and then incubated for 72 h at 35 °C (Nassos et al., 1984).

HPLC analysis

Lactic and acetic acids were analyzed by a Dionex HPLC system (Dionex Corp, Sunnyvale, CA) equipped with a GP50 Gradient pump, an AS50 Auto-sampler, and a PDA-100 Photodiode Array detector. The organic acids were separated on an ion exclusion column, Aminex HPX-87H (300 mm x 7.8 mm i.d.) with guard column containing a cartridge of the same ion exclusion resin (Bio-Rad Laboratories, Hercules, CA). The analysis was performed using mobile phase 0.005 M H_sSO_4 with flow rate of 0.6 ml/min and UV detector set at 210 nm. A 20 μL sample was injected into the HPLC system by the automatic sampler

and the data were collected with PeakNet software (Dionex Corp, Sunnyvale, CA) on a personal computer interfaced with the HPLC system.

Lactic acid (Sigma, St. Louis, MO) and acetic acid (Across Organics, New Jersey) standard solutions were injected into the HPLC system under the same conditions as the samples were analyzed to establish standard curves. The concentrations of lactic and acetic acids in the samples were calculated based on regression line analysis of the established standard curves. Peak identity was confirmed when peak retention times were identical to those of pure standards of each LA and AA (1000 ppm) (figure II.1).

Statistical analysis

The relationship between the amount of acetic and lactic acid and their effect on LM among different states and different products was tested using Dummy regression analysis (SAS 1999). Dummy regression analysis allowed class variables, which included products (such as beef, pork, poultry, and mixed) to be used in regression analysis. Differences were considered significant when the associated p value was less than 0.05. A completely randomized design (SAS, 1999) was also used to compare acetic acid, lactic acid, and acetic + lactic acid means in RTE meat and poultry products among different manufacturers, products types, and product curing. Two replications were performed of the preliminary study examining LAB counts and LA levels in RTE meat and poultry products stored for 6 weeks.

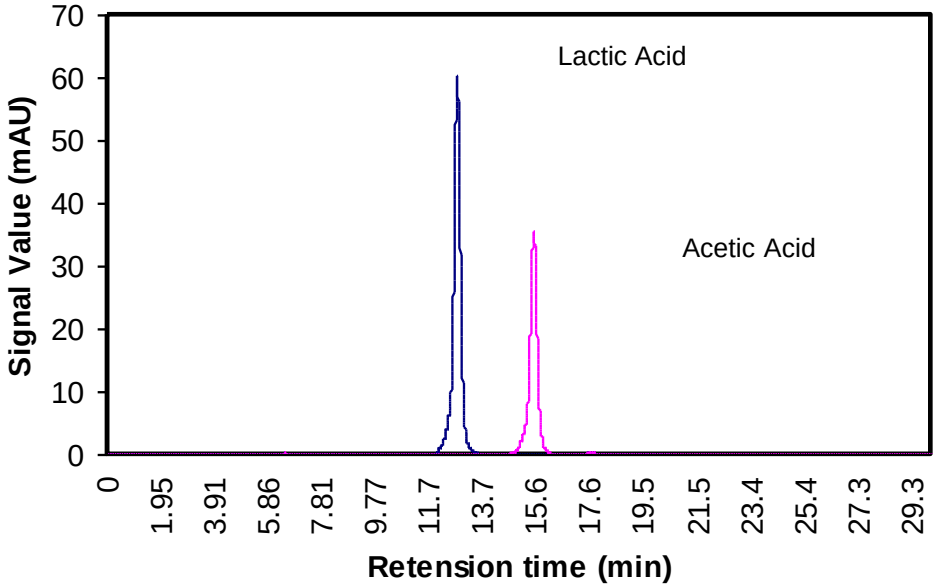


Figure II.1 HPLC chromatograph obtained during ion-exclusion analysis of Lactic acid and acetic acid standards. First peak (blue) with the retention time of 12.7 min represents lactic acid. Second peak (pink) with Retention time of 15.5 min represents acetic acid.

Results and Discussion

Acetic acid and LA are used in the processing of RTE meats and poultry to reduce microbial growth and serve as an additional lethality step in control of *Listeria monocytogenes*, it is important that levels are consistently high enough in products to be effective. Maximum levels of AA and LA permitted under USDA regulations in processed meats are 0.25% (2.5 mg/g) and 4.8% (48 mg/g), respectively (Federal-register, 2000).

To achieve optimal inhibition of LM and other microorganisms, levels of AA and LA need to be as high as permitted without harming flavor of the RTE meat and poultry products.

Preliminary Study – Changes in LA at 4°C over 6 wks in RTE Meat and Poultry

These experiments were conducted to evaluate the relationship between the counts of LAB (CFU/ml) and the amount of lactic acid existing (mg/g) in RTE meat and poultry products from the time of manufacture through 6 weeks storage. LAB plate count increased over time ($P<0.05$), and was not affected by type of meat or poultry samples. Lactic acid and LAB levels in RTE meat and poultry products stored for six weeks at 4 C are shown in Table II-1. Amount of lactic acid extracted from the samples did not change with increased LAB counts ($P> 0.05$) and with storage time of six weeks ($P>0.05$). Based on these results, the data showed that the age of the processed ready-to-eat (RTE)

Table II.1 Mean of lactic acid bacterial counts (log $_{10}$ CFU/ g) and the corresponding mean lactic acid (mg/g) in RTE meat and poultry stored at 4°C for six weeks

Weeks	***Beef***		***Turkey***		***Bologna***		***Ham***	
	Count [a]	**LA [b]**	**Count**	**LA**	**Count**	**LA**	**Count**	**LA**
0	1.25 bc	18.98 x	0 d	9.78 z	0 d	10.13 z	0 d	12.8 z
1	1.00 bc	17.7 xy	0 d	10.66 z	0.75 cd	10.46 z	0 d	13.63 yz
2	0.65 bc	18.93 x	0 d	9.20 z	0.70 cd	10.72 z	0 d	12.51 z
3	0.70 b	17.50 xy	0 d	6.52 z	0 d	10.60 z	0.75 cd	13.31 z
4	3.65 a	12.60 xy	0 d	10.24 z	0 d	11.12 z	0 d	12.66 z
5	2.95 ab	18.85 xy	0 d	7.91 z	0 d	9.16 z	1.05 bcd	13.36 z
6	3.15 a	18.99 x	2.5 abc	8.71 z	0 d	11.02 z	0 d	12.96 z

[a] Means for LAB counts (log CFU/g) in a column followed by different letters are significantly different ($p<0.05$).

[b] Means for LA (mg/g) in a column followed by different letters are significantly different ($p<0.05$).

meat or poultry did not significantly affect the total level of lactic acid present in RTE meat and poultry products.

Levels of Acetate and Lactate in RTE Meat and Poultry by manufacturer

All data presented refer to manufacturers by letters of the alphabet (A-Z). If a sample was positive, it was designated as a letter with the addition of a “p” for positive and a number indicating the sequential order in which was discovered (1 up to 5). For manufacturers having <10 samples, all data were grouped and that group was called “ZZ”. For positive LM samples, all manufacturers having a positive sample were given a letter designation regardless of the number of samples in the data set.

Out of ~1800 samples, approximately 1200 samples came from manufacturers that had no positive LM samples in the data collected during this study (Table II.2). In this group of manufacturers having all negative LM RTE meat and poultry products, three had over 100 samples collected nationwide in a 12 month period so they were well represented. Over 500 negative LM samples came from manufacturers who had 10 or less samples in the data set. The remaining ~600 samples collected came from manufacturers having one or more positive LM samples. Two manufacturers who had at least one positive sample had over 100 samples collected in 12 months. Twenty six samples (17 positive for LM) came from manufacturers who had less than 10 samples in the data set.

