Investigating the Effects of Food Grade Antimicrobial Ingredients on the Microbiota of a Meat Model System

By

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THESIS ABSTRACT

The microbial spoilage of meat is a major problem due to product and economic losses. Therefore, it is important to understand how to address spoilage bacteria to reduce meat and meat product losses. Thus, the objectives of this study were 1) to investigate the antimicrobial effect of organic acids (sodium lactate (SL), sodium diacetate (SD), sodium propionate (SP) and their combinations (SL+SD, SL+SP, SD+SP, SL+SD+SP) on the populations of the lactic acid bacteria (LAB) involved in Ready-to-Eat (RTE) meat spoilage, and 2) to examine how these organic acid salts influence the changes in the spoilage LAB microbiota. Cooked vacuum-packaged deli-style turkey breasts manufactured with the different organic acids were inoculated with five spoilage LAB species (namely *Lactococcus lactis*, *Leuconostoc mesenteroides* (bacon and ham isolates), *Enterococcus faecium,* and *Lactobacillus sakei*) and stored at 4ºC for 42 days. Studies were designed to analyze the effect of organic acids on spoilage LAB population using classical microbiology techniques in the model meat system and using Bioscreen C automated Growth Curve Analysis System in broth. A third study was designed to demonstrate the growth dynamics and microbial community composition changes of inoculated bacterial communities in the model meat system study through the use of 16S rRNA sequencing.

Results from microbiological challenge testing revealed the incorporation of SL (SL, SL+SD, SL+SP, SL+SD+SP) in meat formulations extended the lag phase of LAB mixture for up to a week ($P < 0.05$), whereas SD, SP, and SD+SP, did not provide any antimicrobial effect different from control for 42 days of storage ($P > 0.05$). In the microbiota analysis, SD did not show any effect on the composition of LAB mixture compared to the control for 35 days, whereas the addition of SL to the SL+SD containing formulation slowed down the changes in the relative abundance of inoculated LAB microbiota for the first 14 days ($P < 0.05$). Among the LAB species, *L. sakei* was

noted to be competitive and highly adaptive to the environment, being predominant regardless of treatments throughout the 35-day time period. The results demonstrated that the incorporation of SL into formulations satisfactorily impacted microbial spoilage dynamics.

1. CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Meat is an essential nutrition source for humans due to being rich in vitamins, minerals, and proteins, but at the same time, it is easily perishable. Throughout history, humans have developed many techniques to keep meat and meat products fresh and safe while also minimizing waste caused by biochemical and microbiological changes. Cooking, smoking, drying, salting, refrigerating, or packaging are methods that have been used to preserve these products longer. The use of some of these techniques dates back to 2000 BC (Jay et al., 2005). The spoilage microorganisms and their role in meats were probably unknown until A. Leeuwenhoek observed their presence via a microscope in 1683 (Jay et al., 2005). At that era, Nicholas Appert wanted to develop a method to store products where the foods were placed into bottles with cork and heated in a water bath to destroy bacteria and reduce further contaminations, which created a highly effective food preservation by canning practice (Appert, 1810, 1831). In 1810, he published his patented findings which proved that products can maintain their flavor, color, quality, and reduce spoilage with that method (Appert, 1810). In 1864, Louis Pasteur was accepted as the first person who demonstrated that food spoilage was caused by microorganisms, that heat application could destroy these microbes, and that recontamination from the air was reduced by using sealed containers to preserve food products.

In addition to heat application, refrigeration has evolved as a fundamental food storage method whereas the low temperature effectively reduces the growth rate of the bacteria, reducing the spoilage rate of the food/meats (Borch et al., 1996). In the past, ice was used as a cold storage material while with the technology developments, the first refrigeration machine in 1834, and the first refrigerators for home use were manufactured in the 1910s (Holladay, 1994). Inexpensive

household refrigerators have become purchasable and available for normal home-use since 1940 such that the storage of foods has been significantly increased. Later, other innovations for food preservation techniques have been arisen including artificial drying, vacuum packaging, ionizing, radiation, biopreservation, and chemical preservation (Soomro et al., 2002).

Further developments in preservation methods, changing consumption habits due to modern lifestyle, and a greater demand for processed foods have increased the availability and consumption of meat and meat products in connection with globalization and overseas shipments. Fifty-two billion pounds of meat and 48 billion pounds of poultry were produced only to feed Americans in 2017 (NAMI, 2017). During that time, 2.2 million sheep and lambs, 32.2 million cattle and calves, 121 million hogs, 241.7 million turkeys, and 9 billion chickens were processed by meat and poultry industry in US (NAMI, 2017). However, waste and losses have become more common and the most severe ones have been especially seen in developed countries with the high availability of meat and meat products (Gustafsson et al., 2013). This is particularly explained by retailers and consumers in Europe and the United States, with high consumer availability of the meat and meat products. The waste at consumer stage in EU and US accounts for about half of the overall meat losses and waste (Gustafsson et al., 2013). Meanwhile, about 820 million people worldwide have suffered from hunger, and about 2 billion people cannot reach regularly to safe, sufficient and nutritious food (WHO, 2019).

The major factor for processed meat product losses arises from microbial contamination and related deterioration of the meat and poultry products, which causes significant economic losses for the food industries. Improvements in preservation of food systems has necessitated new antimicrobial approaches in response to the latest consumer demands and the food industry needs

without sacrificing important sensory or quality properties. Therefore, the food preservation process starts with the elimination or control of microorganisms.

Many of the sliced and vacuum-packaged meat products like hams, sausages, and turkey are still under the risk of recontamination with spoilage and pathogenic flora during slicing and packaging even though cooking or pasteurization is applied. Despite extensive food safety research and improved food preservation methods such as heating, drying, freezing, packaging, and the addition of antimicrobial agents, or other chemicals, microbial spoilage problems in the meat and poultry products are still encountered. (Brul et al., 2002; Guerrero-Legarreta, 2014). This has created a demand for efficient preservative methods and technologies that will help improve the safety, freshness, and wholesomeness for meat and poultry products for a long time.

The application of organic acids as antimicrobial agent is a commonly used approach for pathogens in the food industry to increase shelf life. Their usage has been evaluated for decontamination and preservation of RTE meat products, and promising results have been achieved in inactivating post-processing bacterial contamination (Barmpalia et al., 2004; Geornaras et al., 2005; Byelashov et al., 2008). The use of organic acids resulted in the reduction of bacterial flora and extension in the shelf life of meat and meat products with prolonged bacteriostasis during storage (Smulders et al., 1986; Palumbo & Williams, 1994; Barmpalia et al., 2004; Dubal et al., 2004). This bacteriostatic effect gains significant importance for processed meat products owing to being RTE and the long shelf life. However, the effects of organic acids on lactic acid spoilage bacteria in commercial practice are not well known. As such, the use of organic acids in meat and poultry products that are sliced or packaged after a heat treatment (Debevere, 1989) and understanding the behaviors of spoilage bacteria against organic acids (Brul & Coote, 1999) might be promising to improve current preservation methods and to extend shelf life.

Further, with the application of new technologies such as next-generation sequencing techniques, the characteristics and dynamics of microbial spoilage of the processed RTE meat and poultry products will be better understood under selected conditions.

The hypothesis of the dissertation is that each microorganism has different sensitivity or response against different antimicrobial ingredients. The use of different organic acid salts in the RTE meat product formulations can affect the growth dynamics and microbial community structure of spoilage lactic acid bacteria and helps to improve shelf life and meat safety.

The objective of this study was to demonstrate the differences in the growth dynamics and microbial community structure of lactic acid bacteria commonly found in RTE meat products while applying similar concentrations of organic acids used for Listeria monocytogenes and to correspondingly help the extension of shelf life. More specifically, the study targeted to accomplish the following objectives:

1. Determine the influence of different organic acids on the shelf life and the inoculated spoilage lactic acid bacteria population of the cured, cooked, sliced, and vacuum packaged turkey breast throughout the storage time by utilizing classical microbiology techniques.
2. Determine the effect of various level of organic acids on the growth dynamics of individually inoculated spoilage lactic acid bacteria in a modified broth by using Bioscreen C automated Growth Curve Analysis System.
3. Determine the impact of different organic acids commonly used in meat products on the microbiota of inoculated spoilage lactic acid bacteria during the storage time by utilizing high throughput 16S rRNA genetic sequencing.

In the long term, this research will contribute to developing new methods to extend shelf life of processed meat products while maintaining their high quality by reducing the meat product

losses due to microbial contamination risk. Moreover, with the combination of new technologies such as the application of next-generation sequencing techniques, metabolomics, and bioinformatics, the effect of organic acids and the interactions between spoilage LAB in meat microbiota will be better understood.

ORGANIC ACIDS AS ANTIMICROBIAL

Organic acids have been used as antimicrobials for a long time to avoid spoilage and extend the shelf life of perishable foods (Ricke, 2003). They have been utilized effectively in controlling microbial contamination and transmission of foodborne pathogens in post-harvest food production and processing when used as food antimicrobials or sanitizers of food and/or equipment surfaces (Dubal et al., 2004; Samara & Koutsoumanis, 2009; Toldrá, 2010). In addition to food preservatives, organic acids are considered also as food ingredients, widely found in nature as normal constituents of plants and animals, and mostly and naturally produced by microorganisms (Gauthier, 2005). FDA accepts organic acids as antioxidants, flavoring agents, acidulants, pH adjuster, and even nutrients in addition to preservation purposes (USDA-FSIS, 2013).

Mainly, there are two forms of organic acids; pure acids and salts. While lactic acid, propionic acid, and acetic acid are pure acids, their calcium, sodium, or potassium components are salt forms (Smulders & Greer, 1998). Similar to organic acids, the salt forms also have the same antimicrobial characteristic. Organic acids exhibit both bactericidal and bacteriostatic effects whereas some salt form, lactic acid, and sorbic acid salts, tend mainly to behave bacteriostatically (Smulders et al., 1986). Whereas the salt forms are directly added in formulations of different ready-to-eat (RTE) meats, organic acids are applicable to decontaminate the surface of fresh meat and meat products by spray, dip or wash to eliminate outgrowth of spoilage and pathogenic

microorganisms. Additionally, salt forms have some advantages over acids. They are odorless, less corrosive, and safer to handle and easier to apply in the machinery due to their solid and less volatile structure (Huyghebaert et al., 2011). Organic acids as an aqueous solution at the level of up to 2.5% have been accepted as a part of carcass wash applied pre-chill for the elimination of microbial pathogens on meat carcasses in the US (USDA, 2015). Various research findings have indicated the form of dipping solutions of organic acids may improve the microbiological safety and quality of raw meat and poultry products and carcass parts prior to packaging (Barmpalia et al., 2004; Geornaras et al., 2005; Byelashov et al., 2008). Meat carcasses decontaminated with lactic acid have had a negligible effect on the sensory properties (Pipek et al., 2004, 2005).

The application of organic acid salts is also adequate in limiting the growth of bacteria during storage of RTE meat products. Sodium lactate (SL), sodium acetate (SA), or their combinations, for example, are commonly applied in RTE meat and poultry products to restrict the growth of pathogenic bacteria (*Listeria monocytogenes*) (Tompkin, 2002). The addition of SL or the combination of SL and sodium diacetate (SD) in the RTE meat product formulations such as wieners, cooked bratwurst (Glass et al., 2002), beef bologna (Mbandi & Shelef, 2002), beef sausage (Bingöl & Bostan, 2007), and cooked beef roasts (Miller & Acuff, 1994a), extend shelf-life and inhibits the growths of pathogens such as *Listeria monocytogenes*, *Salmonella enterica, Salmonella typhimurium*, and *Escherichia coli* 0157:H7. Glass et al. (2007) demonstrated that propionate containing ingredients limits the growth of *L. monocytogenes* in cured deli-style turkey breast and can be used as another option to lower sodium-based salts while sustaining food safety (Glass et al., 2013). Adding 0.9% SL and 0.09% lactic acid to marinated chicken showed that the proliferation of spoilage microorganisms would be delayed and the shelf life would be prolonged during storage (Smaoui et al., 2012). In summary, individual or combination of salt of organic

acids delays the growth of psychrotrophic spoilage microorganisms such as lactic acid bacteria and extends the shelf life of meat products (Serdengecti et al., 2006).

MODE OF ACTION

Antimicrobial properties of the organic acids are impacted by many factors; types and physiological status of microorganisms (growth phase; lag, log or stationary), possible interactions between microorganisms in the system, the type and concentration of the acid, the exposure time, available nutrition to microorganisms, and the physicochemical conditions (temperature, pH, water activity, etc.) of the meat and meat products (Ricke, 2003; Lianou et al., 2012; Odeyemi et al., 2020). The exact role of the antimicrobial activity of the organic acids are hard to determine due to the mentioned factors and also the complex nature of molecular interaction affecting cell metabolism in connection with external factors. In the majority of applications, the action has been shown bacteriostatic rather than bactericidal, inhibiting growth instead of destroying the bacteria. However, if the concentration of acid is high enough, that induces cell death rather than growth inhibition (Stratford & Eklund, 2003; Serrazanetti et al., 2013). There are various hypotheses offered to explain the mode of action of the antimicrobial activity of organic acids. Some possible ways suggested demonstrating the growth inhibition of organic acids; the intracellular pH drop, anion accumulation, and energy depletion (Russell, 1992).

Effects of pH

Early studies have suggested understanding intracellular and extracellular pH is the key (Booth & Stratford, 2003; Davidson et al., 2012), which defines not only the bacterial community that will predominate during shelf life but also their activities as spoilage organisms (Huyghebaert

et al., 2011). Although all bacteria have their optimum, minimum, and maximum pH value for growth, they are usually fastidious and favor to grow at a pH near neutrality (pH 6.5 to 7.5). For instance, bacteria mainly spoil protein-rich foods like meat and meat products with a pH range of between 5.5 and 6.5. Reducing the intracellular pH below neutrality is an efficient way of restricting microbial growth and metabolic activity (Booth & Stratford, 2003). Organic acids play a role as effective food preservatives below pH 4.5 with both bacteriostatic and bactericidal effects.

The antimicrobial effect of organic acid highly depends on their dissociation level (Serrazanetti et al., 2013). Organic acids and their salt forms are known to be weak acids which partially dissociate in the water into undissociated/protonated/acid or dissociated/anion form. Dissociation of the organic acid is controlled by environmental pH and pKa of the acid (Davidson et al., 2012). pKa is the pH at which 50% of the acid is dissociated. Antimicrobial activity usually occurs when pH is lowered to or below pKa. This is maybe because low pH leads to an increase in the proportion of undissociated acid molecules (Mani-López et al., 2012).

Undissociated acids are the most effective as antimicrobials. They are lipophilic and uncharged, allowing them to pass through the microbial cell membrane by simple diffusion. Their behavior is simply illustrated in Figure 1.1. Salmond et al. (1984) and Russell (1992) proposed the primary toxic effect of organic acids comes from undissociated acid accumulation. Low extracellular pH increases the proportion of undissociated acid molecules outside the cell. That creates a proton gradient and triggers the flow of the undissociated acid into the cell. Upon reaching the neutral pH inside the cell, the undissociated acids dissociate into charged ions (lipid insoluble anions and protons) that cannot pass the plasma membrane on their own and acidify the cytoplasm (Ricke, 2003; Stratford & Eklund, 2003; Davidson et al., 2012). This process continues until equilibrium is reached by the pH gradient across the membrane (when the inner and outer

concentrations of the undissociated acid are equal) that eventuates in the aggregation of anions and protons in the cytoplasm (Booth et al., 1989; Hirshfield et al., 2003).

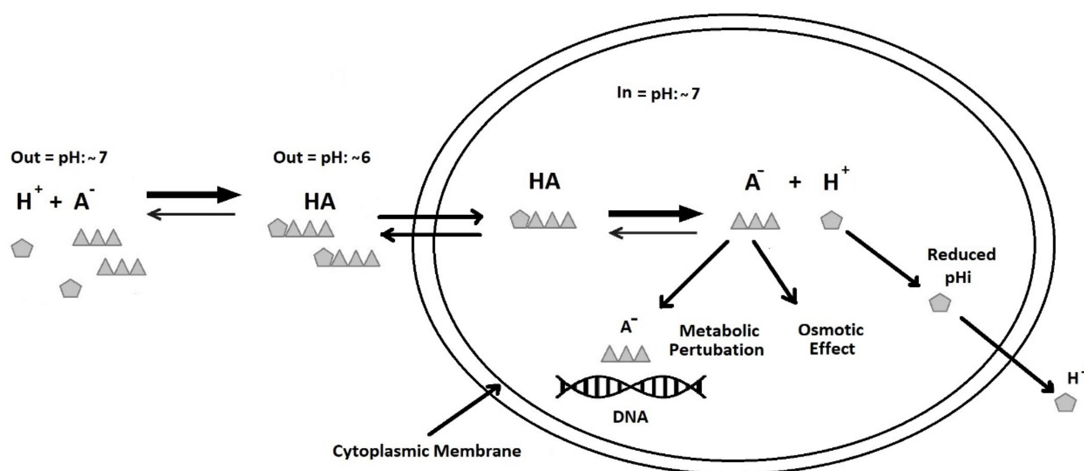

Figure 1.1 Behavior of the organic acids and ions inside and outside the microbial cell

As these two ions accumulate, protons generate pH stress by reducing internal pH, while anions create osmotic stress, so bacteria may experience metabolic problems and perturbation, resulting in growth inhibition or cell death depending on the severity of internal and external conditions. pH hemostasis inside cells is crucial for cells that need to maintain a near-neutral pH cytoplasm for growth and metabolic activities such as molecule translocation, DNA replication, the regulation of ATP, RNA and protein synthesis, (Booth et al., 1989; Davidson et al., 2012). However, the exact role of the organic acids inside the cell is hard to determine due to the complex nature of the molecular interactions (Ricke, 2003).

pH gradient (ΔpH) and pH Regulation in LAB

The pH gradient (ΔpH) between the cytoplasm and the medium determines the concentration of the proton released into the cytoplasm. A greater inhibitory impact is expected at

greater ΔpH. However, large ΔpH can be avoided by allowing a lower intracellular pH by bacteria. However, bacterial growth still can be limited despite this adaptation mechanism since most of the biologically important molecules such as proteins and enzymes related to ATP production and biosynthetic reactions have optimum activity or stability around neutrality (Padan et al., 1981).

The greatest life-threatening event for LAB is being unable to generate energy. Fermentative glycolysis is disrupted by cytoplasmic acidification, which inhibits enzymes and decreases energy synthesis (Konings, 2002). LAB can generate energy, in addition to glycolysis, through the application of proton-motive force (PMF) generating membrane transport systems. Most of these transport mechanisms keep the internal and external pH from lethal decrease and play an important role in pH homeostasis and control. The internal pH for most LAB is above pH 7 during glycolysis (Poolman et al., 1987), which allows all lactate to dissociate anionic form (lactate$^-$) and creates a concentration gradient between inside and outside the cell. A specific protein transport system is required for lactate- transportation because it cannot pass via diffusion but by an electrical gradient until the PMF dissipate (Russell, 1992). Lactate$^-$ is thrown out the cell, accompanied by at least two protons during the efflux (Otto et al., 1980; Driessen & Konings, 1990). This excretion is beneficial for both alkalization of cytoplasm and gaining energy (from 0.66 to 0.5 ATP per lactate). When the internal pH decreases, the undissociated form will increase and the lactate$^-$ concentration will be low in the cytoplasm. Consequently, lactate- efflux and energy gain will decrease (Konings, 2002).

Overall, a decrease in the intracellular pH can limit the bacterial growth and energy synthesis (Cook & Russell, 1994). The importance of regulating pH and maintaining a small ΔpH is crucial at this point (Siegumfeldt et al., 2000). Accumulated protons must be removed to establish pH hemostasis and overcome pH stress. Therefore, bacteria have evolved in different

ways to survive under pH stress; decrease the intracellular pH or maintain a constant pH gradient(ΔpH). While neutrophilic bacteria tend to keep their internal pH close to neutral even though large proton gradients between cell cytoplasm and environment, many acid-tolerant bacteria such as lactic acid bacteria regulate internal pH to keep pH gradient constant (Booth, 1985; Cook & Russell, 1994; Siegumfeldt et al., 2000).

Microorganisms are generally more resistant to external pH variations than internal pH variations (Booth & Stratford, 2003). The microorganism may eventually adapt to the environment and regulate its internal pH to a point where the cell may be able to continue its growth. This adaptation period can be referred to as the "lag phase" (Lambert & Stratford, 1999). There are several possible mechanisms by which a bacterium can resist the negative impact of the pH stress including loss of glycolytic enzyme activity responsible for producing ATP, damage of cell membrane structure, and macromolecules such as DNA and proteins (Cotter & Hill, 2003). These mechanisms counteract the cytoplasmic pH decrease by the removal of protons, alkalization of the external environment, cell membrane changes, stress proteins, and chaperones production (Cotter & Hill, 2003).

Proton pumps (F_1F_0-ATPase)

Free protons in the cytoplasm must be exported outside of the cell. However, as stated in chemiosmotic theory, the cell membrane has a low proton permeability regardless of external pH generating a charge gradient across the membrane resulting in an electrochemical potential (ΔΨ), which is a proton-motive force (PMF) (Miller & Acuff, 1994; Ricke, 2003; Davidson et al., 2012). Protons cannot pass cytoplasmic membrane freely on their own and their elimination from the cell requires ATP depletion by active transport, but this limits the energy amount that could be used

for growth (Russell, 1992). Acidification of the cytoplasm cannot be stopped in the presence of large concentrations of organic acid in the cell despite continued proton extrusion because protons may no longer be expelled by the cells quickly enough to alkalinize the cytoplasm (Kashket, 1987; Russell, 1992). The most important mechanism for proton export in fermentative bacteria appears to be the proton-translocating ATPase (H+-ATPase, F_1F_0-ATPase). It has been suggested that the activation of H+-ATPase proton pumps activation and the fraction of synthesized subunits assembled into H+-ATPase increase at low intracellular pH (Kobayashi et al., 2000; Cotter & Hill, 2003). The optimum internal pH range for this enzyme was found to vary between 5 to 7.5 in lactic acid bacteria (Nannen & Hutkins, 1991). Lambert and Stratford (1999) showed in their mathematical model that the duration of the lag phase increases along with the time required for proton export depending on H+-ATPase activity. As experimentally verified, gradual internal pH increases with proton removal from the cell ends the metabolic inhibition and the lag phase, and that stimulates the cell growth (Theron & Lues, 2007). Stratford and Anslow (1996) demonstrated very extended lag phases in microorganisms by acetic acid at low pH, however, these lag phases were shorter at neutral pH (Lianou et al., 2012).

