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Evaluation of Microbial Community Dynamics Impacting the Shelf-Life of Processed Meats

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EVALUATION OF MICROBIAL COMMUNITY DYNAMICS IMPACTING THE SHELF-LIFE OF PROCESSED MEATS

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The objective of this study in its entirety was to utilize high next-generation genetic sequencing to evaluate the microbial communities involved with processed meat spoilage. High throughput 16S rRNA gene sequencing on the Illumina MiSeq© platform was used alongside traditional plating methods to characterize the growth and composition of bacterial communities in processed meats. Previous results from this lab indicated a relatively high prevalence of *Pseudomonas* in cooked, sliced, and vacuum packaged deli meats, which was contrary to conventional wisdom. Therefore, four studies were designed to further evaluate the factors that may influence microbial communities in processed meats. Study 1 aimed to identify differences between the processing environment in which sliced deli-ham is produced, handled, and packaged. Products within the same category description from three separate processors had significantly different bacterial community profiles, however, all had prevalence of *Pseudomonas*, to varying degrees. Study 2 was designed to determine differences in the bacterial communities of various phases throughout processing, from raw ground beef to cooking, slicing, and applying an antimicrobial or post-lethality treatment. Raw ground beef and sliced bologna had similar bacterial community profiles, having the least

microbial diversity with a high prevalence of *Pseudomonas*, while both cured and uncured links, and bologna with high pressure processing (HPP) or with organic acid salts had a higher proportion of various *Firmicutes* and *Proteobacteria*. Study 3 aimed to determine the differences in microbial community composition of sliced bologna caused by different clean-label and traditional antimicrobials. Increased growth and a higher prevalence of *Pseudomonas* were observed in the control treatment with no antimicrobial, while all antimicrobial treatments had greater microbial diversity, with increased amounts of various organisms compared to the Control. Study 4 aimed to identify differences in the microbial community composition between franks smoked with natural hardwood smoke, dipped in liquid smoke, or unsmoked. Minimal bacterial growth was observed in all three treatments throughout 14 weeks of refrigerated storage, and there were minuscule differences in their bacterial community composition. Differences in the microbial community composition of processed meats are vital to extending the shelf-life of products and further understanding their relationship with meat spoilage.

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INTRODUCTION

The purpose of this study was to identify key differences in the spoilage bacterial communities of meat products that can aid in extending shelf-life. Shelf-life of a meat product is determined by the amount of time until spoilage, which can be simply identified by gross discoloration, slime, or off-odors (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Spoilage can be caused by both chemical and microbiological factors, but in the case of cooked or processed meats, the latter is usually the main cause. In order to increase shelf-life, most processors aim to reduce the microbiological load on the product, either by preventing microbial contamination, by preventing or delaying bacterial growth, or by eliminating vegetative bacterial cells. Recently, research tools have been developed that allow scientists to take an alternative approach to combat spoilage through microbial community dynamics. Through the use of high throughput genetic sequencing, researchers can gain a high-definition, in-depth view of the intricacies involved with meat spoilage and bacterial growth, and uncover key differences that would otherwise be unknown with traditional plating methods.

It is well understood that different species of bacteria behave differently in the presence of certain growth parameters, and this can play a role in meat spoilage. For instance, shifting the microflora toward lactic acid bacteria will result in delayed spoilage caused by souring and acid, rather than putrid or cheesy odors (Borch, Kant-Muermans, & Blixt, 1996). Whether intentional or not, many of the methods in place to extend the shelf-life of meat products not only reduce growth but somehow shift microbial populations toward slower growing and less detrimental species that will delay spoilage. It has been traditionally thought that most deli meats to have a spoilage microbiota made

of mostly lactic acid bacteria species (Borch et al., 1996). However, previous research has shown high prevalence of *Pseudomonas* growth in sliced, vacuum packaged deli meat (Bower, Stanley, Fernando, Burson, & Sullivan, 2018a; Bower, Stanley, Fernando, & Sullivan, 2018b). This leads to the question of what exactly determines which species dominate and are involved with meat spoilage, and what can be done to extend shelf-life by altering the spoilage community.

The working hypothesis for the dissertation is that altering the bacterial communities involved with meat spoilage can extend shelf-life beyond just minimizing bacterial growth. Many factors are involved with the bacterial community, including the initial load quantity and composition and any processes or ingredients that may alter bacterial growth.

The objective of these studies was to utilize high throughput 16S rRNA genetic sequencing to characterize the microbial communities involved with processed meat spoilage to determine the impact of various processes and ingredients and identify steps that can be taken to extend shelf-life. More specifically, the research aimed to accomplish the following objectives:

1. Determine the role microbial community composition of the post-lethality contamination load plays in the spoilage microbiota throughout storage time.
2. Determine the effect of common processes used throughout the production of processed meat products on meat spoilage and bacterial communities.
3. Determine the influence of various antimicrobial ingredients commonly used in meat products on the microbial community dynamics involved with meat spoilage.

The long-term goal of this research is to determine strategies to increase shelf-life through a combination of altering the bacterial community to select for less detrimental species in combination with decreasing overall microbial growth. The methods used in this research are fairly novel in the past 5-10 years, therefore a large portion of the understanding and characterization of bacterial communities involved in meat processing needs to be accomplished. This research could provide for the basis of strategies used to increase shelf-life of processed meats, and developing methods to address specific problems surrounding meat spoilage.

REVIEW OF LITERATURE

1. Introduction

Processed meat products undergo one or multiple processing steps and have added ingredients to alter the flavor, texture, and most importantly improve shelf-life of the product. Throughout history, meat products have been salted, smoked, cooked, or dried for means of preservation to be eaten year-round. Since the dawn of refrigeration for preservation, these processes have become somewhat less important, however, they are still often utilized for the characteristic flavor, texture, and eating experience that these products offer. With the large scale of production and distribution of meat products globally, improving shelf-life is still a key priority of many meat processors. With improved technology available for the analyses of shelf-life, we can look far beyond color or bacterial growth to determine the shelf-life of product. While consumers still identify spoilage using the tools available to them, which are limited to appearance and/or smell of the meat or dates indicated on packaging, researchers and producers are developing the tools to look much deeper into spoilage and observe its cause from a microbiological and chemical point of view.

Typically, meat products are refrigerated to decrease the growth of mesophilic pathogenic organisms, thus, allowing for spoilage organisms to grow which can be detrimental to quality but are not harmful if ingested. Most consumers would agree that gross discoloration, strong off-odors, and the development of slime would constitute meat spoilage (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). From a microbiological viewpoint, shelf-life is typically defined as the number of days to reach 7 log bacteria/g

or bacteria/cm^2 (Borch, Kant-Muermans, & Blixt, 1996), but can also be defined as the production of certain by-products that cause discoloration, odors, or slime. Although product spoilage is inevitable, certain storage environments or treatments may help to delay spoilage, either from a total reduction in bacterial growth or by selecting for certain bacteria that are slower growing or less detrimental to product quality.

2. The Microbiota of Meat

Since healthy muscle is inherently sterile, the microbiota associated with fresh meat originates in the intestines of the animal, on the hide of the animal, or processing surfaces in the abattoir or cutting areas (Koutsoumanis & Sofos, 2004). After initial contamination, many processes and treatments are applied to alter the growth environment, reducing overall growth, and selecting for certain bacteria, typically in the interest of pathogen reduction. Reducing overall bacterial growth is the main goal of most processors, however, most interventions simply select for a certain group of bacteria that may be more favorable due to slow growth, less offensive by-products, and reduced risk of pathogenicity.

While the microbiota of raw and cooked meats typically varies greatly, it is important to evaluate the types of bacteria commonly found in both raw and cooked meat products and determine their origination and point of contamination. Table 1 demonstrates the different Genera of bacteria typically found in raw meat packaged under various conditions (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Most meat products are stored under refrigeration, and packaged in either vacuum or modified atmosphere packaging, which shifts to anaerobic, gram-positive bacteria becoming

Table 1. Genera of bacteria commonly found in raw meat stored in different conditions (adapted from Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015)

Gram-positive	Storage Conditions			Gram-negative	Storage conditions		
	Air	MAP	VP		Air	MAP	VP
Bacillus	+		+	*Achromobacter*	+		
Brochothrix	+	+	+	*Acinetobacter*	+	+	+
Carnobacterium	+	+	+	*Aeromonas*	+		+
Corynebactenum	+			*Alcaligenes*	+	+	+
Clostridium			+	*Alteromonas*	+	+	+
Enterococcus	+	+		*Campylobacter*	+		
Kocuria	+			*Chromobacterium*	+		
Kurthia	+			*Citrobacter*	+	+	
Lactobacillus	+	+	+	*Enterobacter*	+	+	
Lactococcus	+			*Escherichia*	+		
Leuconostoc	+	+	+	*Flavobacterium*	+		
Listeria	+	+		*Hafnia*	+	+	+
Microbacterium	+	+	+	*Klebsiella*	+		
Micrococcus	+	+		*Kluyvera*	+		
Paenibacillus	+			*Moraxella*	+		
Staphylococcus	+	+	+	*Pantoea*	+		+
Streptococcus	+	+		*Proteus*	+	+	
Weisella	+	+	+	*Providencia*	+	+	+
				Pseudomonas	+	+	+
				Serratia	+	+	+
				Shewanella	+		
				Vibrio	+		
				Yersinia	+		+
				Moraxella	+		

MAP: modified atmosphere packaging
VP: vacuum packaging

prevalent in spoilage rather than the aerobic, gram negative species seen in raw, unpackaged meat (Doulgeraki, Ercolini, Villani, & Nychas, 2012). Hultman, Rahkila, Ali, Rousu, & Björkroth, (2015) aimed to characterize the unique microbiota associated with man-made meat processing environments and the resulting contamination of meat products. The researchers collected and analyzed samples from a commercial production plant of raw meat handling areas, cooked meat packaging areas, raw materials and cooked sausages using quantitative methods to determine lactic acid bacteria and total plate counts, as well as 16s rRNA sequencing to characterize the microbiome (Hultman et

al., 2015). Results indicated that Firmicutes and more specifically *Leuconostoc* dominated the microbiota of cooked sausages despite relatively low abundance of *Leuconostoc* in the raw product and in the RTE processing environment (Hultman et al., 2015). In the raw processing environment, *Pseudomonadales*, *Actinomycetales*, *Bacillales*, and *Lactobacillales* were most prevalent while *Actinomycetales*, *Pseudomonadales*, *Lactobacillales*, *Clostridiales*, and *Enterobacteriales* were most prevalent in the RTE environment (Hultman et al., 2015). Also using 16S rRNA sequencing, *Carnobacterium*, *Lactobacillus*, and *Leuconostoc* were dominant at the time of expiration in the microbiota of both cooked poultry meat (Geeraerts, Pothakos, De Vuyst, & Leroy, 2018) and cooked pork meat (Geeraerts, Pothakos, De Vuyst, & Leroy, 2017) in MAP packaging from retail markets. Conversely, Bower, et al. (2018b) reported a spoilage community dominated by *Pseudomonadaceae* in cooked, sliced, vacuum packaged deli turkey and roast beef with varying salt concentrations. Furthermore, Mertz et al. (2014) identified *Pseudomonas* as the most common bacteria found on meat slicers in ready to eat (RTE) processing environments. Thus, the microbiota of meat products is a complex ecosystem that is affected by many intricate factors and cannot simply be characterized in a one size fits all manner.

3. Factors Affecting Meat Spoilage

There are various intrinsic and extrinsic factors that affect microbiological growth and thus, spoilage in meat products. Manipulating various combinations of these factors in order to increase the shelf-life of a meat product is commonly known as hurdle technology (Leistner, 1978). The most important factors in food preservation are

temperature, water activity, pH, redox potential, preservatives, and competitive microorganisms (Leistner, 2000). While the idea of hurdle technology is for the purpose of microbiological preservation, many hurdles applied to meat products also affect quality and contribute to unique characteristics of a product, such as a dried and fermented salami.

3.1 Temperature

Temperature is one of the most important factors involved with meat spoilage. Throughout history, many food preservation methods were developed in the absence of refrigeration in order to keep food from spoiling throughout the warmer seasons. While heat treatment is associated with the elimination or reduction of pathogenic organisms, storage temperature plays a vital role in spoilage growth as well. Most pathogenic organisms are mesophiles, hence the well-known "danger zone", or 4 – 60 °C (40 – 140 °F), the range which should be avoided when storing or holding meats. According to USDA FSIS (2017) Appendix A guidelines for RTE products, meat products should be cooked to an internal temperature of at least 70 °C, or if cooked to a lower temperature, it must be held at that temperature for a given

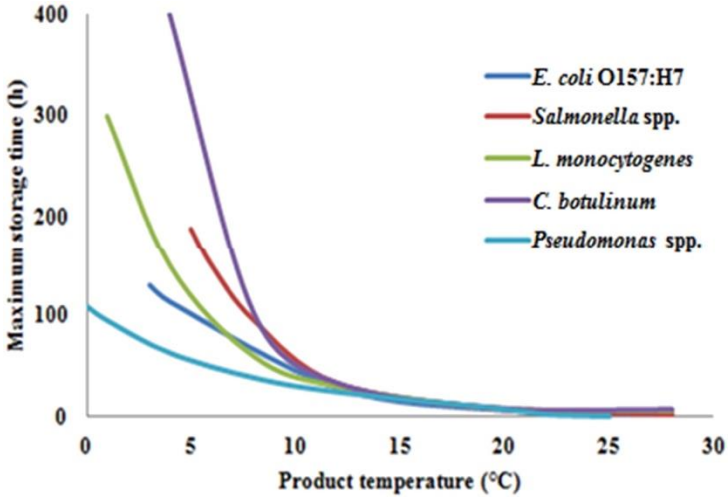

Figure 1. ComBase model predictions for the influence of product temperature on the maximum storage time for different microorganisms assuming intrinsic conditions similar to beefsteak, predicted by ComBase Predictor. The maximum allowable increase was 2.5-log for *Pseudomonas* spp. and 1 log for all other microorganisms. (Adapted from Karthikeyan et al., 2015)

amount of time in order to achieve a sufficient lethality. Under Appendix A, red meat must meet a 6.5 $\log_{10}$ reduction of *Salmonella,* while poultry must meet a 7.0 $\log_{10}$ reduction of *Salmonella,* and if using an internal validation study or peer-reviewed journal article, a 5.0 $\log_{10}$ reduction must be demonstrated. During storage, however, temperature can greatly affect not only the spoilage rate but also determine which organisms are allowed to grow. Karthikeyan et al., (2015; Figure 1) reported the predicted maximum storage times at various given temperatures using the ComBase growth modeling program. While it is generally understood that under refrigeration, reduced temperatures will result in reduced bacterial growth, minimum growth temperature limits and growth rates will vary among bacterial species. *Listeria monocytogenes* is a major concern in ready-to-eat meats, as it continues to grow, although slowly, at normal refrigerated temperatures (Barria, Malecki, & Arraiano, 2013).

3.2 Moisture and Water Activity

Fresh meat is considered a high moisture food, with a typical water activity (a_w) between 0.98-0.99, allowing for optimal water availability for bacterial growth. Often times processed meats will contain added humectants, such as sodium chloride, to reduce the water activity. Blickstad & Molin (1983) reported that the addition of 4% sodium chloride in the water phase will decrease the a_w value from 0.99 to about 0.97, reducing the growth of salt-sensitive microorganisms, such as *Pseudomonas* spp. and *Enterobacteriaceae,* and the microflora developing will shift to more salt-tolerant

microorganisms such as lactic acid bacteria and yeasts. Bower et al. (2018b) reported a negative linear relationship between water activity and salt concentration in sliced, deli-style roast beef and turkey breast, as well as a negative linear relationship between salt and total plate counts, indicating that the decrease in water activity caused by increasing salt suppressed microbial growth. According to USDA Food Safety and Inspection Service (2014a), the critical limit for pathogen growth in dried meats is 0.85 for aerobic storage and 0.91 for anaerobic storage. In other words, *Staphylococcus aureus*, one of the most osmotolerant meat pathogens, will not grow below the given water activity, allowing for a shelf-stable product (USDA FSIS, 2014a). For other common pathogens, the critical a_w is even greater, listed in table 2 (ICMSF, 1996). Although a minimum water activity threshold is essential for bacterial growth, it is nearly always used in combination with other hurdles, such as salt concentration, pH, or thermal treatment to delay or eliminate bacterial growth.