Interestingly, a sample where there was only one sample collected from that manufacturer during the study was a positive LM sample that is designated

Table II.2 Acetic and lactic acid (mg/g) in RTE meat and poultry products by manufacturer with only negative LM samples

Total number	Codes	LA means	LA range (min to max)	AA means	AA range (min to Max)	LA+AA means
121.00	A	16.13 cdeh ±0.07	1.29 59.53	1.04 efg ± 0.92	0.0 8.10	17.21 bcg
124.00	B	21.53 ab ±5.43	5.93 50.46	1.56 b ± 0.60	0.0 3.76	23.09 a
117.00	E	12.84 fg ±5.73	1.06 26.85	1.06 def ±1.87	0.0 7.39	13.92 ef
26.00	F	10.79 gi ±4.17	1.59 19.08	1.52 bch ±2.10	0.0 9.59	12.31 efh
39.00	G	16.65 cde ±5.15	1.21 26.89	1.17 cde ±0.60	0.0 3.09	17.83 bcg
47.00	H	22.15 ab ±4.89	1.60 33.59	1.48 bc ±1.64	0.0 5.05	23.65 a
25.00	I	13.65 efg ±7.15	2.48 28.35	0.66 g ±0.56	0.0 2.03	14.31 def
15.00	J	14.95 cdef ±5.35	2.07 19.85	1.11 bcdefg ±0.50	0.0 1.41	16.06 bcde
30.00	M	23.03 a ±5.73	7.46 34.63	1.06 defgh ±0.50	0.0 2.45	24.09 a
31.00	P	12.79 fgi ±1.75	9.38 16.25	0.72 fg ±0.27	0.0 1.15	13.54 efh
39.00	R	11.90 fgi ±6.19	1.14 28.56	1.44 bchi ± 1.10	0.0 3.42	13.35 efh
24.00	V	10.71 gi ±4.25	1.12 17.17	0.70 fgj ±0.91	0.0 3.47	11.41 fh
17.00	W	12.20 fgi ±4.71	5.17 23.50	0.73 efg ±0.41	0.0 1.30	12.93 efh
561.00	ZZ	15.23 cdeh ±6.92	1.08 46.29	1.06 ej ±0.86	0.0 5.91	16.31 bcd

Sp1 (Manufacturer S, positive sample 1) in Table II.2. There was no data available as to which Alternative (1, 2 or 3) the manufacturer had chosen for the HACCP program for that product.

Since the RTE meat and poultry samples were collected from stores randomly based on random numbers weighted by population in the state, the total number of samples from a single manufacturer occurred due to random chance since different retail grocery stores carry different inventories and tend to favor certain manufacturers depending on regional preferences and retail store contracts. The total number of samples from a single manufacturer ranged from 1 to 239 samples. Any assumptions based on number of samples collected and identity of a manufacturer based on size would be in error due to randomization of the data collection process. Approximately 75% of samples were collected from major retail grocery chains (the top 50) and 25% of samples came from smaller or more regional grocery stores.

Levels of acetate expressed as acetic acid (AA) and lactate expressed as lactic acid (LA) in products from meat manufacturers having no positive LM samples in approximately 1800 RTE meat and poultry samples collected in California, Georgia, Minnesota and Tennessee are shown in Table II.2.

Variation in lactic acid content of samples ranged widely even within a single manufacturer's products. For example, lactic acid levels in RTE meat and poultry samples from Manufacturers A and B ranged from 1.29 to 59.53 mg/g and 5.93 to 50.46 mg/g, respectively (Table II.2). Acetate levels for products within a single manufacturer ranged from 0 to 9.59 mg/g (0.96%). Since some

products may not have lactate/acetate/diacetate added during formulation or processing, ranges in the acetic acid levels are not particularly surprising. However, what is surprising is that the maximum levels of diacetate permitted in meat formulations is 0.25% and many manufacturers had samples that exceeded that level (Table II.2, and II.3) at the high end of the range.

The mean LA content of RTE meat and poultry samples varied significantly ($p<0.001$) among manufacturers and ranged from 10.71 to 23.03 mg/g for negative LM samples (Table II.2). For positive LM samples, the mean LA content ranged from 4.23 to 21.28 mg/g (table II.3). Mean AA content ranged from 0.66 to 1.56 mg/g for manufacturers with negative LM samples and from 0.7 to 5.74 mg/g for manufacturers including positives. The means for LA and AA were within regulatory levels except for the 5.74 mg/g level which was for a single sample from one manufacturer - incidentally a positive LM sample.

Since no significant differences ($p<0.05$) were found between LA and AA levels in manufacturers having negative and positive LM samples due to the wide range and variation in LA and AA (both within manufacturer and from one manufacturer to another) among samples, manufacturers having positive samples were separated and individual positive samples within each manufacturer identified as to LA and AA content (Table II.3). It is important to note that only 0.14% of pre-packed RTE meat and poultry and 1.4% of deli sliced samples tested nationally were positive for LM in the NAFSS study from which our samples were taken (Oyarzabal *et al.*, 2005), therefore, over 98.5% were negative for LM.

Table II.3 Acetic and lactic acid (mg/g) in RTE meat and poultry in LM-positives and LM-negatives RTE meat and poultry products in manufacturers with positive LM samples

#	Code	LM (+)	LA	LA range (min to max)		AA	AA range (min to max)		LA + AA
121	C	total	9.16 i ±4.21	1.13	25.17	0.7 efg ±0.74	0.00	4.12	9.82 h
	Cp1	+	7.34			0.06			7.4
	Cp2	+	8.29			1.67			9.96
	Cp3	+	1.13			0.00			1.13
	Cp4	+	18.20			3.35			21.55
	Cp5	+	9.88			0.00			9.88
239.	D	total	12.31 defgi ±7.05	1.22	41.11	1.94 bcd ±0.87	0.00	8.23	14.26 bcdefh
	Dp1	+	5.68			2.35			8.03
34	K	total	18.24 bcd ±3.60	11.38	25.44	1.30 bcdefg ±0.46	0.36	2.65	19.53 abc
	Kp1	+	15.73			0.87			16.6
	Kp2	+	14.44			0.85			15.29
	Kp3	+	15.55			1.01			16.56
55	L	total	10.21 fghi ±4.48	5.87	28.08	0.99 bcdefg ±0.45	0.00	2.07	11.22 defgh
	Lp1	+	9.45			1.17			10.62
11	N	total	21.28 abc ±3.00	14.11	24.92	1.41 bcdefg ±1.78	0.00	6.07	22.68 ab
	Np1	+	19.77			0.68			20.45
7	O	total	11.18 defgi ±6.08	3.84	19.63	1.05 bcdefg ±0.89	0.06	2.71	12.25 cdefh
	Op1	+	3.84			0.59			4.43

Table II-3 continue

#	Code	LM (+)	LA	LA range (min to max)		AA	AA range (min to max)		LA + AA
6	Q	total	12.61 cdefgi ±2.24	7.93	14.26	1.60 bcdefg ±2.07	0.00	5.32	14.22 bcdefh
	Qp1	+	7.93			5.32			13.25
	Qp2	+	10.22			0.55			10.77
	Qp3	+	10.41			0.60			11.01
	Qp4	+	9.18			0.00			9.18
	Qp5	+	8.62			3.09			11.71
1	S	+	4.23 fghi ±0.00	4.23	4.23	5.74 a ±0.00	5.75	5.75	9.97 bcdefh
3	T	total	17.97 abcdefg ±2.88	13.38	19.09	0.95 bcdefg ±0.25	0.34	0.83	18.90 abcdef
	Tp1	+	15.65			0.83			16.48
76	U	total	11.38 fgi ±5.74	1.14	24.63	1.06 cdefg ±1.27	0.00	6.24	12.45 efh
	Up1	+	20.24			0.55			20.79
	Up2	+	9.12			0.22			9.34
	Up3	+	11.37			0.17			11.54
	Up4	+	9.70			2.73			12.43
	Up5	+	5.62			0.15			5.77
33	X	total	11.01 fgi ±5.53	1.17	24.05	1.27 bcdefg ±1.21	0.00	6.01	12.28 efh
	Xp1	+	24.05			1.47			25.52
	Xp2	+	8.56			1.18			9.74
	Xp3	+	2.45			0.85			3.3
37	Y	total	16.31 bcdefg ±7.53	1.32	29.05	1.01 bcdefg ±0.76	0.00	2.85	17.33 abcdef
	Yp1	+	11.80			0.20			12.00

Table II-3 continue

#	Code	LM (+)	LA	LA range (min to max)		AA	AA range (min to max)		LA + AA
34	Z	total	11.01 defgi ±4.23	4.25	23.06	0.72 bcdefg ±0.75	0.00	3.26	11.74 defgh
	Zp1	+	9.12			0.22			9.34
10	ZZp	total	11.08 fgi ±4.04	7.65	18.91	0.83 efgi ±0.46	0.00	1.48	11.91 efh
	ZZp1	+	12.95			0.47			13.42
	ZZp2	+	18.01			1.01			19.02
	ZZp3	+	18.91			1.12			20.03
	ZZp4	+	7.95			0.00			7.95
	ZZp5	+	7.91			0.35			8.26
	ZZp6	+	7.65			0.55			8.2
	ZZp7	+	12.41			1.48			13.89
	ZZp8	+	10.45			0.33			10.78
	ZZp9	+	9.18			0.50			9.68
	ZZp10	+	10.88			0.18			11.06

To determine the efficacy of LA and LA + AA for reduction in the occurrence of LM in RTE meat and poultry, the question that needed to be answered is whether the levels of LA or LA + AA in positive LM samples was lower compared to overall or negative LM samples for those manufacturers having at least one positive sample. When LA levels of individual positive LM samples were ranked and compared to negative samples by Spearman's rank correlation coefficient, positive samples were found to have a significantly ($p<0.01$) inverse relationship with LA levels in samples. Therefore, as LA level in samples was reduced, they were significantly ($p<0.01$) more likely to be positive for LM.