Cytoplasm Buffering Capacity

Cytoplasm buffering capacity is offered as an additional mechanism to eliminate protons. Proton concentration also changes as a result of its consumption during metabolic reactions in the cell. The proton coming from the acid may be absorbed by cytoplasm buffering capacity via amino acid decarboxylation (Hutkins & Nannen, 1993). Glutamate decarboxylase (GAD) has been found that it was associated with controlling the pH of gram-positive cells. GAD combines an internalized glutamate with a proton inside the cell and converts into more alkaline GABA (γ-

aminobutyrate) which then will be exported outside the cell via an antiporter. As a result, an increase in cytoplasmic pH and extracellular pH is observed. However, the increase in extracellular pH is very low. The buffering capacity in most bacteria is thought to have a slight effect on the internal pH regulation (Cotter & Hill, 2003).

Alteration of Metabolic Pathways

Meat and products are not rich in carbohydrates. Carbohydrates can be found in meat products if they are added to the formulation during production. Even though a rich carbohydrates environment, low pH presents a particular challenge. The bacterium can shift energy metabolism under acid stress from carbohydrate utilization to amino acid catabolism (Cotter & Hill, 2003; Serrazanetti et al., 2011). Nonetheless, the uptake of amino acids requires a large fraction of the metabolic energy, consequently, priority is given to survival rather than growth. The higher amino acid conversion instead of carbohydrate utilization has been shown by the decrease of lactate in both *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* metabolism (Serrazanetti et al., 2011; Vrancken et al., 2011). Additionally, the proteins related to amino acid metabolism have been upregulated while some proteins related to cell division and carbohydrate degradation were downregulated in *L. plantarum* (Even et al., 2002; Vrancken et al., 2011). Furthermore, amino acid carriers are also sensitive to pH. In *Lactococcus lactis*, the transport rate of alanine increased as the intracellular pH decreased. However, glutamate transport can reduce by pH drop. *L. lactis* does not allow intracellular pH significantly reduced as glutamate is essential for growth. Despite the sensitivity of the glutamine and asparagine transport system to pH*, Lactobacillus casei* allows intracellular pH reduction because these amino acids are not as important for *L. casei* (Kashket, 1987; Strobel et al., 1989).

Nevertheless, if the acid amount is high enough, then none of these mechanisms can handle excess protons resulting in a severe decrease in internal pH (Booth & Stratford, 2003). This decrease in pH causes stress on intracellular pH homeostasis resulting in drainage cellular energy resources by catalytically impairing proton-motive force, membrane disruption, and inhibition of essential metabolic reactions (Ricke, 2003; Axe & Bailey, 1995; Diez-Gonzalez & Russell, 1997).

Growth Inhibition by Anion

Later studies, however, indicated that lower pH could not be the sole determinant of growth inhibition. The minimum growth pH was 4.05 when used HCl and citric acid; however, it was 4.4, 5.4, and 5.5 for lactic, acetic, and propionic acids respectively (Chung & Goepfert, 1970). That shows that the organism can change its external surroundings to a more favorable range when used HCl and citric acids compared to the other acids (Jay et al., 2008). It has also been shown that different weak acids prevent *L. monocytogenes* growth to varying degrees, even though the internal pH is the same. Growth inhibition may also result from the accumulation of the weak acid anion inside the cell cytoplasm (Russell & Diez-Gonzalez, 1998). When the ΔpH increases, the concentration of organic acids within the cell increases. The concentration of anions increases via acid dissociation in the more alkaline cytoplasm. In addition, an increase in ΔpH will also induce an accumulation of fermentation acid anions. Cook and Russell (1994) reported a logarithmic correlation between ΔpH and anion accumulation. Only a small increase in ΔpH can cause a drastic increase in the concentration of anions to reach a toxic level. Accumulation of the anion at a toxic level can cause a variety of potential problems for the cell. First, it causes osmotic stress increasing cytoplasmic osmolarity, and as the flow of water into the cell increases, the turgor pressure can potentially reach a lethal level. The cell may compensate for this by reducing the concentrations

of other solutes in the cytoplasm (Roe et al., 1998). Secondly, the high levels of anions accumulation in the cytoplasm might inhibit DNA synthesis and cell division and disrupt metabolic reactions by influencing the enzyme activity (Salmond et al., 1984; Russell, 1992; Hirshfield et al., 2003).

THE CHEMICAL PROPERTIES OF WEAK ACID SALTS

Sodium Lactate (SL)

Sodium lactate ($C_3H_5NaO_3$, Mw =112.06 g/mol), also known as E325, Sodium DL-lactate, or sodium 2-hydroxypropanoate, is a sodium salt of inactive lactic acid (PubChem, 2020b). It is obtained from natural L (+) lactic acid which has pKa=3.86. SL is manufactured using a chemical process where sodium hydroxide (an alkali material) is added to lactic acid by neutralizing it to create sodium salt (Houtsma, 1996). SL is hygroscopic and primarily found in water with a lactate content of 60% (w/w) with neutral pH (Shelef, 1994; Houtsma, 1996). It is an odorless and colorless liquid (PubChem, 2020b). SL is accepted as generally recognized as safe (GRAS) at 21 CFR 184.1768 for use in foods as direct food ingredients with no restriction except good manufacturing practices (Shelef, 1994; USDA, 2015). SL is utilized as a pH control agent, emulsifier, humectant, and flavor enhancer with a slight salty taste in meat and poultry products and helps to improve cooking yields, water-holding capacity, juiciness, and color stability (Shelef, 1994; Doores, 2005; USDA, 2015). The recommended level of lactate based on the final weight of the product is 2.0% (3.3 of the 60% solutions), and it is applied in ready-to-eat products (Shelef, 1994). When SL was added to cooked beef roast up to 4.0%, it affected positively the meat with improved juiciness and tenderness and resulted in darker red with a less gray surface (Houtsma, 1996).

SL has been employed effectively as an antibacterial substance, and there is increasing evidence that it has been effective in limiting the growth of aerobes and anaerobes in meat products (Shelef, 1994; Doores, 2005). It is possible to see recontamination of cooked meat products with a flora during handling, such as slicing and packaging after heat application despite the application of salt, nitrite, and refrigeration, therefore; the inclusion of SL in the process contributes to the extension of the shelf life further (de Wit & Rombouts, 1990). SL has been permitted in fully cooked meat, meat food products, poultry, and poultry food products no more than 4.8% of the total product weight to eliminate the growth of certain pathogens such as *Clostridium botulinum* and *L. monocytogenes* (USDA-FSIS, 2000). De Wit and Rombouts (1990) showed SL inhibited the growth of *Streptococcus faecalis, Staphylococcus aureus,* and *Salmonella typhimurium* by prolonging the lag phase and lowering the maximum growth rate. 3.0% and 4.0% SL in the cooked beef roast was successful in limiting the proliferation of *E. coli* 0157:H7, *S. typhimurium*, and *L. monocytogenes* (Miller & Acuff, 1994). It has been shown that 1.8% SL reduced the growth rate of *Salmonella enteritidis* and *L. monocytogenes* in beef with pH 6.3 and 79% moisture (Mbandi & Shelef, 2001). Houtsma et al. (1994) demonstrated that the inhibition of toxin production from *C. botulinum* was dependent on the incubation temperature in peptone-yeast extract medium (pH 6.1). 2.0% and 2.5% SL retarded the toxin production at 15 and 20°C respectively while 3.0%, 4.0%, and > 4.0% SL completely inhibited toxin production at 15, 20, and 30°C respectively. 2.0% SL prolonged the latency time and reduce the growth rate of *L. monocytogenes* on vacuum-packaged turkey bologna artificially contaminated (2 to 3 log CFU/g) (Wederquist et al., 1994, 1995).

SL is also promising for the inhibition of spoilage bacteria in addition to pathogens. The usage of 2.5% to 3.3% SL in cooked cured ham products stopped the growth of *L. curvatus* and extended the shelf life without negative sensory changes (Stekelenburg & Kant-Muermans, 2001).

They concluded the presence of SL in the product prolonged the lag time of *L. curvatus* and reduced the growth rate. Drosinos et al. (2006) also showed that the level of 4.0% SL or higher completely inhibited the growth of *L. curvatus* in MRS at 4°C for 14 days. The concentrations of 2.0% SL and 3.0% SL had a less inhibitory effect on *L. curvatus*, whereas 1.0% SL did not stop the growth like higher concentrations, however, it delayed the growth of *L. curvatus*. McDonald et al. (1990) found when the intracellular pH decreased to between 4.6 and 4.8, *Lactobacillus plantarum* stopped growing and these pH values were between 5.4 and 5.7 for *Leuconostoc mesenteroides.*

Sodium Diacetate (SD)

Sodium diacetate ($CH_3COOH.CH_3COONa$, Mw= 141.08 g/mol), also known as 126-96-5, M913, or E262, is a white water-soluble powder salt with a crystalline structure and bound compound of sodium acetate and acetic acid (42.25%). In water, 42.25% acetic acid available in SD is released (Shelef & Addala, 1994; PubChem, 2020a). There are two types of production methods for SD. The reaction of sodium carbonate with acetic acid is the technical grade production method, while the reaction between anhydrous sodium acetate and acetic acid results in special grade SD. It has an acetic acid odor and is used as a seasoning to impart salt and vinegar flavor.

This food additive meets the specifications of the Food Chemicals Codex and is generally recognized as safe (GRAS) to be used as an adjuvant, pH control, flavoring, and more importantly antimicrobial agent (CFR, 2019). The growth of *L. curvatus* was inhibited by the addition of SD to MRS broth at level 0.5%, 0.75%, and 1.0% resulting in the same inhibition degree, however, 0.25% SD did not stop the growth of *L. curvatus* (Drosinos et al., 2006). Stekelenburg & Kant-

Muermans (2001) reported although the addition of 0.2% SD in the formulation of ham product inhibited *L. monocytogenes*, the application of 0.1% and 0.2% SD did not stop the growth of *L. curvatus*. Additionally, the usage of 0.2% SD in the formulations showed an undesirable effect on the taste and odor of the ham product. Shelef & Addala (1994) determined the minimum inhibitory concentration (MIC) of SD for *L. monocytogenes* in the BHI broth system, where interaction was demonstrated between acid concentration and temperature. MIC reduced with the decrease in the incubation temperature. Broth pHs containing 35mM at 35°C, 32mM at 20°C, and 28mM at 5°C were 5.25, 5.40, and 5.60 respectively. They demonstrated that SD was more successful than acetic acid alone in suppressing the growth of microorganisms over the pH range of 5 to 6, and the inhibitory effect was attributed to the SD rather than the only pH. Along with the *L. monocytogenes*, SD inhibited the growth of *Salmonella enteritis, Escherichia coli, Shewanella putrefaciens*, and *Pseudomonas fluorescens*, whereas; *Enterococcus faecalis, Staphylococcus aureus, Lactobacillus fermentis, P. fragi,* and *Yersinia enterocolitica* were insensitive to the antimicrobial compound (Shelef & Addala, 1994).

(USDA-FSIS, 2000) (9 CFR 424.21) has approved SD (pKa, 4.75) for use in processed meat and poultry products no more than 0.25% by weight of the total formulation. FSIS concluded that the growth of *L. monocytogenes* in meat and poultry products was effectively inhibited by the SD level of up to 0.25 (CFR, 2019). *L. monocytogenes* populations in blue crab meat washed with 2M SD reduced 2.6-log10 units/g within six storage days at 4ºC (Degnan et al., 1994). It has been reported the use of 0.25% SD in cook-in-bag turkey breasts retarded the spoilage to occur until 12 weeks (Meyer et al., 2003).

SD is also in usage with other potential antimicrobials in addition to individual usage. Barmpalia et al. (2004) demonstrated frankfurters containing a combination of SL (1.8%) and SD

(0.25%) did not allow *L. monocytogenes* to grow throughout the shelf life, however; SL (1.8%) and SD (0.125%) inhibited *L. monocytogenes* completely for the first 8 days of storage. Additionally, they also reported subsequent growth was markedly decreased. Bedie et al. (2001) tested the effects of sodium lactate (3.0% or 6.0%), sodium acetate (0.25% or 0.5%), and sodium diacetate (0.25% or 0.5%) in frankfurters which were surface inoculated (10^3 to 10^4 CFU/cm^2) with *L. monocytogenes*. It was observed 6.0% SL and 0.5% SD in the frankfurter formula were bactericidal for *L.monocytogenes* throughout the shelf life at 4ºC (120 days), while 3.0% SL was bacteriostatic for at least 70 days. On the other hand, others (SD at 0.25% and SA at 0.5 and 0.25% in decreasing order of effectiveness) that were less effective inhibited the pathogen only for 20 to 50 days. Mbandi & Shelef (2001) tested SL (1.8 and 2.5%), SD (0.1% and 0.2%), sodium acetate (SA) (0.2%), and their combinations in comminuted sterile beef inoculated with *L. monocytogenes* and *S. enteritidis*. They demonstrated that the growth rate of both *L. monocytogenes* and *S. enteritidis* was reduced by the addition of 1.8% SL. Moreover, 0.2% SD was more successful than sodium lactate in inhibiting *L. monocytogenes*. The mixture of 0.2% SD and 2.5% SL was bactericidal to *S. enteritidis*, and bacteriostatic to *L. monocytogenes* after 20 days at 10°C. The combination of 0.1% SD and 1.8% SL produced a bacteriostatic effect, whereas *S. enteritidis* had less than 10 cells/g after storage at 5ºC for 30 days.

Sodium Propionate (SP)

Sodium propionate (C3H5O2Na, Mw = 96.06), also known as sodium propanoate, 137-40-6, or propionic acid sodium salt, is a salt form of propionic acid and consist of equal numbers of sodium and propionate. The propionates are obtained by the neutralization of propionic acid with sodium hydroxides. It is freely soluble in water and ethanol and has a faint acetic-butyric odor

and a slight cheese-like flavor (PubChem, 2020c). SP has a pKa 4.87 and at a pH 6, only 6.7% is undissociated, while at pH 4, 88% of SP is undissociated (Jay et al., 2008). SP is used in low dosage to keep the organoleptic quality of food due to pungent odor (Søltoft-Jensen & Hansen, 2005). SP (21 CFR 184.1784) is approved as generally recognized as safe (GRAS) materials for various food and meats. Additionally, it is listed as antimycotics when migrating from food-packaging material (21 CFR 181.23). SP is used mainly as mold and rope inhibitors in bread. Although most mold is killed during cooking, products can be contaminated during packaging, and spoilage can be seen during storage (Doores, 2005). SP has been demonstrated to stop the growth of some gram-positive pathogenic bacteria, such as *L. monocytogenes, Clostridium botulinum* in meat as well as media systems (El-Shenawy & Marth, 1989; Rebollo et al., 1997). Cherrington et al. (1991) demonstrated that *Escherichia coli* K12 and *Salmonella* spp. were inhibited by 0.5 to 0.7 M propionic acid at pH 5.0 in 1 hour. When 5mM propionic acid into incubating cultures, it produced bacteriostasis on *E. coli* lasting 30 min, and the rate of DNA, RNA, protein, lipid, and cell wall synthesis was reduced. After 30 min, the growth resumed in the presence of the acid, however, cells from the cultures treated with acid were larger than controls (Cherrington et al., 1990). The combination of SL and 0.1% or 0.2% SP in both beef top rounds and patties extended the shelf life and improved the sensory properties (Maca et al., 1997a, b). The growth of *L. monocytogenes* was significantly inhibited in cured ham and uncured turkey breast stored at 4°C for 12 weeks when the RTE products were formulated with $\geq$ 0.2% propionate or a combination of 0.1% propionate and 0.1% sorbate (Glass et al., 2007). Hu & Shelef (1996) studied the behavior of *L. monocytogenes* in pork liver sausages made with different fat contents and 1.8% SD and 0.2% SP. They demonstrated the antilisterial effect of propionate and lactate raised by the increment in fat content.

LACTIC ACID BACTERIA (LAB)

Microorganisms display varied tolerances to organic acids. Some fermentative microorganisms such as lactic acid bacteria (LAB) may be more tolerant to reduced internal pH than other bacteria species. LAB can naturally acidify their environment to below 5.0. Extracellular pH drops more than intracellular pH because protonated lactic acid produced by fermentation are exported quickly with the carrier-mediated process (Hutkins & Nannen, 1993). LAB is a group of gram-positive bacteria and an important part of the fermentation and preservation process. They are not only tolerant of weak acids but also produce them as a by-product of their metabolism. Some acids, such as acetic acid, are critical to the metabolism of the lactobacilli but inhibitory to bacilli.

LAB are commonly considered safe in the food industry and are used as a starter for the production of safe, high quality, fermented, or cooked meat products. Besides their potential benefits, certain strains are responsible for meat spoilage, off-odors and off-flavor formation, color alteration, gas production, ropy slime formation, and shelf life reduction (Korkeala & Björkroth, 1997). There are two major types of LAB, homofermentative lactobacilli and heterofermentative leuconostocs frequently responsible for spoilage of vacuum-packaged cooked cured meat products (Holley, 1997; Korkeala & Björkroth, 1997; Chenoll et al., 2007), entailing a significant financial loss to manufacturers. Three LAB species, *Lactobacillus curvatus, Lactobacillus sakei,* and *Leuconostoc mesenteroides ssp mesenteroides* have been regularly reported to dominate in the spoilage of cooked vacuum-packaged meats (Dykes et al., 1994; Yang & Ray, 1994; Samelis et al., 2000). Samelis et al. (1998) investigated the composition and origin of the spoilage flora of refrigerated vacuum-packaged cooked ham, as a consequence, it was seen that the major causative agents of spoilage LAB were *L. sakei* and *L. mesenteroides ssp mesenteroides* due to

recontamination in the cutting room. Kalschne et al. (2015) investigated the colonization of spoilage LAB in sliced vacuum-packaged cooked ham stored at 4 °C and 8 °C for 45 days. *Leuconostoc/Weissella* sp (60.0%), *Enterococcus* sp. (24.0%), and homofermentative *Lactobacillus* sp. (16.0%) were the predominant LAB on the first day of storage; however, the predominant LAB was homofermentative *Lactobacillus sp.* (73.0% at 4 °C and 63.0% at 8 °C) followed by Leuconostoc/Weissella sp (27.0% at 4 °C and 37.0% at 8 °C) at 45 days of storage. They also reported that the predominant species were mainly *L. curvatus* followed by *L. sakei and L. mesenteroides*, nevertheless, the *Enterococcus sp.* was not present in the samples at 45 days of storage. Chávez-Martínez et al. (2016) showed that the strains isolated from the spoilage flora of sliced cooked ham were 23.8% thermophilic Lactobacillus, 23.8% mesophilic Lactobacillus, 28.6% Enterococcus, 14.3% Lactococcus, and 9.5% Streptococcus. Chenoll et al. (2007) demonstrated that LAB consisted of Lactobacillus (37.0%), Leuconostoc (43.0%), Carnobacterium (11.0%), Enterococcus (4.0%), and Lactococcus (2.0%) as dominant microorganisms in vacuum-packaged cooked and refrigerated meat products. It is also reported that the microbial population of spoiled products was dominated by *L. mesenteroides*, was always present at the moment bloating occurred, and also *L. sakei*, *L. plantarum,* and *L. curvatus* were found in the decreasing order of abundance. Barakat et al. (2000) also reported that *L. lactis* and *E. faecalis* were found to be part of the dominant microbiota of cooked and modified atmosphere packaged chicken meat stored at 3.5°C for up to 7 weeks. Samelis et al. (2000b) demonstrated that *L. sakei subsp. carnosus* predominated in the spoilage flora of sliced, vacuum-packaged, smoked, oven-cooked turkey breast, while *L. mesenteroides subsp mesenteroides* was predominant in the spoilage flora of sliced, vacuum-packaged, unsmoked, boiled turkey breast fillets stored at 4ºC. They also reported that the unsmoked turkey breasts flora, *L. sakei, Weissella viridescens,* and an

atypical group of leuconostoc-like bacteria had more diversity and grew faster than the smoked breast. The slicing and vacuum packaging operations were the main reason for the contamination of both smoked and unsmoked turkey breasts manufactured at the same plant. Three different types of lactic acid bacteria, *L. mesenteroides subsp. mesenteroides, L. lactis subsp. lactis and Leuconostoc citreum* were isolated from spoiling cooked meat products stored below 10°C (Hamasaki et al., 2003) Samelis et al. (2000a) showed that the *L. sakei/curvatus* group and *L. mesenteroides subsp. mesenteroides* were formed the predominant microflora of six different vacuum-packaged, cooked, cured meat products. It was reported that the product type highly impacted the growth rate and composition of LAB flora during refrigerated storage. The LAB under vacuum had more prolific growth on the products in order: ham > turkey breast fillet > smoked pork loin > pariza > mortadella > bacon. It was also demonstrated that although the *L. sakei/curvatus* group was dominant almost in all products (pork loin, bacon, pariza, mortadella, and frankfurters), the spoilage flora of the non-smoked, boiled whole-meats, such as cooked ham and turkey breast fillet, were predominated by *L. mesenteroides subsp mesenteroides*. Samelis et al. (2000a) noted that emulsion-type meat products (pariza, mortadella, and frankfurters) had more diverse LAB flora. *L. citreum* and *L. carnosum* were found in emulsion sausages and boiled whole-meats respectively, whereas *W. viridescens* was isolated from smoked meat products only (Samelis et al., 2000a). Nowak & Krysiak (2005) indicated that psychrotrophic LAB (*L. mesenteroides ssp. mesenteroides, Lactobacillus fermentum,* and *W. viridescens*) dominated in the spoiled vacuum-packaged frankfurters stored at 4°C, 8°C, and 15°C. They demonstrated that the sensory changes were not linked with bacterial numbers but with the kind of spoiling flora.