Table 2. Critical a_w for various pathogens in meat (adapted from ICMFS, 1996)

Organism	Minimum a_w for Growth
Campylobacter	0.98
Pseudomonas	0.97
C. botulinum	0.93
C. perfringens	0.93
Salmonella	0.94
E. coli O157:H7	0.95
L. monocytogenes	0.92

3.3 Atmospheric Gas

The use of modified atmosphere packaging (MAP) is one of the greatest tools in extending the shelf-life of both fresh and cooked meats. The most commonly utilized MAP systems in the meat industry are vacuum packaging, where as much atmospheric air is removed from the package as possible, and gas flush, where atmospheric air is removed from the package and replaced with a controlled gas mixture. The main uses of

MAP are to retain fresh meat color, where high oxygen (O_2) gas can be used to generate desirable red bloom color or low concentrations of carbon monoxide (CO) can be used to create a more stable red color. In cooked meats, both cured and uncured products have a much more stable color than fresh meat, so it is of less concern from a packaging viewpoint. Instead, an anoxic environment is sought in order to minimize lipid oxidation and off-flavors in the cooked product (McMillin, 2017). A common cooked meat MAP gas composition is 70% N_2 and 30% CO_2, with the goal of reducing both lipid oxidation and spoilage due to aerobic microbe growth (Smiddy, Papkovsky, & Kerry, 2002). Still, many processors have utilized various manipulations of the gaseous atmosphere to reduce or alter bacterial spoilage. Aerobic psychrotroph *Pseudomonas* spp. commonly dominate refrigerated spoilage, and their abundance often negatively contributes to overall shelf-life (Dainty & Mackey, 1992; Molin & Ternström, 1982). When oxygen is reduced or eliminated, however, bacterial growth is either limited or very slow (Dainty & Mackey, 1992). This allows for slower growing Lactobacilli to fill the niche and ultimately dominate the bacterial community (Gill & Newton, 1978). Furthermore, Gill & Tan (1980) reported that MAP with 20% CO_2 delayed microbiological growth, but did not alter the spoilage flora. Oxygen is still needed in fresh meats to create the desirable "bloom" color, however, a percentage of CO_2 has been included in many MAP packaged meat products because of its bacteriostatic effects. In-depth research regarding the bacterial community effects of various types of cooked meat is somewhat lacking, however, the removal of oxygen and addition of CO_2 creates favorable shifts and conditions to extend shelf-life.

3.4 pH

The effect of pH on bacterial growth is simple, typically, a lower pH will reduce bacterial growth. Still, some bacteria are more acid resistant than others and thus, reducing pH can create a niche allowing certain bacteria to thrive due to the inhibition of acid-sensitive competitors. For instance, lactic acid bacteria are known to ferment sugars in food and produce lactic acid, lowering the pH and suppressing the growth of other microorganisms (Huis In't Veld, 1996). Normally pH varies between species, and typically decreases in the order poultry > beef ≥ pork (Duffy, Vanderlinde, & Grau, 1994; Gibson, Bratchell, & Roberts, 1988), and tends to vary between animals and even between muscles in a given species. Thus, spoilage patterns tend to match the pH, with the more neutral pH of poultry leading to faster microbial spoilage than beef or pork. Meat pH can also be used as one of many hurdles to decrease or eliminate microbial growth. The critical pH of various pathogens and spoilage organisms are listed in table 3 (ICMSF, 1996). Like most growth factors, these limits vary depending on temperature, water activity, and even the type of acids present, and pH is typically combined with other hurdles for the purpose of food preservation.

Table 3. Critical pH for various pathogens and spoilage organisms in meat (adapted from ICMSF, 1996)

Organism	Minimum pH for Growth
C. perfringens	5.0
Campylobacter	4.9
C. botulinum	4.6
E. coli O157:H7	4.0-4.4
L. monocytogenes	4.4
Y. enterocolitica	4.2
S. aureus	4.0
Salmonella	3.8

3.5 Metabolic Substrates

For most bacteria, glucose is the preferred energy source to facilitate growth. However, there is very little glucose available in meat or muscle tissue, yet bacterial spoilage still occurs. The ability of a bacterial species to metabolize alternate substrates is essential to its ability to grow in a low-glucose medium such as fresh meat, and likewise, the availability of glucose in meat products is key in altering the spoilage microbiota (Nychas, Dillon, & Board, 1988). The type and preference of metabolizable

Table 4. Substrates used by meat spoilage bacteria during growth in aerobic storage (A), vacuum packaging (VP) and modified atmosphere packaging (MAP). Adapted from Casaburi et al., 2015.

	Pseudomonas		*Enterobacteriaceae*		*Br. thermosphacta*		Lactic Acid Bacteria		*Clostridium*	
Substrates[a]	A	VP/MAP	A	VP/MAP	A	VP/MAP	A	VP/MAP	A	VP/MAP
Glucose	1	1	1	1	1	1	1	1		1
Glucose-6-P	2	2	2	2	2	2	2	2		2
Lactic acid	3		3							
Pyruvic acid	4	3								
Gluconic acid	5	3								
Gluconate-6-P	6									
Acetic acid		3		3						
Amino acids	7	3	4		3			3		
Ribose					4					
Glycerol					5					

The numbers reported indicate the order of substrate utilization.

substrate (Table 4) varies among spoilage bacteria (Casaburi et al., 2015). As shown in Table 4, *Pseudomonas* has the capability to utilize many more substrates than other common spoilage organisms, many of which include waste products from other spoilage organisms, which explains one of the factors allowing *Pseudomonas* to commonly dominate meat spoilage. Other organisms, such as lactic acid bacteria and *Enterobacteriaceae,* have a more narrow range of substrates, and therefore, require other conditions to create a niche where they can dominate spoilage. In processed meats, spoilage can also be affected from a substrate perspective because glucose, sucrose, or other sugar-containing ingredients are often included in the formula, and special care should be taken to consider the spoilage implications of these ingredients.

4. Microbial Interventions to Increase Shelf-life

Various interventions have been used throughout history with the goal of preserving meat products. For centuries, meats were smoked, salted, dried, fermented and cooked with the goal of keeping meat edible throughout the warmer months when spoilage more readily occurred. Until the advent of refrigeration, fresh meat would not last unless eaten immediately or processed in some way. Today, many historical methods are still used or imitated but are applied more for the flavor and eating characteristics rather than solely for preservation. In many products, however, these hurdles are documented and used as scientific justification for a safe product in Hazard Analysis and Critical Control Point (HACCP) plans or other forms of food safety verification.

4.1 Cooking

Cooking is one of the most common and well-known microbial interventions. Products which undergo sufficient heat treatment will have little to no vegetative cells remaining, other than those from post-lethality exposure or from spores. In most raw meat products, *Salmonella* and Shiga toxin producing *E. coli* are the main pathogens of concern, and the USDA Food Safety and Inspection Service has clearly outlined the internal temperature requirements for elimination of these bacteria, as 70 °C, or any time and temperature combination from Appendix A which extends all the way to 54.4 °C internal temperature held for 112 or 121 min. in order to achieve a 6.5 or 7.0 log CFU/g reduction, respectively (USDA FSIS, 2017). There are also a number of relative humidity guidelines to select from, to ensure that the surface of the product does not get too cool from evaporation, as well as to reduce the concentration of solutes on the surface due to drying (USDA FSIS, 2017). Research has shown that drying and increased solute concentrations can allow some bacteria to become more heat resistant (Riemann, 1968).

Since cooking kills nearly all vegetative cells present in meat, the microbiome undergoes a complete shift after cooking because most bacteria on the product after cooking can be assumed to be the result of post-lethality contamination, commonly from handling the product during peeling, slicing, or packaging. In fresh meat, various *Pseudomonas* species commonly dominate aerobic spoilage, while lactic acid bacteria will commonly dominate vacuum packaged (VP) or modified atmosphere packaged (MAP) meat with other facultative species commonly isolated as well (Casaburi et al., 2015). Benson et al. (2014) designed a study using 16S rRNA sequencing to identify possible microbial successions during storage of pork sausage. The study identified a

population dominated by *Weissella* and *Leuconostoc* on day 0 sampling, with a drastic shift toward two species of *Pseudomonas* by day 15, and also that within the lactic acid bacteria identified, there was a sharp decline in *Lactobacillus gasseri*, and a sharp increase in *Carnobacterium divergens* (Benson et al., 2014). Furthermore, another shift occurred between day 15 and day 30, where the dominant pseudomonads were replaced by *Lactobacillus graminis, Carnobacterium divergens*, *Yersinia mollaretti*, *Serratia*, and *Buttiauxella brennerae* (Benson et al., 2014). The results from this study indicate that not only does the flora of fresh meat vary greatly, but there may even be successions throughout storage time.

The microbiota of cooked meats, however, typically varies much more than that of fresh meat and can vary based on specie, processing environment, packaging type, and ingredients. Borch et al., (1996) state that the flora of cooked meat products typically consists of various species of lactic acid bacteria, and varies based on the product composition and the manufacturing site. Miller, Liu, & Mcmullen (2015) evaluated the microbiome of various ready to eat sliced turkey products available in the retail market and determined that *Leuconostoc*, *Carnobacteria*, *Brocothrix*, and *Lactobacillus* were the dominant flora, and that reduced sodium products had more diverse bacterial species than did regular sodium products. Similarly, in the Belgian retail, Geeraerts et al. (2018) identified *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* as the dominant genera, in addition to various other lactic acid bacteria. In pork ham products, Geeraerts et al. (2017) also identified *Carnobacterium*, *Lactobacillus*, and *Leuconostoc* as the dominant flora. In contrast, Bower et al. (2018b) observed a flora dominated by *Pseudomonas* in sliced, vacuum packaged uncured sliced turkey breast and roast beef, and generally saw

an increase in *Pseudomonas* and a decrease in bacterial diversity as salt was reduced. These studies indicate that the microbial ecology of cooked meats may be relatively predictable in some cases, but varies greatly depending on a number of factors.

4.2 Smoking

Smoke is typically applied during the cooking process of meat products, however, it contributes its own, unique antimicrobial functions, and in some cases may be performed without cooking. Smoke itself has an acidic pH but it is likely a combination of acids, formaldehydes, and phenols that contribute to the antimicrobial functions of smoke (Hui, Nip, Rogers, & Young, 2001). Smoke is made of a mixture of thousands of compounds, many of which are due to the pyrolysis of cellulose, hemicellulose, and lignin, and include, but are not limited to hydrocarbons, alcohols, aldehydes, ketones, acids, and phenols (Tóth & Potthast, 1984). Messina et al. (1988) reported a greater than 99.9% reduction in *Listeria monocytogenes* in vacuum packaged beef franks dipped in a commercially available liquid smoke and stored for 72h. Niedziela, MacRae, Ogden, & Nesvadba (1998) designed a study to look more in depth into the antimicrobial effects of smoke, by evaluating *Listeria monocytogenes* growth in salted, smoked salmon compared to salmon that was salted only, as well as the effects of salt, phenols, and formaldehydes on the growth of *Listeria monocytogenes* cultures. Results indicated that dry salted salmon fillets increased 2-5 log cycles of *Listeria monocytogenes*, while growth was inhibited on smoked fillets (Niedziela et al., 1998). Brined salmon displayed similar results, where smoked salmon delayed *Listeria monocytogenes* growth for at least three weeks, compared to *Listeria monocytogenes* related spoilage after two weeks in the

brined-only fillets (Niedziela et al., 1998). Neither salt nor smoke phenols prevented *Listeria* growth, however, formaldehyde suppressed growth for two weeks before an increase in *Listeria* was detected (Niedziela et al., 1998). This indicates that the antimicrobial effectiveness of smoking meats likely is attributed to the formaldehyde produced by wood smoke. Clearly, the technical science involved with meat smoking has not been a common topic in current research, especially in red meats. It is worthwhile, however, to examine and evaluate the effects of smoke when dealing with microbiological and shelf-life research in meat and poultry products, as smoke clearly provides some antimicrobial function. The effects of smoking should not be overlooked in microbial community research, and may even be the basis for microbial research in the future.

4.3 Fermentation

Similar to other common preservation hurdles, fermented meats can be traced nearly to the beginning of recorded history. The microbiology involved with fermented meats is itself an entire area of research within food and meat science. While the basics of dairy fermentation are fairly well understood and controlled, meat fermentations are less understood, generally, are much more heterogeneous, and involve a mixture of bacteria, yeasts, and molds. Most modern fermented meats, especially in the United States, use a controlled starter culture for safety and quality reasons, to ensure the fermentation is started with the most productive fermentative organisms (Franciosa, Alessandria, Dolci, Rantsiou, & Cocolin, 2018). The foremost method of preservation involved with fermentation is pH. As stated previously, pH is one of the more important

factors involved with shelf-life and meat spoilage, and many spoilage and pathogenic bacteria are pH sensitive, especially when combined with salt, drying, and other hurdles (Adams & Nicolaides, 1997). The basics of modern meat fermentation involve mixing the meat with salt, spices, curing ingredients, starter culture, and a sugar source, and the added culture will metabolize the sugar fermenting glucose into lactic acid and lowering the pH (Hugas & Monfort, 1997). In a complex fermentation like meat, however, mixtures of microbial species, conditions, and other factors lead to various products being produced, the most relevant of these being bacteriocins. Bacteriocins are extracellularly released bioactive peptides which have antimicrobial or bacteriostatic activity (De Vuyst & Leroy, 2007). Some common bacteriocins produced by fermentation cultures include nicin, sakacin, pediocin, plantaricin, carnocin, and leucocin; most bacteriocins are produced by gram positive organisms and are inhibitory toward gram negative bacteria (Chikindas, Weeks, Drider, Chistyakov, & Dicks, 2018). Furthermore, some bacteriocins are approved to be added directly to certain food products. In addition to pH and bacteriocin production, the concept of competitive inhibition comes into play with fermented products as well. The organisms used for fermentation are better suited to metabolize the available substrates and energy sources compared to pathogens or spoilage organisms, and thus out compete these organisms for energy. The combination of pH reduction by acid production, bacteriocin production, and competitive inhibition contribute to the selective antimicrobial functions associated with fermented foods and help regard fermented foods as some of the safest products made from a bacterial perspective.

4.4 Drying

Drying is one of the simplest concepts of food preservation used today. In essence, drying involves removing moisture from the product and reducing or inhibiting bacterial growth due to increased osmotic pressure and reduced available water. Typically, gram positive bacteria are more osmotolerant than gram negatives due to their thicker peptidoglycan wall, but growth during drying varies between each species. The microbiota of dried products is reflected as such, and typically resembles that of fermented products, including a mix of lactic acid bacteria as well as some *Staphylococcus* (Correia Santos, Fraqueza, Elias, Salvador Barreto, & Semedo-Lemsaddek, 2017; Fontana et al., 2016; Połka, Rebecchi, Pisacane, Morelli, & Puglisi, 2014). As with most methods of preservation, drying is commonly used as one of multiple hurdles in a food product but is probably one of the most important factors contributing to shelf-stability of a product (USDA FSIS, 2011). Typically, drying will be combined with cooking and/or smoking (beef jerky), fermentation (salami), salting (dry cured meats), and packaging, which typically plays a role in all food preservation. Meat drying is determined by the relationship between the water activity of the meat and the relative humidity in the chamber or air surrounding the product. Typically, some amount of heat will be applied to the product to increase the rate of drying. Since moisture can only evaporate from the surface, the rate of drying cannot be too rapid or else case hardening can occur, where the surface of the product seals up and inhibits moisture migration from the center of the product to the surface for evaporation (Toldrá et al., 2007). Drying is one of the oldest and simplest methods of food preservation, and when

combined with other microbial hurdles can be very effective at increasing shelf-life and achieving shelf stability.

4.5 Packaging

Packaging technology is one of the most commonly utilized bacterial interventions in the meat industry. Nearly every meat product comes in some sort of package, depending on the composition and the designated use for the product. The most basic antimicrobial function of packing is to provide a physical barrier against contamination, however meat packaging has advanced well beyond just that. Modified atmosphere packaging (MAP), which consists of removing atmospheric air and gas flushing, and vacuum packaging (VP) are two of the more commonly used methods, especially in cooked meats. In some cases, oxygen permeable packaging is still used for retail display of fresh meats in order to provide desirable red color (Polkinghorne et al., 2018), however, the shelf-life of both cooked and fresh meats can be greatly extended with other packaging techniques. Aerobic storage of meats typically leads to spoilage dominated by *Pseudomonas*, while vacuum packaged and anaerobic MAP (N and/or CO_2) meats select for the growth of lactic acid bacteria (Blickstad & Molin, 1983). Using 16S sequencing, Mansur et al. (2018) determined that when comparing beef stored under air and vacuum packaged, a significant shift occurred where *Pseudomonas* was most prevalent in aerobically stored beef, while vacuum packaged beef saw more predominant growth of *Lactobacillus*. This was accompanied by a shift in the volatile organic compounds (VOCs) associated with each product that ultimately defined spoilage (Mansur et al., 2018). Kameník et al., (2014) reported no significant difference in total

bacterial growth between vacuum packaged, vacuum skin packaged, and gas flushed steaks, however proportions of lactic acid bacteria were increased in vacuum and vacuum skin packaged meats, while aerobic MAP packages (80% O_2 / 20% CO_2) enabled the growth of *Pseudomonas*, similar to overwrapped or aerobically stored meat. In cooked meats, high oxygen environment is not necessary for color maintenance, therefore high-oxygen packaging is rarely used, and instead vacuum, nitrogen-flushed, or nitrogen and carbon dioxide-flushed packages are favored as a method to minimize oxygen in the package. While packaging may not be the most important factor involved with bacterial growth and spoilage of meats, it does play an important role in all types of meat products.