There were other trends noted in the data set. For manufacturers that had zero prevalence for LM, level LA + AA (A,B,G,H,J,M and ZZ) was more than 15 mg/g for 77% of the samples (937/1216) (table II.2). While concentration of LA + AA in most of manufacturers that have positive-LM samples were less than 15.00 mg/g such as in manufactures C, D, L, O, Q, S, U, X, Z, and PP (table II.3). Samples represented by the mentioned manufacturers had 33 positive samples for LM from the total 39 positive-LM samples. Thus, Data by manufacturing level in this study supported the higher concentration of antimicrobials (acetic plus lactic acids), the better inhibition of LM.

For every rule there are exceptions, as some manufacturers have small total of acetic plus lactic acids antimicrobials and all their samples were negative for LM and the same for the opposite. This might be explained as negative-LM samples with small concentration of acetic plus lactic acids might did not come in

contact with any listerial contamination. On the other hand, LM positive samples with relatively high amount of acetic plus lactic acids might be explained by the function of antimicrobial. It is reported that food preservation by antimicrobials is best achieved when the microorganism to be inhibited are low in number (Davidson & Taylor, 2007). High LM count (> 100 MPN/g) in some positive LM samples in this study (table II.4) might affect the function of acetic and lactic acids even with their high concentration. Antimicrobial function could also be affected by other microbial factors (resistance, growth rate, and interaction with other microorganisms), or intrinsic factors (food nutrients, pH, oxidation reduction potential, and water activity, and/or processing (heat, high pressure, and post-lethality contamination) (Davidson & Taylor, 2007).

Within a single manufacturer's samples, there was significant variation in acetic, lactic, and acetic plus lactic acids concentrations among LM-positive samples and LM-negative samples were identified. In all manufacturers that have prevalence of LM, means of AA, LA, and AA + LA were separated for LM-negative samples (C, D, K, …) and for LM-positive samples (Cp, Dp, Kp, …) except for manufacturer "S, and PP" as all their samples were LM-positives (table II.5). Lower means of AA + LA were associated with LM-positive samples compared to LM-negatives in the same manufacturer such as in "C, D, K, O, Q, Y, and Z". Other manufacturers showed no differences (P>0.5) such as "L, N, T, and X. Variation of organic acid among different manufacturers was expected due to differences in formulations, while it was not under the same manufacture. These differences might be a result of uneven distribution of these antimicrobials

Table II.4 Acetic and lactic acid levels (mg/g) in LM-positive RTE meat and poultry products categorized by LM levels (MPN/g) for RTE meat and poultry products

Products	***LM levels (MPN/g)***							
	< 0.3		**0.3-10**		**10-100**		**> 100**	
	LA and AA levels (mg/g)							
	AA	**LA**	**AA**	**LA**	**AA**	**LA**	**AA**	**LA**
Beef (n=12)	1.36	10.57	1.88	15.70	1.01	15.55	_ [a]	_
Pork (n=9)	0.98	8.51	0.22	9.12	_	_	0.57	10.31
Poultry (n=12)	0.70	11.66	_	_	0.33	10.45	3.09	8.62
Mixed (n=1)	1.67	8.29	_	_	_	_	_	_

[a] No products of this type were positive for LM at this MPN/g.

Total 5 samples (1 beef, 2 pork, and 2 poultry samples) were not tested for LM count (MPN/g), thus they were not included in the table.

during processing, or due to changes in formulation.

Level of acetate and lactate in RTE meat and poultry products by products

Data were classified by product into four categories beef, poultry, pork, and mixed. Concentrations of AA, or LA was not significantly different ($p>0.05$) in cured compared to uncured products (table II.6). The major difference in cured vs. uncured products is the nitrite level and occasionally the addition of sugar. Since RTE meat and poultry products were collected within their normal shelf-life and were not spoiled by lactic acid bacteria that might have produced lactic acid, it is not unusual that LA and AA were not different in cured vs. uncured products. Samples in each product category was then sub-divided into different types, for example beef samples were represented by roast beef, beef franks, salami, corned beef, pastrami, and beef bologna (table II.7). Levels of AA, LA, and AA + LA were each significantly different ($p<0.0001$) among all products.

In beef products, means of LA were significantly higher ($p<0.05$) in roast beef and beef pastrami (17.55, 18.84 mg/g, respectively) compared to beef bologna and corned beef (11.84, 11.79 mg/g, respectively). Since both roast beef and beef pastrami are whole-muscle beef product, the higher concentration of LA in these products might be explained by lactic acid remaining after glycolysis or the residual effect of LA being used as sanitizer on beef carcasses. Many beef slaughter plants now use lactic acid washes on carcasses after slaughter to reduce contamination. It had been reported that warm (55°C) 2% lactic acid spray was effectively used in reducing aerobic plate counts and counts

TableII.5 Means of acetic and lactic acid (mg/g) in LM-positives and LM-negatives RTE meat and poultry products in manufacturers with positive LM samples

Total number	Codes	LM	LA means	AA means	AA + LA Means
121.0	C	-	10.43	0.66	11.00
	Cp	+ (n=5)	7.89	0.74	8.63
239.0	D	-	17.94	1.22	19.18
	Dp	+ (n=1)	6.68	2.66	9.34
34.0	K	-	19.43	1.34	20.77
	Kp	+ (n=3)	17.04	1.25	18.33
55.00	L	-	10.71	0.53	11.26
	Lp	+(n=1)	9.71	1.46	11.17
11.00	N	-	20.98	1.80	22.78
	Np	+ (n=1)	21.57	1.01	22.58
7.00	O	-	16.66	1.31	17.98
	Op	+(n=1)	5.71	0.79	6.51
6.00	Q	-	16.07	1.04	17.11
	Qp	+ (n=5)	9.15	2.16	11.32
1.00	S	+ (n=1)	4.23	5.74	9.98
3.00	T	-	17.75	0.72	18.47
	Tp	+ (n=1)	18.19	1.17	19.34
76.00	U	-	10.62	1.13	11.76
	Up	+ (n=5)	12.15	0.99	13.14
33.00	X	-	10.76	1.12	11.89
	Xp	+ (n=3)	11.26	1.41	12.67
37.00	Y	-	20.06	1.48	21.56
	Yp	+ (n=1)	12.53	0.55	13.11
34.00	Z	-	12.64	0.92	13.58
	Zp	+ (n=1)	9.37	0.51	9.89
10.00	PP	+ (n=10)	11.08	0.83	11.91

TableII.6 Acetic and lactic acid (mg/g) in RTE meat and poultry products by cured and uncured products

Product (n=1883)		Beef (n=364)	Mixed (n=77)	Pork (n=546)	Poultry (n=896)	Total / Means
Cured	Number	162	77	540	505	1285
	LA	15.49 ±6.49	15.19 ±6.00	14.63 ±7.04	14.65 ±6.92	14.39 a [a]
	AA	0.94 ±0.78	0.91 ±0.69	0.81 ±0.86	0.81 ±1.30	1.28 b
	Positive -LM	6	1	11	9	27
Uncured	Number	202	0	6	391	600
	LA	18.96 ±7.10	-[b]	17.40 ±6.50	15.15 ±7.80	13.65 a
	AA	1.20 ±0.88	-	0.38 ±0.51	0.86 ±1.22	1.28 b
	Positive -LM	7	0	0	5	12

[a] Means followed by the same letter within a type of organic acid are not significantly different (p>0.05)

[b] No products of this type were not available.