Lactobacillus sakei

Lactobacilli are microaerophilic, usually non-motile, nonsporulating, gram-positive bacillus, which groups by pair or short-chain (Champomier-Vergès et al., 2001; Lahtinen et al., 2011; Bajpai et al., 2016). *L. sakei* is a psychrotrophic, facultative anaerobic species, and can form a heme-dependent catalase, which prevents rancidity and discoloration caused by hydrogen peroxide (Knauf et al., 1992; Zagorec & Champomier-Vergès, 2017). *L. sakei* grows best between pH 6.0 and 6.5, while it can also grow in pH range 4.06 and 8.40 (Leroy & de Vuyst, 1999). The GC content is between 42–44% (Champomier-Vergès et al., 2001). Lactobacilli can be commonly detected in various environments, including nutrient-rich dairy and meat environments, natural ecological niches such as soil and plants (Lahtinen et al., 2011). *L. sakei* becomes dominant in different raw fermented foods representing different environments such as sauerkraut, sourdough, fish and it forms the main flora of fresh meat, and gain dominance in meat products under cold storage and oxygen-depleted environments such as vacuum packaging (Champomier-Vergès et al., 2001, 2002; Zagorec & Champomier-Vergès, 2017). Meat has a highly competitive environment during the fermentation process, therefore, some of the *L. sakei* strains are commercially applied as starter culture owing to its competitiveness and play a major role in preserving meat products by outcompeting undesired microorganisms, including pathogenic species (Hammes et al., 1990; Chaillou et al., 2005, 2013). Meat products are rich sources in terms of myofibrillar proteins, while they have poor carbohydrate content (Champomier-Vergès et al., 2001). *L. sakei* has various nutritional requirements and amino acids are significant in explaining *L. sakei* growth and survival in meat. The species are prototrophic for aspartic and glutamic acids, which can be obtained by the deamination of asparagine and glutamine, respectively (Chaillou et al., 2005). Its high adaptation to the meat environment has been explained by genome analysis

(Claesson et al., 2007). The purine nucleoside metabolism and the ability to catabolize arginine, an abundant amino acid present in meat have been shown (Aristoy & Toldrá, 1998; Rimaux et al., 2011, 2012). *L. sakei* can benefit additional energy sources giving a competitive advantage in matrices with a low fermentable sugar concentration by the utilization of the ribose present in nucleosides and the activation of the arginine deiminase (ADI) pathway that produces ornithine, ammonia, and carbon dioxide and concomitantly generates ATP (Rimaux et al., 2013; McLeod et al., 2017). The arcABCTDR, PTP gene cluster encodes the ADI pathway in *L. sakei* (Zúñiga et al., 1998, 2002). Usage of arginine by *L. sakei* is subjected to catabolite repression, is sensitive to pH, and is induced by the presence of arginine and anaerobiosis, which are associated with the conditions of meat storage (Champomier Vergès et al., 1999; Zúñiga et al., 2002; Rimaux et al., 2012). Although the utilization of arginine does not provide an obvious advantage for growth (Zúñiga et al., 2002), it enhances the survival of *L sakei* due to the ATP and ammonia production during arginine degradation and resulting in internal pH increase (Champomier Vergès et al., 1999). *L. sakei* has been also detected in large quantities in spoiled products (Zagorec & Champomier-Vergès, 2017). In addition to the arginine deiminase pathway, some of the *L. sakei* strains possess a second operon encoding enzymes an operational agmatine deiminase (AgDI) pathway (Rimaux et al., 2012). Rimaux et al. (2012) demonstrated that the biogenic amine putrescine was generated from agmatine through the AgDI pathway in *L. sakei*, thereby producing extra ATP, and ammonia against acid stress to improve competitiveness. This property may contribute bacteria to become more competitive during spoilage. *L. sakei* are the facultative heterofermentative species: they convert sugars to lactic acid mainly and generates the same amount of CO_2 and ethanol if there is no other electron acceptor present (Lahtinen et al., 2011). Even though raw meat containing a limited amount of carbohydrates has nutrients for *L. sakei* to

grow, it can only use glucose and ribose out of the few sugars found in meat (McLeod et al., 2011). Hexose fermentation in *L. sakei* is homolactic and advances through anaerobic glycolysis. Glucose is taken into the cell through the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) (Champomier-Vergès et al., 2001). Fermentation of pentoses such as ribose, arabinose, and gluconate occurs via the phosphoketolase pathway, also called the heterolactic pathway (Champomier-Vergès et al., 2002). During that process, their use for growth requires thiamine in the medium, a precursor of thiamine pyrophosphate synthesis, which is a cofactor in phosphoketolase. *L. sakei* produce both D- (−) and L- (+) lactic acid when only L-lactate dehydrogenase (L-LDH) is present in the bacteria, and lactate racemase catalyzes the conversion of L-to D-lactate (Hiyama et al., 1968; Maleret et al., 1998; Champomier-Vergès et al., 2001; Holzapfel & Wood, 2014).

Lactococcus lactis

Lactococcus lactis is a spherical-shaped, catalase-negative, facultative anaerobe, mesophilic gram-positive cocci of 0.5–1.5 µm in size, forming short chains and have GC content between 33.8–36.9 mol% (Teuber & Geis, 2006; Von Wright, 2011; Passerini et al., 2013). It has two subspecies, *lactis* and *cremoris*, which have been researched for their usage in fermented dairy products manufacturing such as milk, cheese, and yogurt. However, *L. lactis* spp *lactis* has been also able to colonize different environments such as plant and animal material (Passerini et al., 2013). *L. lactis* spp. *lactis* is more salt-tolerant (4.0%) and more temperature resistant (40°C) than *L. lactis* spp. *cremoris* (Teuber & Geis, 2006). The genus Lactococcus grows best at a temperature around 30°C; on the other hand, they can also grow at a temperature between 10-40°C (Lee & Collins, 1976; Teuber & Geis, 2006). Although *L. lactis* can grow at a pH range of as low as 4.0

to 5.0 depending on the strain and the medium composition, the best pH growth range is between 6.3 and 6.9 (Sánchez et al., 2008). There has long been known of the adverse impact of low pH on the growth of Lactococcus cells. *L. lactis* reduces the external pH to around 4.5 as a result of the acidic end products build up during growth with excess glucose. However, it was unable to grow when the external pH below 5.3. *L. lactis* allow their internal pH to decline as the external pH decreases, however, it could not glycolyze glucose when the intracellular pH was less than 5.6 and reduces ATP synthesis (Cook & Russell, 1994). *L. lactis* cells can maintain intracellular pH values greater than 7.0, even if the extracellular pH was as low as 5.0. *L. lactis* utilize hexoses homofermentatively producing L (+) lactic acid, and complex media containing at least glucose, glutamate, arginine, valine, and methionine is required for their growth. The anaerobic pathway, fermentation, is the main metabolism of *L. lactis* that generates lactic acid form the available carbon sources including glucose, lactose, galactose, glucosamine, fructose, maltose, mannose, sucrose, mannitol, ribose, and trehalose. Nevertheless, each carbon source affects differently the cell growth rate. Glucose, galactose, lactose, mannose, sucrose, and glucosamine impact the growth rate to the same degree, whereas mannitol and fructose result in lower growth rates compared to glucose (Oliveira et al., 2005).

Leuconostoc mesenteriodes

Leuconostocs are gram-positive, catalase-negative, facultative anaerobic, non-thermophilic, and ovoid-shaped cocci; they grow optimally in temperatures between 20°C and 30°C (Björkroth & Holzapfel, 2006; Endo et al., 2014). When it is above 40ºC, almost no growth is seen (Johanna Björkroth et al., 2014). Leuconostocs prefer an initial optimal environment pH of 6.5; on the other hand, *L. mesenteroides subsp. mesenteroides* cannot grow at pH 4.8 (Björkroth

& Holzapfel, 2006; Huys et al., 2012). They need rich and complex media containing nicotinic acid, thiamin, biotin, and pantothenic acid for growth. Glutamic acid and valine are also required for the growth of *L. mesenteroides subsp. mesenteroides* (Björkroth & Holzapfel, 2006). However, Leuconostocs, nonproteolytic and nonhemolytic do not reduce nitrate and cannot hydrolyze arginine due to the absence of arginine deaminase (Björkroth & Holzapfel, 2006; Huys et al., 2012). In terms of energy production, they are obligatory heterofermentative under microaerophilic conditions and use a combination of pentose phosphate (aka hexose monophosphate shunt) and phosphoketolase pathways. Glucose and other hexose sugars are fermented to an equimolar amount of D-lactate, CO_2, and ethanol or acetate (DeMoss et al., 1951; Björkroth et al., 2014). The G+C content of *L. meseneteroides* is between 37-45 mol% and their cell wall has a peptidoglycan type, L-Lys-L-Ser-L-Ala2. In terms of distinguishing Leuconostoc species from other LAB, characteristics such as the ovoid appearance, inability to hydrolyze arginine, and specifically the production of gas and D (–)-lactic acid from glucose play a significant role (Björkroth & Holzapfel, 2006; Björkroth et al., 2014).

Enterococcus faecium

Enterococci are gram-positive, catalase-negative (some strains produce pseudocatalse), microaerophilic, non-motile, non-spore-forming, spherical or ovoid cells found in pairs or chains (Murray, 1990; Byappanahalli et al., 2012). They are fastidious and can be found in different habitats such as water, soil, plant, silage, gastrointestinal tract (GIT) of humans and animals, and rumen. Moreover, enterococcus contaminates the foods most likely as a result of the contamination of animal and plant sources (Lahtinen et al., 2011). The colonization level differs depending on product type, production season, and other factors, and Enterococci seem to become part of the

fermentation in many fermented foods (Holzapfel et al., 2002; Bhardwaj et al., 2008). They are widely found in nature and this is because of their persistence and their resistance to growth-inhibiting factors such as salt, heat, drying, acidity, and chemical sanitizing agents (Holzapfel et al., 2002). Enterococci can grow in temperatures ranging from 10ºC to 45ºC; however, *E. faecalis* and *E. faecium* can also grow at 50ºC and in 6.5% salt, while the growth between 0ºC and 6ºC has been also reported for them (Murray, 1990; Jay et al., 2008). *E. faecalis* and *E. faecium* can grow up to pH 9.6. However, *E. faecalis* can adapt to more acidic environments (pH 2.9 - 4.2). *E. faecium* has a cell wall peptidoglycan of the Lys-D-Asp type, and the G+C content of the DNA ranges from 37.0 to 40.0% mol (Švec & Franz, 2014; Mubarak & Soraya, 2018). *E. faecium* is a facultative anaerobe and obligatory homofermentative microorganism (Huycke et al., 1998; Lebreton et al., 2014). It can utilize lactose, arabinose, salicilin, mannitol, and glycerol to produce acid (Jay et al., 2008).

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2. CHAPTER 2: CONTROLLING MEAT SPOILAGE LACTIC ACID BACTERIA IN A CURED DELI-STYLE TURKEY BREAST USING FOOD – GRADE ANTIMICROBIALS

ABSTRACT

The salt forms of organic acids are widely and successfully used against controlling pathogenic bacteria such as *Listeria monocytogenes* in ready-to-eat (RTE) products. However, there is very limited knowledge about the effect of organic acids on lactic acid bacteria (LAB) responsible for meat spoilage. Therefore, the purpose of this study was to investigate the growth dynamics and behavior of LAB spoilage bacteria when weak organic acids are included in the formulations at similar concentrations to those used for controlling *L. monocytogenes*. 0.125% sodium diacetate (SD), 0.125% sodium propionate (SP), 2.5% sodium lactate (SL), and all combinations of SD, SP, and SL were tested against the mixture of spoilage LAB in a cured, deli-style turkey breast system (75.0% moisture, 1.7% salt, pH 6.3) study. Sliced cooked products were surface inoculated with a mixture of LAB (*Lactobacillus sakei, Leuconostoc mesenteroides* (bacon and deli-shaved ham isolates), *Lactococcus lactis, and Enterococcus faecium*) at 3 log CFU/g, vacuum-packaged, and stored at 4ºC for 42 days. Results demonstrated that treatments with SL, SL+SD, SL+SP, SL+SD+SP significantly ($P < 0.05$) slowed the growth of the LAB mixture, resulting in extension of the lag phase approximately up to a week. On the other hand, SD, SP, and the combination of SD and SP did not reveal any significant effect ($P > 0.05$) on the LAB throughout the shelf life. In addition, the relative ability of individual SL, SD, and SP to impact the growth of meat spoilage bacteria described above was compared using modified APT broth with an adjusted pH of 6.3 at 10ºC for 14 days. All strains were sensitive to concentration changes

in SL, whereas SP and SD were ineffective or less effective depending on the strain. This study confirmed that the use of SL in the formulation helped improve the shelf life of RTE meat products.

INTRODUCTION

Meat is an essential food source for humans. Changing consumption habits with a modern and busier lifestyle have brought an increased demand for processed meat products due to their accessibility, convenience, and practicality (Leroy & Degreef, 2015). However, meat is highly vulnerable to microbial deterioration due to physicochemical characteristics (near-neutral pH, high water activity, and high nutrient content) providing for an ideal growth medium for spoilage bacteria. As such, meat and poultry products can be stored for only a limited time until spoiled by subsequent growth of the microorganisms introduced before, during, or after processing (Hugas, 1998). According to FAO (2012), 20.0% of the produced meat and meat products from animal slaughter to consumption is lost or wasted globally each year. If not controlled, microbial contamination responsible for spoilage can result in significant economic damages for industries (Nychas et al., 2008). Improvements in meat preservation techniques such as packaging, irradiation, and high-pressure processing have contributed to increases in storage time up to several months for refrigerated meat and poultry products. Despite comprehensive food safety research and advanced food preservation techniques, these problems are still experienced due to recontamination of spoilage and pathogenic flora on ready-to-eat (RTE) meat products during slicing and packaging processes even when cooking or other forms of pasteurization are applied (Brul et al., 2002). Therefore, the elimination or control of microorganisms is a main requirement to positively impact meat preservation and subsequent shelf life improvement.

The main cause for the spoilage of vacuum-packaged meat and meat products is the growth of lactic acid bacteria (LAB) species (Holley, 1997; Korkeala & Björkroth, 1997; Sarmento et al., 2015). Moreover, it has been demonstrated that the bacterial spoilage flora in RTE meats has commonly consisted of *Lactobacillus, Leuconostoc, Enterococcus, and Lactococcus* (Chenoll et al., 2007; Kalschne et al., 2015; Chávez-Martínez et al., 2016). Additionally, the predominant species in the composition of spoilage flora of meat products have been reported to primarily be *Lactobacillus sakei* and *Leuconostoc mesenteroides ssp mesenteroides* (Dykes et al., 1994; Yang & Ray, 1994; Samelis et al., 1998, 2000), while *Lactobacillus lactis* and *Enterococcus faecalis* have also been found to be part of the dominant microbiota (Barakat et al., 2000).

The utilization of organic acids, sodium lactate (SL), sodium diacetate (SD), sodium propionate (SP), and their combinations have been applied to prevent spoilage and prolong the shelf life of perishable foods (Ricke, 2003). Their growth restricting capabilities are well known against pathogenic bacteria such as *L. monocytogenes* (Glass et al., 2002, 2007). However, the knowledge related to their effects on spoilage LAB in meat products remains very limited. Therefore, the aim of this study was to investigate the growth dynamics and behavior of spoilage LAB when weak organic acids are included in the formulations at similar concentrations used for controlling *L. monocytogenes*.

MATERIAL AND METHODS

Product Manufacture

Cured, deli-style turkey breasts were manufactured following good manufacturing practices at the University of Wisconsin-Madison, Meat Science and Muscle Biology Laboratory. Frozen, whole, skinless, and boneless turkey breasts were purchased from a local supplier and

stored as frozen (-25ºC) until needed. Since all raw meats were obtained in a frozen state, all meat was subject to a single freeze/thaw cycle and the resulting purge was discarded. After thawing at 2.2 to 4.4ºC, the breasts were ground using a grinder (Hobart Model 4732, Hobart Corporation, Troy, OH) to 9.53 mm (3/8”).

Product formulations for microbiological analysis were targeted to achieve a 100% cook yield (no-cook loss) after thermal processing. The base formulation (1.7% salt, 0.30% sodium tripolyphosphate (STPP), 1.0% modified food starch, 100 mg/kg sodium nitrite, and 547 mg/kg sodium erythorbate) was used in all treatments to mimic a typical formulation used in the industry for a cured deli-style turkey breast. Cured deli-style turkey breast formulations (TRTs; n=8), with an adjusted pH of 6.3 and moisture of 75.0%, were prepared with 2.5% sodium lactate (SL) (syrup, 60.0% (w/w) (Sigma-Aldrich, St. Louis, MO), 0.125% sodium diacetate (SD) (Sigma-Aldrich, St. Louis, MO), 0.125% sodium propionate (SP) (Sigma-Aldrich, St. Louis, MO), and their combinations (SL+SD, SL+SP, SD+SP, SL+SD+SP) as well as a control containing no antimicrobial for microbiological challenge testing. Formulations for cured, deli-style turkey breast reported as a percentage of the total formula (Table 2.1). Sodium bicarbonate was used in formulations containing SL, SD, SL+SD, SL+SP, SD+SP, and SL+SD+SP to adjust the product pH to 6.3. Each treatment was manufactured in 3 replications, on three different days of production to demonstrate different raw turkey breast batches.

The Solver function in Microsoft Excel 2007 was used to calculate the details of treatment formulations (Microsoft Corporation; Redman, WA, USA). This function makes use of the approximate moisture levels of meat and all added ingredients while the required ingoing levels of each formulated ingredient serve as limitations (i.e., 1.7% salt, 0.30% STPP, 1.0% modified food starch, 100 mg/kg sodium nitrite, 547 mg/kg sodium erythorbate, 2.5% SL, 0.125% SD and

0.125% SP were constraints for final product levels in all formulations). The treatments were "solved" by changing ingoing amounts of ingredients to the preferred limits while aiming for a wanted final product moisture level. Appendix 1 demonstrates the formulation spreadsheets adjusted by using the Solver function.

All weighed ingredients for all formulations were dissolved in water to provide uniform ingredient distribution. The ingredients were added into a solution in the following order: STTP, salt, sodium nitrite, sodium erythorbate, antimicrobials, sodium bicarbonate (if necessary), and modified food starch. After combining the ingredients, the solution was mixed with ground turkey meat for 2 min in a stand mixer (Hobart A120, The Hobart manufacturing Co., Troy, OH, USA). The mixture was then transferred to a rotary vane vacuum filler (Handtmann VF 608 Plus vacuum filler, Handtmann CNC Technologies Inc., Lake Forest, IL) and stuffed into 90 mm flat width moisture impermeable plastic casings (Nova X, Visko Teepak USA, Kenosha, WI, USA) to produce chubs that were approximately 65 cm in length. A clipper (Poly-clip model EZ 6080, Poly-clip System Corp., Mundelein, IL, USA) was used to close tightly the ends of each chub.

Thermal Processing and Slicing

Cooking was accomplished using a steam-jacketed kettle (Groen model N30, Groen MFG. CO., Chicago, IL) preheated to 80ºC until the internal temperature of the chubs reached 73.9ºC. After cooking, products were immediately chilled in ice water for 20 min and then placed in cooler (~ 4ºC) until sliced the following day.

All cooked turkey chubs were sliced using a manual deli slicer (Berkel Model 919E, Berkel Incorporated, Troy, OH, USA). To minimize contamination risk with background microflora, a 70:30 ethanol / distilled water solution was sprayed on all product contact surface areas and the

exterior of the plastic casings of each treatment chub prior to slicing. The ethanol solution was allowed to evaporate for 15 s before product contact to ensure that ethanol's residual effects did not inhibit the growth of subsequent inoculated species. The peeling process for each chub was done immediately before slicing to reduce the possibility of recontamination. Since slice thickness was inherently affected by the density of varied product formulations, the products were sliced to a target thickness of 25 ± 1 g per slice. All treatments were immediately vacuum sealed (45.7 cm x 71.1 cm, 3-mil high-barrier pouches; Ultra Source LLC, Kansas City, MO, USA) after slicing and transferred to the Food Research Institute at the University of Wisconsin-Madison for microbial challenge testing. Microbiological challenge testing started one to two days after slicing, and products were stored at 4ºC until inoculation.