4.6 Antimicrobials

There are numerous antimicrobial compounds used in meat products, the most common of which are in the broad category of organic acids. Organic acids are an extensive category of antimicrobial agents which include, but are not limited to, lactic, acetic, and propionic acids and their respective salts. The main function of organic acids as antimicrobials is related to pH and pKa. The undissociated form of most organic acids can penetrate the cell membrane of bacteria, and once in the cytoplasm with a neutral pH, acids will dissociate and acidify the cell (Doyle, Beuchat, & Montville, 2001). Constant acidification of the cytoplasm will deplete cellular energy in order drive the ATP pump and remove protons from the cytoplasm in an attempt to normalize cytoplasmic pH and the proton gradient (Doyle et al., 2001). Although organic acids are typically more effective in acidic foods, due to the increased pH gradient between the food and bacterial cytoplasm, they are commonly used in meat products and are effective at inhibiting

certain microorganisms from proliferating, especially *Listeria monocytogenes.* Organic acid ingredients such as sodium or potassium lactate and diacetate or propionic acid are commonly used as antimicrobial agents under the USDA FSIS *Listeria monocytogenes* Guideline (USDA FSIS, 2014b) for ready to eat meat and poultry products. Their effectiveness against the growth of *L. monocytogenes* is well documented (Ahmed et al., 2015; Seman, Borger, Meyer, Hall, & Milkowski, 2002; Stekelenburg, 2003), but processors typically validate *Listeria monocytogenes* inhibition based on each product formulation and application. Beyond *Listeria monocytogenes* control, organic acids can alter the spoilage microbiota of meats due to selective inhibition. Benson et al. (2014) reported a shift in the microbiota of fresh pork sausage throughout storage time when sodium lactate and diacetate was added, where untreated sausage saw multiple complex waves of bacterial growth from various species, while sausage with sodium lactate and sodium diacetate added were characterized by the growth of a single species of *Lactobacillus graminis*. Similarly, Bouju-Albert, Pilet, & Guillou (2018) found that fresh sausages with and without potassium lactate and sodium acetate were both dominated by lactic acid bacteria, however, untreated samples saw an increase in *Brochothrix* spp. and *Pseudomonas spp.*, while addition of lactate/diacetate resulted in an increase in *Leuconostoc mesenteroides* and *Lactobacillus* spp. These studies indicate a common shift toward lactic acid bacteria when organic acids are used, as well as less complex and less diverse spoilage community.

Salt and sodium nitrite can both be considered antimicrobials, even though they both serve additional primary purposes in meat products. Salt primarily works as a preservative by decreasing water activity and drawing moisture out of the cells of

bacteria (Albarracín, Sánchez, Grau, & Barat, 2011). While most modern meat products do not rely on salt as the main preservative agent, it can be very effective and preserving and improving the safety and shelf-life of meat products when combined with other hurdles, such as cooking, fermenting, drying, etc. Salt is typically less effective at inhibiting gram positive than gram negative bacteria due to the difference in thickness of the peptidoglycan wall. In meat products, this is commonly demonstrated by a shift from *Pseudomonas* spp. toward lactic acid bacteria (Blickstad & Molin, 1983; Ruusunen & Puolanne, 2005). However, Bower et al. (2018b) observed a decrease in microbial growth as well as a decrease in microbial diversity as salt increased in roast beef and turkey breast, with communities dominated by *Pseudomonas* spp. regardless of salt concentration (1.0 to 2.5% meat block basis). In products obtained from the retail market, Miller et al., (2015) determined that reduced-sodium sliced deli products had greater bacterial diversity than their regular-sodium counterparts. These results show that although salt is not inhibitory by itself, it does still affect microbial growth and may alter the microbiota associated with meat spoilage. Furthermore, salt is used in combination with other hurdles to inhibit spoilage or bacterial growth.

Sodium nitrite is a commonly used meat ingredient, known for its role in the development of the characteristic color, flavor, and texture of cured meats, but is also a well-known antimicrobial. The main antimicrobial function of nitrite is its bacteriostatic effects toward *Clostridium* spp. Although the mechanisms are not completely known, it's thought that the conversion of nitrite to the free radical nitric oxide during the curing process disrupts the functional proteins and enzymes by creating sulfur bridges, and is especially effective against *C. botulism* and *C. perfringens* (Doyle et al., 2001). As

reported by (O'Leary & Solberg, 1976), nitrite inhibition of *C. perfringens* is likely due to interaction with sulfhydryl containing glycolytic enzymes in the bacterial cell, specifically glyceraldehyde-3-phosphate dehydrogenase and aldolase. Furthermore, Gardner, Costantino, Szabó, & Salzman (1997) reported a similar inhibition of *E. coli* through interference with glycolytic and tricarboxylic acid cycle (TCA) enzymes. In other bacteria, nitrite has been shown to interfere with active transport and the electron transport chain (Yarbrough, Rake, & Eagon, 1980). Although various factors come into play, lactic acid bacteria are not typically affected by sodium nitrite to the degree of other spoilage organisms, while *Enterobacteriaceae,* on the other hand, are completely inactivated by nitrite (Duranton et al., 2012). While nitrite is not considered an inhibitory or antimicrobial agent for *Listeria monocytogenes* control, it can alter the growth of *Listeria monocytogenes* in cooked meats. Duffy et al., (1994) reported that *Listeria monocytogenes* took nearly twice as long to reach a 3 log increase in products containing nitrite compared to no nitrite. Results from Myers et al., (2013) are in agreement, where nitrite slightly suppressed the growth of *Listeria monocytogenes,* where products formulated without nitrite had ~1 log increase in *Listeria monocytogenes* growth after 28 d storage compared to those containing nitrite. Similarly, 200 ppm sodium nitrite from a conventional source reduced *Listeria monocytogenes* growth in sliced hams, however 50 or 100 ppm of nitrite from either conventional or celery powder did not reduce *Listeria monocytogenes* growth compared the no nitrite control (Myers et al., 2013). These studies indicate that although nitrite does not inhibit *Listeria monocytogenes* growth completely, it can reduce *Listeria monocytogenes* growth and can thus be used in combination with other tools or hurdles to reduce the risk of *Listeria monocytogenes*

prevalence, and similar to salt, can help shift the microbiota of cooked meats toward lactic acid bacteria.

4.7 Bioprotective Cultures

The elimination of all bacterial growth on a meat product is not practical; processes to reach commercial sterilization are either detrimental to product quality or not well-accepted by consumers–e.g. irradiation. Rather, many preservation methods select for the growth of slower-growing or less detrimental species as a way of delaying spoilage as long as possible. This concept has brought forth the idea of using bioprotective cultures—known bacteria which demonstrate antagonism toward pathogenic or otherwise harmful bacteria—as a means of extending the shelf-life of meat products. One study evaluated 91 strains of lactic acid bacteria isolated from meat products and their feasibility and effectiveness as protective cultures on cooked, cured meats (Vermeiren, Devlieghere, & Debevere, 2004). Of these strains, 12 were identified to have the greatest antibacterial properties, with regard to their inhibition of *Listeria monocytogenes*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum*, and *Brochothrix thermosphacta*, and furthermore were characterized by a fast growth rate, and except for a bacteriocin producing strain and a lactocin S producing strain, rapid acidification caused by acetic acid production (Vermeiren et al., 2004). Finally, none of these strains negatively affected sensory properties or acceptance of the hams (Vermeiren et al., 2004). Rapid spoilage of meat products is often caused by slime or discoloration associated with bacterial growth. Comi, Andyanto, Manzano, & Iacumin, (2016) determined that bacon spoilage and green discoloration is caused by *Leuconostoc mesenteroides*, and also that

Lactococcus lactis and *Lactobacillus sakei* decreased the growth and the risk of spoilage associated with *Leuconostoc mesenteroides*. Although these cultures may delay spoilage caused by certain bacteria, it's well known that lactic acid bacteria will inevitably spoil meat products as well, albeit slower and with less repulsive characteristics, such as acidification and souring. Nonetheless, there may be some practical application for the use of lactic acid bacteria as protective cultures against the growth of the psychrophilic pathogen, *Listeria monocytogenes*. One study determined that a combination of three lactic acid bacteria strains was effective at reducing *L. monocytogenes* growth throughout 8 weeks of shelf-life, and was even more effective when these cultures were combined with lactate/diacetate in the frankfurter formula (Koo, Eggleton, O'Bryan, Crandall, & Ricke, 2012). The concept behind protective cultures is somewhat complex, as a combination of the production of organic acids and bacteriocins, as well as competitive inhibition by way of competition for substrate and other nutrients5 play a role. The key, however, is to identify bacterial strains that can inhibit harmful or pathogenic bacteria while not contributing to spoilage growth. Because of this challenge, protective cultures have not been widely applied in cooked meat products with the exception of fermented meats.

5. Quality of Processed Meats

Many ingredients are multi-functional, imposing both antimicrobial and quality effects on meat products. At the same time, some antimicrobials are detrimental to product quality at amounts sufficient to provide microbial control and must be addressed as such. When using certain antimicrobials, it is up to the processor to determine how

much emphasis to place on safety/shelf-life versus quality. Ingredient suppliers have worked to lessen the negative effects of some ingredients, such as offering buffered organic acids or acid salts. Regardless, formulations may need to be adjusted in order to address the effects of these ingredients.

5.1 Salt

Salt is a multifunctional ingredient that provides function beyond just its preservative capacity. Sodium chloride is essential to the flavor and quality associated with meats and cured meat products. Salt concentration is one of the most noticeable flavor characteristics in meat products, a small change in salt can result in a significant change in overall flavor and saltiness. In bacon, ham, and sausages, salt content typically ranges from 1.5 to 3.0% (Desmond, 2006), but in recent years producers have slowly reduced typical salt concentrations. Salt adds its own flavor to meat products, but also acts as a flavor enhancer, increasing characteristic meat flavors (Ruusunen, Sàrkkà-Tirkkonen, & Puolanne, 1999). In addition to a reduction in salty taste, sausage flavor, smoky flavor, spicy flavor, and even sausage odor were impacted by a 50% reduction in formulated salt in hotdog sausages (Aaslyng, Vestergaard, & Koch, 2014). Furthermore, bacon and salami sensory attributes were significantly affected by only a moderate 20-25% reduction in salt concentration (Aaslyng et al., 2014). Salt is clearly essential to the flavor of processed meat products, and a core ingredient in meat formulations. Besides flavor, salt is also essential to the texture of meat products, which is accomplished by the solubilization or “extraction” of myofibrillar proteins within the meat. As described by Desmond (2006), salt-solubilized myofibrillar proteins form a sticky exudate on the

surface of meat products, and once these proteins are heat coagulated, they form a matrix, entrapping water and binding meat pieces together. This concept allows for products such as boneless hams, chicken nuggets, and other comminuted products where smaller pieces are held together by extracted proteins to make up one larger piece. Using instrumental texture profile analysis, 2.5% salt resulted in harder, more cohesive, and less deformable beef sausages, while 1% salt produced a relatively weak bind (Xiong, Noel, & Moody, 1999). Similarly, salt soluble proteins allow for meat emulsions as well. According to Aberle, Forrest, Gerrard, & Mills (2012), salt causes protein solubilization and swelling to take place, increasing water holding, but also allowing for proteins to interact with and surround fat droplets. Hydrophobic portions of proteins bind and surround fat droplets, while hydrophilic tails interact with water and other proteins, and upon cooking create a stable gel suspending fat droplets in the water phase (Aberle et al., 2012). A visual representation of salt concentration in relation to protein extraction and emulsion stability is seen in Figure 2 (Horita, Messias, Morgano, Hayakawa, & Pollonio, 2014). Horita et al. (2014) describe the reduced salt formulation (C) as having an open and spongy texture compared to the control (A) due to increased protein extraction resulting in greater emulsion stability. The effects of salt concentration on emulsion quality and stability are well documented (Horita et al., 2014; Tobin, O'Sullivan, Hamill, & Kerry, 2012a) and generally a greater salt concentration results in more protein extraction and greater emulsion stability. However, most meat products are limited by taste to around 2-3%

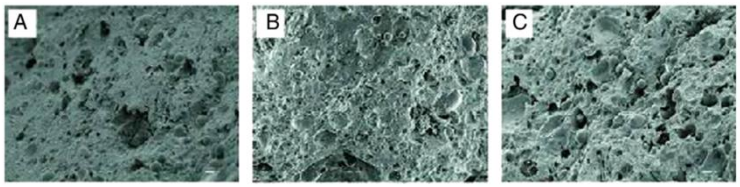

Figure 2. Scanning electron microscopy of beef frankfureters formulated with: A) 2.0% NaCl, B) 1.5% NaCl, or C) 1.0% NaCl. (Horita, Messias, Morgano, Hayakawa, & Pollonio, 2014)

ingoing salt (Tobin, O'Sullivan, Hamill, & Kerry, 2012b). Thus, other strategies are often combined with salt such as mixing, tumbling, grinding, or other forms of mechanical action in order to reach peak myofibrillar protein extraction.

While the preservative mode of action of salt relies on dehydration, at typical concentrations salt actually helps bind water in meat products. In most meat products, cooking yield is directly related to water retention, and with increased water retention, also comes increased moisture from a sensory perspective. The most probable theory of water binding is summarized by Ruusunen & Puolanne, (2005) where the negative chloride ions of salt bind more tightly to meat proteins, and the repulsion of these negative charges causes muscle swelling. With the increased space between muscle fibers, more water is allowed to interact with polar side chains as well as the added chloride ions, increasing water binding (Ruusunen & Puolanne, 2005). Adding salt to meat products has advantages in fresh meats, but the most obvious benefits are through increased cooking yields in processed meats. Improving cook yield not only means increasing the amount of sellable product but also can lead to increased moisture in the product from a sensory perspective, in turn, improving product quality. In frankfurters, salt concentration is negatively correlated with cooking loss (Horita et al., 2014) as well as consumer perception of toughness and juiciness (Tobin et al., 2012a) indicating water holding effects of salt concentration affect both the processor and the consumer. Likewise, cooking yield decreased as salt decreased in beef patties, as did consumer perception of juiciness, however a 50% decrease in salt resulted in patties with the highest overall consumer acceptance (Tobin et al., 2012b). These results indicate that salt aids in the water holding ability of meat, and furthermore, that salt concentration and

water holding negatively affect the product from a cooking yield perspective as well as from a decreased perception of juiciness by consumers.

5.2 Nitrite

Sodium nitrite is one of the basic ingredients in cured meat products and is specifically what makes them "cured". While the antimicrobial properties of nitrite in cured meats have been previously described, nitrite also plays a role in the quality of meats, being the primary ingredient responsible for the characteristic color, flavor, and texture of cured meats. The color chemistry of cured meats is well-known and is based on the conversion of nitrite (NO_2) to nitric oxide (NO) and its interaction with the sarcoplasmic protein, myoglobin (Hui et al., 2001). Whereas oxygen bound to myoglobin creates the cherry red color of fresh oxygenated meats, nitric oxide bound to myoglobin creates nitrosylmyoglobin/nitrosylmetmyoglobin, and upon cooking creates nitrosylhemachrome, which is the light pink, typical cured meat color (Hui et al., 2001). Put simply, cured meats cannot be made without nitrite or other nitric oxide compounds. In addition to color, nitrite is responsible for the characteristic flavor of cured meats. It is thought that many of the flavor differences between cured and uncured meats are due to volatile hydrocarbons, which vary between cooked and cured beef, pork, and chicken (Ramarathnam, Rubin, & Diosady, 1991, 1993). These proportions and absence/presence of many of these compounds during cooking depends on nitric oxide compounds, however, they likely contribute more to aroma than flavor (Ramarathnam et al., 1993). Lipid oxidation is also reduced by sodium nitrite in the curing process, contributing to additional flavor differences. By itself, nitrite acts as a prooxidant in solutions, however

in the presence of other pro-oxidants such as Fe^{++}, nitrite converted to nitric oxide during cooking will quench free radicals and significantly decrease oxidation (MacDonald, Gray, & Gibbins, 1980). During the cooking process, non-heme iron is released from heme pigments and causes warmed over flavor by catalyzing lipid oxidation, so the addition of nitrite prior to cooking reduces oxidation and thus alters the flavor of cured meats due to a decrease in oxidation or warmed over flavor (Igene, King, Pearson, & Gray, 1979; MacDonald et al., 1980). Cho & Bratzler (1970) determined that in a blind test, consumers could tell the flavor difference between pork roasts containing nitrite and those containing no nitrite, regardless of salt concentration or smoking. Furthermore, the researchers found that salt alone was not responsible for cured meat flavor, as consumers could correctly identify a reduced salt cured roast compared to an uncured roast containing more salt but no nitrite (Cho & Bratzler, 1970). These qualities, in addition to the antimicrobial effects of nitrite, display the necessity for the usage of nitrite in cured meats. Furthermore, cured meats would not exist without the inclusion of nitrite.