TableII.7 Acetic and lactic acid (mg/g) in RTE meat and poultry products by product type

Product (n=1883)	product name	total #	LM-positives (n=39)	LA means[a]	AA means	LA +AA means
Beef	roast beef	222	6	17.55 ag	1.29 cd	18.83 a
(n=364)	beef franks	5	0	13.96 abcdefh	1.30 bcdef	15.26 abcdefg
	corned beef	11	1	11.79 defh	1.07 cdef	12.87 bcdefg
	beef pastrami	22	0	18.52 ag	1.55 bc	20.09 a
	bologna	104	6	11.84 fh	0.94 f	12.81 egh
Poultry	chicken breast	69	0	16.37 ab	1.29 cde	17.67 a
(n=896)	roasted chicken breast	60	0	14.28 bcde	0.98 f	15.25 acdf
	smoked chicken	7	0	15.67 abcdef	0.94 cdef	16.63 abcdef
	roasted turkey breast	264	2	14.26 cd	0.96 f	15.18 bc
	smoked turkey	193	3	12.92 ef	0.93 f	13.84 def
	turkey breast	251	8	13.22 def	0.98 f	14.21 cdef
	turkey ham	23	1	16.54 abc	1.13 cdef	17.68 ab
	turkey pastrami	14	0	10.33 fh	0.94 def	11.29 fgh
	bologna	15	0	12.01 defh	1.33 cdef	13.35 cdefg

Table II.7 continue

Product (n=1883)	product name	total #	LM-positives (n=39)	LA means	AA means	LA +AA means
Pork	ham	336	6	12.65 ef	0.96 f	13.63 def
(n=546)	smoked ham	80	2	13.39 def	0.99 ef	14.39 cdef
	cooked ham	106	3	13.18 def	0.94 f	14.13 cdef
	bacon	4	0	12.66 bcdefgh	2.41 ab	15.08 abcdefg
	liver loaf	2	0	19.42 abcdef	0.82 def	21.20 abcde
	pork salami	4	0	19.42 abcdef	1.77 bcdef	22.47 a
	bologna	14	0	8.54 h	0.82 def	9.37 g
Mixed (n=77)	bologna	77	1	12.43 ef	1.04 def	13.49 cdef

[a] Means within a column followed by the same letter are not significantly different (p>0.05)

of *Enterobacteriaceae*, total *coliforms*, thermotolerant *coliforms*, and *Escherichia coli* in beef carcass surface regions (Castillo *et al.*, 1999).

In beef products, means of LA were significantly higher ($p<0.05$) in roast beef and beef pastrami (17.55, 18.84 mg/g, respectively) compared to beef bologna and corned beef (11.84, 11.79 mg/g, respectively). Since both roast beef and beef pastrami are whole-muscle beef product, the higher concentration of LA in these products might be explained by lactic acid remaining after glycolysis or the residual effect of LA being used as sanitizer on beef carcasses. Many beef slaughter plants now use lactic acid washes on carcasses after slaughter to reduce contamination. It had been reported that warm (55°C) 2% lactic acid spray was effectively used in reducing aerobic plate counts and counts of *Enterobacteriaceae*, total *coliforms*, thermotolerant *coliforms*, and *Escherichia coli* in beef carcass surface regions (Castillo *et al.*, 1999).

No significant differences in LA concentrations were found among beef franks, corned beef, and beef bologna (13.96, 11.79, and 11.84 mg/g, respectively). In these product types, meat was blended, mixed, and LA was only controlled by the product formulation, which might explain the consistency of LA among these products.

The poultry category included: chicken products (chicken breast, roasted, and smoked chicken breast), turkey products (turkey breast, smoked turkey breast, turkey ham, and pastrami), and chicken and/or turkey bologna. LA was significantly higher in chicken breast (16.37 mg/g) than other turkey products such as roasted turkey breast (14.26 mg/g), smoked turkey (12.92 mg/g), turkey

breast (13.22 mg/g), and turkey pastrami (10.33 mg/g). However, the LA of chicken breast was similar to turkey ham ($p>0.05$). LA can be used as a decontaminant of chicken or turkey carcasses. At levels of 5% or above LA eliminated or inhibited all spoilage bacteria (*Pseudomonas* species, *Shewanella putrefacciens*) on fresh poultry broiler carcasses (Russell, 1998). It was also reported LA used at concentration of 5% combined with steam inactivated *Listeria innocua* inoculated on the surface of chicken skins (Lecompte *et al.*, 2008). The significantly lower ($p<0.05$) levels of LA in turkey pastrami and corned beef compared to most poultry or beef products may be due to the differences in processing of pastrami and corned beef since they may be steamed or brined during manufacturing which may dilute the surface lactic acid.

The pork category included ham, smoked ham, cooked ham, bacon, liver loaf, pork salami, and pork bologna. Lactic acid was significantly lower in bologna (8.54 mg/g) compared to other pork products except bacon. Mixed category included only one type of products which was a bologna made of mixed beef, poultry, and pork and it was positive for LM.

Acetic acid was higher in roast beef, beef franks, beef pastrami, bacon, pork salami and chicken breast (1.29, 1.30, 1.55, 2.41, and 1.77, 1.29respectively) compared to other RTE meat and poultry products. Higher concentrations of AA in these samples were associated with reduced incidence of LM. Concentration of AA + LA was proportional to LA concentration; as AA + LA concentrations were also significantly higher in roast beef, beef pastrami, chicken breast, turkey ham, and pork salami (18.83, 20.09, 17.67, 17.68, and

22.47 mg/g respectively) than beef bologna (12.81mg/g), smoked turkey(13.84 mg/g), turkey breast (14.21 mg/g), ham (13.63 mg/g), smoked ham (14.39 mg/g), cooked ham (14.13 mg/g), and mixed bologna (13.49 mg/g). In general, products with higher concentration of AA + LA had a reduced incidence of LM, and products with lower concentration of AA + LA had an increased incidence for LM such as beef bologna (6/104), smoked turkey (3/193), turkey breast (8/251), ham (6/336), smoked ham (2/80), cooked ham (3/160), and mixed bologna (1/77). Roast beef and turkey ham samples had higher concentration of AA + LA but still had some positive samples (6/222 and 1/23) for LM (table II.7).

Association of LM with RTE meat and poultry samples having a high concentration of AA + LA might be due to post-processing contamination of RTE products with LM. The relatively limited Listeria control interventions at retail may increase the likelihood of introduction of the pathogen into some foods at retail and food service establishments compared to food processed in USDA or state inspected facilities.

Although approximately 50% of the samples in this study were prepackaged products and 50% were sliced in the deli department, most (89%) of the LM-positive samples were from RTE meat and poultry sliced in the deli section of the grocery. Some samples with very high levels of acetic acid (0.57%) or lactic acid (2.3%) were positive for LM; thus, post-process contamination of RTE meat and poultry is not always prevented by antimicrobials that may be added during slaughter and/or formulation.

High LM contamination levels have been documented in RTE foods and

may reduce inhibitory effectiveness of AA or LA against LM. The likelihood of contamination of RTE foods at deli in supermarkets has been reported by several authors (Gombas *et al.*, 2003b; Handa *et al.*, 2005; Vitas *et al.*, 2004). The prevalence of LM in in-store-packaged deli salads, and luncheon meat was 3.6, and 2.7%, respectively, whereas, the corresponding prevalence in manufacture-packaged products was 1.4, and 0.4% respectively (Gombas *et al.*, 2003b).

High incidence of LM in foods served at the deli in supermarkets could be due to several factors including high volume of public traffic, improper handling and storage of food products. Utensils, food contact surfaces, personnel and other items such as brooms may serve as a source of contamination or cross-contamination of RTE food as the result of poor food handling practices, inadequate training, improper serving practices, lack of sanitation, and inadequate cleaning (Sheen & Hwang, 2008).

According to Hudson and Mott (1993), slicing machines may be contaminated with the pathogens from meat packaging materials which can be a source of cross-contamination of delicatessen products during slicing (Hudson & Mott, 1993).The association of slicing equipment with transmission of LM in retail and food service environments is due to the ability of the pathogen to adhere to surfaces and form biofilms on surfaces of the equipment (Lianou & Sofos, 2007). Given the ubiquitous nature of *L. monocytogenes*, other sources of the pathogen in stores could include the environment, food handlers, customers' traffic, and incoming raw ingredients or processed products that have been contaminated after the lethality treatment at the manufacturing facility. Lianou and Sofos (2007)

outlined a comprehensive food safety system which was based on the philosophy of HACCP systems for control of LM in retail and food service operations (Lianou & Sofos, 2007).

Significant finding

The results of this study indicated that acetic acid and lactic acid are interacting and significantly ($p<0.01$) associated with lower occurrence of LM in RTE meat and poultry products. Although almost all major RTE meat or poultry processors currently claim to use acetate, lactate and/or diacetate, the data show that some products had wide variations in levels of these organic acids and some did not have detectable levels.

In conclusion, residual levels of acetates, and lactate in a large national sampling of RTE meat and/or poultry products varied widely. This indicates a wide disparity in product formulation in meat manufacturing in the United States and/or uneven mixing of acetates or lactates in formulations. LA and AA are helpful for control of *Listeria monocytogenes* in RTE processed meat and poultry products. More consistent and even application of organic acids in formulations may provide safer RTE meat and poultry products although the best method of LM control is still environmental control and prevention. Competent delivery of food safety at both processing and at retail must be implemented to provide safe RTE meat and poultry products.