Microbiological Challenge Testing

Samples (each containing two 25 g slices) were inoculated with a mixture of 5 LAB species. Two different strains of *L. mesenteroides* (bacon and deli-shaved ham isolates), *L. sakei* (meat isolate), *L. lactis*, and *E. faecium* (meat isolate) originating from two different commercial meat-related companies were grown individually in 9 mL All Purpose Tween (APT) broth (BD Difco, Sparks, MD, USA) at 30ºC for 20 - 22 hours. LAB species were harvested by centrifugation (4000 x g, 20 min) and suspended in 4.5 mL of Butterfield's phosphate buffer. Equivalent populations of each strain were combined to obtain a five-strain blend of LAB for the inoculation of products. Each LAB strain population and their mixtures were validated by plating on APT agar (BD Difco, Sparks, MD, USA). The two turkey breast slices (~50 g) per package were surface inoculated with a target of 3 log CFU/g of five different LAB strain blends (5 log CFU/50 g package) in total by applying 0.25 mL inoculum onto various surface areas. Both uninoculated and

inoculated slices were placed in gas-impermeable vacuum chamber pouches (3 mil, 7 x 9", UltraSource, Kansas, MO, USA) and vacuum-packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany) prior to being stored at 4ºC for up to 42 days. Uninoculated and triplicate inoculated samples were tested for the enumeration of LAB populations, pH, odor, and appearance changes at each sampling point. Microbial populations were enumerated on days 0 (inoculation day), 3, 7, 10, 14, 21, 28, 35, and 42 by diluting rinse material obtained after washing and hand massaging the contents of each package for 3 min in 50 mL of sterile Butterfield's phosphate buffer solution. 1.5 mL of rinse material from each technical replicate were collected into 1.5 mL Eppendorf tubes and stored at -80ºC later for the microbiota analysis. All LAB populations were enumerated in triplicate samples by surface plating 0.1 mL diluted rinse on APT agar (BD Difco, Sparks, MD, USA) with 0.01 g/L bromcresol purple (5 mL per 500 mL agar) spread using a sterile bent glass rod, and incubating at 30ºC for 48 h.

Physiochemical Analysis

Proximate composition analysis of turkey products was carried out in triplicate using CEM smart 5-turbo moisture and fat analyzer (CEM Corporation, Matthews, NC), and CEM sprint protein analyzer (CEM Corporation, Matthews, NC). pH (1:10 dilution for 5 g homogenized portion, Thermo Scientific Orion Star benchtop pH meter and Electrode, pH ATC Combination; Thermo Scientific Orion; ROSS Ultra; Glass triode), water activity (Decagon AquaLab TE-4 water activity meter, Pullman, WA), NaCl (measured as % Cl^-, AgNO3 potentiometric titration, Mettler DL22 auto titrator) and nitrite (Colorimetric method 973.31) (AOAC, 1990) were assayed for triplicate samples for each treatment.

Preparation of Broth System and Inoculation for Bioscreen C

Two different strains of *L. mesenteroides* (bacon and deli-shaved ham isolates), *L. sakei* (meat isolate), *L. lactis*, and *E. faecium* (meat isolate) originating from two different commercial meat-related companies were grown individually in 9 mL All Purpose Tween (APT) broth (BD Difco, Sparks, MD, USA) at 30ºC for 20 - 22 hours. For all lactic acid bacteria, species were harvested by centrifugation (4000 x g, 20 min) and suspended in 4.5 mL of Butterfield's phosphate buffer. Populations of each strain were validated by plating on APT agar (BD Difco, Sparks, MD, USA) for the LAB. A stock APT broth (BD Difco, Sparks, MD, USA) containing 100 mg/kg sodium nitrite (SN), 547 mg/kg sodium erythorbate (SE), and a total concentration of 1.7% salt was prepared. Depending on the concentrations (wt/vol) of weak organic acids used (0.3, 1.2, 2.1, and 2.7% SL; 0.05, 0.15, 0.25, and 0.50% SD; and 0.05, 0.15, 0.25, and 0.50% SP), antimicrobials were added into bottles containing 100 mL stock broth to get the concentrations. pHs were adjusted to 6.3 using NaOH or HCl to mimic a turkey breast system environment. After pH adjustments, the broths were sterilized using 0.2µm Nalgene Rapid-Flow disposable Filter Units (Thermo Scientific, Waltham, Massachusetts, U.S.). 10 mL of the filter-sterilized broths with antimicrobials were inoculated with a target of equivalent to 3 log CFU/mL of individual strain by applying 0.05 mL inoculum. 200µL of both uninoculated and inoculated samples were placed into a 100-well honeycomb plate in duplication. The changes in optical density (OD) in Bioscreen C (Growth Curves USA, Piscataway, NJ) were measured every 30 min for 14 days at 10ºC. In this study, two different controls, C1 (sterile APT broth) and C2 (sterile APT containing 1.7% salt, 100 mg/kg SN, and 547 mg/kg SE), were used. All microbiological data for the broth system were reported as an average of the duplicate sample optical density (OD) at each measurement point.

Statistical Analysis

Data analysis was performed using a linear mixed models, which can be escribed as:

$$Y_{ijk} \sim \mu + T_i + D_j + r_k + (TD)_{ij} + e_{ijk}$$

where Y_{ij} is the response variable (natural log transformed CFU count), μ is the model intercept, T_i is the fixed effect of treatment i, D_j is the fixed effect of sampling day j, r_k is the random effect of replicate k (k= 1 to 3), $(TD)_{ij}$ is the fixed effect of interaction between treatment i and day j, and e_{ijk} is a random residual term, assumed independent and normaly distributed as $e_{ijk} \sim N(0, \sigma^2_e)$. The analysis was performed using MIXED procedure (PROC MIXED) of SAS software (SAS Institute Inc., Cary, NC, USA). Least squares means (LSM) of fixed effects were calculated with the LSMEANS option. The multiple comparison adjustment of the p-values for differences of the LSM were determined using the–Tukey-Kramer adjustment procedure. Significance was established at $P < 0.05$.

Fitting Growth Curves: After collecting data from the meat system for microbial enumeration throughout 42 days of storage for each treatment, the data was analyzed using DMfit (Baranyi & Roberts, 1994) web-based application (developed by The ComBase consortium; http://www.combase.cc/index.php/en/) to fit growth curves to data. The growth curves were then used to estimate the lag times and population growth rates between sampling time points where physical enumeration was not present. Such values were considered as obtained experimentally and used for the model formation. R-squared output value was utilized to determine the "goodness of fit" for each curve and yielded reasonable growth rates and lag-times while fitting a classical sigmoidal shape to growth curves (estimating the lag, exponential growth, and stationary phase) in the current study.

RESULTS

Physiochemical Properties

For all treatments in the meat system, the physiochemical properties were within acceptable limits and very similar to experimental design targets as well as those found in commercial production. Actual pH values for all treatments at day 0 were the same as targeted pH levels, 6.3, and differed only $\pm$ 0.01 units for some treatments (Table 2.2). The pH values of all treatments decreased to an average of 6.11 at the end of the shelf life (Table 2.5 and Figure 2.2). Mean percent moisture for all formulations was 74.70% $\pm$ 0.17% and ranged from 74.50% to 75.06%. Analytical water activity, salt, protein, and fat results are displayed in Table 2.2. Mean salt levels for all treatments were 1.75% $\pm$ 0.02% NaCl with a range of 1.72 - 1.78%. Water activity (a_w) levels ranged from 0.972 to 0.979, while those treatments containing lactate had a mean a_w values of 0.973, which was approximately 0.005 units lower than other treatments. Protein values ranged from 23.57% to 25.50%, while fat had a mean value of 0.10% + 0.04%. Mean residual nitrite values after thermal processing (day 0) were 45 $\pm$ 3 mg/kg. No changes were observed in odor, appearance, or pH for the uninoculated samples during the 6-week storage time at 4°C across all treatments. Moreover, background microflora growth was not observed during the experiment. On the other hand, pH decreased for the inoculated samples during the 6-week hold time as a result of lactic acid accumulation due to microbial growth, while changes were not observed for odor or appearance.

In the broth system study, undissociated acid levels calculated using Henderson–Hasselbalch equation (Po & Senozan, 2001) and water activity measurements are illustrated in Table 2.4. SD and SP were not observed to have an effect on the water activity level (0.982),

whereas the concentration increase of SL decreased the water activity from 0.981 to 0.972 due to its hygroscopic property.

Growth Curves for Lactic Acid Bacteria Mixture

The growth curves for the outputs of DMfit (only enumeration results from the samples were used) from each treatment inoculated with the mixture of LAB are shown in Table 2.3. The data provide for strong fitment of the curves since having the r-squared value closer to 1 means a better fit of the growth curve to collected data values. Therefore, the adjustment was not needed since all treatments supported approximately the same level of growth. The fitted curves had a mean r-squared 0.998, mean standard error 0.094, mean lag period 4.3 days, and mean growth rate 0.39 log CFU/mL.

Microbiological Data - Control of LAB Mixture in Turkey Breast Meat System

The results from this study demonstrate that the addition of SL in the formulation of cured deli-style turkey breast was effective in controlling and extending the lag phase of LAB compared to control, SD, SP, and the combinations of SD and SP. LAB population changes (*L. sakei* (meat isolate)*, L. lactis, E. faecium* (meat isolate), and *L. mesenteroides* (bacon and deli-shaved ham isolates)) in cured, deli-style turkey breast samples stored at 4ºC for 42 days. The data was reported as an average of the three biological replications (including technical triplicate for each) at each sampling point (Table 2.6). The average starting LAB population of rinse was 3.22 $\pm$ 0.02 log CFU/mL. There was no difference between treatments not containing SL (0.125% SD, 0.125% SP, and SD+SP) and the control, and those treatments reached populations of > 8.00 log CFU/ml by day 21. On the other hand, while other antimicrobials did not show any effect against LAB, all

treatments including SL (2.5% SL, SL+SD, SL+SP, and SL+SD+SP) significantly ($P < 0.05$) slowed the growth of LAB by extending the lag phase and reached populations of > 8.00 log CFU/mL at day 28 (Table 2.6 and Figure 2.1).

Microbiological Data – Control of Individual LAB in Broth System

APT broth system was modified to mimic turkey breast meat environment with addition of organic acid salts, salt, SN and SE. Then, the growth behaviors of LAB in modified APT broth was compared to 2 controls without organic acids; only APT broth (C1) and APT broth containing salt, SN, and SE (C2). C1 was used to provide an ideal environment for the LAB growth as a positive control. C2 was used to investigate how salt, SN and SE affect the growth of LAB without organic acid salts as a second control.

The growth patterns of *L. sakei* in modified APT broth containing various amounts of SL, SD, and SP stored at 10ºC for 14 days are shown in Figure 2.3. *L. sakei* was not affected by the addition of the combination of salt, SN, and SE in APT broth (C2). The lowest concentration of SL increased the lag time for up to 2 days compared to C1 and C2; however, the growth rate was similar to C1 and C2. The effect of SL on *L. sakei* in a broth system at pH 6.3 was observed to be concentration-dependent whereas the higher concentrations also had the longer lag phases and the lower growth rates (OD). Interestingly, the growth of *L. sakei* was completely inhibited by the use of 2.7% SL with no sign of growth at least for 14 days at 10ºC. The addition of SD in the broth system extended the lag phase of *L. sakei* but did not inhibit it completely. The 0.05 and 0.15% SD displayed a similar growth rate for *L. sakei* compare to C1 and C2. 0.50% SD had the most significant effect on reducing the growth rate. As the concentration of SP increased, the lag time also increased, but the growth rate of *L. sakei* did not reduce with 0.05, 0.15, and 0.25% SP.

Although the most effective concentration of SP was 0.50%, it did not completely inhibit the growth of *L. sakei*; however, a significantly reduced growth rate was observed.

The growth patterns of *L. mesenteroides* (deli-shaved ham isolate) in modified APT broth containing various amounts of SL, SD, and SP stored at 10ºC for 14 days are shown in Figure 2.4. *L. mesenteroides* (ham isolate) was slightly sensitive to the mixture of salt, SN, and SE in C2 in the broth system at 10ºC. Also, the effect of SL was observed to be concentration-dependent. The increase in concentration caused an extension in lag time while reducing the growth rate. Concentrations of 2.1% SL and higher significantly reduced the growth rate of *L. mesenteroides* (ham isolate). *L. mesenteroides* (ham isolate) was resistant to SD and it did not differ from C2 even at 0.5% level. The concentrations of 0.05, 0.15, and 0.25% SP also did not reveal any significant effect on *L. mesenteroides* (ham isolate). On the other hand, 0.5% SP increased the lag time along with reducing the growth rate.

The growth patterns of *L. mesenteroides* (bacon isolate) in modified APT broth containing various amounts of SL, SD, and SP stored at 10ºC for 14 days are shown in Figure 2.5. The combination of salt, SN, and SE in C2 slowed the growth of *L. mesenteroides* (bacon isolate), whereas the length of lag time and growth rate were not affected at 10ºC for 14 days. *L. mesenteroides* (bacon isolate) was observed to be sensitive to SL as concentrations of 1.2% SL and higher inhibited the growth of *L. mesenteroides* (bacon isolate). On the other hand, SP and SD were highly ineffective against controlling *L. mesenteroides* (bacon isolate) with little reduction in the growth rate at 0.5% SP level.

The growth patterns of *L. lactis* in modified APT broth containing various amounts of SL, SD, and SP stored at 10ºC for 14 days are shown in Figure 2.6. Although it is difficult to make a clear conclusion about the potential effects of the organic acids investigated in this experiment

since *L. lactis* did not grow well in APT broth (C1) compared to other LAB, the results still give some clues about the behavior of the microorganism and its response to organic acids. The combination (C2) of salt, SN, and SE in the APT broth showed some effect on controlling *L. lactis* as it increased the lag phase while reducing the growth of *L. lactis*. The SL concentrations of 0.3 and 1.2% allowed some growth of *L. lactis* while increasing the lag phase. As such, it is difficult to infer any inhibitory effects of SL at these concentrations due to the low growth rates in the C1 and C2. However, it appears that growth was slightly reduced at these concentrations. The SL concentrations of 2.1% and higher completely inhibited the growth of *L. lactis* at 10ºC for 14 days. Therefore, higher lactate concentrations of at least 2.1% might be suggestive evidence for the growth inhibition effect of lactate. Although SD also increased the lag phase, it did not completely inhibit the growth. Even though 0.05, 0.15, and 0.25% SD had a similar level growth rate as the C2, 0.5% was more effective than other concentrations. In terms of SP, all concentrations were clustered around the C2 line. SP increased the lag time of *L. lactis*, but no growth inhibition was observed at any concentrations except for the highest concentration at 0.50%.

The growth patterns of *E. faecium* in modified APT broth containing various amounts of SL, SD, and SP stored at 10ºC for 14 days are shown in Figure 2.7. *E. faecium* also displayed a similar growth rate in C1 and C2 as *L. Lactis.* Although *E. faecium* also did not grow well in APT compared to other lactic acid bacteria, the results still give information about the behavior of the microorganism impacted by organic acids. Salt, SN, and SE in C2 revealed a slightly negative effect on *E. faecium*. The effect of SL was concentration-dependent and levels higher than 1.2% SL completely inhibited the growth of *E. faecium* at 10ºC for 14 days. All concentrations of SD increased the lag time of *E. faecium* to the same extent, whereas the growth rate was not affected except with the decrease in 0.50% SD. As the concentration of SP increased, the lag phase of *E.*

faecium was also increased and the growth pace slowed. At the end of the experimental shelf life (14 days at 10ºC), 0.05, 0.15 and 0.25% SP had almost the same growth rate as C2, while 0.50% SP completely inhibited the growth of *E. faecium*.

DISCUSSION

It is important to be kept the RTE meat products in the shelves longer for producers due to significant economic concerns by controlling the spoilage of lactic acid bacteria in RTE meat products while maintaining conditions inhospitable to meat borne pathogens. Therefore, we tested the effect of organic acids similar concentrations used for *L. monocytogenes* to test their effect on the growth of LAB.

According to the findings in the turkey breast meat system experiment, the use of SL in the turkey breast formulations exhibited the highest antimicrobial effect among the organic acid salts tested without any negative impact on the color and odor of the turkey breasts, and prolonged the lag phase up to 1 week. Our results were consistent with the previous findings. Cegielska-Radziejewska & Pikul (2004) reported that 2.0% SL inhibited the growth LAB in refrigerated-stored, sliced poultry sausages while maintaining their initial quality. The cold storage period of these products with SL has been found to often be prolonged 1 or 2 more weeks (de Wit & Rombouts, 1990). Stekelenburg & Kant-Muermans (2001) also demonstrated the application of 2.5% to 3.3% SL in ham products reduced the growth rate and increased the lag phase of LAB (*L. curvatus*) while extending shelf life without noticeable sensory changes. Additionally, the usage of SL at varying concentrations in the range of 2% to 4% has been also reported as successful in limiting the proliferation of pathogens in various RTE turkey, ham, and beef meat products (Miller & Acuff, 1994; Wederquist et al., 1994; Stekelenburg & Kant-Muermans, 2001).

In the current study, SL combinations with SP, SD and SP+SD showed antimicrobial effect on the growth of LAB and this effect was similar to application of SL by itself. Barmpalia et al., (2005) reported that the combination of 1.8% SL and 0.25% SD inhibited the growth of LAB in pork bologna. As mentioned earlier, SL reported as effective at 2% and higher concentration. Its combination with SD might help increase its impact under this concentration. In addition to SL combination with SD, various studies reported antimicrobial effect of SL with sodium acetate (SA). The lag time of LAB was extended when 3.0% SL combined with 0.5% SA resulting in greater inhibition of *L. plantarum* (Sarmento et al., 2015). A combination of 2% SL and 0.5% SA were able to inhibit the growth of *L. curvatus* in MRS broth (Drosinos et al., 2006). Another combination, 2% SL and 0.2% SP, extended the shelf life of the vacuum-packaged patties by decreasing the microbiological growth of mostly consisting of LAB (Maca et al., 1997). Combined effects of organic acid salts mostly studied on pathogens. SL has been commonly combined with other organic acid salts especially with SD and its antimicrobial effectiveness against pathogens increased in processed meat products (Glass et al., 2002; Serdengecti et al., 2006). As reported in previous studies, their combination significantly inhibited the growth of pathogens in RTE meat products (Mbandi & Shelef, 2001; Glass et al., 2002, 2007).

On the other hand, SD, SP, and the combination of SD and SP did not show any inhibitory effect on LAB growth compared to control in the current turkey breast experiment. Stekelenburg & Kant-Muermans (2001) also noted that 0.2% SD did not impact the growth of *L. curvatus* while it showed a negative effect on the taste and odor of the ham product. SA reported as inhibitory on the growth of *L. curvatus* in MRS broth at 0.5%, 0.75%, and 1.0% concentration, whereas 0.25% SA was ineffective (Drosinos et al. 2006). However, Iino et al. (2002) reported that SA increased the activity of lactate dehydrogenase's (LDH) in *L. sakei* and *L. plantarum*, resulting in more

production of lactic acid in the presence of SA. Ray (1996) also reported the addition of SA caused the noticeable growth of LAB and the enhancement of the production of L-lactic acid. As such, 0.125% SD was chosen to test its possible antimicrobial effects while preventing any possible negative impact on the taste and odor of the turkey breast. However, the amount used in this study showed neither a positive nor a negative effect on the LAB mixture growth pattern and was likely due to the low experimental concentration. Although very limited information is available in the literature regarding the effect of SP on LAB, Glass et al. (2007) indicated that 0.2% and 0.3% propionate significantly inhibited the growth of *L. monocytogenes* in ham stored at 4ºC for 12 weeks, whereas 0.1% propionate delayed the growth up to 10 weeks. Maca et al. (1997) showed the combinations of 2% SL and 0.2% SP extended the shelf life of the vacuum-packaged patties by decreasing the microbiological growth of mostly consisting of LAB, whereas individual 0.3% sodium acetate and the combination of 2% SL and 0.1 SP did not differ too much from control. In our study, SP and its combinations did not impact the growth of the LAB mixture except in combination with SL. While SP, SD, and their combinations were effective on pathogens, the LAB population was found to be more resistant to these organic acids. LAB continuously acidifies their environment during lactic acid fermentation and is more likely to survive under the more acidic condition compared to pathogens like *L. monocytogenes* due to pH regulation systems such as protons export channel and acid transport mechanisms (Hutkins & Nannen, 1993). Ouattara et al., (1997) also used the non-salt form of lactic acid, propionic and acetic acids and found lactic acid most was effective, while propionic and acetic acids were slightly effective.

In the broth system experiment, the effect of SL was generally observed to have longer lag times, lower growth rates, and/or lower number of bacteria. The growth of *L. mesenteroides* (bacon isolate), *L. lactis*, and *E. faecium* were significantly reduced for 14 days by the concentrations of

at least 1.2% SL, while the growth rates for *L. sakei* and *L. mesenteroides* (ham isolate) were less affected compared to the other 3 LAB strains. Additionally, *L. mesenteroides* (ham isolate) was found to be the most resistant strain. Its growth rate started to decline significantly after 1.2% SL. Overall, we observed *L. mesenteroides* (ham isolate) to be more resistant to growth compared to *L. mesenteroides* (bacon isolate). In terms of sensitivity, *L. mesenteroides* (bacon isolate) was the most sensitive while others, *E. faecium, L. lactis*, *L. sakei*, and *L. mesenteroides* (ham isolate) were less sensitive in decreasing order. Additionally, it appears most of the strains were resistant to SD and SL at the tested levels except at the concentration of 0.50%. Only the growth of *E. faecium* was inhibited by SP with the highest concentration (0.50%). The concentration of 0.50% SD showed a pronouncing impact on *L. sakei* and delayed its growth until 9 days. Overall, effect of SD and SP was not concentration-dependent.

Overall, tests in APT broth and meat systems showed only SL and its combinations with SD or SP providing a decrease in the LAB growth rate. LAB has various regulation systems to survive if the acidity fluctuates or changes (such as a cytoplasmic pH decrease below a neutral state). The amount of available energy in the cell plays a key role to decide which metabolic pathway way should be followed. Microorganisms in the SL added environment likely used their energy to regulate internal pH rather than growth. Impact of the lactate on the lactate dehydrogenase (LDH) enzyme has been previously reported as a possible explanation for the extension in the lag phase (Desguin et al., 2017).Therefore, lactate needs to be eliminated from the cell to avoid slowing down the LDH enzyme due to the lactate accumulation in LAB (Desguin *et al.*, 2017). Elimination of accumulated lactate and protons in the cell requires energy and this process can be done by membrane-bound electron transfer and/or by the proton- translocating ATPase complex (proton) and carrier-mediated efflux (lactate) (De Wit & Rombouts, 1990).