5.3 Organic Acids

Organic acids and organic acid salts are extremely useful antimicrobials in meat products, however, acids are typically detrimental to meat quality in general. The functions of protein extraction and pH dependent as mentioned earlier, the salt soluble myofibrillar proteins within meat are responsible for the bind and texture associated with processed meats, and these proteins are somewhat sensitive to acids. When organic acids are added to a product, these proteins can be partially denatured, losing some of their functionality in the process. Furthermore, water holding is also pH dependent, where

increased pH causing an increase in negatively charged proteins leads to greater water holding ability due to net charge. As acids are added to a product, we typically lose some of the water holding ability due to a decrease in the negative net charge of proteins. This concept can be supported by the study of Medyński, Pospiech, & Kniat (2000) who demonstrated that salt and lactic acid work antagonistically, where water holding of meat with no added salt increased with lactic acid concentration, while water holding of meat with salt added was negatively affected by lactic acid, and increasingly so as salt increased. Crist et al. (2014) reported a decrease in cooking yield in fresh Italian pork sausage when lactate or an acetic acid/sodium lactate mixture was included, indicating a decrease in water holding caused by the antimicrobials. However, the meat industry still relies on organic acids as one of the primary chemical preservatives and does so by strictly using just enough organic acid to maintain microbial control while still retaining acceptable meat quality.

6. Techniques for Evaluating Microbial Ecology

Culture or plating based microbiological techniques have been around for decades, and are still used and valuable today, however, the advent of genomic methods have greatly advanced the study of microbiology in general. Genomic methods have allowed for a broader and more in-depth analysis of microbial communities beyond what culture based plating methods can offer. Often times with plating methods, we can identify broad groups of bacteria, such as psychrotrophs, aerobes, anaerobes, lactic acid bacteria, *Enterobacteriaceae*, etc. Selective agar and conditions can be applied to differentiate counts of these groups based on known phenotypes, such as

aerobic/anaerobic or mesophilic/psychrotrophic. Genomic methods, on the other hand, allow for a complete characterization of the community and require little prior knowledge of the bacteria being analyzed. Genomic methods are based on the process of polymerase chain reaction (PCR), which is an amplification technique for cloning the specific or targeted parts of a DNA sequence to generate thousands to millions of copies of DNA of interest (Bhatia et al., 2015). Polymerase chain reaction is a fundamental concept to genomic methods of analyses, regardless of the method used. Various methods have been developed for the purpose of genetic sequencing, depending on their desired use. In the case of bacterial ecology, rather than sequencing an entire gene or genome to identify the presence of genetic traits, methods that amplify a specific region of the genome are utilized to differentiate and identify bacterial species. Short regions of DNA are amplified multiple times to characterize and determine relative abundance of the various species present in the community. In recent years, Sanger sequencing has been used to characterize the microbiota in deli meat (Miller et al., 2015) and strain specific identification of lactic acid bacteria (Miller, Chumchalová, & McMullen, 2010). Furthermore, the Roche-454 platform has been used to evaluate bacterial communities and microbial successions associated with pork sausage (Benson et al., 2014). Currently, one of the most widespread methods for community analysis is the Illumina MiSeq platform. Application of the MiSeq platform has yielded results similar in quality to the 454 platform while providing significantly greater sequencing coverage (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). The MiSeq has become popular in genomic labs because it generates longer reads than other methods while maintaining adequate sequencing depth, and the cost of sequencing is more attainable for individual

researchers and smaller projects (Caporaso et al., 2012). Illumina sequencing has become widespread in soil, gut, and other areas of ecology study, but has been lesser utilized in the food industry, especially meat science. Previous research in this lab has utilized the MiSeq platform for the analysis of bacterial community dynamics of roast beef and turkey breast with varying amounts of ingoing salt (Bower, et al. 2018b) as well as sliced deli-ham with varying salt concentration and varying nitrite sources and concentration (Bower, 2018a). While costs somewhat limit this technology to research institutions, DNA sequencing will continue to become more ubiquitous as accessibility and cost continue to become more approachable to individual researchers and food and meat processors alike.

7. Data Analyses for Microbial Ecology

The robust amount of data and information resulting from MiSeq sequencing, while useful and insightful, can be overwhelming to researchers. Transforming raw reads into identifiable groups of bacteria, let alone useful, interpretable data can be a struggle not only for the researcher but in terms of available computing power as well. Often times, large scale patterns and changes in microbiome can only be detected using high throughput sequencing, however, one struggle has been access to software that can handle these large datasets and produce concrete results that can easily be interpreted. Quantitative insights into microbial ecology is an open source software built for dealing with such data, and easily producing and visualizing results (Caporaso et al., 2010). Once reads have been binned into operational taxonomic units (OTUs) the first step in simplifying and comprehending these massive datasets is to identify overall differences

in the bacterial community. Alpha and beta diversity are commonly used as key metrics of determining overall differences in species diversity. Within a community, the addition of species is known as community, within-habitat, or alpha diversity (Whittaker, 1972). Alpha diversity of a sample from a community is characterized by two properties of interest—species number and relative importance—and thus requires two measurements. Richness in terms of species number can simply be reported as the number unique species per unit area (Whittaker, 1972) and in the case of bacterial communities evaluated with 16S sequencing, can be measured as the number of observed OTUs per *n* sequences. To avoid underestimating the actual number of species present caused by analyzing a single sample from a population, adjustments can be made to adjust for the number of species present but not actually detected by sampling. One such method is the nonparametric estimator Chao1, which adjusts the number of observed species to account for singleton and doubleton OTUs, however, Chao1 should still be treated as a minimum asymptotic estimator, as is the case with most richness estimators (Gotelli & Colwell, 2011). In the case of 16S sequencing, steps are taken in laboratory procedures to standardize and normalize the number of individual reads that will be analyzed from each sample, however, we are still typically left comparing samples with varying read counts. To account for this between-sample difference, rarefaction can be performed where samples are subsampled n^* times, where $n^* = n$, the lowest number of reads of all samples (Gotelli & Colwell, 2011). Beta diversity is the extent of differentiation between communities (Whittaker, 1972). Currently, one of the more popular methods for determining beta diversity in bacterial communities is the UniFrac method (Lozupone & Knight, 2005). Many statistical techniques are limited when analyzing 16S sequencing

data, as they do not account for sequence similarity and thus result in a loss of information about the bacterial community (Lozupone & Knight, 2005). The UniFrac method evaluates the community based on a phylogenetic tree generated from 16S sequencing data and creates a distance matrix based on the number and length of unshared branches between two samples within the phylogenetic tree (Lozupone & Knight, 2005). This method is qualitative only; it does not account for the frequency or abundance of each species or OTU within a sample. The weighted UniFrac metric does just that, compares samples similarly but accounts for the relative abundance of each OTU and weights it accordingly in the distance measures (Lozupone, Hamady, Kelley, & Knight, 2007). Both the weighted and unweighted UniFrac metrics are important to microbial ecology; the unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone et al., 2007). These methods can both be useful in the study of meat microbiota, as we are interested in the factors that affect growth, but also in the initial load composition and its effects on spoilage.

8. Conclusion

While meat scientists may not be leading the charge in the study of bacterial ecology, there is much to be gained from evaluated growth and spoilage patterns of meat products utilizing next-generation methods. Most research regarding meat spoilage has centered around preventing or reducing growth, however with new technology researchers are able to gain an in-depth view of *what* is growing. Studies utilizing next-

generation community analysis have recently begun to surface and further research in the area could provide insight regarding spoilage that has yet to be realized. The first step in this area of research is an in depth-characterization of spoilage communities in meat products, including the factors that might affect or alter spoilage. Scientists are aware of the factors that might influence the total growth of bacteria in meat products, and most species can be predictable based on growth conditions. With next-generation sequencing, however, we gain a clearer view of how more species grow and interact, both dominating groups of bacteria as well as those in smaller proportions. Future research in this area should also include using bacterial community data to correlate certain groups or species with the many by-products or metabolites that are either involved in spoilage or delay spoilage. At the current time, this is a somewhat novel research area for the meat industry, however next-generation bacterial community analysis will continue to be a vital tool in evaluating meat spoilage.

There is a clear gap in knowledge regarding the detailed characteristics of the microbial community dynamics involved with meat spoilage. As new technology has developed, some of the common knowledge regarding spoilage communities in processed meats have already been challenged. Throughout four studies, we hope to further develop the knowledge base surrounding the complex bacterial communities involved with meat spoilage. Study 1 aims to characterize key differences in the spoilage community determined by the processing environment. Study 2 aims to determine differences in the microbiota throughout various phases in processing. Studies 3 and 4 aim to identify differences in bacterial communities caused by the use of common ingredients and processes, namely antimicrobials and smoking, respectively. These

studies each serve to fill a void in the literature which will further develop our understanding of meat spoilage, as well as increase the knowledge base for future studies in the area of meat spoilage.

DETAILED MATERIALS & METHODS

This section contains detailed materials and methods used for each study. Journal-appropriate methods are included in each manuscript.

Study 1

1.1 Sample Selection and Procurement

Ham samples were purchased at a local grocery store and selected from products on the retail shelf. Three different brands of smoked ham were evaluated (A, B, C). All three products were labeled as "Ham, Water Added" and were sliced case-ready packages. Three replications were purchased, with a replication consisting of products of the same brand, establishment number, and sell-by date (to have been produced on the same day in the same plant). Furthermore, each of the three replications, respective to each brand, were from the same establishment number to ensure replications were from the same processing plant within brand. Products were stored in the original packaging at the Loeffel Meat Laboratory in a covered plastic lug at approximately 0 °C (+/- 3 °C) until their respective sampling time. Samples were evaluated according to the sell-by date of each replication at the following intervals: 4 weeks prior to sell-by (-4), 2 weeks prior to sell-by (-2), sell-by date (0), 2 weeks after sell-by (+2), and 4 weeks after sell-by (+4). Separate packages were used for each day of sampling.

1.2 Bacterial Enumeration by Plating Methods

For each respective sampling, one package was removed from storage and

processed. Approximately 30-40 g of each sample was aseptically transferred from the vacuum pouch into a WhirlPak bag (Nasco, Fort Atkinson, WI), weight recorded, and combined with 50ml of sterile BBL™ Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate were collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Aerobic plate counts (APC) and anaerobic plate counts (AnPC) were performed using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate on Brain Heart Infusion agar (BHI) plates (Thermo Fisher Scientific, Waltham, MA) and were incubated at 37 °C for 48 h. For AnPC, samples were prepared as described for APC and were incubated at 37 °C for 48 h in an anaerobic box containing 1-2 BD GasPak EZ sachets to create an anaerobic environment (BD Medical Technology, Franklin Lakes, NJ). After 48 h of incubation, colonies were counted manually as described by the EddyJet owner's manual. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

1.3 Bacterial Community Analysis

Bacterial community analysis using high throughput sequencing of the 16s rRNA gene was performed on each sample collection using the MiSeq Illumina Sequencing Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Microbial DNA extraction from homogenized meat samples was performed using a modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed,

and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR) amplification in a 20 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc.), approximately 1-5 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). The PCR reaction was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 58 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD), and further gel purified using the Pippin Prep system (Sage Science, Inc., Beverly, MA). Final concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and the 16S libraries were sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller,

Kononoff, & Fernando, 2016), using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed to 251bp. Sequences were binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

1.4 Physicochemical Analyses

After samples for microbial analyses were removed, the remaining product was used for laboratory analyses of pH and objective color. On initial (-4) sampling, water activity, salt concentration, and proximate composition were also evaluated.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt concentration was measured according to Sebranek, Lonergan, King-Brink, Larson, & Beerman (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining extract to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

1.5 Proximate Composition

Moisture, fat, protein, and ash of pulverized samples were determined. Samples were manually diced, submerged in liquid nitrogen until completely frozen, and pulverized using a Hobart commercial blender (Model 51BL32; Waring Commercial, Torrington, CT). In duplicate, 2 g of pulverized tissue were used to quantify moisture and ash content using a LECO thermogravimetric analyzer (Model TGA701, LECO Corporation, St. Joseph, MI). In triplicate, total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure. In duplicate, protein content was measured using a LECO Nitrogen/Protein analyzer (Model FP528, LECO Corporation, St. Joseph, MI).

1.6 pH

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of homogenized cooked meat sample in 90ml of double distilled water.

1.7 Objective Color

Objective color (*L**, *a**, *b**) was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The instrument was calibrated using a white tile (Y:93.15, x:0.3165, y:0.3330). The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from

dirtying the colorimeter lens. A total of six readings were taken from two slices from each sample and averaged for color values.

1.8 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2017). For salt, water activity, and proximate composition (measured on initial sampling only), data were analyzed using R (lm and anova functions), and means were separated using the agricolae package (HSD.test function; De Mendiburu, 2017) For pH, color, APC and AnPC, data were analyzed as a 3 (brand) by 5 (storage time) interaction, with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the lsmeans package in R (lsmeans and cld functions; Lenth, 2016). Significance was determined at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 3,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Good's coverage test was performed to ensure adequate sampling depth was achieved. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2017) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the lsmeans package in

R (function lsmeans and cld; Lenth, 2016). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analyses. Both weighted UniFrac and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree. The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

Study 2

2.1 Treatment Design

Beef from a single production day was ground and subdivided into seven treatment groups representing different phases of processing from raw ground beef to cooked, sliced deli meat: T1-Raw ground beef; T2-Raw fresh beef sausage; T3-Cooked, linked beef sausage; T4-Cooked, cured, beef franks; T5-Sliced beef bologna; T6-Sliced beef bologna with high pressure processing (HPP) treatment: T7-Sliced beef bologna with potassium lactate/sodium diacetate as an antimicrobial. Each treatment was produced in three replications, on three separate days of processing and representing raw materials from three different days of processing. Raw treatments (T1, T2) were evaluated every three days for a total of 21 days, with day 0 being the day of raw processing. Cooked treatments (T3-T7) were evaluated every 14 days for a total of 112 days, with day 0 being the day of slicing and packaging. Details on production and storage parameters are outlined below.

2.2 Beef Processing Production

Boneless beef shoulder clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen (-20 °C) until use. Replications were purchased as three different production days, with the goal of having a similar baseline raw meat block within each replication but separation between replications. Beef shoulder clods were frozen three days after the production date (date of fabrication) listed on the box, and each replication was tempered at 4 °C four days prior to its respective grinding and

processing day. On three separate processing days, thawed shoulder clods were course ground using a 12.5 mm plate, mixed, and separated into 11.34 kg batches. Meat was then randomly assigned to one of seven treatments (T1-T7).

Production of each treatment was done as follows:

- T1: course ground meat was fine ground through a 3.2 mm plate using a Hobart Meat Grinder (Model #4734, Hobart MFG. Co., Troy, OH), stuffed into approximately 226 g poly meat bags using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA), and sealed using plastic tape.
- T2: course ground meat was mixed with salt and seasoning blend (2.0% salt, 0.5% dextrose, 0.3% garlic powder, and 0.3% white pepper) and 3% water (on a meat block basis) in a double action mixer (100DA 70, Leland Southwest, Fort Worth, TX), fine ground through a 3 mm plate, stuffed into approximately 226 g poly meat bags, and sealed using chub tape.
- T3: course ground meat was mixed with salt and seasoning blend, 10% water (meat block basis), fine ground through a 3 mm plate, stuffed into approximately 58 g links in 22 mm edible collagen casings (Brechteen Company, Chesterfield, MI), cooked to an internal temperature of 71 °C in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI) followed by a 30 min cold shower, chilled overnight at 0 °C, 4 links were placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), and vacuum sealed to approximately 1.4 kPa (Multivac Model C500; Multivac Inc., Kansas City, MO).
- T4: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, and 550 ppm sodium erythorbate (meat block basis), chopped

to a temperature of 18 °C using a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany), stuffed into edible collagen casings, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, chilled overnight at 0 °C, 4 links placed into a pouch, and vacuum sealed.

- T5: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, and 550 ppm sodium erythorbate (meat block basis), chopped in a bowl chopper, stuffed into fibrous casings (90 mm x 24" pre-stuck, Kalle, Gurnee, IL), pulled and clipped using a Tipper Clipper (Model PR465L; Tipper Tie, Inc., Apex, NC), cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, chilled overnight at 4 °C, sliced into 2 mm slices using a deli slicer (Bizerba Model SE12, Balingen, Germany), 10 slices (approximately 225 g) were stacked and placed into a pouch, and vacuum sealed.
- T6: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, chopped in a bowl chopper, stuffed into fibrous casings, pulled and clipped, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, chilled overnight at 0 °C, sliced into 2 mm slices, 10 slices (approximately 225 g) were stacked and placed into a pouch, vacuum sealed, and subject to HPP at 600 mPa for 3 min with a pressure ramp rate of 300MPa/min, near instantaneous (<3 s) release time, and process fluid temperature maintained below 15 °C (Hiperbaric 55, Hiperbaric USA, Miami, FL).
- T7: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, and 3.5% potassium

lactate/sodium diacetate blend (Opti.form PD4; Corbion Purac, Lenexa KS), chopped in a bowl chopper, stuffed into fibrous casings, pulled and clipped, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, chilled overnight at 0 °C, sliced into 2 mm slices, 10 slices (approximately 225 g) were stacked and placed into a pouch, and vacuum sealed.