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III. HACCP VALIDATION FOR USE LACTIC ACID ON BOLOGNA, HAM, AND RED-HOT RTE MEAT AND POULTRY PRODUCTS

Abstract

The frequent incidence of *Listeria monocytogenes* (LM) in ready-to-eat (RTE) meat and poultry products led to a USDA / FSIS final rule for the post-processing lethality control of LM (9 CFR 430). RTE meat and poultry products processing plants must include control programs for LM in their HACCP plans and verify their effectiveness against LM. The objective of this study was to evaluate 2% lactic acid (LA) for its effect as a post-lethality treatment and *Listeria* inhibitor on RTE meat and poultry products produced by a Southeastern Meat Manufacturing Company. Bologna, Ham (souse), and Red Hots (miniature frankfurter) samples were provided by the manufacturer. Samples were dip inoculated with LM with approximately log 5 CFU/g using an inoculum in 0.1% peptone water at 25 °C for 20 sec. LM recovery and enumeration after direct platting on PALCAM and/or USDA enrichment (when no growth) from samples after inoculation was approximately log 5 CFU/g depending on size and type of product. Half of the inoculated samples were surface sprayed with 2% LA for 20 sec and the other half kept as controls. All samples were individually placed in vacuum-sealed bags and stored at 4°C (three replicates) for 0, 7, 30, 60, and 90 days. Surface treatment of RTE meat and poultry products by 2% LA caused a significant reduction ($P<0.001$) immediately after treatment (day 0) in the initial LM counts by ≥1 log CFU/g compared to the controls. LM counts decreased to undetectable levels in Souse Roll and Red Hots frankfurters after 7 and 60 days, respectively, with 2% LA treatment. LM in Bologna remained at ≥ 1 log reduction

from initial inoculation for up to 30 days but after 60 and 90 days storage increased to levels similar to untreated controls. Therefore, the effect of 2% LA on LM count differed according to meat type and time of storage; however ≥ 1 log CFU/g reduction was achieved with all three products after application for at least 30 days.

Introduction

Ready-to-eat (RTE) meat and poultry products contaminated with LM have been implicated in several outbreaks of listeriosis in the United States (Table III.1) (CDC, 1998; CDC, 2000; CDC, 2002). LM does not survive the thermal treatment involved in RTE meat and poultry processing (Zaika et al., 1990; Carlier et al., 1996). However, contamination may occur through direct contact of the cooked product with contaminated surfaces in the processing environment during slicing, peeling, repackaging and other procedures (Zhu et al., 2005).

The U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) enforces a zero-tolerance and series of rules concerning LM in RTE meat and poultry products (Table III.1). In addition to proper sanitation, FSIS requires the food-processing industry to apply control measures for LM in RTE products if they are exposed to the processing environment after the lethality processing step (USDA & FSIS, 2003).

The industry is required to use one of three alternatives: (1) a post-lethality inactivation treatment and a LM growth inhibitor, (2) a post-lethality inactivation

Table III.1 Timeline of Events Related to Listeria monocytogenes (LM) adapted from (FSIS, 2007)

Time	***Events related to Listeria monocytogenes***
1987	FSIS initiates regulatory microbiological testing for LM in RTE meat and poultry products and "**zero tolerance**" established.
1997	CDC, FDA and FSIS partnership establishes **Healthy People 2010** goal for LM. With the 1997 baseline year, the target to reduce infections caused by LM by half from 0.5 cases per 100,000 people to 0.25 case per 100,000 by 2010.
1998	Major LM **outbreak** in which hotdogs and possibly deli meats are implicated. CDC reports 101 illnesses, 15 deaths, and 6 stillbirths or miscarriages associated with the outbreak.
1999	In response to 1998 and 1999 outbreaks. FR Notice: "*Listeria* Contamination of RTE Products; compliance with the **HACCP** system regulations" and *Listeria* Guidelines for Industry issued (May 1999).
January, 2000	Direct Rule "Food **Additives** for Use in Meat and Poultry Products: Sodium Diacetate, Sodium Acetate, Sodium Lactate and Potassium Lactate" issued.
May, 2000	**Healthy People goal** for LM set to 0.25 cases per 100,000 by 2005.

Table III-1 continued

Time	***Events related to Listeria monocytogenes***
December, 2000	**Outbreak** spread over 10 states, linked to turkey deli meat.
2001	Performance Standards for the Production of Processed Meat and Poultry Products: Proposed Rule" (includes testing food contact surfaces for *Listeria* spp.) and Draft Compliance Guidelines issued.
2002	Multi-state **outbreak** linked to turkey deli meat products,
Dec, 2002	“Microbial Sampling of Ready-to-Eat (RTE) Products for the FSIS Verification Testing Program” issued.
June, 2003	**Interim Final Rule** "Control of *Listeria monocytogenes* in Ready-to-Eat Meat and Poultry Products" and Compliance Guidelines issued.
2006	FSIS Directives 10,240.4 and 10,240.5 issued. Under this program, establishments are selected based on a risk-ranking model and products, environmental and food-contact surface samples are collected.
April, 2006	FoodNet Data show LM levels are approaching national health objectives.
May, 2006	Compliance Guidelines to Control LM in Post-Lethality Exposed RTE Meat and Poultry Products and Questions and Answers for the interim final rule updated.

treatment or a growth inhibitor, or (3) sanitation measures and environmental testing (USDA & FSIS, 2003). The chosen alternative must be included in the Hazard Analysis and Critical Control Point plan (HACCP) or prerequisite programs, and its effectiveness should be validated by FSIS (USDA & FSIS, 2003).

FSIS developed a compliance guideline to assist processors in meeting the regulatory requirements of the final rule (FSIS, 2006). The guidelines states that the post-lethality treatment must reduce pathogens by at least 1 log, and processing plants that use treatments that cause a reduction of the pathogen by at least 2 log should be subjected to less frequent microbial testing by the FSIS (Table III.2) (FSIS, 2006).

Lactic acid (LA) has a long history of use as an acidulants in a wide variety of food and is currently used by the meat industry for decontamination of beef and pork carcasses (Castillo *et al.*, 2001; Pipek *et al.*, 2006; Vannetten *et al.*, 1995).

Generally, treatments with lactic acid at varying concentrations result in bacteria reductions ranging from 1 to 3 log CFU/g on meat surfaces (Anderson *et al.*, 1992). The effectiveness of lactic acid for controlling meat borne pathogens varied between studies and may be attributable to differences in acid concentration as well as methods for acid delivery, contact time, sampling techniques, tissue type or organisms (Greer & Dilts, 1992).

Table III.2 Expected Levels of Control for Post-lethality Treatments adapted from FSIS compliance guidelines (FSIS, 2006)

	Levels of reduction or inhibition achieved to control LM		
	Higher level [1]	***Lower level*** [2]	***Not eligible***[3]
Post lethality treatment (log_{10}) reduction of LM	≥ 2 Equal to or greater than 2	≤ 2 Greater then 1 and Less than 2	< 1 Less than 1

[1] Relatively less sampling by FSIS

[2] Relatively more sampling by FSIS

[3] Unless there is supporting documentation

LA was effective against LM when applied as a surface treatment of RTE meat and poultry products (Byelashov *et al.*, 2008; Gonzalez-Fandos & Dominguez, 2006a). Byelashov *et al* (2008) reported that spraying frankfurters with 5% LA (v/v) for 10 seconds after inoculation reduced count of LM by 1.8 log CFU/cm^2. LA suppressed growth of LM for 39-41 days in frankfurter samples stored at 4°C (Byelashov *et al.*, 2008). Also a similar effect was found when LA was used as a dipping solution (Geornaras *et al.*, 2006b). Since initial levels of LM on the surface of frankfurters were reduced by 1.8 log CFU/cm^2 when they were dipped in a 2.5% aqueous solution of LA (v/v).

The objective of this study to evaluate 2% LA for its effect as a post-lethality treatment and *Listeria*-inhibitor on the survival of LM on RTE Red Hots (miniature frankfurter), Bologna, and Souse Roll samples produced by a Southeastern Meat Manufacturing Company.

Materials and methods

Inoculum preparation

The *Listeria monocytogenes* used in this study was previously isolated and identified from RTE meat and poultry products that were collected from four different FoodNet states. This isolate was preserved by freeze-drying and stored at - 4°C. To revive the LM isolate, it was transferred to 9 ml of Brain Heart Infusion broth (BHI) (Difco, Becton Dickinson, Sparks, Md.), and incubated at 35°C for 24 h. After two consecutive transfers, inoculum was diluted (1 x 10^8

CFU/ml) in 0.1% peptone water (Difco, Becton Dickinson) to obtain approximately 10^6-10^7 CFU/ml for inoculation.

LM was enumerated after direct platting on PALCAM. If no growth occurred after direct platting, samples were enriched in LEB (Listeria Enrichment Broth).