Actively growing cells can handle this energy loss by utilizing the energy used for synthesis and growth resulting in reduced growth rate and possibly growth yield. On the other hand, cells in the lag phase cannot properly respond to energy requirements due to lack of energy and that will result in lag phase extension (De Wit & Rombouts, 1990).

CONCLUSIONS

The determination of the shelf life of meat products requires the consideration and assessment of the growth responses of spoilage bacteria to antimicrobials. LAB reacts differently to the existence of various weak organic acids due to their heterogeneous nature. Therefore, this study was informative to investigate and better understand the effect of organic acids had on LAB flora in a turkey breast and broth system. However, these studies need to be expanded for a better understanding of LAB metabolisms and possible antagonist and symbiosis effect on each other in the microbial community in meat systems in a connection with the effect of organic acids. Therefore, more comprehensive studies need to be conducted for the development of preservation methods to inhibit the growth of microorganisms longer. A molecular-level analysis may also be useful to help better understand how they interact with each other when organic acid salts are included in the meat formulations. Moreover, extra protection against the pathogenic microorganisms in vacuum-packaged products may be realized from a better understanding of LAB. This study confirms the use of SL in the meat formulations slows down the changes in the LAB populations and prolongs the lag phase at the beginning of shelf life and helps improving the shelf life of RTE meat products.

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TABLES AND FIGURES

Table 2.1 Formulations[a] for cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments reported as a percentage of the total formula.[b]

Treatments	Water (%)	Sodium Lactate (%)	Sodium Diacetate (%)	Sodium Propionate (%)	Sodium Bicarbonate (%)
1 – C	7.94	0	0	0	0
2 – SP	8.32	0	0	0.125	0
3 – SL+SP	11.95	2.5	0	0.125	0.02
4 – SL	11.56	2.5	0	0	0.02
5 – SD+SP	8.88	0	0.125	0.125	0.06
6 – SD	8.49	0	0.125	0	0.05
7 – SL+SD	12.19	2.5	0.125	0	0.10
8 – SL+SD+SP	12.56	2.5	0.125	0.125	0.10

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate (syrup, 60.0% (w/w)), SD: Sodium diacetate.

[a] All formulations had an equal percentage of sodium chloride (1.7%), sodium tripolyphosphate (STPP) (0.30%), modified food starch (1.0%), sodium nitrite (100 mg/kg), and sodium erythorbate (547 mg/kg). Numbers demonstrated as a percent of finished weight basis of the total formula.

[b] The Solver Add-on in Microsoft Excel (Microsoft Corporation; Redman, Wash., U.S.A.) used to calculate formulations.

Table 2.2 Physiochemical analysis[a] of cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments before inoculation with lactic acid bacteria

Treatments	pH[b]	Moisture[c] (%)	Fat[d] %	Protein[e] %	Nitrite[f] (mg/kg)	NaCl[g] (%)	a_w[h]
1 – C	6.30 ± 0.00	74.75 ± 0.32	0.12 ± 0.08	24.87 ± 0.56	41 ± 5	1.73 ± 0.03	0.979 ± 0.003
2 – SP	6.30 ± 0.00	74.77 ± 0.06	0.08 ± 0.06	24.51 ± 1.10	44 ± 7	1.76 ± 0.06	0.978 ± 0.003
3 – SL+SP	6.31 ± 0.02	74.58 ± 0.29	0.08 ± 0.07	23.93 ± 0.83	48 ± 3	1.78 ± 0.01	0.973 ± 0.003
4 – SL	6.30 ± 0.02	74.68 ± 0.25	0.04 ± 0.04	23.57 ± 0.56	42 ± 5	1.77 ± 0.02	0.973 ± 0.003
5 – SD+SP	6.31 ± 0.02	75.06 ± 0.99	0.13 ± 0.10	25.16 ± 0.01	44 ± 5	1.72 ± 0.09	0.978 ± 0.004
6 – SD	6.29 ± 0.02	74.51 ± 0.39	0.10 ± 0.08	25.50 ± 0.20	46 ± 6	1.73 ± 0.04	0.979 ± 0.004
7 – SL+SD	6.31 ± 0.02	74.72 ± 0.30	0.04 ± 0.06	23.66 ± 0.53	43 ± 5	1.77 ± 0.05	0.972 ± 0.004
8 – SL+SD+SP	6.31 ± 0.01	74.50 ± 0.63	0.18 ± 0.11	23.71 ± 0.08	50 ± 11	1.74 ± 0.08	0.973 ± 0.004
Mean[i]	6.30 ± 0.01	74.70 ± 0.17	0.10 ± 0.04	24.36 ± 0.70	45 ± 3	1.75 ± 0.02	0.976 ± 0.003

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

[a] Shown are average compositional data based on cured, cooked, deli-style turkey ± standard deviation for triplicate.

[b] pH of fully cooked deli-style turkey measured using Orion ROSS Ultra Glass Triode ATC combination pH probe and Orion star benchtop pH meter, 5 g meat to 45 mL of distilled water.

[cd] Percentage of moisture and Fat in fully cooked cured deli-style turkey – CEM smart 5-turbo moisture and fat analyzer.

[e] Percentage of protein in fully cooked cured deli-style turkey - CEM sprint protein analyzer.

[f] Residual nitrite in fully cooked cured deli-style turkey - (Colorimetric method 973.31) (AOAC, 1990).

[g] Percentage of sodium chloride in fully cooked cured deli-style turkey measured as Cl- using Mettler Toledo DL-22 Food and Beverage analyzer, silver nitrate titration.

[h] Water activity of fully cooked cured deli-style turkey measured using an AquaLab 4TE water activity meter.

[i] Mean across all treatment

Table 2.3 DMfit[a] outputs for growth curves developed for the mixture of lactic acid bacteria

Treatments	R^2	SE	Initial	Lag[b]	Rate[c]	Max[d]
1 – C	0.999	0.057	3.20	3.5	0.48	8.36
2 – SP	0.997	0.112	3.22	3.7	0.53	8.34
3 – SL+SP	0.999	0.053	3.21	5.7	0.32	8.30
4 – SL	0.998	0.106	3.18	6.1	0.32	8.29
5 – SD+SP	0.999	0.082	3.17	3.3	0.44	8.31
6 – SD	0.998	0.092	3.18	2.9	0.46	8.33
7 – SL+SD	0.995	0.153	3.14	4.5	0.29	8.28
8 – SL+SD+SP	0.998	0.100	3.15	5.0	0.29	8.25
Mean[e]	0.998 $\pm$ 0.001	0.094 $\pm$ 0.030	3.18 $\pm$ 0.03	4.3 $\pm$ 1.1	0.39 $\pm$ 0.09	8.31 $\pm$ 0.03

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

[a] Data reached > 8.00 log CFU/mL rinse maximum growth through 42 days stored at 4°C.

[b] Lag presented in days

[c] Rate demonstrated in log CFU/mL rinse per day

[d] Maximum value (log CFU/mL rinse through 42 days stored at 4°C) used in growth curve calculation

[e] Mean across all treatments

Table 2.4 The corresponding levels of the calculated molar concentrations of undissociated acid (mM) and measured water activity (a_w) of sodium lactate (SL), sodium propionate (SP), and sodium diacetate (SD) depending on the concentration (% [wt/vol]) at initial pH 6.3 in a modified APT broth system.

Sodium Lactate (pKa=3.86)			**Sodium Propionate** (pKa=4.87)			**Sodium Diacetate** (pKa=4.76)		
Used (%)	SL[a]	a_w[b]	Used (%)	SP[a]	a_w[b]	Used (%)	SD[a]	a_w[b]
0.3	0.16	0.981	0.05	0.19	0.982	0.05	0.20	0.982
1.2	0.64	0.979	0.15	0.58	0.982	0.15	0.60	0.983
2.1	1.12	0.976	0.25	0.97	0.982	0.25	1.00	0.983
2.7	1.45	0.972	0.50	1.93	0.980	0.50	2.01	0.980

[a] Molar concentration of undissociated acid form

[b] Water activity of the modified APT broth system measured using an AquaLab 4TE water activity meter

Table 2.5 pH changes of lactic acid bacteria inoculated cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments[a] stored at 4°C for 42 days.

	Average pH (Inoculated)							
Timepoints	**Treatments**							
(Day)	**Control**	**SP**	**SL+SP**	**SL**	**SD+SP**	**SD**	**SL+SD**	**SL+SD+SP**
0	6.30 ± 0.00	6.30 ± 0.01	6.31 ± 0.01	6.30 ± 0.01	6.31 ± 0.01	6.29 ± 0.03	6.31 ± 0.02	6.31 ± 0.02
3	6.32 ± 0.01	6.32 ± 0.01	6.31 ± 0.00	6.31 ± 0.00	6.31 ± 0.00	6.32 ± 0.01	6.32 ± 0.01	6.32 ± 0.01
7	6.29 ± 0.03	6.28 ± 0.03	6.31 ± 0.01	6.27 ± 0.04	6.26 ± 0.04	6.26 ± 0.04	6.27 ± 0.04	6.28 ± 0.02
10	6.29 ± 0.01	6.29 ± 0.03	6.31 ± 0.02	6.28 ± 0.02	6.29 ± 0.02	6.29 ± 0.02	6.30 ± 0.01	6.30 ± 0.01
14	6.20 ± 0.01	6.20 ± 0.03	6.30 ± 0.02	6.27 ± 0.03	6.20 ± 0.04	6.18 ± 0.05	6.25 ± 0.04	6.27 ± 0.02
21	6.07 ± 0.01	6.09 ± 0.01	6.23 ± 0.08	6.17 ± 0.07	6.08± 0.03	6.06 ± 0.05	6.24 ± 0.01	6.24 ± 0.02
28	6.08 ± 0.02	6.10 ± 0.02	6.15 ± 0.07	6.10 ± 0.02	6.09 ± 0.02	6.09 ± 0.03	6.16 ± 0.02	6.20 ± 0.04
35	6.07 ± 0.01	6.12 ± 0.02	6.13 ± 0.04	6.09 ± 0.01	6.11 ± 0.05	6.07 ± 0.03	6.13 ± 0.02	6.13 ± 0.03
42	6.09 ± 0.03	6.10 ± 0.03	6.14 ± 0.04	6.08 ± 0.03	6.11 ± 0.03	6.10 ± 0.03	6.12 ± 0.02	6.13 ± 0.03

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

[a] Shown are average pH changes of lactic acid bacteria inoculated cooked, cured deli-style turkey ± standard deviation for triplicate.

Table 2.6 Change in lactic acid bacteria populations[a] in cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments stored at 4°C for 42 days.

	Average of Populations of Lactic Acid Bacteria (log CFU/mL)							
Timepoints	**Treatments[b]**							
(Day)	**Control**	**SP**	**SL+SP**	**SL**	**SD+SP**	**SD**	**SL+SD**	**SL+SD+SP**
0	3.21 ± 0.12	3.22 ± 0.03	3.24 ± 0.07	3.23 ± 0.09	3.24 ± 0.03	3.24 ± 0.06	3.19 ± 0.09	3.18 ± 0.10
3	3.44 ± 0.01	3.47 ± 0.06	3.27 ± 0.11	3.25 ± 0.13	3.37 ± 0.01	3.46 ± 0.01	3.30 ± 0.04	3.31 ± 0.06
7	4.97 ± 0.17	4.88 ± 0.19	3.85 ± 0.21	3.61 ± 0.21	4.88 ± 0.20	5.13 ± 0.12	3.77 ± 0.14	3.74 ± 0.17
10	6.23 ± 0.09	6.67 ± 0.40	4.59 ± 0.35	4.49 ± 0.42	6.16 ± 0.08	6.54 ± 0.20	4.99 ± 0.39	4.75 ± 0.31
14	7.97 ± 0.16	7.95 ± 0.15	5.94 ± 0.55	5.88 ± 0.42	7.62 ± 0.14	7.84 ± 0.31	5.81 ± 0.61	5.86 ± 0.51
21	8.40 ± 0.02	8.37± 0.03	7.81 ± 0.38	7.65 ± 0.42	8.26 ± 0.05	8.31 ± 0.09	7.62 ± 0.32	7.57 ± 0.34
28	8.32 ± 0.01	8.34 ± 0.04	8.24 ± 0.06	8.24 ± 0.05	8.37 ± 0.04	8.36 ± 0.07	8.21 ± 0.09	8.21 ± 0.09
35	8.37 ± 0.02	8.38 ± 0.04	8.31 ± 0.03	8.32 ± 0.03	8.28 ± 0.03	8.34 ± 0.04	8.29 ± 0.05	8.24 ± 0.02
42	8.35 ± 0.05	8.37 ± 0.03	8.34 ± 0.01	8.34 ± 0.01	8.32 ± 0.04	8.35 ± 0.10	8.31 ± 0.08	8.30 ± 0.10

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

[a] Shown are average population numbers (log CFU/mL) ± standard deviation for triplicate.

[b] Rinse material collected by washing and hand massaging for 3 min with 50 mL of sterile Butterfield's phosphate buffer solution.

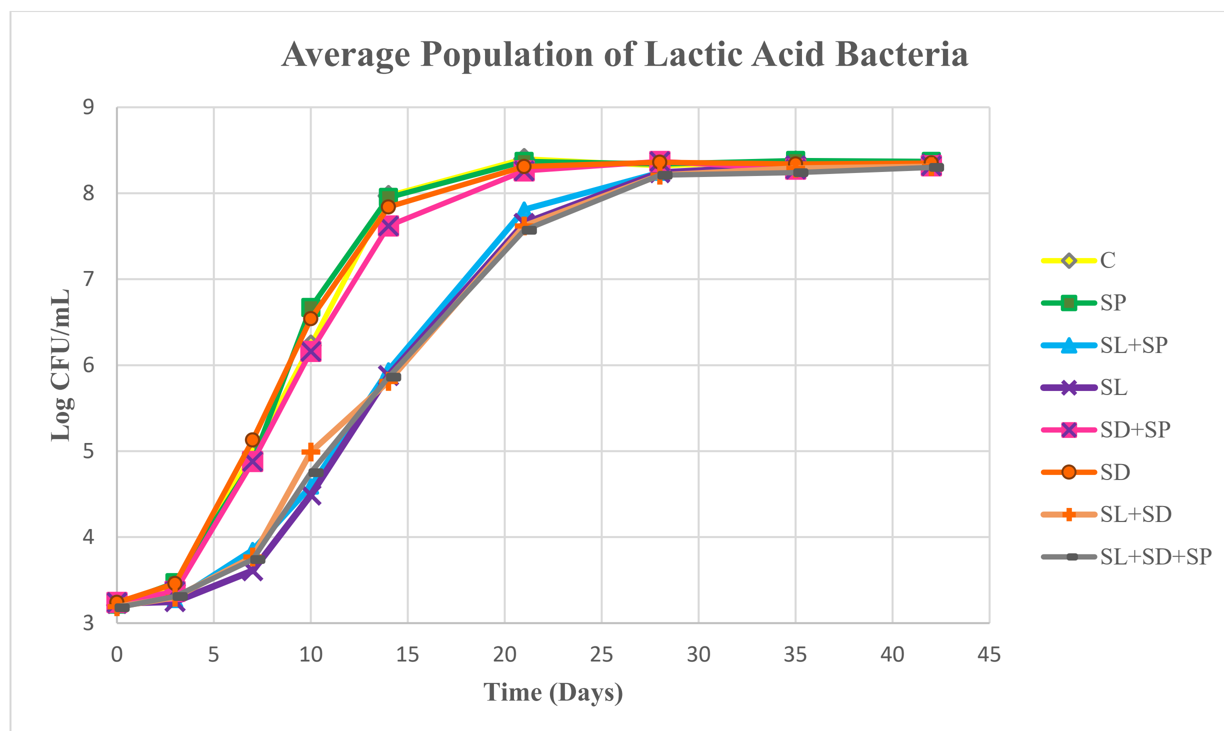


Figure 2.1 Change in lactic acid bacteria populations in cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments stored at 4ºC for 42 days, drawn from Table 2.6.

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

Error bars are not shown to avoid visual cluttering in the figure.

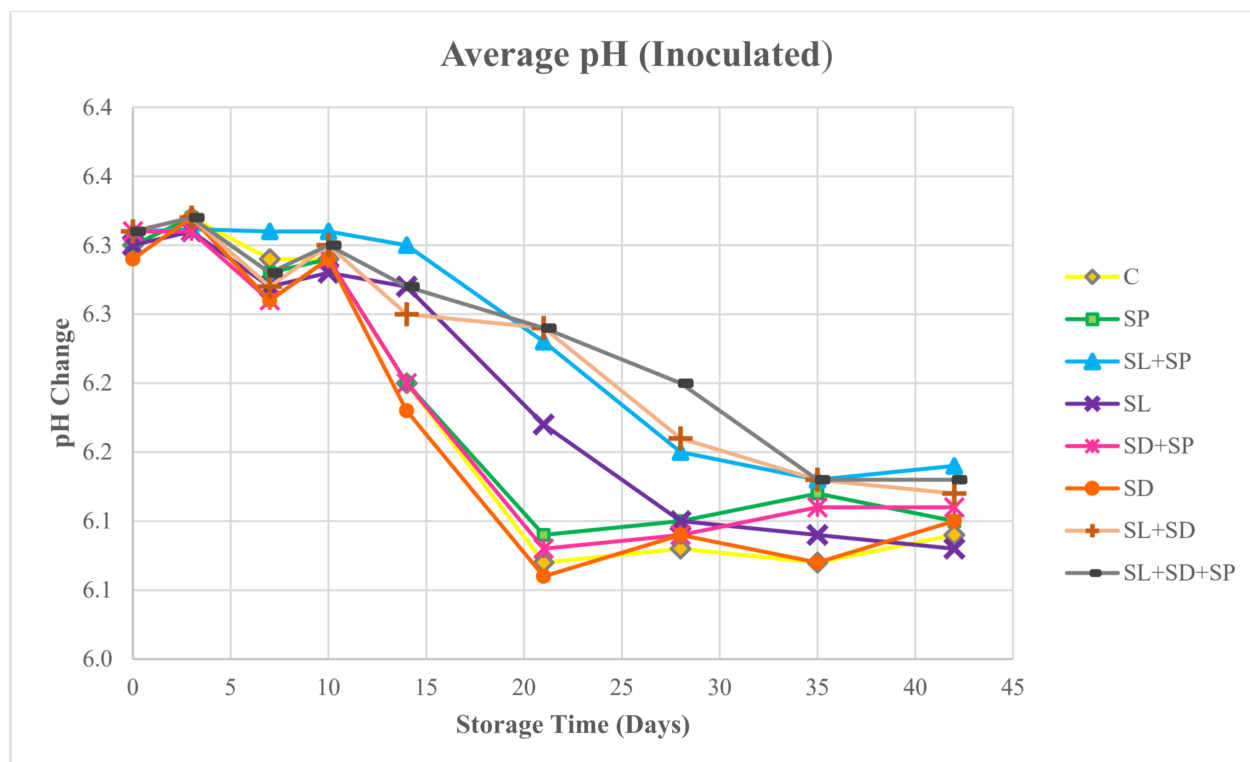


Figure 2.2 pH changes of lactic acid bacteria inoculated cured, cooked, sliced, and vacuum-packaged deli-style turkey breast treatments stored at 4ºC for 42 days, demonstrated from Table 2.5.

C: control with no antimicrobials, SP: Sodium propionate, SL: Sodium lactate, SD: Sodium diacetate.

Error bars are not shown to avoid visual cluttering in the figure.

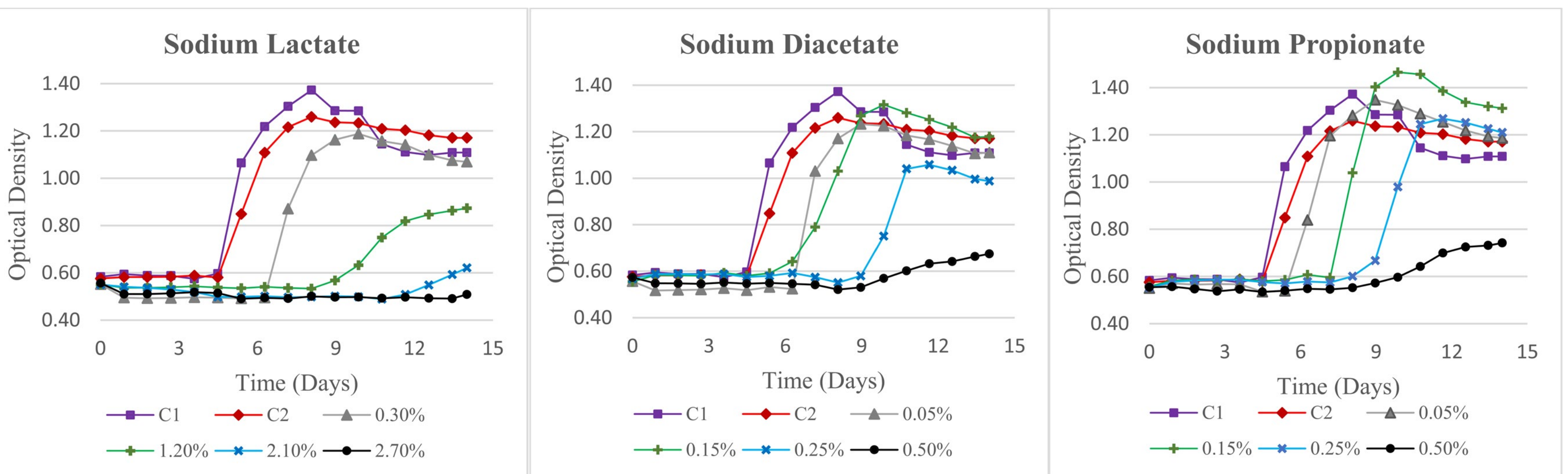


Figure 2.4 The growth patterns of *Lactobacillus sakei* in modified APT broth containing various amounts of sodium lactate, sodium diacetate, and sodium propionate at 10ºC for 14 days.