All samples were stored covered at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

2.3 Bacterial Enumeration Through Traditional Plating

Approximately half of each sample package (80-120 g) was aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate was collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB) and psychrotrophic plate counts (PPC) were evaluated using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. Brain Heart Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. Aerobic plate counts and LAB counts were incubated at 37 °C for 48 h and enumerated manually following Eddy Jet directions.

Anaerobic plate counts were incubated at 37 °C for 48 h in an anaerobic box containing 1-2 BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ) and enumerated manually following Eddy Jet Directions. Psychrotrophic plate counts were incubated at 4 °C for 96 h and enumerated manually following Eddy Jet Directions. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

2.4 Bacterial Community Analysis

Bacterial community analysis using high throughput sequencing of the 16S rRNA gene was performed on each sample collection using the MiSeq Illumina Sequencing Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Due to the scale of this study, cooked sample weeks 2 and 4 were not subject to 16S sequencing after reviewing culture based growth data. Microbial DNA extraction from homogenized meat samples was performed using a modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed, and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR), amplification in a 25 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA), approximately 20 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich et al. (2013). The

PCR reaction was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 58 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Due to low DNA concentration, purified DNA was subject to additional PCR using the same process listed above with 5 amplification cycles. Following secondary amplification, DNA was applied to a 1.5% agarose gel, and the target band was manually excised, and recovered using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Final size and concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and library concentration was confirmed using a DeNovix QFX Fluorometer with the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc, Wilmington, DE). The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller, Kononoff, & Fernando, 2016), using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed

to 251bp. Sequences were binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

2.5 Physicochemical Analyses

The remaining half of each sample package (80-120 g) was used for physicochemical analyses. Measures of salt, water activity, and proximate composition were measured on day 0 only, while objective color and pH were measured throughout storage time.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt concentration was measured according to Sebranek, Lonergan, King-Brink, Larson, & Beerman (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining extract to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

2.6 pH

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of homogenized meat sample in 90ml of double distilled water.

2.7 Objective Color

Objective color (*L**, *a**, *b**) was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from dirtying the colorimeter lens. Six readings were averaged from either a flattened ground sample, interior and exterior of two split links, or the surface of two slices.

2.8 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For salt and water activity (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package (lsmeans and cld functions; Lenth, 2019). For all shelf-life measures, cooked and raw samples were analyzed separately. For pH, color, and plate counts, data were analyzed as a factorial arrangement with 2 treatments by 8 sampling times for raw samples, and 5 treatments by 9 sampling times for cooked samples, with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans

package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 3,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All subsequent statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2019) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the emmeans package in R (function lsmeans and cld; Lenth, 2019). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analyses. Both weighted UniFrac and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree. The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters

(Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

Study 3

3.1 Treatments and Experimental Design

Beef bologna was formulated and produced to include one of five common food grade antimicrobial organic acids: potassium lactate and sodium diacetate (LD), propionic acid (P), buffered vinegar (BV), and cultured sugar (CS), as well as a control (C) containing no antimicrobial. Due to various concentration and composition between antimicrobials, each treatment was formulated based on the supplier's recommendation for 90 days of *Listeria monocytogenes* inhibition. Each treatment was produced in three replications, on three separate days of processing. Each treatment was evaluated every 2 weeks for a total of 14 weeks, with day 0 being the day of slicing and packaging. Details on production and storage parameters are outlined below.

3.2 Bologna Production

Boneless beef clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen at -20 °C until use. On three separate processing days, shoulder clods were thawed for approximately 3 days at 4 °C, tempered clods were hand cut into pieces, and placed into 11.34 kg batches and randomly assigned to one of 5 treatments. Each treatment was mixed with 2.0% salt 0.5% dextrose, 0.15% garlic powder, 0.30% white pepper, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, 10% water, and 10% ice (on a meat block basis). Meat and ingredients were chopped in a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany), and antimicrobial was added to the chopper near the end of chopping. Antimicrobial inclusions were as follows: LD: 3.81%

Optiform PD4 (Corbion, Lenexa, KS); P: 0.5% BactEASE 6 (Kemin Industries, Des Moines, IA) BV: 0.93% BactoCEASE NV (Kemin Industries); CS: 2.51% Verdad N70 (Corbion). Batter was stuffed using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA) into fibrous casings (90 mm x 24" pre-stuck, Kalle, Gurnee, IL), casings were pulled and clipped using a Tipper Clipper (Model PR465L; Tipper Tie, Inc., Apex, NC), cooked to an internal temperature of 71 °C in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI), followed by a 30 min cold shower, and chilled overnight at 0 °C. The following day, bologna logs were sliced into 2 mm slices (Model SE12, Bizerba USA Inc., Joppa, MD), 10 slices were stacked and placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), and vacuum sealed to approximately 1.4 kPa (Multivac Model C500; Multivac Inc., Kansas City, MO). All samples were stored covered at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

3.3 Bacterial Enumeration Through Plating Methods

From each sample package, 5 slices (100-120 g) were aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate were collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB) and psychrotrophic aerobe plate counts (PPC) were performed using the

homogenized samples. Brain Heart Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. For APC and LAB, plates were incubated at 37 °C for 48 h, AnPC were incubated at 37 °C for 48 h in an anaerobic box containing BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ), PPC were incubated at 4 °C for 96 h. All plates were enumerated manually following instructions from the Eddy Jet manual. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

3.4 Bacterial Community Analysis

Community analysis was performed as described in study 2.

3.5 Physicochemical Analyses

The remaining half of each sample package (100-120 g) was used for physicochemical analyses. Measures of salt, water activity, and proximate composition were measured on day 0 only, while objective color and pH were measured throughout storage time.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt

concentration was measured according to Sebranek, Lonergan, King-Brink, Larson, & Beerman (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining extract to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

3.6 Proximate Composition

Moisture, fat, protein, and ash of pulverized samples were determined. Samples were manually diced, submerged in liquid nitrogen until completely frozen, and pulverized using a Hobart commercial blender (Model 51BL32; Waring Commercial, Torrington, CT). In duplicate, 2 g of pulverized tissue were used to quantify moisture and ash content using a LECO thermogravimetric analyzer (Model TGA701, LECO Corporation, St. Joseph, MI). In triplicate, total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure. In duplicate, protein content was measured using a ThermoFisher Flash SMART Elemental Analyzer (Waltham, MA).

3.7 pH

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of homogenized cooked meat sample in 90ml of double distilled water.

3.8 Texture Profile Analysis

Texture profile analysis (TPA) was performed by cutting a 13mm slice into a 4.0 cm × 4.0 cm square and measured using a 2500 kg load cell on an Instron (Model number

1123; Instron Worldwide, Norwood, MA) with a 140mm plate. Each slice was compressed two times to 75% of its original thickness with a head speed of 30 mm/min, and the characteristics of hardness, springiness, cohesiveness, and chewiness were determined according to Bourne (1978). Briefly, hardness is the maximum force (N) during the first compression cycle. Springiness is the ratio of the duration (s) of the second compression cycle compared the first cycle, measuring the elastic recovery of the product. Cohesiveness is the ratio of the positive force area under the curve of the second compression cycle compared to that of the first cycle. Chewiness is the product of hardness, springiness, and cohesiveness multiplied.

3.9 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For salt, water activity, and proximate composition (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Data were analyzed as a 5 (treatment) by 8 (storage time) interaction with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot2 and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 5,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All

statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Good's coverage test was performed to ensure adequate sampling depth was achieved. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2019) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the emmeans package in R (function lsmeans and cld; Lenth, 2019). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analyses. The weighted and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences, and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree. The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for a permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al.,

2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

Study 4

4.1 Treatments & Experimental Design

Beef frankfurters were produced using one of three smoking methods: liquid smoke (LS), natural smoke (NS), and unsmoked (US). Each treatment was produced in three replications, on three separate days of processing. Each treatment was evaluated every 2 weeks for a total of 14 weeks, with day 0 being the day of peeling and packaging. Details on production and storage parameters are outlined below.

4.2 Frankfurter Production

Boneless beef clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen at -20 °C until use. On three separate processing days, clods were tempered at 4 °C, and thawed clods were hand cut into pieces. One 34 kg batch mixed with 2.0% salt 0.5% dextrose, 0.15% garlic powder, 0.30% white pepper, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, 10% water, and 10% ice, on a meat block basis. Meat and ingredients were chopped in a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany) and batter was stuffed using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA) into 24.5 mm cellulose casings (27 Caliber USA, Viscofan USA, Montgomery, AL) into approximately 70 g links. Stuffed links were split into three approximately equal batches to be cooked independently, and each treatment was cooked to an internal temperature of 71 °C in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI), followed by a 30 min cold shower, and chilled overnight at 0 °C. Unsmoked (US) franks were placed directly in the smokehouse and cooked using no

smoke; LS franks were dipped in a 20% liquid smoke mixture (CharSol Select 24P, Red Arrow, Manitowoc, WI) for one minute prior to entering the smokehouse; NS franks were smoked using natural wood smoke from an Alkar smoke generator (Alkar-RapidPak Inc., Lodi, WI) set to a temperature of 246 °C with Frantz Hickory Sawdust (Frantz Company Inc., Butler, WI) for approximately 45 minutes during the cook cycle. The same cooking cycle was used for each treatment, with the exception of the addition of natural smoke to the NS franks. The following day, franks were peeled and placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), 4 franks per pouch, and vacuum sealed (Multivac Model C500; Multivac Inc., Kansas City, MO). All samples were stored covered at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

4.3 Bacterial Enumeration Through Traditional Plating Methods

Two links from each sample package (approximately 110-130 g) was aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate was collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB) and psychrotrophic aerobic plate counts were performed using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. Brain Heart

Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. For APC and LAB, plates were incubated at 37 °C for 48 h, and enumerated manually following Eddy Jet directions. The AnPC plates were incubated at 37 °C for 48 h in an anaerobic box containing BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ) and enumerated manually following Eddy Jet Directions. The PPC plates were incubated at 4 °C for 96 h, and enumerated manually following Eddy Jet Directions. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

4.4 Bacterial Community Analysis

Bacterial community analysis was performed as described in study 2.

4.5 Physicochemical Analyses

The remaining two links of each sample package (110-130 g) were used for physicochemical analyses. Measures of salt, water activity, and proximate composition were measured on day 0 only, while objective color and pH were measured throughout storage time.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt

concentration was measured according to Sebranek, Lonergan, King-Brink, Larson, & Beerman (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining extract to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

4.6 Proximate Composition

Moisture, fat, protein, and ash of pulverized samples were determined. Samples were manually diced, submerged in liquid nitrogen until completely frozen, and pulverized using a Hobart commercial blender (Model 51BL32; Waring Commercial, Torrington, CT). In duplicate, 2 g of pulverized tissue were used to quantify moisture and ash content using a LECO thermogravimetric analyzer (Model TGA701, LECO Corporation, St. Joseph, MI). In triplicate, total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure. In duplicate, protein content was measured using a ThermoFisher Flash SMART Elemental Analyzer (Waltham, MA).

4.7 pH

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of homogenized cooked meat sample in 90ml of double distilled water.

4.8 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For salt, water activity, and proximate composition (measured day 0 only), data

were analyzed using R (lm and anova functions), and means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). For pH, color, and plate counts, data were analyzed as a 3 by 8 interaction with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

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between each pair of samples by determining the ratio of shared to unshared branches in the phylogenetic tree created from sample sequences, and the weighted UniFrac adjusts for relative abundance of species. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for a permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

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STUDY 1. EVALUATION OF THE SPOILAGE MICROBIOTA ASSOCIATED WITH DIFFERENT BRANDS OF PRE-SLICED, PACKAGED DELI-STYLE HAM

Abstract

This study evaluated the spoilage microbiota associated with sliced, packaged, deli-style ham available in the retail market. Three different brands of pre-sliced ham, water added were purchased at local markets and evaluated every two weeks beginning four weeks prior to the sell-by date until four weeks beyond the sell-by date. Using 16S rRNA gene sequencing, brand A had a different bacterial structure compared to brands B and C, according to the weighted UniFrac distance matrix. Brand A had a greater proportion of *Carnobacterium*, *Bacillus*, and *Prevotella*, and B and C had greater proportions of *Pseudomonas*, *Photobacterium*, and *Lactococcus*. Brand A also had a lower salt concentration, greater moisture percentage, and less fat percentage, and increased APC. Differences in spoilage microbiota can in part be attributed to the factors involved with different processing locations, as shown by three different brands of ham, as well as slight differences in formulation including salt concentration and organic acid use.

1. Introduction

Meat spoilage is characterized as a change in the product rendering it unacceptable to the consumer, whether from chemical, biological, or physical change. Bacterial spoilage manifests itself as visible growth, textural changes, or off-odors and off-flavors caused by bacterial growth (Gram et al., 2002). Refrigeration and packaging type are two of the biggest contributors in selective growth of microorganisms (Doulgeraki, Ercolini, Villani, & Nychas, 2012), however, when placed under similar storage conditions, more precise factors may cause significant changes in the spoilage community, thus altering the length of time as well as the severity of spoilage. Lactic acid bacteria (LAB) are typically identified as the primary contributors to spoilage in cooked meats stored under refrigeration and modified atmosphere packaging (Geeraerts, Pothakos, De Vuyst, & Leroy, 2017, 2018), however, there may still be great variability of the bacterial community composition within LAB. Even so, under refrigeration and vacuum packaging, aerobic psychrotrophs from the genus *Pseudomonas* may be allowed to dominate spoilage of cooked and sliced deli meats (Bower, Stanley, Fernando, & Sullivan, 2018). With these mixed results regarding the dominant spoilage communities of cooked meats, steps should be taken to further characterize the predicted microbiome associated with cooked meat products under similar storage conditions.

The purpose of this study was to evaluate the spoilage microbiota of case-ready sliced and packaged ham available in the retail market. Since most microorganisms are inactivated during cooking, bacteria on the cooked product are likely attributed to post-lethality contamination. Miller, Liu, & Mcmullen, (2015) suggest that when comparing deli products of varying sodium concentration, the genera present on meat samples is

specific to individual processing facilities. In cooked sausage, however, Hultman, Rahkila, Ali, Rousu, & Björkroth, (2015) suggest that the microbiome of spoilage organisms is more similar to that of the meat batter than of the processing environment. The aim was to determine differences in the microbiota of similar products based on variation between the post-lethality processing environment of various manufacturers in the United States.

2. Methods and Materials

2.1 Sample Selection and Procurement

Ham samples were purchased at a local grocery store and selected from products on the retail shelf. Three different brands of smoked ham were evaluated (A, B, C) originating from three different establishments. All three products were labeled as "Ham, Water Added" and were sliced case-ready packages. In the interest of maintaining brand anonymity, Table 1 demonstrates select functional ingredients of each individual brand which may affect microbial growth and/or community composition. Three separate replications were purchased for each brand. A replication consisted of products of the same brand, establishment number, and sell-by date (to have been produced on the same day in the same plant). Furthermore, each of the three replications, respective to each brand, had the same USDA establishment number to ensure replications were from the same processing plant within brand. Products were stored in the original packaging at the Loeffel Meat Laboratory in a covered plastic lug at approximately 0 °C (+/- 3 °C) until their respective sampling time. Samples were evaluated according to the sell-by date of each replication at the following intervals: 4 weeks prior to sell-by (-4), 2 weeks prior to

sell-by (-2), sell-by date (0), 2 weeks after sell-by (+2), and 4 weeks after sell-by (+4).

2.2 Microbial Analyses

For each respective sampling, one package was removed from storage and processed. Approximately 30-40 g of each sample was aseptically transferred from the retail package into a WhirlPak bag (Nasco, Fort Atkinson, WI), weighed,combined with 50ml of sterile BBL™ Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate was collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Aerobic plate counts (APC) and anaerobic plate counts (AnPC) were performed using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate on Brain Heart Infusion agar (BHI) plates (Thermo Fisher Scientific, Waltham, MA) and were incubated at 37 °C for 48 h. For AnPC, samples were prepared as described for APC and were incubated at 37 °C for 48 h in an anaerobic box containing 1-2 BD GasPak EZ sachets to create an anaerobic environment (BD Medical Technology, Franklin Lakes, NJ). After 48 hrs of incubation, colonies were counted manually as described by the EddyJet owner's manual. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

Bacterial community analysis using high throughput sequencing of the 16s rRNA gene was performed on each sample collection using the MiSeq Illumina Sequencing Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Microbial DNA extraction from homogenized meat samples was performed using a

modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed, and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR) amplification in a 20 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc.), approximately 1-5 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). The PCR reaction was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 58 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD), and further gel purified using the Pippin Prep system (Sage Science, Inc., Beverly, MA). Final concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and the 16S libraries were sequenced using the Illumina

MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller, Kononoff, & Fernando, 2016), using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed to 251bp. Sequences were binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

2.3 Physicochemical Analyses

After samples for microbial analyses were removed, the remaining product was used for laboratory analyses of pH and objective color. On initial (-4) sampling, water activity, salt concentration, and proximate composition were also evaluated.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt concentration was measured as described by Sebranek, Lonergan, King-Brink, Larson, & Beerman (2001) using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

Moisture, fat, protein, and ash of pulverized samples were determined. Samples were manually diced, submerged in liquid nitrogen until completely frozen, and pulverized using a Hobart commercial blender (Model 51BL32; Waring Commercial, Torrington, CT). In duplicate, 2 g of pulverized tissue were used to quantify moisture and ash content using a LECO thermogravimetric analyzer (Model TGA701, LECO Corporation, St. Joseph, MI). Using triplicate 2 g samples in a filter paper thimble, total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure. In duplicate, protein content was measured using a LECO Nitrogen/Protein analyzer (Model FP528, LECO Corporation, St. Joseph, MI).