Inoculation of samples

Bologna (mixed meat and chicken), Souse (mixed pork snouts, hearts, tongues, and skin), and Red-hots miniature frankfurters (Mixed chicken, pork, and beef) rolls were provided by the manufacturer. All samples were formulated by the manufacturer with lactate/diacetate at 2.5% as an antimicrobial agent during processing. Each product (three replicates) was inoculated with LM by dipping product into a suspension of LM (log 6-7 CFU/ml) for 20 sec. at room temperature (25 °C). After inoculation, samples were removed and drained on a sterile metal grid for 30 min at room temperature to allow attachment of inoculated cells before treatment and vacuum packaging. For bologna and souse entire roll of product (approximately 5 kg) was inoculated in a suspension (10^6-10^7 CFU/ml) in a deep sterile tray. Red-hot frankfurters chains (about 10 individuals in each chain) were cut into singles and inoculated in the same suspension.

Treatment of samples

After inoculation, samples were transferred into a class II Biohazard cabinet, placed on sterile grill wire netting, and sprayed with a hand-activated

squeeze bottle. Freshly prepared solution of 2% (v/v) LA (supplied by the manufacturer) was applied after inoculation, and inoculated samples (three replicates) that were not treated served as controls. Samples were sprayed with 2% LA (Purac) for 20 sec (about 17 g) and then drained for 5 min (Byelashov *et al.*, 2008). Following treatment, all samples (three replicates) were sliced (about 125 g each) and individually placed in vacuum-sealed bags and stored at 4°C for 0, 7, 30, 60, and 90 days.

Microbial analysis

Bologna, souse, and red-Hot frankfurter RTE meat and poultry products samples (three replicates) weighing 125 g each were aseptically placed in a sterile stomacher bags. According to Zhang et al, using 125-g with 1: 5 dilution rate sample increased the detection limit, and delete problems associated with large volume without compromising *listeria* recovery (Zhang *et al.*, 2007). Sufficient volume (500 ml) of 0.1% peptone water was added to each sample to obtain dilution ratio of 1:5 (Zhang *et al.*, 2007). Samples were homogenized by stomaching for 2 min (Seward Stomacher 400, Seward Ltd., Worthington, UK).

Ten-fold serial dilutions were prepared in 0.1% peptone water, consequently, this series of tubes contained 0.02, 0.002 etc g of sample from the original 1:5 (0.2) dilution (Zhang *et al.*, 2007). Aliquots of appropriate dilutions were surface plated onto PALCAM agar (Difco, Becton Dickinson) for enumeration of LM and pour-plated in MRS Agar (Difco, Becton Dickinson) for total lactic acid bacteria (LAB) initial counting.

All plates were left for 2 h at room temperature and then incubated for 24 h at 35 °C, and bacteria colonies were counted; counts were expresses as log CFU/g. When no growth occurred, samples were re-enriched in Listeria Enrichment Broth (LEB) (Difco, Becton Dickinson), and re-plated. However, none of the samples showing no growth of LM had recovery of LM after enrichment.

pH determination

Samples (5 g) were mixed with 10 ml of distilled water (Gonzalez-Fandos & Dominguez, 2006a). The pH of the homogenized sample was determined by homogenizing a sample in a whirl-Pak bag with distilled water for 1 min in a stomacher. The pH was measured with a pH meter (Accumet, Cole-parmer, Fisher Scientific) by immersing a pH electrode in the bag containing the homogenate. Determination of pH was performed in triplicates.

Statistical analysis

The study had replicates (three trials) and for each replicate duplicate sample for every treatment was analyzed at each sampling day. Colony counts were converted to $\log_{10}$ CFU/g and if LM was not detected after enrichment, the count was represented by 0. Completely randomized design (SAS, 1999) was used blocked by product.

Results and discussions

Antimicrobial effects

Surface treatment of bologna, souse, and red-hot frankfurter RTE meat and poultry product by 2% LA caused significant ($p<0.001$) and immediate reduction in LM counts compared with untreated controls.

After application, time 0 reduction was observed in LM counts by ≥ 1 log CFU/g in all types of RTE products and after 7 days the reduction changed according to type of product and days of storages. However, greater than 1 log reduction occurred in all treatments up to 30 days (Table III.3, Figure III.1, III.2, and III.3).

The overall mean of LM counts in bologna, and red-hot significantly ($p<0.05$) after LA application by more than one log CFU/g reduction to 2.5, and 2.1 CFU/g, respectively (figure III.4). In souse, the overall mean of LM counts (0.7) were small compared with bologna, and red-hot and reduction after application of LA was less than one log CFU/g. However, about 2 log CFU/g reduction was achieved immediately after application (Table III.3, figureIII.2).

Storage

Significant reduction ($P<0.001$) of the overall means of LM counts was achieved with 2% LA over storage days (Figure III.5). The initial mean counts of Lactic acid bacteria (LAB) at day 0 in Bologna, souse, and Red-Hot were 5.1, 4.8, and 6.4 CFU/g, respectively. LM counts decreased to undetectable levels in souse quickly (after 7 days) and LM was undetectable in Red-Hots (after 60

days) with 2% LA treatment (Table III.3, Figures III.1, III.2, and III.3). However, LM in Bologna was not significantly different than ($p<0.05$) samples stored 60 or 90 days (Figure III.1).

Products pH

The initial pH of the inoculated bologna, ham, and red-hot that were not sprayed with LA were 7.73, 5.76, and 6.54, respectively (Table III.4).

The treatment with the LA reduced the products pH by 0.12, 0.12, and 0.05 respectively (table III.4). The pH of bologna, ham, and red-hot products were relatively stable through out the entire storage period.

The pH values of RTE meat products that have been published varied widely in their pH values but mostly ranged from 5.00 to 6.00. Fermentation and smoking can slightly reduce these pH values (Ingham *et al.*, 2004). The pH of bologna was higher than normally seen in this product which normally ranges from 5.00 to 6.00. The higher pH could perhaps be due to a unique formulation containing an ingredient such as sodium phosphate.

Meat processors are responsible for validating the safety of their products as part of a HACCP program by providing scientific data. However, the cost of validation is limiting for small manufacturers. The objective of this study was to evaluate 2% lactic acid (LA) for its effect as a post-lethality treatment and *Listeria* inhibitor on the survival LM on RTE meat and poultry products produced by a Southeastern Meat Manufacturing Company.

Table III.3 Average of LM (log CFU/g) [1]counts at different storage days on Bologna, Ham (Souse), and Red-Hot samples that were inoculated and either left untreated controls or sprayed with 2% LA

Product	***Storage days***	***LM (log CFU/G)***	
		Untreated controls	**Sprayed with 2% LA**
Bologna	0	4.3 bc	2.5 ghi
	7	3.5 cdefg	1.1 kl
	30	4.1 bcd	3.1 efgh
	60	3.8 cde	3.3 defgh
	90	2.4 hi	2.7 fghi
Souse	0	3.2 defgh	1.2 jkl
	7	0.3 lm	0 m
	30	0 m	0 m
	60	0 m	0 m
	90	0 m	0 m
Red-Hot francfurter	0	6.3 a	5.0 b
	7	3.6 cdef	3.2 defgh
	30	3.6 cdef	2.1 ij
	60	1.8 ijk	0 m
	90	2 ijk	0 m

[1] value in a column followed by different letters are significantly different (P<0.05)

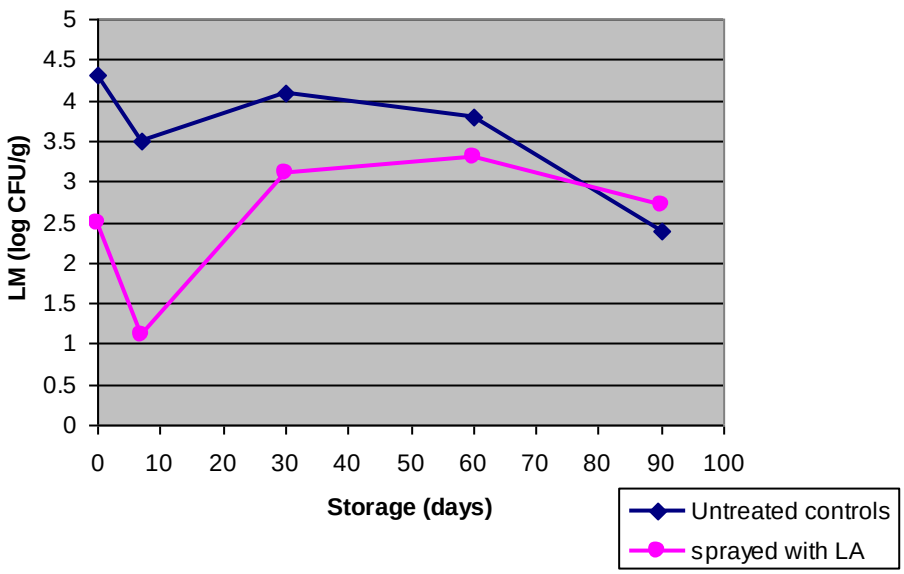


Figure III.1 Average of LM (log CFU/g) counts on Bologna that were inoculated and either left untreated controls or sprayed with 2% LA and stored at 4°C for 90 days