C1: APT broth, C2: APT broth containing salt, sodium nitrite, and sodium erythorbate.

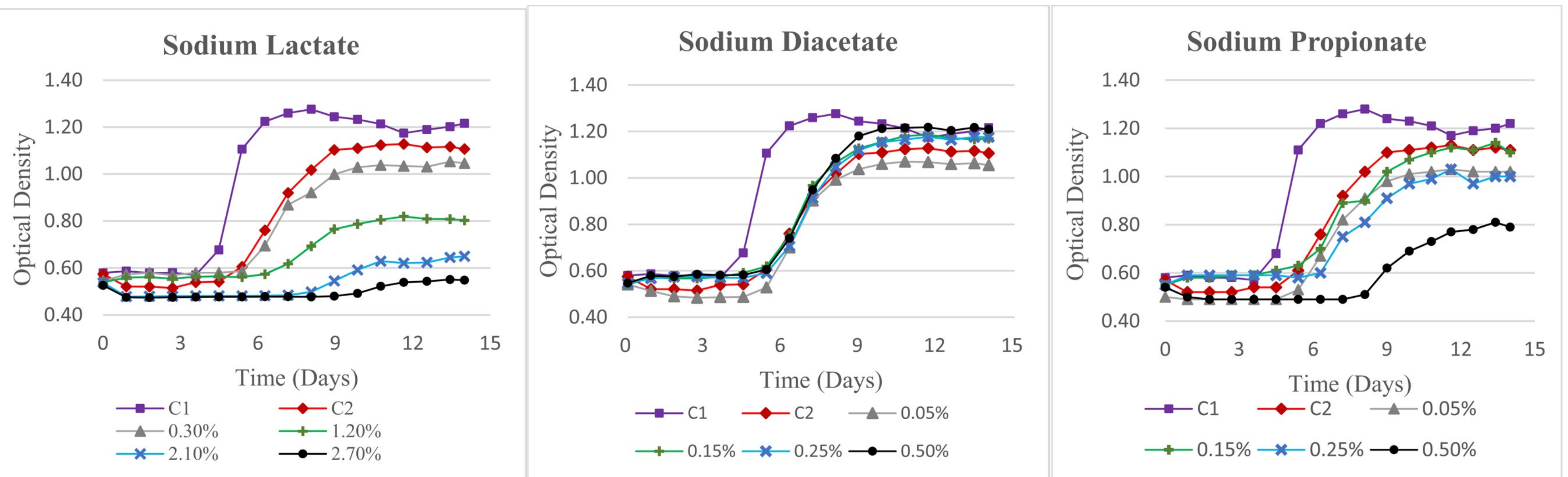


Figure 2.5 The growth patterns of *Leuconostoc mesenteroides* (deli-shaved ham isolate) in modified APT broth containing various amounts of sodium lactate, sodium diacetate, and sodium propionate at 10ºC for 14 days.

C1: APT broth, C2: APT broth containing salt, sodium nitrite, and sodium erythorbate.

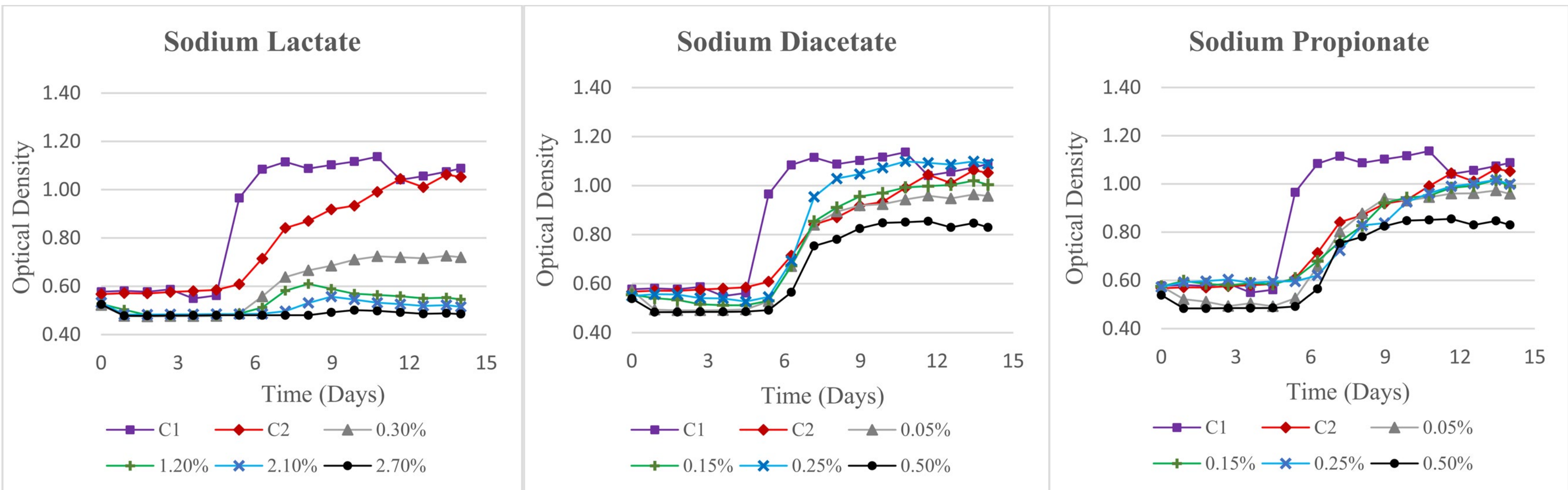


Figure 2.6 The growth patterns of *Leuconostoc mesenteroides* (bacon isolate) in modified APT broth containing various amounts of sodium lactate, sodium diacetate, and sodium propionate at 10°C for 14 days.

C1: APT broth, C2: APT broth containing salt, sodium nitrite, and sodium erythorbate.

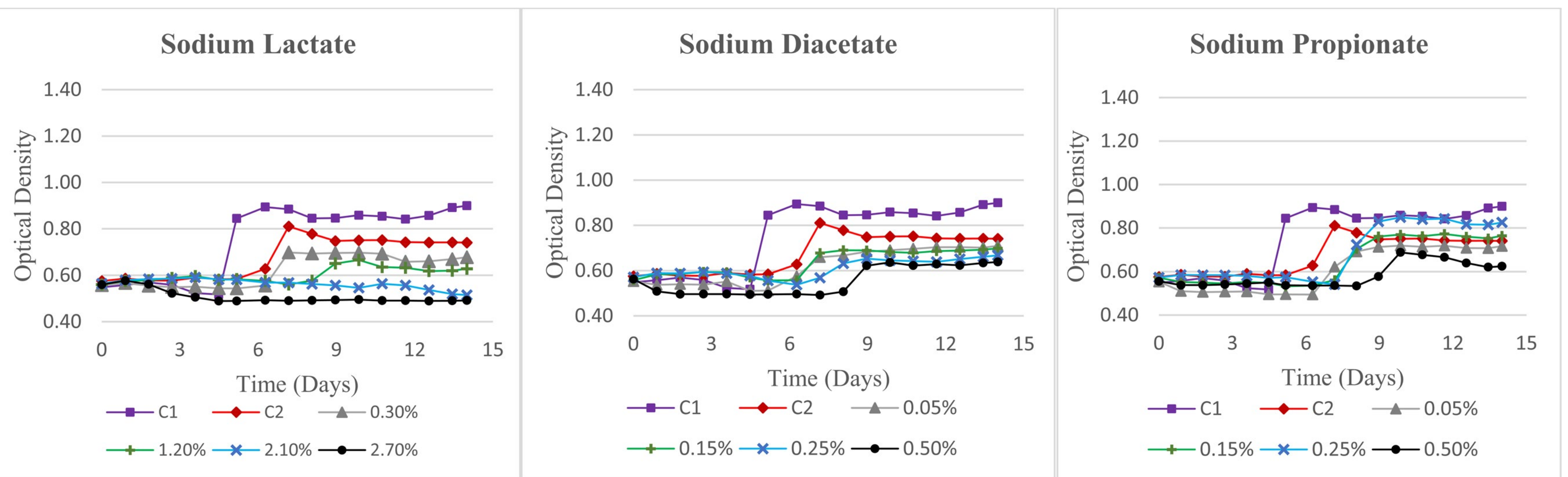


Figure 2.7 The growth patterns of *Lactococcus lactis* in modified APT broth containing various amounts of sodium lactate, sodium diacetate, and sodium propionate at 10ºC for 14 days.

C1: APT broth, C2: APT broth containing salt, sodium nitrite, and sodium erythorbate.

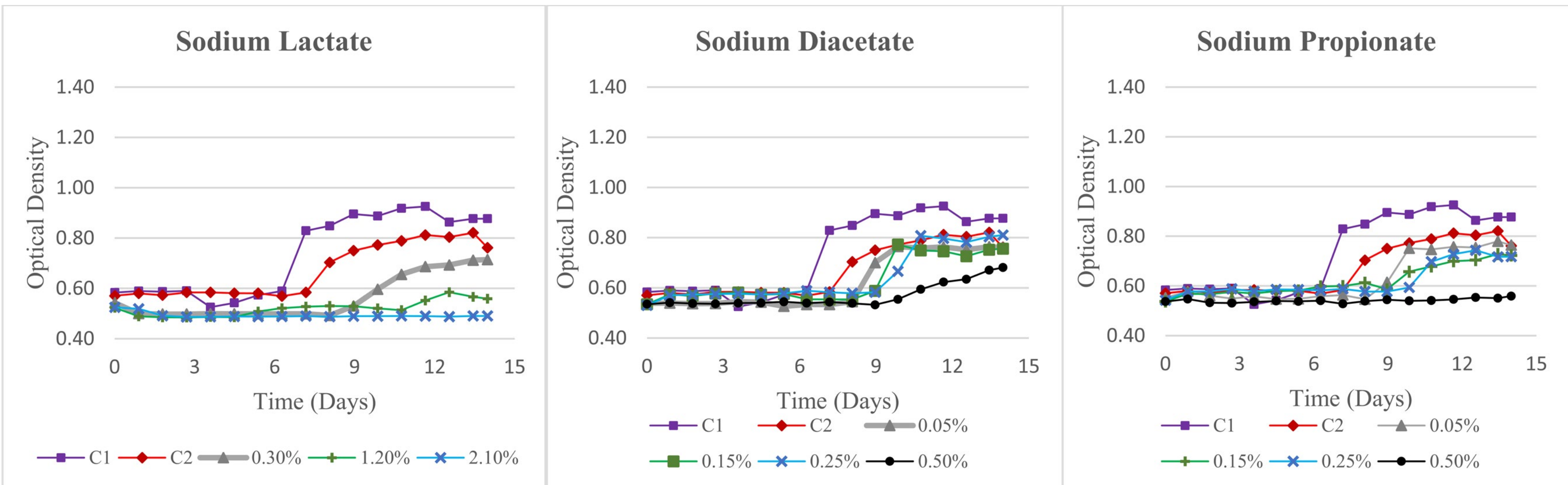


Figure 2.8 The growth patterns of *Enterococcus faecium* in modified APT broth containing various amounts of sodium lactate, sodium diacetate, and sodium propionate at 10ºC for 14 days.

C1: APT broth, C2: APT broth containing salt, sodium nitrite, and sodium erythorbate.

3. CHAPTER 3: THE EFFECT OF COMMONLY USED ORGANIC ACIDS ON THE MICROBIOTA OF CURED DELI-STYLE TURKEY BREAST

ABSTRACT

Sodium lactate (SL) and the combination of SL and sodium diacetate (SD) are commonly used for controlling pathogenic microorganisms in ready-to-eat (RTE) meat products. However, there is very limited information on the impact of these organic acids on the spoilage microbiota of RTE meat products. Therefore, the purpose of this study was to investigate changes in the microbial community structure consisting of spoilage lactic acid bacteria (LAB) when SD and SL and SD are included in the formulations at concentrations similar to those used for controlling *Listeria monocytogenes*. Sliced cooked, vacuum-packaged turkey breast samples containing no SD or SL (C, control), 0.125% SD, and the combination of 2.5% SL + 0.125% SD (SLSD) were inoculated with five different LAB species (*Lactobacillus sakei*, *Leuconostoc mesenteroides* (bacon and deli-shaved ham isolates), *Lactococcus lactis*, and *Enterococcus faecium*) and stored at 4°C for 35 days. Microbial community changes were analyzed utilizing 16S rRNA gene sequencing for the V4 region from the samples collected at days 0, 7, 14, 21 and 35. No significant difference was observed between the richness of microbial community for all treatments ($P > 0.05$). According to Bray-Curtis dissimilarity matrix and permutational ANOVA, a significant difference in ß-diversity was observed only between C and SLSD ($P < 0.05$) due to the antimicrobial effect of SL that slowed down the changes in microbial community composition until day 14. *L. sakei* remained a dominant strain throughout 35 days of storage regardless of treatment while *L. mesenteroides* existed with a very low abundance. Two of the five strains were not seen after day 7. No significant effect ($P > 0.05$) was observed for the SD treatment compared to C. According to the results from this study, only SLSD affected the microbial community

structure at the beginning of the storage. This study demonstrated the incorporation of SL in the formulation slowed down the changes in the microbiota of RTE meat products.

INTRODUCTION

Meat spoilage is a serious problem that affects different parts of the food chain including production, storage, and consumption, and storage. It makes meat products undesirable for consumption resulting in the decrease in shelf life and meat quality due to undesirable changes in physical and chemical characteristics such as the formation of off-flavors, discoloration, off-odors, and slime (Pellissery et al., 2020). It is a process involving various bacteria species and environmental factors such as the composition of the product itself, storage temperature, and product pH. The nutrition-rich composition of meat and meat products further supports the microbial spoilage process allowing the establishment of the microbial community. If the richness and abundance of spoilage-related species in this community reach an unacceptable level, it threatens to negatively impact shelf life and even microbial safety (Zhao et al., 2015). Due to microbial spoilage, excessive amounts of meat products have been wasted in the meat industry supply chain or discarded by customers. Meat processing establishments want to minimize and ideally eliminate microbial contamination to reduce industrial and economic losses, minimize or prevent meat waste, as well as to keep their products on the shelves longer without a reduction in quality. Concomitantly, consumers expect access the meat products that are wholesome, free of contamination, nutritious, and easy to prepare (e.g. ready-to-eat, RTE). RTE meat products are especially preferred due to consumers' busier lifestyles; however, they are also highly open to increasing opportunities for contamination (USDA, 2016). In general, properly cooked RTE meat and poultry products are expected to be free of microbiological contamination after thermal process. However, many post-cooking handling steps increase the risk of microbial contamination

which can be traced back to workers, environment, equipment, and processes such as packaging and slicing (Dempster et al., 1973; Korkeala & Björkroth, 1997; Samelis et al., 2000a, 2000b; Pothakos et al., 2015; Odeyemi et al., 2020). Even though RTE meats are processed in highly hygienic conditions and typically begin with undetectably low bacteria amounts (under 10 CFU/g) at the beginning of shelf life, spoilage can still occur quickly (Hamasaki et al., 2003).

The spoilage of CO_2-modified atmosphere or vacuum-packaged RTE meat products is primarily caused by lactic acid bacteria (LAB) and is considered as an economic loss rather than a food safety concern (Sarmento et al., 2015; Iulietto et al., 2015). *Lactobacillus, Leuconostoc, Enterococcus, Lactococcus,* and *Streptococcus* have been demonstrated to be the predominant flora in vacuum-packaged cooked and refrigerated meat products (Chenoll et al., 2007; Chávez-Martinez et al., 2016). These LAB detected in the high-hygiene packaging area have been reported to constitute the major microbiota of the cooked sausages (Hultman et al., (2015). These researchers further identified the LAB species as *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc gelidum subsp. gasicomitatum*, *Lactobacillus curvatus*, and *Lactococcus lactis.* In the microbiota of RTE meat products, *Lactobacillus sakei* and *Leuconostoc mesenteroides* have been regularly reported as the main predominant species (Dykes et al., 1994; Yang & Ray, 1994; Samelis et al., 1998, 2000a, 2000b). *L. lactis* and *Enterococcus faecalis* have also been found to be part of the predominant microbiota of RTE meat products (Barakat et al., 2000).

The application of organic acid salts is one of the most common and effectively used methods for pathogenic bacteria in controlling post-thermal process contamination in RTE meat products. (Glass et al., 2002; Mbandi & Shelef, 2002; Maks et al., 2010; Stopforth et al., 2010). However, limited studies are available on RTE meat products to reveal the effect of organic acids

on the mixture of spoilage LAB. Drosinos et al. (2006) applied different organic acid salts (sodium lactate, sodium acetate, potassium sorbate, and their combinations) in different concentrations to both broth system and cooked, vacuum-packaged RTE meat products stored for 40 days at 4°C. They reported an antimicrobial effect on the growth of spoilage LAB that also increased with an increase in concentration. Similar results were reached with sodium lactate regarding LAB in cooked, vacuum-packaged ham stored at similar conditions (Stekelenburg & Kant-Muermans, 2001). It has been also reported the shelf life doubled when the SL concentration increased from 2.5% to 3.3% (Stekelenburg & Kant-Muermans, 2001). These type studies are valuable to serve as fundamental investigations to the elude the effect of organic acids on spoilage LAB; however, they were limited to only bacterial colony enumeration in microbiological challenging tests and were not able to demonstrate how microbial community structure changed during storage.

Most recently, 16S rRNA gene sequencing has enabled the characterization of microbial diversity and relative abundances in meat systems revealing microbial community dynamics during storage time (Ercolini, 2013; Rouger et al., 2017). It is important to evaluate microbiota dynamics since each bacterial group may contribute to meat spoilage; however, spoilage contribution might change depending on environment and microbial community structure (Zhao et al., 2015; Cauchie et al., 2020). Therefore, a strong knowledge of microbial community composition and its dynamics under certain circumstances is crucial for the elimination or control of spoilage microorganisms in the preservation of meat products. However, how LAB species behave in microbial communities and interact with their environment is not well understood, especially in RTE meat products. Therefore, more information is needed about how the spoilage communities are changing throughout storage with the application of the different antimicrobials to improve the microbiological safety and the shelf life of RTE meat products. According to our

knowledge, there is no study specifically focused on the effect of different antimicrobials on inoculated spoilage microbiota of vacuum-packaged, cooked, and cold-stored RTE meat products. Therefore, the objective of this study was to investigate how the most commonly used antimicrobials sodium diacetate, and the combination of sodium lactate and sodium diacetate, affect the microbial community structure of spoilage lactic acid bacteria in the deli-style turkey breast during 35 days of storage when applied at similar concentrations as sued for controlling *Listeria monocytogenes*.

MATERIAL AND METHODS

Product Manufacture

Cured, deli-style turkey breasts were manufactured following good manufacturing practices at the University of Wisconsin-Madison, Meat Science and Muscle Biology Laboratory. Frozen, whole, skinless, and boneless turkey breasts were purchased from local suppliers and stored as frozen (-25ºC) until needed. Since all raw meats were obtained in a frozen state, all meat was subject to a single freeze/thaw cycle and the resulting purge was discarded. After thawing at 2.2 to 4.4ºC, the breasts were ground using a grinder (Hobart Model 4732, Hobart Corporation, Troy, OH) to 9.53 mm (3/8”).

Product formulations for microbiological analysis were targeted to achieve a 100% cook yield (no-cook loss) after thermal processing. The main formulation (1.7% salt, 0.30% sodium tripolyphosphate (STPP), 1.0% modified food starch, 100 mg/kg sodium nitrite, and 547 mg/kg sodium erythorbate) was used in all treatments to mimic typical formulations used in the industry for a cured deli-style turkey breast. Cured deli-style turkey breast formulations (TRTs; n=8), with an adjusted pH of 6.3 and moisture of 75.0%, were prepared with 2.5% sodium lactate (SL) (syrup,

60% (w/w) (Sigma-Aldrich, St. Louis, MO), 0.125% sodium diacetate (SD) (Sigma-Aldrich, St. Louis, MO), 0.125% sodium propionate (SP) (Sigma-Aldrich, St. Louis, MO), and SL+SD, SL+SP, SD+SP, and SL+SD+SP as well as a control containing no antimicrobial for microbiological challenge testing. Formulations for cured, deli-style turkey breast reported as a percentage of the total formula (Table 2.1, Chapter 2). For the current microbiota study, only samples collected from turkey breast containing SD, SD+SL, and control with no antimicrobial were used. Each treatment was manufactured in 3 replications, on three different days of production to demonstrate different raw turkey breast batches.

The Solver function in Microsoft Excel 2007 was used to calculate the details of treatment formulations (Microsoft Corporation; Redman, WA, USA). This function makes use of the approximate moisture levels of meat and all added ingredients while the required ingoing levels of each formulated ingredient serve as limitations (i.e., 1.7% salt, 0.30% STPP, 1.0% modified food starch, 100 mg/kg sodium nitrite, 547 mg/kg sodium erythorbate, 2.5% SL, 0.125% SD, and 0.125% SP were constraints for final product levels in all formulations). The treatments were "solved" by changing ingoing amounts of ingredients to the preferred limits while aiming for a wanted final product moisture level. Appendix 1 demonstrates the formulation spreadsheets adjusted by using the Solver function.