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of cooked meat sample in 90ml of double distilled water. Objective color (*L**, *a**, *b**) of was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The instrument was calibrated using a white tile (Y:93.15, x:0.3165, y:0.3330). The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from dirtying the colorimeter lens. A total of six readings were taken from two slices from each sample and averaged for color values.

2.4 Statistical Analyses and Experimental Design

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2017). For salt, water activity, and proximate composition (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using

the agricolae package (HSD.test function; De Mendiburu, 2017) For pH, color, APC and AnPC, data were analyzed as a 3 (brand) by 5 (storage time) interaction, with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the lsmeans package in R (lsmeans and cld functions; Lenth, 2016). Significance was determined at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 3,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Good's coverage test was performed to ensure adequate sampling depth was achieved. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2017) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the lsmeans package in R (function lsmeans and cld; Lenth, 2016). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analysis. Both weighted UniFrac and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree.

The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

3. Results and Discussion

Means for APC and AnPC are presented in Figure 1. There was a brand by storage time interaction for AnPC ($P = 0.032$), but no interaction for APC ($P = 0.441$). For brand A, AnPC generally increased throughout storage time, ranging from 0.89 log CFU/g at week -4 to 5.13 log CFU/g at week +4. Brand B remained under 2.00 log CFU/g throughout storage time, and brand C remained under 0.90 log CFU/g AnPC. There was a brand effect on APC ($P = 0.017$) where brand A had the greatest mean APC at 2.97 log CFU/g, while brands B and C were less at 0.40 and 0.36 log CFU/g, respectively.

In order to ensure adequate sampling depth, goods coverage was performed on the rarefied OTU table, and revealed that the depth used was able to characterize $\geq$ 95.8% of the total bacterial community. Diversity estimates Chao1 and observed OTUs were analyzed to determine differences in community richness, the number of different species

in a sample (Figure 2). There was a brand by storage time interaction for Chao1 (P = 0.043), however, there were no significant interactions or main effects for observed OTUs ($P > 0.099$). Using the weighted and unweighted UniFrac distance matrices, overall differences in bacterial community structure were determined. There was a main effect of brand on the weighted ($P < 0.001$) and unweighted ($P = 0.006$) UniFrac, where brands B and C had a more similar community structure than brand A, as shown in Figure 3. As displayed in Figure 4, brand A had a greater proportion of *Carnobacterium*, *Bacillus*, and *Prevotella* than both B and C, and B and C had greater proportions of *Pseudomonas*, *Photobacterium*, and *Lactococcus* compared to brand A.

Measures of meat pH, and objective color (CIE L^*, a^*, b^*) were recorded throughout storage time. There was a brand by storage time interaction for pH, where brand B at week 0 had a lower pH than all other storage times and brands. There were no main effects or interaction for L^* or a^* ($P \geq 0.244$), but there was a main effect of brand for b^* ($P = 0.017$), where brand A displayed greater b^* (yellowness) than both brands B and C. Salt and water activity were measured on the initial week of sampling (week -4) only. Both salt and water activity were different between brands ($P \leq 0.007$), where brand A had a lower salt concentration than B and C, and brand B had a lower water activity than A and C.

Proximate composition was measured on the initial day of sampling from each brand and replication. Moisture, fat, and ash were all significantly different between brand ($P < 0.012$), while there were no differences in protein ($P = 0.304$). Brand A had greater moisture and lower fat content than brands B and C, while brand B had a greater ash content compared to A and C.

Brand A had greater bacterial growth as well as a significantly different bacterial community structure compared to brands B and C. Given that brand A had less salt than both B and C, it is likely that the increased growth and the shift in community structure seen in brand A are related to the difference in salt concentration. Salt is one of the main preservative ingredients added to meat products, and as such typically decreases bacterial growth and increased concentrations (Borch, Kant-Muermans, & Blixt, 1996; Bower et al., 2018). Furthermore, brand A contained sodium propionate, an antimicrobial, whereas brand B contained potassium lactate and sodium diacetate antimicrobials, and C contained no organic acid antimicrobial. Organic acids are one of the more commonly used antimicrobial agents to prevent the growth of *Listeria monocytogenes* in ready-to-eat products, but also inhibit other organisms and may shift the bacterial community structure (Ahmed et al., 2015; Benson et al., 2014). Thus, differences in organic acids could in part explain the differences seen in the current study. Another explanation to the differences observed is simply the difference between the post lethality environment in which each of the brands was handled and packaged. The cooking process inactivates most of the microorganisms present on the raw meat, therefore most bacteria present in the cooked product are assumed to be introduced through post-lethality processing or handling. In the case of these products, they would be sliced and packaged before being shipped, and thus would each be handled in a different, unique post-lethality processing environment.

4. Conclusion

Similar to how terroir plays a large role in the microbiome associated with wine and grape production (Canfora et al., 2018), it is likely that a processing plant environment contributes a unique initial contaminating bacterial community structure that shapes the eventual spoilage microbiome. Differences between brands or establishments that affect microbial composition in RTE products are numerous but could include geographical location, sanitation practices including detergents or sanitizing chemicals, employee hygiene practices, temperature of processing and storage environments, line speed, and post-lethality exposure time, among others. While it is difficult to identify any one factor individually, the results presented indicate that one or a combination of these factors influence the microbial community to cause differences between brands.

Traditional wisdom would suggest that the conditions and environment of cooked ham either in vacuum packaging or low-oxygen modified atmosphere packaging would suppress the growth of spoilage aerobes like *Pseudomonas.* However, it's been shown that *Pseudomonas* is commonly found on the contact surfaces of meat slicers (Mertz et al., 2014), and the current study demonstrates that *Pseudomonas* was prevalent in all three brands of commercial products analyzed. Data from this study support that theory, demonstrating that similar products still have unique spoilage bacterial communities, which are likely impressed on them from the environment in which they were handled and packaged. Since it is difficult to find products from various companies that are produced exactly the same with identical ingredients, further research with a similar aim as this study should be performed in order to validate the idea that the spoilage community associated with RTE meat products is determined by the processing

environment and define precisely how influential the environment is in determining spoilage communities.

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Tables

Table 1. Product category and selected functional ingredients that may influence microbial growth of the three brands of ham evaluatedin the study.

Brand	A	B	C
Label	Ham, Water Added	Ham, Water Added	Ham, Water Added
Selected Functional Ingredients	Dextrose, Propionic Acid, Salt, Sodium Erythorbate, Sodium Nitrite, Sodium Phosphate, Sugar	Dextrose, Potassium Lactate, Salt, Sodium Diacetate, Sodium Erythorbate, Sodium Nitrite, Sodium Phosphate	Dextrose, Salt, Sodium Erythorbate, Sodium Nitrite, Sodium Phosphates

Table 2. Least squared means for main effect of brand on chemical and microbiological analysis of retail ham products.

	Brand				
	A	B	C	SEM[1]	*P* value
Salt %	1.74[b]	2.49[a]	2.59[a]	0.13	0.007
Water Activity	0.98[b]	0.96[a]	0.98[b]	0.001	<0.001
Protein %	15.98	15.68	16.80	0.48	0.304
Moisture %	78.18[b]	74.65[a]	74.66[a]	0.33	<0.001
Fat %	2.64[b]	5.16[a]	4.96[a]	0.45	0.012
Ash %	3.2[b]	4.5[a]	3.59[b]	0.11	<0.001
pH[†]	6.43	6.22	6.44	0.04	--
L*	67.34	68.37	67.53	0.57	0.405
a*	9.38	10.29	9.58	0.35	0.182
b*	6.26[b]	5.34[a]	5.54[a]	0.16	<0.001
APC[1]	2.97[b]	0.4[a]	0.36[a]	0.34	<0.001
AnPC[†1]	3.19	0.87	0.17	0.36	--

[1] SEM: Standard error of the overall mean, APC: aerobic plate count, AnPC: anaerobic plate count

[†] Indicates a significant ($P < 0.05$) brand by storage time interaction, therefore main effects cannot be analyzed.

[a,b] Means in the same row lacking a common superscript are significantly different ($P < 0.05$)

Figures

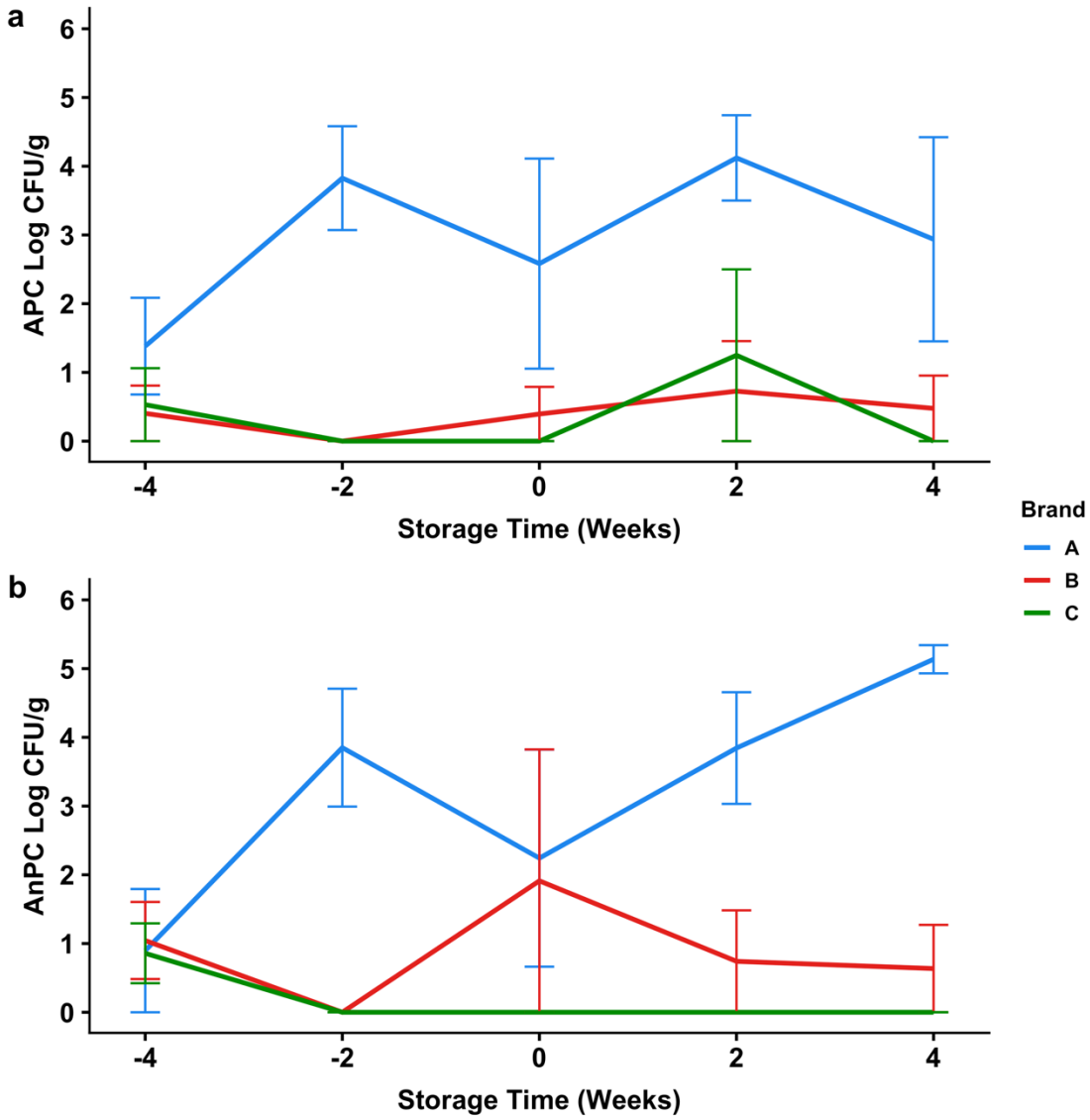

Figure 1. Aerobic plate counts (a) and anaerobic plate counts (b) of three brands of sliced, pre-packaged deli ham throughout storage time.

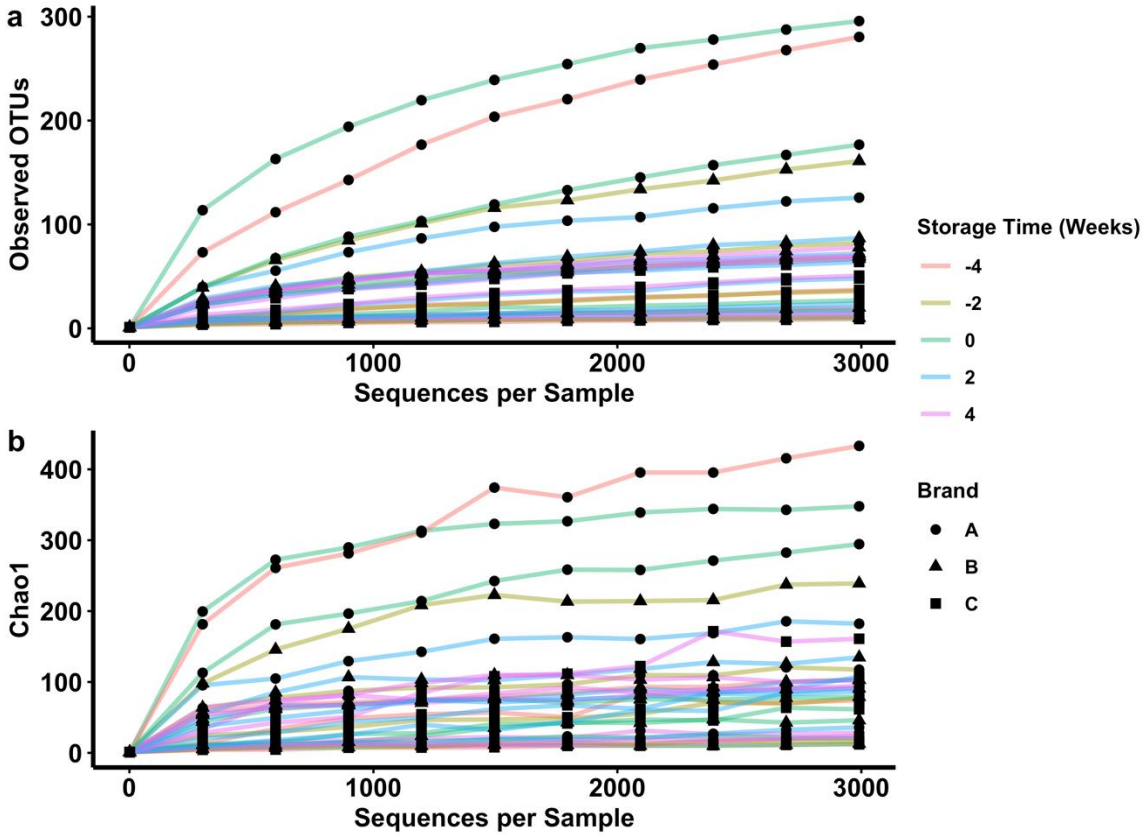

Figure 2. Observed OTUs (a) and Chao1 (b) estimates of community richness at various sampling depths for different brands and days of sampling of sliced, pre-packaged deli ham. All individual samples are displayed, colored by storage time with the marker shape representing brand. All samples were rarefied to an even depth of 3000 reads.