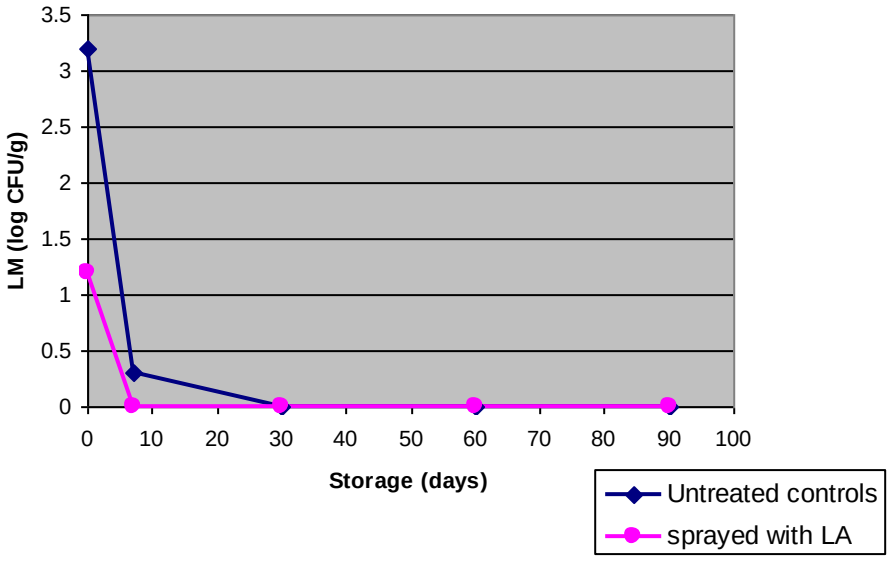


Figure III.2 Average of LM (log CFU/g) counts on Ham Souse that were inoculated and either left untreated controls or sprayed with 2% LA and stored at 4°C for 90 days

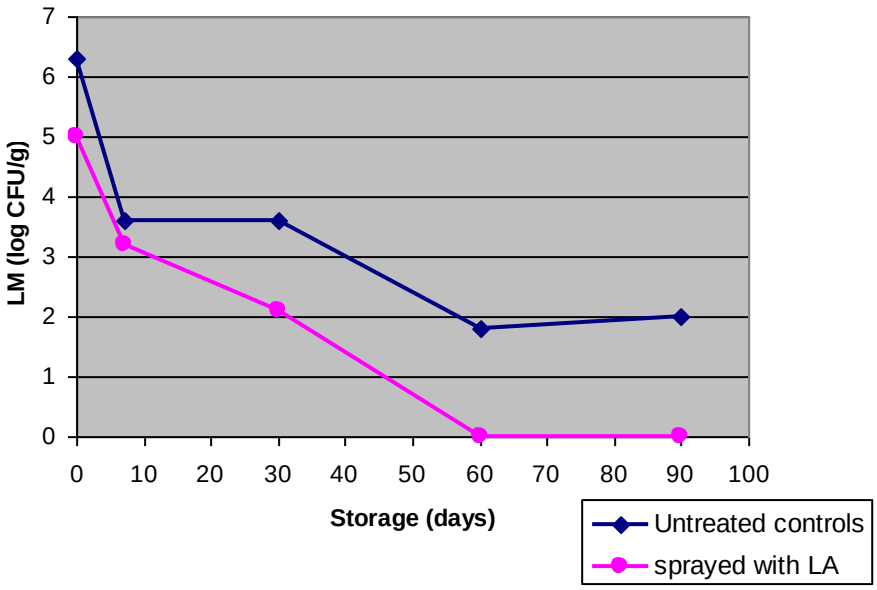


Figure III.3 Average of LM (log CFU/g) counts on Red-Hot frankfurters that were inoculated and either left untreated controls or sprayed with 2% LA and stored at 4°C for 90 days

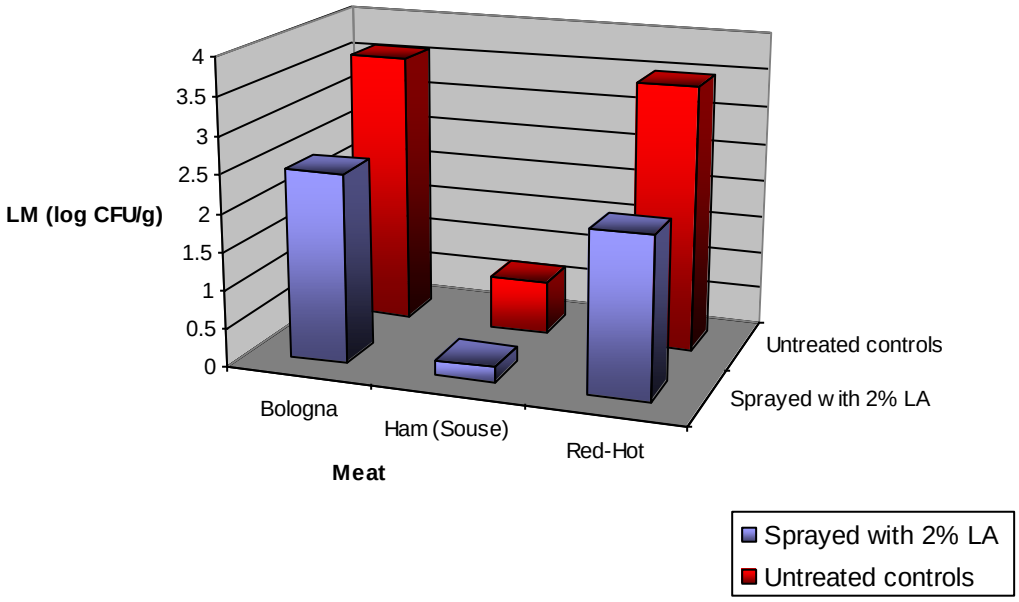


Figure III.4 Average of LM (log CFU/g) counts on Bologna, Ham (Souse), and Red-Hot samples that were inoculated and either left untreated controls or sprayed with 2% LA over the 90 day storage period at 4°C. Lactic acid (2%) significantly reduced LM in all treatments.

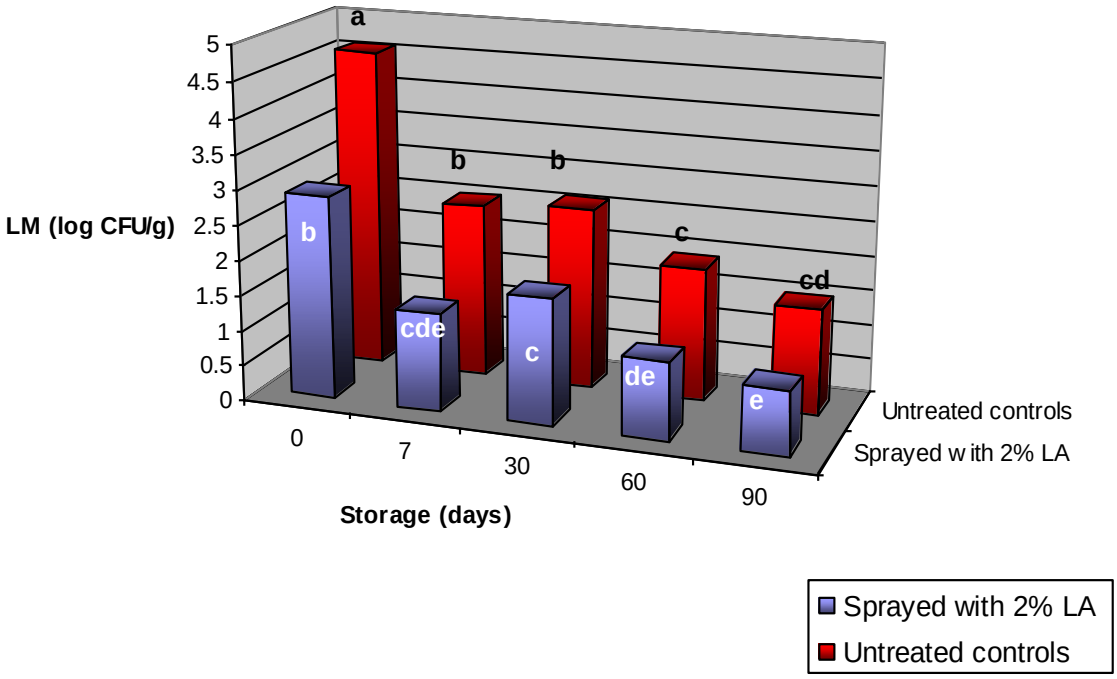


Figure III.5 Means LM (log CFU/g) counts on all products either left untreated controls or sprayed with 2% LA and stored at 4°C for 90 days. Means followed by different letters are significantly different ($p<0.05$).