All weighed ingredients for all formulations were dissolved in water to provide uniform ingredient distribution. The ingredients were added into a solution in the following order: STTP, salt, sodium nitrite, sodium erythorbate, antimicrobials, sodium bicarbonate (if necessary), and modified food starch. After combining the ingredients, the solution was mixed with ground turkey meat for 2 min in a stand mixer (Hobart A120, The Hobart manufacturing Co., Troy, OH, USA). The mixture was then transferred to a rotary vane vacuum filler (Handtmann VF 608 Plus vacuum

filler, Handtmann CNC Technologies Inc., Lake Forest, IL) and stuffed into (90 mm flat width) moisture impermeable plastic casings (Nova X, Visko Teepak USA, Kenosha, WI, USA) to produce chubs that were approximately 65 cm in length. A clipper (Poly-clip model EZ 6080, Poly-clip System Corp., Mundelein, IL, USA) was used to close tightly the ends of each chub.

Thermal Processing and Slicing

Cooking was accomplished using a steam-jacketed kettle (Groen model N30, Groen MFG. CO., Chicago, IL) preheated to 80ºC until the internal temperature of the chubs reached 73.9ºC. After cooking, products were immediately chilled in icy water for 20 min and then placed in cooler (~4ºC) until sliced the following day.

All cooked turkey chubs were sliced using a manual deli slicer (Berkel Model 919E, Berkel Incorporated, Troy, OH, USA). To minimize contamination risk with background microflora, a 70:30 ethanol / distilled water solution was sprayed on all product contact surface areas and the exterior of the plastic casings of each treatment chub prior to slicing. The ethanol solution was allowed to evaporate for 15 s before product contact to ensure that ethanol's residual effects did not inhibit the growth of subsequent inoculated species. The peeling process for each chub was done immediately before slicing to reduce the possibility of recontamination. Since slice thickness was inherently affected by the density of varied product formulations, the products were sliced to a target thickness of 25 ± 1 g per slice. All treatments were immediately vacuum sealed (45.7 cm x 71.1 cm, 3-mil high-barrier pouches; Ultra Source LLC, Kansas City, MO, USA) after slicing and transferred to the Food Research Institute at the University of Wisconsin-Madison for microbial challenge testing. Microbiological challenge testing started one to two days after slicing, and the vacuum-sealed master packs of the sliced samples were stored at 4ºC until inoculation.

Microbiological Challenge Testing

Samples (each containing two 25 g slices) were inoculated with a mixture of five LAB strains. Two different strains of *L. mesenteroides* (bacon and deli-shaved ham isolates), *L. sakei*, *L. lactis* (meat isolate), and *E. faecium* (meat isolate) originating from two different commercial meat-related companies were grown individually in 9 mL All Purpose Tween (APT) broth (BD Difco, Sparks, MD, USA) at 30°C for 20 - 22 hours. LAB species were harvested by centrifugation (4000 x g, 20 min) and suspended in 4.5 mL of Butterfield's phosphate buffer. Equivalent populations of each strain were combined to obtain a five-strain blend of LAB for the inoculation of products. Each LAB strain population and their mixtures were validated by plating on APT agar (BD Difco, Sparks, MD, USA). The two turkey breast slices per package were surface inoculated with a target of 3 log CFU/g of five different LAB strain blends (5 log CFU/50 g package) in total by applying 0.25 mL inoculum onto various surface areas. Both inoculated and uninoculated slices were placed in gas-impermeable vacuum chamber pouches (3 mil, 7 x 9", UltraSource, Kansas, MO, USA) and vacuum-packaged (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany) prior to being stored at 4°C for up to 42 days.

Rinse material from each sample for microbiota analysis were collected on days 0 (inoculation day), 3, 7, 10, 14, 21, 28, 35, and 42 by washing and hand massaging for 3 min with 50 mL of sterile Butterfield's phosphate buffer solution. 1.5 mL of rinsate from each technical replicate was collected into 1.5 mL Eppendorf tubes and stored at -80°C for later microbiota analysis. For this microbiota study, the effect of only control, SD, and the combination of SL and SD were tested from the samples collected at days 0, 7, 14, 21, and 35.

DNA Extraction, PCR, and Library Preparation

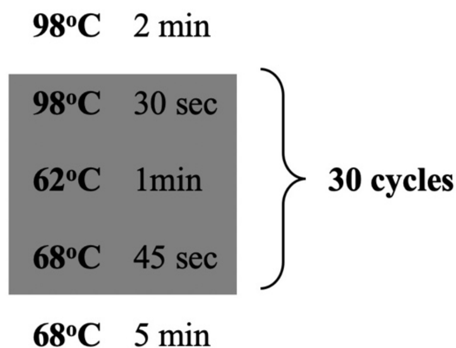
Frozen samples – (-80ºC) were thawed on ice for the DNA extraction process applying a mechanical cellular disruption method and hot/cold phenol. Bead-beating tubes with 0.5 g of 0.1 mm beads were filled with 1 mL of the sample, 700 µL equilibrated phenol, and 50 µl 20% SDS were added and bead-beaten for 2 min. Following incubation in a water bath at 60ºC for 10 min, the tubes were bead-beaten for an additional 2 min followed by centrifuging (Hermle Benchmark - Z 216 MK, HERMLE Labortechnik GmbH, Wehingen, Germany) at 15000 rpm speed at 4ºC for 10 min. The aqueous phase of samples was carefully transferred into phenol safe 1.5 mL Eppendorf tubes and DNAs of the samples were extracted with 500 µL phenol:chloroform:isoamyl alcohol 25:24:1. Tubes were briefly vortexed and centrifuged for 10 min at 4ºC at 15000 rpm. The previous phenol:chloroform:isoamyl alcohol wash step was then repeated to get the DNAs as much as pure and followed with vortexing and centrifugation.

The aqueous phase of samples was collected into new tubes containing 0.1 vol 2M sodium acetate and 0.6 vol (including acetate) isopropyl alcohol. Tubes were mixed by inversion and stored at -20ºC overnight for DNA precipitation. After the precipitation process, samples were centrifuged at the 15000 rpm speed at 4ºC for 20 min and the supernatants were discarded. Pellets were carefully washed with 1 mL 70% ethanol and centrifuged at 15000 rpm at 4ºC for 1 min. The ethanol wash step was repeated using 500 µL ethanol followed by additionally centrifuging at 15000 rpm at 4ºC for 3 min followed by air drying in the fume hood until no ethanol remained. The dried pellets were suspended in 40 µL of Zymogen DNA elution buffer (Zymo Research, D3004-4).

The DNA concentrations of the samples were determined using the Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen - Thermo Fisher Scientific, Waltham, MA) by a Qubit®

Fluorometer (Invitrogen, San Diego, CA). A black tube rack was used to keep the master mix and assay tubes out of the light as much as possible since the Qubit reagents were light-sensitive. Qubit HS reagent (n x 1 µL) was mixed by Qubit HS buffer (n x 199 µL) in a covered microcentrifuge tube to make a master mix. 190 µL of the master mix was aliquoted into each of two Qubit tubes. 198 µL of the master mix was aliquoted into each of the assay tubes. 10 µL of HS standard 1 and 10 µL of HS standard 2 were added into Qubit standard tube 1 and Qubit standard tube 2 respectively. 2 µL of sample DNA was added into each assay tube. After having a final volume of 200 µL, all tubes were briefly vortexed and kept for 2 min in a dark place. Qubit fluorometer was calibrated with standards 1 and 2 by making sure that standards were as prompted by the Qubit. The concentrations were recorded after changing units to ng/µl and the DNA volume to 2 µL on Qubit. After quantifying DNA amounts, concentrations were standardized to 5 ng/µL by diluting with ultrapure water for PCR reactions.

The V4 variable region of the 16S rRNA gene was amplified using barcoded universal bacterial primers (F- GTGCCAGCMGCCGCGGTAA; R- GGACTACHVGGGTWTCTAAT) demonstrated by (Kozich et al., 2013). The primers also contained adapters suitable for sequencing on Illumina platforms (F- AATGATACGGCGACCACCGAGATCTACAC; R- CAAGCAGAAGACGGCATACGAGAT). PCR reactions were performed in 20 µL reaction mixtures containing 12.5 µL 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.6 µL Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA), 10ng template DNA, 0.75 µL forward and reverse primers (10µM/µL), and water to a total volume. A Bio-Rad S1000 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for PCR reactions following this protocol:



Nuclease free water was used as a no-template PCR negative control. After amplification, PCR products were run on a 1.0% w/v low melt agarose gel with SYBERSafe DNA gel stain (Invitrogen, Waltham, CA). Amplicon bands at ~ 380 bp were cut and purified using a ZR 96 Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). If negative control showed a band, all samples in that group were redone starting at PCR set-up and amplification. Clean negative controls indicating no band had the approximate location of the amplicon (~ 380 bp) cut and sequenced as further validation that no contamination was present. Purified products were then quantified using the Qubit® dsDNA HS Assay Kit on a Qubit fluorometer and a 96-well plate spectrophotometer and were equimolar pooled into a 4nM library. The final library was sequenced on an Illumina MiSeq using a 500-cycle PE MiSeq v2 reagent kit (Illumina, San Diego, USA) with a 10% PhiX control and custom sequencing primers (Kozich et al., 2013) at the University of Wisconsin-Madison Biotechnology Center.

Sequence Analysis

Sequences were demultiplexed on the Illumina MiSeq and were further processed using mothur v.1.44.3 (Schloss et al., 2009) following a protocol developed by Kozich et al., (2013). In short, sequences with ambiguous base pairs, homopolymers greater than 8bp, and lengths shorter than 200bp and greater than 500 bp were removed. Sequences were aligned against the SILVA

16S rRNA gene database v138 (Pruesse et al., 2007) and those not aligning to the V4 region were removed. Preclustering was performed (diffs = 2) for error reduction and chimeras were detected and removed (Edgar et al., 2011). Sequences were then taxonomically classified using the mothur-formatted SILVA taxonomic database v138 with a bootstrap cutoff of 80. Unclassified sequences and sequences classified as mitochondria, chloroplasts, Eukarya, or Archaea were removed. Singletons were also removed. Sequences were then grouped into operational taxonomic units (OTU) at 97% sequence similarity. Good's coverage (Good, 1953) was calculated, and samples were normalized to 489 sequences per sample. Chao1 richness (Chao, 1984) and Shannon's diversity index (Shannon, 1948) were then estimated for each normalized sample using mothur. OTUs were classified using SILVA taxonomic database v138 for final classification.

Statistical Analysis

Rare OTUs, probably due to sequencing and PCR errors, were removed. The OTU table was subset to include only OTUs classified as the order "Lactobacillales" for subsequent analyses due to our specific interest in the community structure of the inoculated five different spoilage LAB species. 30 OTUs out of 289 OTUs remained after data cleaning. Statistical analyses were performed in R (v4.0.2, https://www.r-project.org/; R Core Team, 2020).

A table of OTU counts of all treatments (C, SD, and SLSD) including their 3 replicates was created. For a quality control, three OTU (abundance) tables were derived from this OTU table to investigate whether the bacterial community of a randomly selected replicate differed significantly from the mean and median values of the other replicates; OTU table of a random replicate from every three replicates, OTU tables of means and OTU tables of medians. QQ-plots and the Shapiro-Wilk test were used for normality check. The OTU data was not normally

distributed before and after log and inverse log transformations were applied (Skarlupka et al., 2019). A two one-sided test (TOST) of equivalency and a robust TOST (RTOST) test were performed applying the “equivalence” R package (Robinson, 2016) to determine whether all three biological replicates for all treatments were equivalent. TOST requires normality while RTOST makes no assumption on normality and is more robust to long-tailed distributions and outliers (Yuen & Dixon, 1973; Yuen, 1974). The paired RTOST was used on the random replicate vs. the mean OTU and the random replicate vs. the median OTU datasets for all days splitting into treatments.

For alpha diversity, Chao1 richness (number of species) and Shannon’s diversity index (a measure of how evenly microbes are distributed) estimates were used. Alpha statistics were calculated using vegan and phyloseq packages in R (McMurdie & Holmes, 2013). Before running statistical analyses, the Shapiro-Wilk test was used to evaluate the normality of Chao1 ($W = 0.63$, $P = 1.752e-09$) and Shannon ($W = 0.67$, $P = 9.92e-09$) indexes. Being non-normally distributed, the significance of the differences between treatment groups was assessed using the Kruskal-Wallis test for Chao1 and Shannon’s diversity. Furthermore, the paired Wilcoxon rank-sum test was used with false discovery rate (FDR)-corrected p-values to determine which groups are different.

β-Diversity is used to determine how the overall bacterial community differs between samples. For β-Diversity, it is hard to test the microbiota composition directly using OTUs or abundances due to the high dimensionality and phylogenetic structure of the microbiota data. The significance of separation in β-diversity space was assessed by permutational multivariate ANOVA (PERMANOVA). Variance homogeneity assumption for PERMANOVA, which is similar dispersion within each group, was tested applying “betadisper” and then “permutes” in R

"vegan" package (Oksanen et al., 2019). The Bray-Curtis (diversity based on abundance; community structure) and Jaccard (richness based on OTU presence-absence; microbial community composition) distance metrics were calculated beforehand. Data were analyzed as three treatments (C, SD, SLSD) by five-time points (Day: 0, 7, 14, 21, 35), including interaction with the day as a repeated measure. PERMANOVA test was stratified by sampling day with strata argument to account for external variability between treatment groups to see if treatments differ while controlling for time (adonis; vegan package). H_0: Microbiota composition is the same across sampling days. H_1: Microbiota composition differs between sampling days. False discovery rate (FDR)-corrected p-values calculated using "vegdist" (vegan package). A Non-metric multidimensional scaling (nMDS) plot was used to visualize β-Diversity. Community taxonomy composition, β-Diversity, and alpha diversity metrics were visualized using the R package ggplot2 (Wickham, 2009) and phyloseq. Significance was established at $P < 0.05$.

RESULTS

Sequencing generated, 4,308,005 raw sequences, of which 2,752,303 were high-quality reads after the cleanup process, were generated. All samples had a Good's coverage > 0.95. The number of sequences in each sample ranged from 489 to 128,482 with a mean of 60,246 and a median of 80,623 sequences. When separated by treatment, control with no antimicrobial ranged from 575-102,809 sequences, diacetate ranged from 506-126,255, and the combination of lactate-diacetate ranged from 489-128,482 sequences.

Replicates of each sampling day within each treatment were compared applying the TOST and RTOST, with a trim level of 20% and equivalency margin of 0.25, to test whether there is a significant difference between a randomly selected replicate against the mean or median of the

associated three biological replicates. Data were not normally distributed according to the Shapiro-Wilk test and QQ-plot visualization. Inverse log transformation was applied but it did not improved normality. The test results were significant ($P < 0.0001$), rejecting the null hypothesis of not being equivalent at the 5% level. These results demonstrate that comparisons of random against mean and median data sets favor the alternative hypothesis of them being equivalent. RTOST was preferred over TOST due to no assumption on normality and its robustness.

Alpha diversity: Shapiro-Wilk normality test showed that the Alpha diversity metrics, Chao1 and Shannon, did not have normal distributions ($P < 0.001$). Therefore, the Kruskal-Wallis rank sum test was used to test differences of the Chao1 Richness Estimate and Shannon's Diversity metrics. No significant differences were found between replicates ($P = 0.217$). Both Chao1 and Shannon demonstrated reduced bacterial richness and diversity as the storage period increased. The differences between treatments for Chao1 were not significant in the Kruskal-Wallis rank sum test ($P = 0.077$), while significant for Shannon ($P = 0.020$) (Figure 3.1). Further, the paired Wilcoxon rank sum test applied on Shannon and revealed that SLSD treatment had a significant difference compared to C ($P = 0.021$), whereas SD did not show any significant difference from C ($P = 0.507$) and SLSD ($P = 0.085$).

Beta Diversity: The dispersions within each treatment were similar ($P = 0.57$) satisfying the assumption to run PERMANOVA. PERMANOVA test results using Bray-Curtis and Jaccard distance matrix showed that there were no significant treatment main effects ($P > 0.53$). There was a treatment by day interaction ($P = 0.001$) demonstrating that the microbial community composition was affected significantly by treatment over storage time. Most variability was explained by the day between SLSD and C ($P < 0.001$), while other interactions were insignificant. Very similar results were seen when using the Jaccard metric instead of Bray-Curtis. The

differences between both the turkey breast samples within the group and across groups by plotting each day of sampling for all treatments, based on treatment type, were examined using non-metric multidimensional scaling (nMDS) of the Bray-Curtis dissimilarity index (Figure 3.2). nMDS analysis indicated that the microbiota composition of samples in day 0 had a little diverse and spread-out pattern with a similar overall composition, while samples after day 14 clustered closely together. Analysis of turkey breast samples with different treatments showed a clear separation of SLSD from C and SD for especially day 0 and day 7 while C and SD were closely clustered. All treatments for days 14, 21, and 35 clustered at almost the same region.

Composition: Taxonomic bar plots of family and genus level classifications of OTUs (bacterial communities) based on different antimicrobial treatments and sampling day was demonstrated in Figure 3.3. Although the products were inoculated with equal concentrations of each strain of lactic acid bacteria, the relative abundances of each at day 0 were not found to be equal. From the family classification, all treatments were characterized by a high increase in Lactobacillaceae from day 7, while Streptococcaceae and Enterococcaceae disappeared immediately after day 7. Leuconostocaceae population was significantly diminished through the storage time but remained almost the same after day 7. From genus classification, the most obvious change was a dramatic increase in Lactobacillus and a drastic decrease in all other genera for all treatments just after day 0. This decrease was a little slower for samples with SLSD compared to C and SD, which allowed SLSD samples to remain with a diverse community structure until day 14, but overall Lactobacillus (*L. sakei*) occupied the majority through 35 days of storage. Taxonomic analysis indicated that SD and C had similar microbial community structure changes at all sampling points.

DISCUSSION

At the beginning of the shelf life, there was a mixture of 5 equally added different strains containing *L. sakei, L. lactis*, *E. faecium,* and 2 different *L. mesenteroides* strains (bacon and ham isolates) in the microbial community composition of turkey breast. Differences in relative abundances were observed at day 0, which might have occurred in the time interval from inoculation to sampling time due to the differences between adaptation capabilities of bacteria to the new environment. The mixture of the strains was still in a phase of rapid cell division when they were inoculated to turkey breast. Therefore, unequal distribution of bacteria might have occurred in a short time after inoculation although plate counts validated that similar and enough bacteria populations were added before inoculation. On the other hand, the ratio of bacteria abundance across samples was similar after day 14. If there is any significant antimicrobial effect of the organic acids, microbial growth and community composition would be expected to be different between control and treatments following days. Overall, the changes in the microbial composition were similar for SD, SLSD and control at the end of the 35 days. Only *L. sakei* and *L. mesenteroides* remained in the microbial community composition while *E. faecium* and *L. lactis* totally disappeared after day 14. Possible high adaptation similarity of chosen bacteria to SD and SLSD or similar and weak mode of action of SD and SLSD might cause very similar community diversity and abundance as in control. Current study findings were overlapped with the results of previous studies. Kalschne et al., (2015) reported that the predominant species in the vacuum-packaged RTE meat product were mainly *Lactobacillus sp.* followed by *Leuconostoc sp.*, nevertheless, the *Enterococcus sp.* was not present in the samples at the end of storage. It has been also reported that in a mixed culture dominance test, when *L. mesenteriodies* and *L. sakei* were present in equal initial numbers, *L. sakei* outcompeted than *L. mesenteroides* at refrigeration

temperatures (pH ≥ 6) (Zhang & Holley, 1999). Comi et al. (2016) also reported when *L. mesenteroides* were found with *L. sakei* or *L. lactis* bioprotective cultures, *L. sakei* or *L. lactis* grew faster than *L. mesenteroides* and the ratios were 1/1000 (*L. mesenteroides/L. sakei*) or 1/100 (*L. mesenteroides/L. lactis*) at the end of shelf life in bacon, respectively. Therefore, the microbial richness of the meat and poultry products decreased during storage in the current and cited studies. Only a few commonly reported species inside the community remained dominant to spoil the meat, which can be explained by competition for food and adaptation to the environment.

For treatments C, SD, and SLSD, *L. sakei* dominated the community by day 7, while relative abundances of *L. lactis*, *E. faecium*, and *L. mesenteroides* were dramatically decreased. SLSD slowed down the changes in microbial community structure until day 14. After day 14, SLSD had similar changes in relative abundances to C and SD. SLSD mixture might have delayed the lag phase of LAB for a short time until adaptation. If so, this effect probably came from the SL component itself since no effect was observed for SD. Similar to the current study, Drosinos et al., (2006) tested the antimicrobial effect of sodium lactate on spoilage LAB. They observed a limitation of bacterial growth when they used a concentration of 3% SL for 40 days at 4°C in cooked vacuum-packaged meat products. Besides, a significant change in the limitation effect of SL was observed in 4% and 5% SL concentration compared to 2% SL during 15 days in the broth system. However, bacteria behaved similarly to the control after day 15. Stekelenburg & Kant-Muermans (2001) reported similar effects with the application of SD (0.1% and 0.2%) and SL (2.5% and 3.3%) for spoilage LAB in cooked vacuum-packaged ham stored up to 40 days at 4°C. They reported an increase in lag time and shelf life with SL application while no effect was observed with SD. Furthermore, shelf life doubled by 3.3% SL compared to 2.5% SL. The results in this study and literature offered the incorporation of SL in meat formulations had some

antimicrobial effect on spoilage LAB microbiota and can extend shelf life delaying the microbial growth. Higher lactate concentration may result in a more significant effect on growth dynamics on LAB. However, due to mimicking the industry practices in this study, similar amounts of antimicrobials preferred for *L. monocytogenes* in RTE meat products were used. Additionally, it was hard to make any clear conclusion both on the interaction between other bacteria and the possible effect of SLSD due to the quick predominance of *L. sakei*.