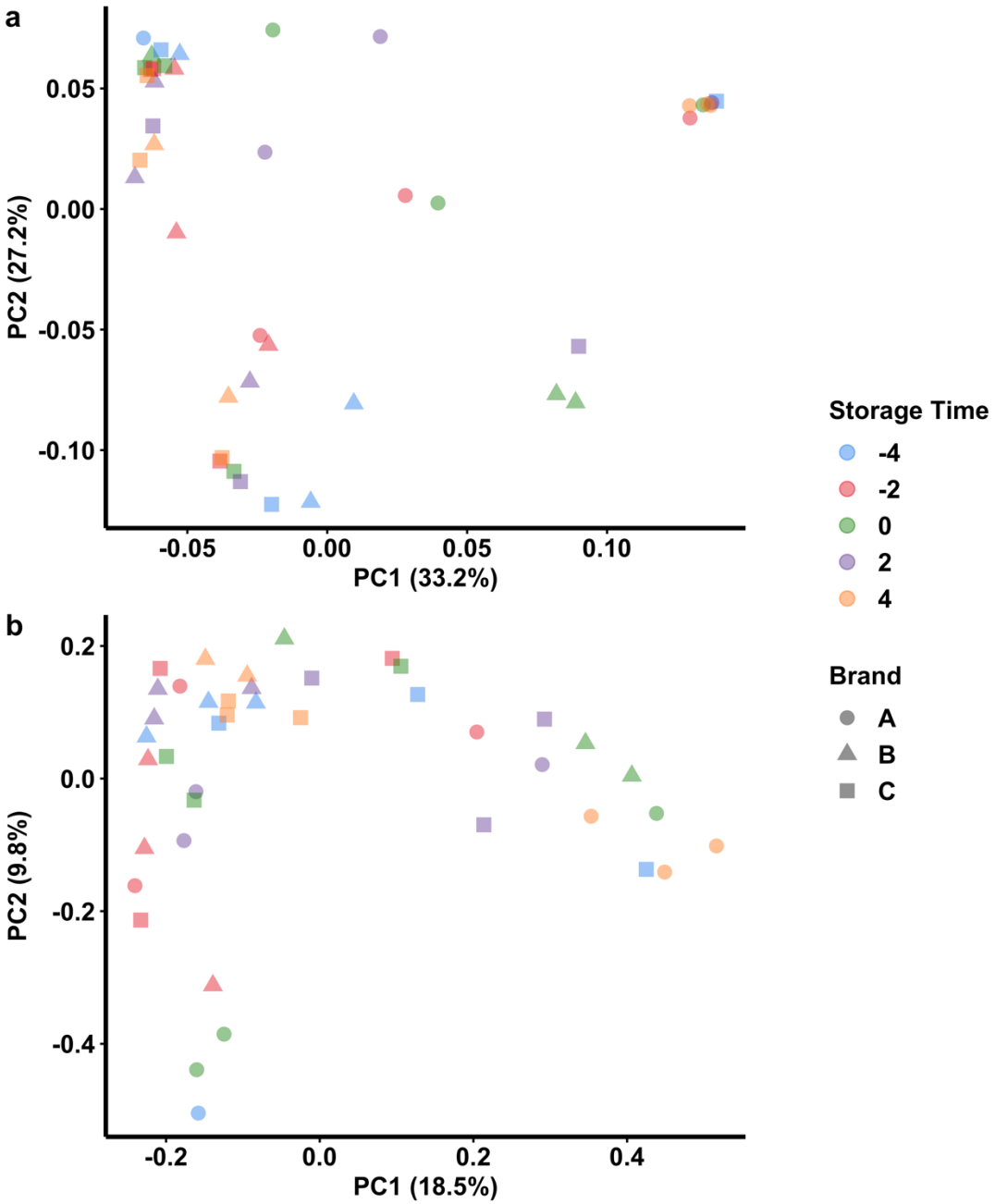

Figure 3. Principal coordinate analysis (PCoA) plot of all sliced, pre-packaged deli ham samples using the weighted (a) and unweighted (b) UniFrac distance matrix. Relative distance between samples indicates dissimilarity between overall bacterial community structure.

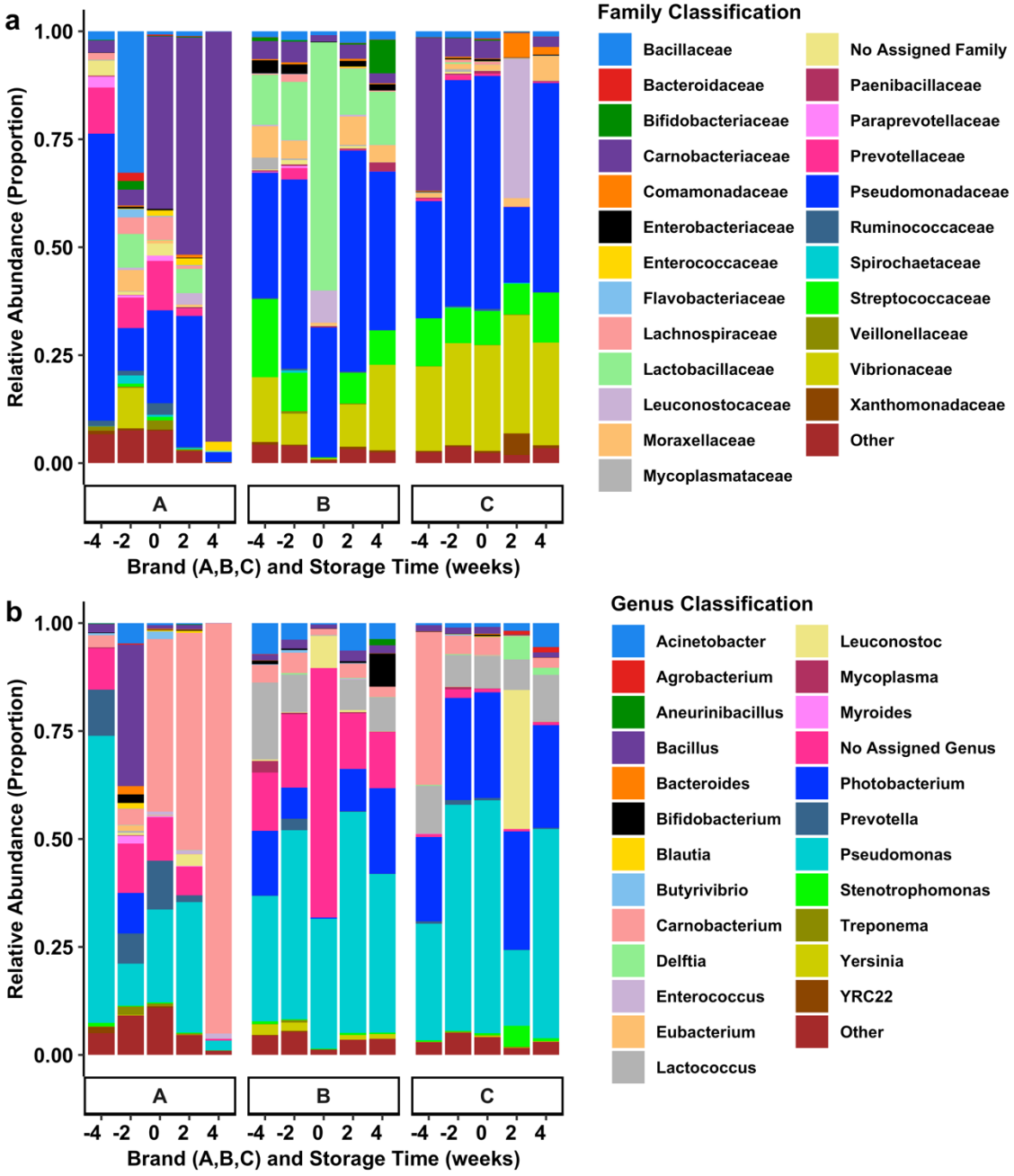

Figure 4. Relative abundance (proportion) of family (a) and genus (b) classification of bacterial community according to brand of sliced, pre-packaged deli ham. The top 24 most prevalent genus according to maximum relative abundance across all three treatments are represented.

STUDY 2. CHANGES IN THE MICROBIOTA ASSOCIATED WITH BEEF PRODUCTS FROM DIFFERENT STEPS OF PROCESSING THROUGHOUT STORAGE

Abstract

The microbial community structure of beef products was evaluated throughout various phases in processing. Raw ground beef (T1), fresh beef sausage (T2), cooked links (T3), cured franks (T4), sliced bologna (T5), and sliced bologna with high pressure processing (HPP) (T6) or organic acid (T7) were evaluated. Storage times of 21 days for raw products and 112 days for cooked products were used to simulate shelf-life of the products. Traditional plating methods and 16S rRNA gene sequencing were used to evaluate microbial community structure. Of the cooked products, sliced bologna had the most bacterial growth, reaching a plateau near 56 d of storage. Ground beef and sliced bologna had similar bacterial communities, dominated by *Pseudomonas*, while T3, T4, T6, and T7 treatments had more diverse community structures. Fresh beef sausage had an increase in *Lactobacillus* compared to the other treatments. This study revealed the microbial community structure of beef varies throughout processing, including phases associated with salting, cooking, slicing, and antimicrobial treatments.

1. Introduction

Shelf-life of beef products can be influenced by a number of factors. Microbial growth is one of the biggest causes of meat spoilage, and yet, it is nearly impossible to completely sterilize meat products while still creating products acceptable to consumers. Instead, most products are cooked to achieve pasteurization, or to eliminate pathogens and increase shelf-life. Thus, most of the preservation methods used today aim to simply reduce overall growth, and to select for microorganisms that are non-pathogenic and less detrimental than spoilage microbes that may produce off odors, colors, and slime rather quickly. Healthy muscle is inherently sterile, thus the microbiota of fresh meat originates in the intestines, on the hide, or in the processing environment (Koutsoumanis & Sofos, 2004). Likewise, most vegetative cells are destroyed during the cooking process, so much of the microbiota of cooked meats originates from the post-lethality processing environment where meats are stored, handled, peeled, sliced, and/or packaged. It is well known that the microbiota of cooked and fresh meats differs greatly, however, a deeper understanding of how each step throughout processing might affect the microbiota and meat spoilage should be explored.

Traditional plating methods have been supplemented with the use of genomic methods to evaluate microbial communities in soil, gut, and other ecological studies, but have only recently been used in meat microbiology. The 16S rRNA gene is highly conserved (Stackebrandt & Goebel, 1994), and thus 16S sequencing has become popular for the widespread characterization of microbial communities for its efficiency and accuracy in taxonomic identification. The development of the Illumina MiSeq platform has led to significantly greater sequencing coverage and longer reads without sacrificing

sequencing depth (Caporaso et al., 2012). The application of this technology can be seen in the meat industry by evaluating the differences caused by various ingredients, as well as evaluating how microbial communities shift over time, however, this study is the first to take a step-wise approach in evaluating how the microbiota changes throughout each phase of beef processing. The results from this study are relevant to the meat industry and the scientific community in order to further characterize the microbiological communities associated with meat spoilage and their origin, with respect to processing environment and processing steps.

2. Methods and Materials

2.1 Treatment Design

Beef shoulder clods from a single production day was ground and subdivided into seven treatment groups representing different phases of processing from raw ground beef to cooked, sliced deli meat: T1-Raw ground beef; T2-Raw fresh beef sausage; T3-Cooked, linked beef sausage; T4-Cooked, cured, beef franks; T5-Sliced beef bologna; T6-Sliced beef bologna with high pressure processing (HPP) treatment: T7-Sliced beef bologna with potassium lactate/sodium diacetate as an antimicrobial. Treatments are summarized in Table 1. Each treatment was produced in three replications, on three separate days of processing and representing raw materials from three different days of processing. Raw treatments (T1, T2) were evaluated every three days for a total of 21 days, with day 0 being the day of raw processing. Cooked treatments (T3-T7) were evaluated every 14 days for a total of 112 days, with day 0 being the day of slicing and packaging. Details on production and storage parameters are outlined below.

2.2 Beef Processing Production

Boneless beef shoulder clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen (-20 °C) until use. Replications were purchased as three different production days, with the goal of having a similar baseline raw meat block within each replication but separation between replications. Beef shoulder clods were frozen three days after the production date (date of fabrication) listed on the box, and each replication was tempered at 4 °C four days prior to its respective grinding and processing day. On three separate processing days, thawed shoulder clods were course ground using a 12.5 mm plate, mixed, and separated into 11.34 kg batches. Meat was then randomly assigned to one of seven treatments (T1-T7).

Production of each treatment was done as follows: T1: course ground meat was fine ground through a 3.2 mm plate using a Hobart Meat Grinder (Model #4734, Hobart MFG. Co., Troy, OH), approximately 226 g was stuffed into poly meat bags using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA), and sealed using plastic tape. T2: course ground meat was mixed with salt and seasoning blend (2.0% salt, 0.5% dextrose, 0.3% garlic powder, and 0.3% white pepper) and 3% water (on a meat block basis) in a double action mixer (Leland Southwest, Fort Worth, TX), fine ground through a 3 mm plate, approximately 226 g was stuffed into poly meat bags, and sealed using plastic tape. T3: course ground meat was mixed with salt and seasoning blend, 10% water (meat block basis), fine ground through a 3.2 mm plate, stuffed into approximately 58 g links in 22 mm edible collagen casings (Brechteen Company, Chesterfield, MI), cooked to an internal temperature of 71 °C in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI) followed by a 30 min cold shower, and chilled overnight at 0 °C. Four

links were placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO) and vacuum sealed to approximately 1.4 kPa (Multivac Model C500; Multivac Inc., Kansas City, MO). T4: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, and 550 ppm sodium erythorbate (meat block basis), chopped to a temperature of 18 °C using a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany), stuffed into edible collagen casings, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, and chilled overnight at 0 °C. Four links were placed into a pouch, and vacuum sealed. T5: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, and 550 ppm sodium erythorbate (meat block basis), chopped in a bowl chopper, stuffed into fibrous casings (90 mm x 24" pre-stuck, Kalle, Gurnee, IL), pulled and clipped using a Tipper Clipper (Model PR465L; Tipper Tie, Inc., Apex, NC), cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, and chilled overnight at 4 °C. The product was sliced into 2 mm slices using a deli slicer (Bizerba Model SE12, Balingen, Germany), 10 slices (approximately 225 g) were stacked, placed into a pouch, and vacuum sealed. T6: course ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, chopped in a bowl chopper, stuffed into fibrous casings, pulled and clipped, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, and chilled overnight at 0 °C. The product was sliced into 2 mm slices, 10 slices (approximately 225 g) were stacked, placed into a pouch, vacuum sealed, and subject to HPP at 600 mPa for 3 min with a pressure ramp rate of 300MPa/min, near instantaneous (<3 s) release time, and process fluid temperature maintained below 15 °C (Hiperbaric 55, Hiperbaric USA, Miami, FL). T7: course

ground meat was mixed with salt and seasoning blend, 10% water, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, and 3.5% potassium lactate/sodium diacetate blend (Opti.form PD4; Corbion Purac, Lenexa KS), chopped in a bowl chopper, stuffed into fibrous casings, pulled and clipped, cooked to an internal temperature of 71 °C, followed by a 30 min cold shower, and chilled overnight at 0 °C. The product was sliced into 2 mm slices, 10 slices (approximately 225 g) were stacked, placed into a pouch, and vacuum sealed. All samples were stored covered at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

2.3 Microbial Analysis

Approximately half of each sample package (80-120 g) was aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate were collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB), and psychrotrophic plate counts (PPC) were evaluated using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. Brain Heart Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. Aerobic plate counts and LAB counts

were incubated at 37 °C for 48 h, and enumerated manually following Eddy Jet directions. Anaerobic plate counts were incubated at 37 °C for 48 h in an anaerobic box containing 1-2 BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ) and enumerated manually following Eddy Jet Directions. Psychrotrophic plate counts were incubated at 4 °C for 96 h and enumerated manually following Eddy Jet Directions. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

Bacterial community analysis using high throughput sequencing of the 16s rRNA gene was performed on each sample collection using the MiSeq Illumina Sequencing Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Due to the scale of this study, cooked sample weeks 2 and 4 were not subject to 16S sequencing after reviewing culture based growth data. Microbial DNA extraction from homogenized meat samples was performed using a modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed, and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR), amplification in a 25 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA), approximately 20 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich et al. (2013). The PCR reaction was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific,

Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, 58 °C for 30 s, and 68 °C for 45 s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Due to low DNA concentration, purified DNA was subject to additional PCR using the same process listed above with 5 amplification cycles. Following secondary amplification, DNA was applied to a 1.5% agarose gel, and the target band was manually excised, and recovered using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Final size and concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and library concentration was confirmed using a DeNovix QFX Fluorometer with the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc, Wilmington, DE).

The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller, Kononoff, & Fernando, 2016) using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed to 251bp. Sequences were

binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

2.4 Physicochemical Analysis

The remaining half of each sample package (80-120 g) was used for physicochemical analyses. Salt concentration and water activity were measured on day 0 only, while objective color and pH were measured throughout storage time.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt concentration was measured using Quantab high range chloride titration strips (Hach Company, Loveland, CO) as described by Sebranek et al. (2001).

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of sample in 90ml of double distilled deionized water. Objective color (L^*, a^*, b^*) of was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from dirtying the colorimeter lens. Six readings were averaged from either a

flattened ground sample, interior and exterior of two split links, or the surface of two slices.