Table III.4 Changes in pH in Bologna, Souse, and Red-Hot RTE products on different storage days of samples that were inoculated and either left untreated controls or sprayed with 2% LA

Product	***Storage days***	***pH***	
		Untreated controls	**Sprayed with 2% LA**
Bologna	0	7.73 [a]	7.62
	7	7.69	7.7
	30	7.68	7.68
	60	7.61	7.6
	90	7.63	7.61
Ham (Souse)	0	5.76	5.64
	7	5.66	5.7
	30	5.63	5.63
	60	5.63	5.64
	90	5.69	5.67
Red-Hot	0	6.54	6.49
	7	6.57	6.54
	30	6.5	6.46
	60	6.46	6.44
	90	6.39	6.48

[a] pH was not significantly different in a type of meat product over 90 d storage period at 4°C

Recontamination of RTE meat and poultry products during post-processing may be the cause of outbreaks of food-borne disease. Spraying or dipping of peeled or sliced cured meat products in antimicrobial solutions before packaging could offer significant protection against LM that may cross-contaminate the product surface post-cooking (Samelis *et al.*, 2001b; Palumbo & Williams, 1994).

Survival of LM in control samples with no treatment was much higher than 2% LA treated samples. Similar results have been reported in previous studies (Glass & Doyle, 1989; Byelashov *et al.*, 2008; Geornaras *et al.*, 2006b; Samelis *et al.*, 2001b).

Overall, results indicated that post-processing LA treatments as surface spraying (Figures III.1, III.2, III.3, and III.4) may provide better antilisterial protection compared to untreated controls. Although LA had an immediate reduction (⩾1 log) on LM populations in bologna, souse, and red-hot RTE meat and poultry products, the effect of 2% LA differed according to meat type and time of storage.

The growth behavior of LM was different in bologna, souse, and red-hot products (Figures III.1, III.2, and III.3). In bologna samples, the mean reduction in LM at time 0 was 1.8 log CFU/g. The overall mean reduction in LM counts over 90 days in treated samples compared to untreated controls was 1.1 logs CFU/g (figure III.4). Reduction of LM (2 Log CFU/g) was achieved immediately after application of LA and for up to 7 days, and continued for ≥1 log reduction for 30 days. Although reduction of LM was achieved for 60 days, growth was thereafter

restored (figure III.1). Samelis *et al* (2005) reported similar finding after dipping pork bologna with LA (2-5%) and other antimicrobial treatments, and suggested higher concentrations and combination with other antimicrobials such as organic acids or their salts, and nisin were the keys for effective and long-term antilisterial effect (Samelis *et al.*, 2005).

LA reduced LM counts in red-hot (miniature frankfurter) significantly ($p<0.05$) after surface application, overall mean reduction of LM of 1.3 Log CFU/g for the 90 day storage period (TableIII.4). The reduction of LM for ≥ 1 log CFU/g was consistent throughout storage at days 0-90, and LM counts decreased to undetectable level after 60 days (Figure III.3). The effect of LA against LM in red-hot samples was similar to that reported by Byelashov et al, (2008) who found that spraying (for 20 sec, at 23±2 °C) inoculated frankfurters with 5% LA reduced LM population by 1.8 CFU/cm^2 (Byelashov *et al.*, 2008). A 2 log reduction of LM on frankfurter following dipping (for 30 sec at 20°C) in a 3.4% LA solution (de Gonzalez *et al.*, 2004), and the same reduction after dipping (for 120 sec at 23°C) in 2.5% solution of LA (Barmpalia *et al.*, 2004b). These data showed that differences in reduction were variable according to application method and acid concentration.

Survival of LM before and after application of 2% LA was different in souse compared to bologna and red-hot samples. Immediate reduction in LM counts (2 logs CFU/g) occurred after spraying, LM rapidly died off on the surface of souse as it decreased to undetectable limits in both sprayed and untreated control after 7 days(Table III.3, Figure III.2).

Other studies had previously reported that souse meat did not support the growth of LM (Ingham *et al.*, 2004b), at various pH values (4.3, 4.7, and 5.1) or at different storage temperature (5°C and 10°C) (Kim *et al.*, 2006), and concluded that the product chemical properties, and formulation affect survival of bacteria. General description method for the preparation of souse, non-skeletal meats is cooking at 74°C (165°F) and then mixing with gelatin, broth, vinegar (acetic acid) and spices. The mixture is poured into moulds and chilled to solidify (Fiddler *et al.*, 1975). Acetic acid was added to the formulation as a natural ingredient (vinegar) which can play a role as an acidulant or antimicrobial for controlling pathogens in addition to the effects of lactate and diacetate in the formulation.

In this study, souse products had an average pH of 5.6 (Table III.4). The low pH may have contributed to the decreased survival of LM on samples for longer periods of time. Glass and Doyle, 1989 similarly reported that LM grew well on meats with a pH above 6.0, but did not on meats ≤ pH 5.0 (Glass & Doyle, 1989). Growth and survival of LM in the 5.0 to 6.0 pH range has not been well documented prior to this study.

In general, the pH was lower in LA sprayed samples than untreated controls. However the differences were small from 0.05-0.12 units in all products (Table III.4). These results agreed with Gonzalez (2006), who observed that dipping of poultry legs in different lactic acid solutions (1, 2, and 5%) stored at 4°C for 7 days caused a decline in LM counts depending on the concentration.

However, pH differences decreased throughout the storage (Gonzalez-Fandos & Dominguez, 2006a).

Differences in LM growth in bologna, ham souse, and red-hot RTE meat and poultry products before and after application of LA may be due to chemical composition and physical properties, for example different pH, a_w, fat and moisture content, food ingredients, and types and levels of spoilage back-ground micro-flora in addition to meat processing procedures. According to several studies, Lactic Acid Bacteria (LAB), and members mainly of genus *Lactobacillus*, are the main cause of spoilage of processed meat products (Davies *et al.*, 1999; Samelis *et al.*, 2000b; Samelis *et al.*, 2000a). Reducing the reduction-oxidation potential by vacuum-packaging and storage at refrigerated temperature are two of the factors that enhance growth of LAB in this type of products.

The initial counts of LM in bologna, souse, and red-hot were 4.3, 3.2, and 6.3 logs CFU/g, respectively. Whereas, the initial counts for LAB (MRS) agar were 5.1, 4.8, and 6.4 logs CFU/g, respectively. Over time, there was a continuous reduction of LM counts occurred in untreated controls over all storage days (Figure III.5). These findings may indicate that part of the observed decrease in pathogen levels caused by LA could be from the competition with high levels of background micro-flora (LAB), or death of the cells.

Previous researchers have reported similar trends (Geornaras *et al.*, 2006b), for example a strain of LAB (*Lactobacillus sakei*) inhibited growth of LM in cooked ham products (Bredholt *et al.*, 2001). Amezquita & Brashears (2002) concluded that the antilisterial activity of LAB could be competition for nutrients or

byproducts of microbial metabolites with antimicrobial activity, mainly bacteriocin, hydrogen peroxide, and organic acid (Amezquita & Brashears, 2002). Others have found that most of the bacterial population on food products was represented by LM and its growth was not inhibited on surface-treated vacuum-packaged frankfurters during storage at refrigeration temperature (Byelashov *et al.*, 2008). Discrepancies in results may be due to processing producers antimicrobials included in formulations, types and levels of spoilage micro-flora, types of inoculum, and storage conditions of products.

In conclusion, Preventive spraying of RTE bologna, souse, and red hot frankfurters with LA (2%) has antilisterial activity when applied as post-processing antimicrobial solutions in meat products due to its immediate bactericidal activity. This treatment may allow processors to operate under the first alternative of the FSIS final rule (USDA & FSIS, 2003). Because the spraying with LA results in more than 1 log CFU/g reduction of the pathogen, processing plants using this postlethality treatment may be subjected to more frequent FSIS verification testing if a 2 log CFU/g reduction is not achieved (FSIS, 2006). Processors should evaluate higher concentrations and other combinations as they may be more effective (≥ 2 log-reduction) in controlling *L. monocytogenes* during slicing, packaging and storage of processed meats (Samelis *et al.*, 2005). Souse meat processors should determine the typical pH of their product. If the pH is less than 4.39, then 2% LA gives an immediate 2 log reduction in LM (FSIS, 2006). Processors may consider the product formulation to be an effective

antimicrobial agent. Spraying 2% LA adds even greater antimicrobial activity within the first 7 days of storage.

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