The findings of the current study were parallel with the results of the APC (Aerobic plate counts) reported in chapter 2 at which SD and C displayed similar growth pattern in the microbiological challenge test, whereas SLSD had an up to 1-week lag phase extension. The APC results were indicative and did not tell how microbial community composition and abundance of LAB changed. Therefore, the behavior of microbes in RTE meats with different antimicrobials needed to be investigated at the family or genus level. The use of SD in the turkey breast formulation had similar results to the control and did not impact the microbial community composition through the shelf life. On the other hand, the presence of SLSD, compared to C and SD, influenced the relative abundances and slowed down the growth of LAB a little for the first week. In this case, it might be concluded that SLSD had some negative impact on the growth of LAB by extending their lag phase for a short time. The effect of SLSD probably came from SL itself rather than SD or the cumulative effect of both, since no significant difference was observed between samples with C and only SD. As known, lactate added to the meat system can arrest the cell growth by causing intracellular pH drop by increasing the dissociated lactic acid accumulation in the cytoplasm (Axe & Bailey, 1995; Ricke, 2003). It has been reported that lactate accumulation had an effect on the activity of lactate dehydrogenase (LDH) enzyme (Desguin et al., 2017). Therefore, excess lactate in the cell needs to be extruded and this process requires energy. Cells

canalize their energy to regulate these processes. Otherwise, it slows down LDH activity and disrupts pH homeostasis (Desguin et al., 2017). It is crucial for bacteria to survive rather than grow when the cytoplasmic pH is below neutral. In that case, bacteria spend their energy to regulate the cytoplasmic pH instead of proliferation. Bacteria cells in the log phase prefer to use their energy for pH regulation rather than growth in that process. However, cells in the adaptation period cannot properly handle the situation because they do not have enough energy, leading to the lag phase extension (de Wit & Rombouts, 1990).

Even though *L. lactis* had a similar predominancy to *L. sakei* in SLSD compared to C and SD at day 0, later, *L. sakei* took over the control in SLSD which confirmed that its dominance was not due to having a higher abundance at day 0 like in C and SD. It might also be concluded that not only dominancy but also the existence of *L. sakei* might have a restricting factor on the growth of other bacteria. If so, *L. mesenteroides* was the most resistant lactic acid bacteria in this competition among others used in this study surviving up to the end of the storage time. *L. mesenteroides* were also reported as dominant in some other studies and were responsible for spoilage with negative effects on the sausage products, such as off-color and off-flavor (Hultman et al., 2015; Comi et al., 2016). However, in their microbial community composition, there was no *L. sakei*. Similarly, *Leuconostoc sp*. was reported as the most dominant species in minced meat supplemented with 2% SL under modified atmosphere when *L. sakei* was not present. Additionally, *Lactococcus sp.* were also present with *Leuconostoc sp*. in the minced meat at day 0. However, *Lactococcus sp.* had a very low relative abundance in a week and the results were similar to our current study (Stoops et al., 2015). No change was observed in the color and odor of the turkey breast in this study although high growth of *L. sakei*. Possible suppression of *L.*

mesenteroides growth by *L. sakei* might prevent spoilage effect on the meat. Therefore, the presence of *L. sakei* affected the microbiota.

CONCLUSION

The effect of organic acids as antimicrobials is widely known for pathogens but limited data are available for the effect of sodium lactate and sodium diacetate on spoilage lactic acid bacteria in meat products in the literature. The results from this study will help to establish a better understanding of the interaction between bacterial growth dynamics and their behaviors under determined environmental conditions. However, comprehensive parallel studies are also needed to better unravel the effect of organic acids and the interactions between spoilage LAB at the species level in meat microbiota including their wide-scale concentrations. Knowledge from this and similar studies will lead to developing new approaches regarding the preservation of RTE meat products either in controlling meat spoilage or selecting bacteria to compete with spoilage or pathogen bacteria.

This study differs from other microbiota studies in the meat system. Instead of investigating natural background flora, the most common spoilage lactic acid bacteria community was defined (inoculated) beforehand. In this study, it was demonstrated the role of weak organic acid salts, SD and SLSD, on lactic acid bacteria responsible for meat spoilage. The effect of the SD and SLSD ranged from none to little in the spoilage community structure of RTE turkey breast by day 14. The spoilage patterns from day 14 to the end of day 35 were almost the same regardless of the applied organic acid. The overall microbial community was mainly dominated by *L. sakei* and only a small decrease was observed in its growth pace until day 14 in treatment SLSD compared

to others. This study demonstrates the addition of SL into meat formulations slows down the changes in microbial community composition and abundance of its member.

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TABLES AND FIGURES

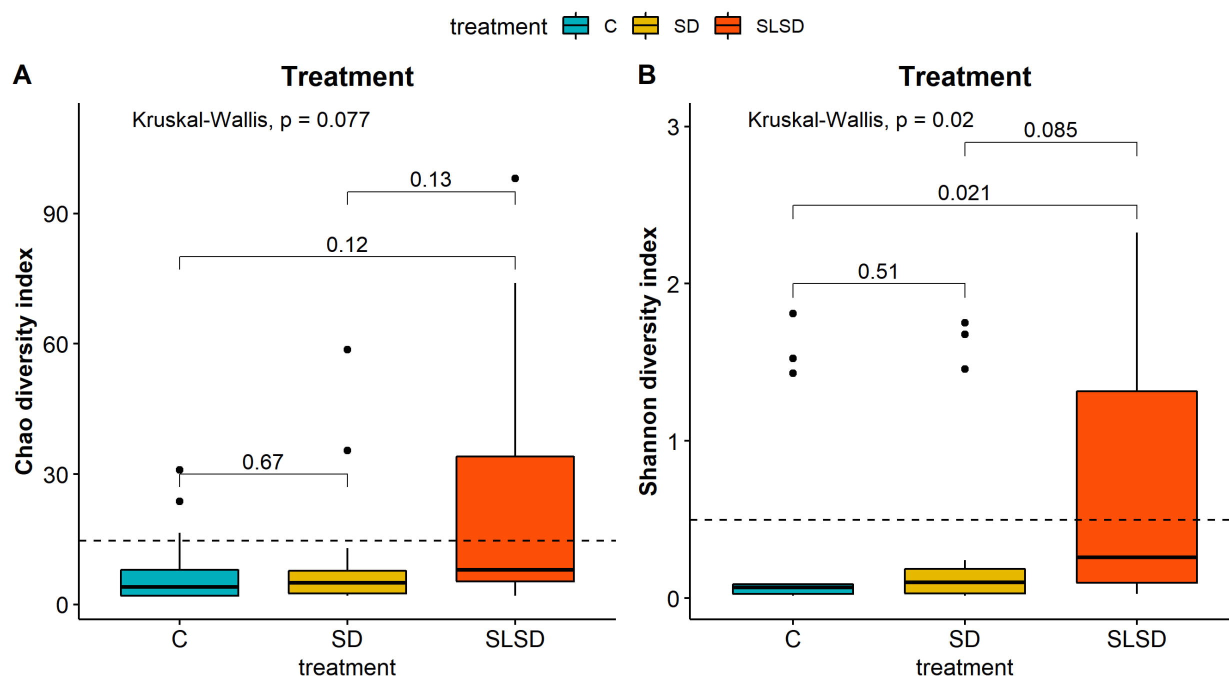

Figure 3.1 Alpha diversity comparisons. Richness estimators (**A**) Chao1 diversity index and evenness estimator (**B**) Shannon's diversity index. P-values for pairwise comparison of the treatments obtained with the pairwise Wilcox rank-sum test (FDR, 0.05). C: Control, SD: Sodium Diacetate, SLSD: Sodium Lactate + Sodium Diacetate.

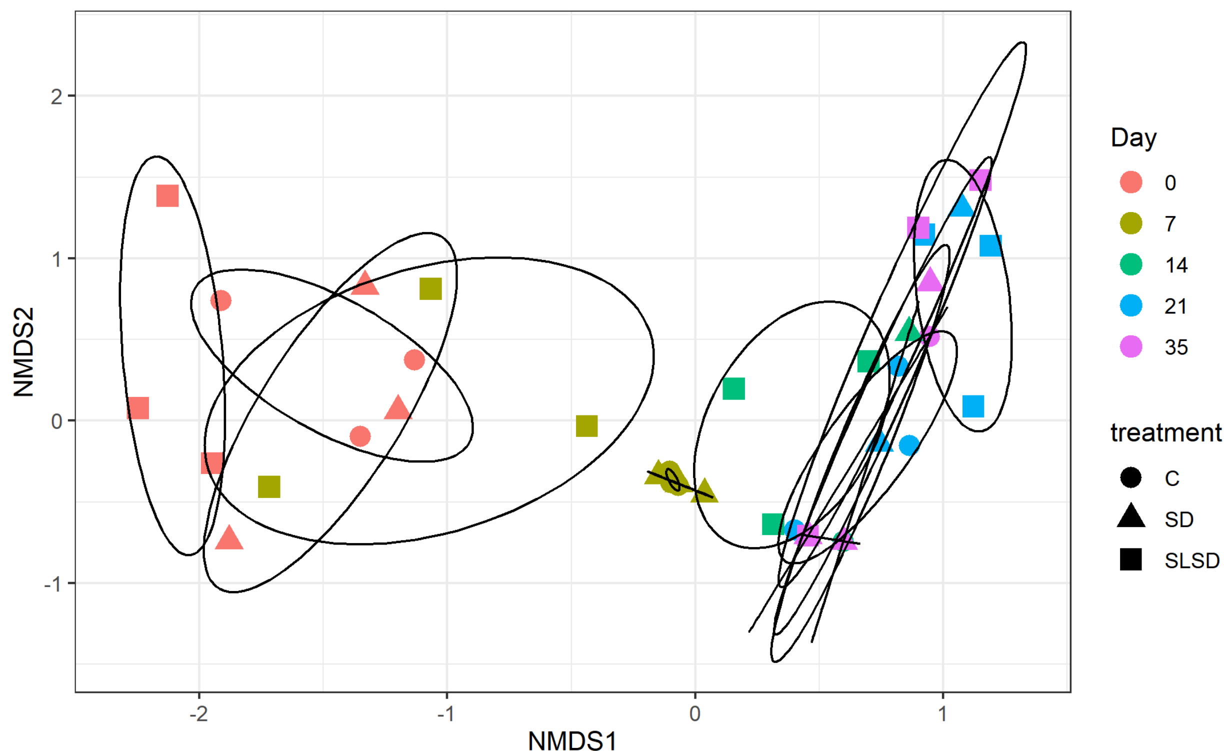

Figure 3.2 Nonmetric multidimensional scaling (NMDS) ordination of beta-diversity of microbiota community in turkey breast samples for three treatments from day 0 to day 35. Bray-Curtis distances were used between samples to generate NMDS to visualize microbiota dissimilarities. Each symbol in the figure represents the microbiota profile of a single sample. Same colored symbol represents groups of samples microbiota belongs to the same sampling day. C: Control, SD: Sodium Diacetate, SLSD: Sodium Lactate + Sodium Diacetate. 0, 7, 14, 21, 35: Sampling days.

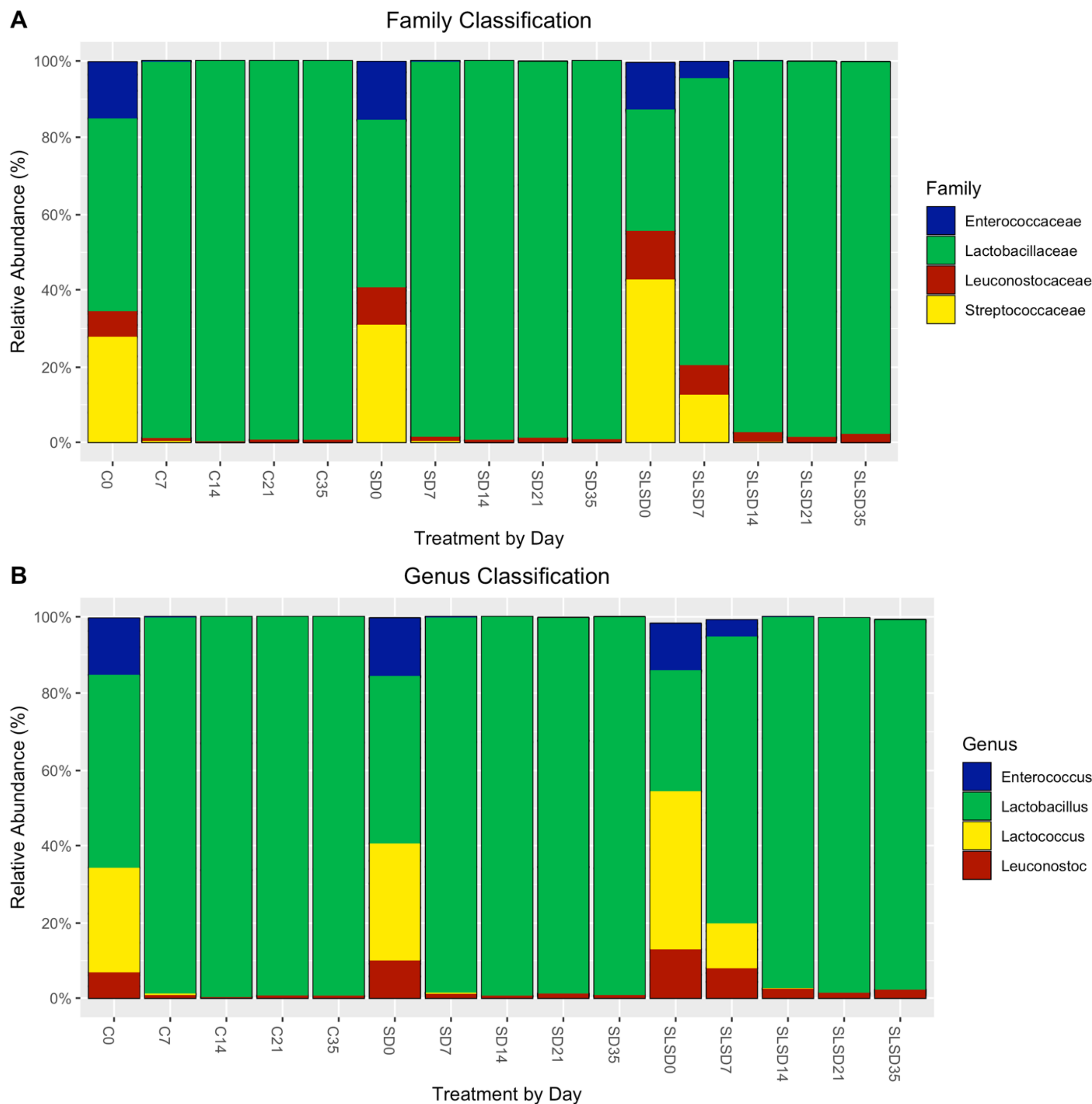

Figure 3.3 Microbial structures of turkey breast samples. (**A**) Family-level classification. (**B**) Genus-level classification. Relative Abundance of the inoculated 5 species used: *L. sakei, L. lactis, E. faecium,* and 2 different *L. mesenteroides* strains. C: Control, SD: Sodium Diacetate, SLSD: Sodium Lactate + Sodium Diacetate. 0, 7, 14, 21, 35: Sampling days.

APPENDICES

Appendix 1: Formulation spreadsheets of cured, deli-style turkey breast treatments (8 Treatments) adjusted by using the Solver function.

Treatment 1 - Control

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.314				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		12.306772						
Total lbs. of Brine		0	1.00					
		0						
Water		0	0.73	330.57	7.94	72.57	1.00	0.73
Salt		1.91	0.16	70.80	1.70	15.54	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.34	0.03	12.49	0.30	2.74	0.00	0.00
Propionate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diacetate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Antimicrobial 4		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Modified Food Starch		1.13	0.09	41.65	1.00	9.14	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.05	0.44	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.14	1.30	0.00	0.00
Total		3.38	9.18	4164.77	11.13	101.74		6.89
							Est Moisture	0.75

Treatment 2 - Propionate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.31				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		12.949421						
Total lbs. of Brine		0	1.06					
		0						
Water		0.00	0.77	348.41	8.32	72.69	1.00	0.77
Salt		1.92	0.16	71.21	1.70	14.86	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.34	0.03	12.57	0.30	2.62	0.00	0.00
Propionate		0.14	0.01	5.24	0.13	1.09	0.00	0.00
Lactate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diacetate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sodium Bicarbonate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Modified Food Starch		1.13	0.09	41.89	1.00	8.74	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.05	0.42	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.14	1.24	0.00	0.00
Total		3.54	9.23	4188.56	11.63	101.66		6.93
							Est Moisture	0.75

Treatment 3 - Propionate - Lactate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.314				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		21.388188						
Total lbs. of Brine		0	1.75					
		0						
Water		0.00	1.19	537.66	11.95	67.92	1.00	1.19
Salt		2.07	0.17	76.52	1.70	9.67	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.36	0.03	13.50	0.30	1.71	0.00	0.00
Propionate		0.15	0.01	5.63	0.13	0.71	0.00	0.00
Lactate		3.04	0.25	112.52	2.50	14.21	0.40	0.10
Diacetate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sodium Bicarbonate		0.02	0.00	0.81	0.02	0.10	0.00	0.00
Modified Food Starch		1.22	0.10	45.01	1.00	5.69	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.04	0.26	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.13	0.75	0.00	0.00
Total		6.86	9.92	4500.90	17.77	101.00		7.44
							Est Moisture	0.75

Treatment 4 - Lactate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.31				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		20.634255						
Total lbs. of Brine		0	1.68					
		0.00						
Water		0.00	1.14	517.01	11.56	67.69	1.00	1.14
Salt		2.05	0.17	76.04	1.70	9.96	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.36	0.03	13.42	0.30	1.76	0.00	0.00
Propionate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactate		3.02	0.25	111.82	2.50	14.64	0.40	0.10
Diacetate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sodium Bicarbonate		0.02	0.00	0.72	0.02	0.09	0.00	0.00
Modified Food Starch		1.21	0.10	44.73	1.00	5.86	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.05	0.27	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.13	0.78	0.00	0.00
Total		6.67	9.86	4473.00	17.25	101.04		7.40
							Est Moisture	0.75

APPENDIX 1 CONTINUED

Treatment 5 - Propionate - Diacetate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.314				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.157536
Injection %		13.914853						
Total lbs. of Brine		0	1.14					
		0						
Water		0.00	0.83	375.21	8.88	72.85	1.00	0.83
Salt		1.94	0.16	71.81	1.70	13.94	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.34	0.03	12.67	0.30	2.46	0.00	0.00
Propionate		0.14	0.01	5.28	0.13	1.03	0.00	0.00
Lactate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diacetate		0.14	0.01	5.28	0.13	1.03	0.00	0.00
Sodium Bicarbonate		0.07	0.01	2.53	0.06	0.49	0.00	0.00
Modified Food Starch		1.14	0.09	42.24	1.00	8.20	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.05	0.39	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.14	1.15	0.00	0.00
Total		3.78	9.31	4224.29	12.38	101.54		6.98
							Est Moisture	0.75

Treatment 6 - Diacetate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.31				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		13.235004						
Total lbs. of Brine		0	1.08					
		0						
Water		0.00	0.79	356.34	8.49	72.74	1.00	0.79
Salt		1.93	0.16	71.39	1.70	14.57	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.34	0.03	12.60	0.30	2.57	0.00	0.00
Propionate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diacetate		0.14	0.01	5.25	0.12	1.07	0.00	0.00
Sodium Bicarbonate		0.06	0.01	2.31	0.05	0.47	0.00	0.00
Modified Food Starch		1.13	0.09	41.99	1.00	8.57	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.05	0.41	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.14	1.21	0.00	0.00
Total		3.61	9.26	4199.13	11.86	101.62		6.94
							Est Moisture	0.75

Treatment 7 - Lactate - Diacetate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.314				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		21.861935						
Total lbs. of Brine		0	1.78					
		0						
Water		0.00	1.21	550.63	12.19	68.05	1.00	1.21
Salt		2.08	0.17	76.81	1.70	9.49	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.37	0.03	13.56	0.30	1.68	0.00	0.00
Propionate		0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactate		3.05	0.25	112.96	2.50	13.96	0.40	0.10
Diacetate		0.15	0.01	5.65	0.13	0.70	0.00	0.00
Sodium Bicarbonate		0.12	0.01	4.38	0.10	0.54	0.00	0.00
Modified Food Starch		1.22	0.10	45.18	1.00	5.58	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.04	0.25	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.13	0.73	0.00	0.00
Total		6.99	9.96	4518.44	18.08	100.98		7.47
							Est Moisture	0.75

Treatment 8 - Propionate + Lactate + Diacetate

Injection Formulation:	ppm	% Ingoing	lb.	gram	% In Final Product	% In Brine	%Moisture in Ingredient	lb water in Product
Meat Block			8.16	3701.31				
Ground Skinless Turkey Breast		100	8.16				0.7546	6.16
Injection %		22.606932						
Total lbs. of Brine		0	1.84					
		0						
Water		0.00	1.26	571.04	12.56	68.24	1.00	1.26
Salt		2.09	0.17	77.28	1.70	9.24	0.00	0.00
Sodium Tripolyphosphate (STPP)		0.37	0.03	13.64	0.30	1.63	0.00	0.00
Propionate		0.15	0.01	5.68	0.13	0.68	0.00	0.00
Lactate		3.07	0.25	113.65	2.50	13.58	0.40	0.10
Diacetate		0.15	0.01	5.68	0.13	0.68	0.00	0.00
Sodium Bicarbonate		0.12	0.01	4.32	0.10	0.52	0.00	0.00
Modified Food Starch		1.23	0.10	45.46	1.00	5.43	0.00	0.00
Sodium Erythorbate	547		0.00	2.02	0.04	0.24	0.00	0.00
Sodium Nitrite (curing salt @ 6.25%)	100		0.01	5.92	0.13	0.71	0.00	0.00
Total		7.18	10.02	4546.01	18.58	100.95		7.52
							Est Moisture	0.75