2.5 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For salt and water activity (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package (lsmeans and cld functions; Lenth, 2019). For all shelf-life measures, cooked and raw samples were analyzed separately. For pH, color, and plate counts, data were analyzed as a factorial arrangement with 2 treatments by 8 sampling times for raw samples, and 5 treatments by 9 sampling times for cooked samples, with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 3,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All subsequent statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha_diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2019) with

storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the emmeans package in R (function lsmeans and cld; Lenth, 2019). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analysis. Both weighted UniFrac and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree. The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2019) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

3. Results and Discussion

3.1. Microbial Analysis

Four traditional plating methods were used to determine viable bacterial cell growth throughout storage time. Due to differing storage time intervals, raw and cooked

samples were analyzed separately. There were no treatment by storage time interactions for APC, AnPC, LAB or PPC in raw samples ($P > 0.05$). For raw treatments, there was a main effect of storage time on APC, AnPC, LAB, and PPC (Figure 1; $P < 0.001$). For APC, counts averaged 2.5 log CFU/g on day 0, and peaked at 5.7 log CFU/g on day 15. For AnPC, growth averaged 2.1 log CFU/g at day 0, and peaked at 5.6 log CFU/g on day 15. Mean LAB count for raw samples was 1.3 log CFU/g on day 0, and peaked at 4.2 log CFU/g at day 15. Psychrotrophic plate counts were 0.0 log CFU/g on day 0, and peaked at 6.52 log CFU/g on day 15. There were no main effects of treatment on any of the plating measures ($P > 0.05$).

There was a treatment by storage time interaction for AnPC in cooked samples ($P = 0.003$), but no interaction for APC, LAB, or PPC ($P > 0.115$). Anaerobic plate counts for T3, T4, and T7 began increasing between day 28 to day 42, while T6, the HPP bologna, had no growth until day 112. In cooked samples, there were main effects of treatment and storage time for APC ($P < 0.001$) and LAB ($P < 0.030$), and a main effect of storage time for PPC ($P < 0.001$). For both APC and LAB, higher counts were observed in T5 sliced bologna compared to all other treatments, and an increase in APC, LAB, and PPC was seen throughout storage time (Figure 2).

Good's coverage test was used to ensure adequate sequencing depth. Good's coverage index indicated that at an even rarefied depth of 3,000 reads, $\geq$ 95.0% of the entire bacterial community was identified. Alpha diversity of bacterial communities was measured using observed OTUs and Chao1 diversity estimates (Figure 3). All treatments reached a plateau before the rarefaction at 3000 sequences, indicating adequate sampling coverage at this depth. For raw samples, there was a main effect of both treatment and

storage time on Chao1 and observed OTUs ($P < 0.023$). Generally, species richness decreased throughout storage time, and T2 had greater species richness than T1 according to both Chao1 and observed OTU estimates. For cooked samples, there was a main effect of treatment for both Chao1 and observed OTUs ($P < 0.009$). For observed OTUs, T5 had less species richness than all other treatments. For Chao1, T5 had the least richness, T3 and T4 had the greatest richness, and T6 and T7 were intermediate. Beta diversity was evaluated using the weighted and unweighted UniFrac distance matrices. There were main effects for treatment ($P = 0.001$) and storage time ($P = 0.002$) for the weighted UniFrac. Treatments T1 and T5 tended to cluster together, separate from all other samples, and T2 somewhat clustered in an additional group, separate from all other treatments. There was no clear independent clustering by storage time, however, later storage times tended to be more widespread, whereas early storage times were more tightly clustered, comparatively (Figure 4a). There was a treatment by storage time interaction for the unweighted UniFrac distance matrix ($P = 0.032$). Upon principal component analysis, T1, T2, and T5 tended to cluster independently from the other treatments, and increased storage times were the outermost samples with respect to the main cluster of observations from all other samples (Figure 4b). Significant shifts in the microbial community can be seen at the phylum classification (Figure 5a). Treatments T1 and T5 had a higher prevalence of *Proteobacteria* which generally increased throughout storage in these treatments. Treatments T2, T4, T6, and T7 had a significant proportion of *Proteobacteria* but were also characterized by a greater proportion of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* compared to other treatments. As shown in Figure 5b, relative abundances of OTUs grouped by genus classification showed

similar behavior between T1 and T5, where *Pseudomonas* was prominent beginning early on in storage and was the dominant genus throughout most of storage time. The ground beef T1 also showed an increase in *Lactococcus* early on in storage and a decrease in *Lactobacillus* over time. Conversely, T3, T4, T6 and T7 saw lower proportions of *Pseudomonas,* and greater proportions of a variety of bacteria, including *Acinobacter*, *Lactobacillus*, and *Psychrobacter*. The fresh sausage T2 displayed a steady increase of *Lactobacillus* and *Brochothrix* over time, as well as a slight increase in *Pseudomonas* occurring later on in storage.

Proper cooking of meat products will destroy most vegetative cells, thus the microbiota of cooked meats is typically assumed to originate from post-lethality, contamination, usually during peeling, slicing, or other post-cook handling (Borch, Kant-Muermans, & Blixt, 1996). Because of this, it would be expected that the bacterial communities associated with cooked and raw products would be vastly different and that raw products would have a more diverse microbiome. Results from this study somewhat contradict that notion and showed that the microbiome of raw ground beef was more similar to that of the T5 sliced bologna than any of the other treatments.

Although minimal growth overall is ideal, it is normally advantageous for processors to take steps to shift spoilage communities away from aerobic psychrotrophs such as *Pseudomonas* and allow for the slower growing LAB to dominate. It has been theorized to use certain bacteria as bio-protective cultures in processed meats (Comi, Andyanto, Manzano, & Iacumin, 2016), as well as isolating by-products from certain LAB to be used as antimicrobials (Woraprayote et al., 2016). It is well established that salt is one of the factors at play that helps select for LAB and suppresses more salt

sensitive organisms such as *Pseudomonas* (Blickstad & Molin, 1983), and results from the raw portion of this study support that claim. While there were no differences between the raw treatments for culture based plating methods, 16S sequencing revealed a drastic difference between T1 and T2, where the raw ground beef T1 microflora had a large amount of *Pseudomonas* growth, and the salted fresh sausage T2 had a greater proportion of *Lactobacillus* growth, which seemed to offset or suppress *Pseudomonas*. It has also been theorized that LAB should also dominate cooked, vacuum-packaged products (Gill & Newton, 1978), however recent studies have shown contrary (Bower, Stanley, Fernando, Burson, & Sullivan, 2018a; Bower, Stanley, Fernando, & Sullivan, 2018b). Mertz et al., (2014) demonstrated that *Pseudomonas* was the most commonly found genera on deli meat slicers, aerobic conditions present on meat equipment in a processing environment. Furthermore, Bower et al. (2018a, 2018b) reported a high incidence of *Pseudomonas* on sliced and vacuum packaged deli meat, regardless of salt or sodium nitrite concentration. The results from T5 in this study are in line with these studies, as a high prevalence of *Pseudomonas* was seen. It is of interest though, that a lower proportion of *Pseudomonas* was identified in both the cured and uncured links, as well as the HPP and lactate/diacetate bologna. The difference in *Pseudomonas* seems to coincide with the difference in plate counts, where T5 had more growth compared to the other cooked treatments. It would seem that the growth seen in T5 was likely attributed to *Pseudomonas* and the lower growth observed in T3, T4, T6, and T7 resulted in a more diverse bacterial community with a lower proportion of *Pseudomonas* and no clear dominant genera. For the linked product treatments, it seems a matter of surface area likely suppressed microbial growth due to less surface that interacts with the post-

lethality environment, as well as less surface area exposed to oxygen during handling and packaging. As for the other sliced treatments, HPP has been well documented to reduce microbial growth in foods (Garriga, Grèbol, Aymerich, Monfort, & Hugas, 2004; Myers et al., 2013), and organic acids have been shown to reduce microbial growth, as well as shift the microbial population toward more acid-tolerant LAB (Benson et al., 2014). When compared to LAB, the main competitive advantage of *Pseudomonas* is its rapid growth rate (Gill & Newton, 1978). This is one explanation of a high prevalence of *Pseudomonas* in the treatments where *Pseudomonas* was not otherwise controlled through other antimicrobial processes or ingredients.

3.2 Physicochemical Analysis

Measures of pH and objective color (CIE L^*, a^*, b^*) were collected throughout shelf-life and due to differing storage times and intervals, raw and cooked samples were analyzed independently. Treatment main effects for pH and objective color are shown in Table 2. There were no treatment by storage time interactions for pH and objective color in raw samples ($P > 0.05$), nor were there interactions in cooked samples for pH, L^*, or b^* ($P > 0.005$). There was a main effect of storage time on pH of both raw ($P = 0.003$) cooked samples ($P < 0.001$) where pH generally decreased over time, ranging from 5.85 on day 0 to 5.61 on day 21 in raw samples, and 6.16 on day 0 to 6.00 on day 98 in cooked samples. A decline in pH is typically expected over time due to the growth of acid producing organisms (Gram et al., 2002). Measures of lightness, L^*, varied throughout storage time ($P < 0.001$) in raw samples, ranging from 49.7 to 53.2. Such small differences were statistically significant, however, they are of little practical value in the

current study. There was a treatment effect on *L** ($P = 0.015$) in cooked samples where the uncured franks (T4) were darker than the cured treatments T4, T5, and T6. There were main effects of treatment ($P = 0.010$) and storage time ($P < 0.001$) on *a** in raw samples. Treatment 1, ground beef, had greater *a** values than treatment 2, fresh sausage. Day 0 had the highest *a** values measuring 17.00, and *a** decreased until day 9, where *a** remained between 10.98 and 9.71 for the remainder of storage time. In cooked samples (T3-T7), there was a treatment by storage time interaction for *a** ($P < 0.001$), displayed in Figure 6. On day 0, the uncured franks (T3) had a much lower *a** value, 8.55, than each of the cured treatments (T4-T7), which ranged from 18.37 to 19.39. An increase in a* was observed in T3 throughout storage time, while T4-T7 each decreased throughout storage. Furthermore, T6 bologna with HPP decreased more rapidly than the other cured treatments and had a lower *a** value than all treatments including T3 uncured franks at day 98 and day 112. For *b**, which measures yellowness, there were main effects for both treatment ($P < 0.001$) and storage time ($P = 0.003$) in cooked samples only. Treatment 3, the uncured franks, had lower *b** values compared to all other treatments. There was a trend of decreasing *b** throughout storage time, ranging from 8.77 at day 0 to 7.62 at day 98. Measured differences in b* values, while statistically significant, are likely of little practical importance.

Salt and water activity were measured on day 0 only, and therefore all treatments were compared. There was a treatment effect for both salt and water activity ($P < 0.001$). Salt concentration ranged from 0.00% in T1 to 2.32 % in treatment 4 (Table 3). Treatment T1 was formulated as ground beef with no salt or spices added, and thus should not have a measurable amount of salt. Treatment T2, fresh sausage, had the next

lowest salt concentration, likely due to a dilution effect from the water retained in the product when compared to the cooked T3-T7. Treatments T3-T7 were all statistically similar in salt concentration which would be expected as they were all based on common formulated salt concentrations and subject to similar cooking schedules. Water activity ranged from 0.9965 in treatment 1 to 0.9708 in treatment 7, and only treatment 1 was statistically different compared to treatments 2-7 (Table 3). It would be expected that treatment 1 had a higher water activity than the other treatments, as all other treatments contained salt and spices, and as solutes increase, water activity typically decreases (Mathlouthi, 2001).

4. Conclusion

The results from this study and other bacterial community studies collectively will help to establish a more developed understanding of how microbes behave and interact in certain environments and will lead to further development of methods to control spoilage based on knowledge of bacterial community dynamics. To our knowledge, this is the first study to directly compare bacterial community dynamics throughout each phase of processing, from raw ground beef to a cooked and sliced product. It was suspected that the largest difference in microbial community structure would be between the raw and cooked treatments, the similarities between raw ground beef T1 and sliced bologna T5 are quite surprising. The results from this study show that exposure to the processing environment without antimicrobial controls yields a similar microbiome on a cooked product compared to raw. If an antimicrobial control is in place, such as HPP or organic acids, not only is microbial growth suppressed, but the bacterial community remains

more diverse without the emergence of a dominant species through 112 days of storage. Likewise, in the linked treatments, due to limited surface area minimal interaction with the processing environment post-lethality, the microbial growth and community characteristics were similar to that of the bologna with antimicrobial treatments. Finally, this study demonstrates the role salt plays in meat spoilage, considering the differences in T1 and T2 and their spoilage microbiomes. This study demonstrates a wide overview of the spoilage characteristics throughout processing, however specific treatments and processes should be further explored to identify and confirm the mechanisms by which spoilage communities can be controlled or manipulated.

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Tables

Table 1. Summary of treatments used in the study

T1	Ground Beef	Raw
T2	Fresh beef sausage with salt and spices	
T3	Linked, cooked sausage	Cooked
T4	Cured beef franks	
T5	Sliced beef bologna	
T6	Sliced bologna with HPP treatment	
T7	Sliced bologna with sodium lactate and sodium diacetate	

Table 2. Least squared means for main effect of treatment for shelf-life measurements of beef products.

	Treatment[1]										
	Raw				Cooked						
	1	2	SEM[2]	P value	3	4	5	6	7	SEM[2]	*P* value
pH	5.81	5.70	0.06	0.223	6.08	6.10	6.10	6.11	6.00	0.03	0.192
L*	52.17	51.02	0.80	0.453	55.75^{x}	60.75^{y}	60.82^{y}	60.63^{y}	59.66^{xy}	0.94	0.015
a*	14.38^{b}	10.54^{a}	0.62	0.010	9.92	16.52	17.34	13.87	17.55	0.37	--[†]
b*	9.81	9.76	0.28	0.907	7.17^{x}	8.79^{y}	8.81^{y}	8.72^{y}	8.42^{y}	0.19	< 0.001

[1] Treatments: 1-Ground Beef, 2-Fresh Beef Sausage, 3-Cooked Link, 4-Beef Frank, 5-Sliced Bologna, 6-Bologna w/HPP, 7-Bologna w/ antimicrobial.

[2] SEM: Standard error of the overall mean.

[†] Indicates a significant ($P < 0.05$) brand by storage time interaction, therefore main effects cannot be analyzed.

[a,b; x,y,z] Means in the same row within either raw or cooked treatments lacking a common superscript are significantly different ($P < 0.05$) after Tukey's HSD adjustment.

Table 3. Least squared means for day 0 physicochemical measurements of beef products.

	Treatment[1]								
	1	2	3	4	5	6	7	SEM[2]	*P* value
Salt (%)	0 .00[c]	1.29[b]	1.92[ab]	2.32[a]	2.01[ab]	1.93[ab]	1.95[ab]	0.17	<0.001
Water Activity	0.9965[b]	0.9787[a]	0.9737[a]	0.9729[a]	0.9782[a]	0.9752[a]	0.9708[a]	0.0020	<0.001

[1] Treatments: 1-Ground Beef, 2-Fresh Beef Sausage, 3-Cooked Link, 4-Beef Frank, 5-Sliced Bologna, 6-Bologna w/HPP, 7-Bologna w/ antimicrobial.

[2] SEM: Standard error of the overall mean.

[a,b,c] Means in the same row within either raw or cooked treatments lacking a common superscript are significantly different ($P < 0.05$) after Tukey's HSD adjustment.

Figures

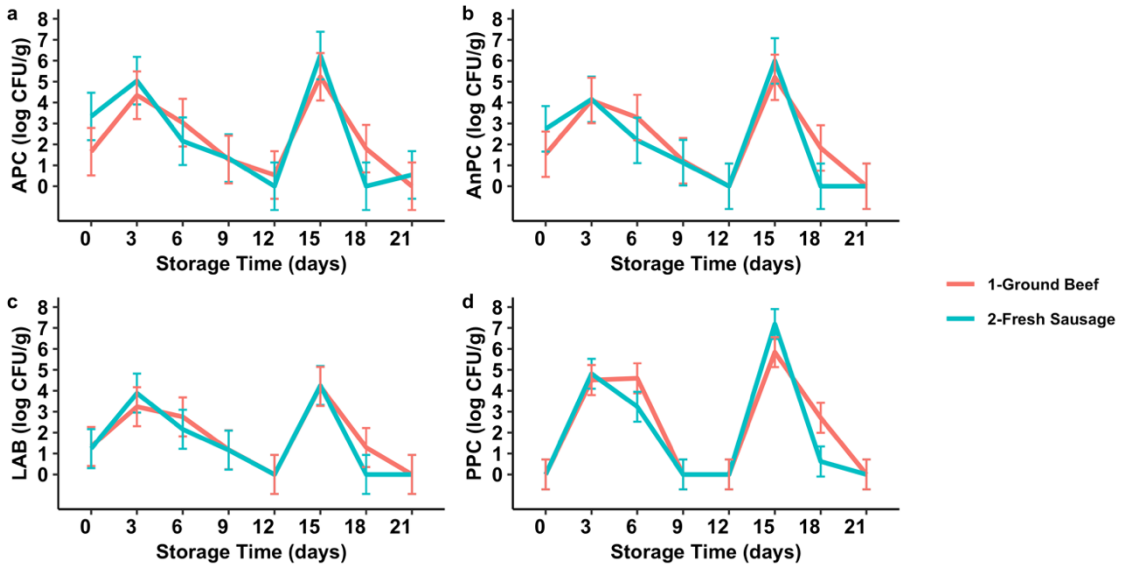

Figure 1. Mean plate counts for raw samples (T1, T2) throughout storage time. Plating methods: a) aerobic plate count (APC); b) anaerobic plate count (AnPC); c) lactic acid bacteria count (LAB); d) psychrotrophic plate count (PPC).

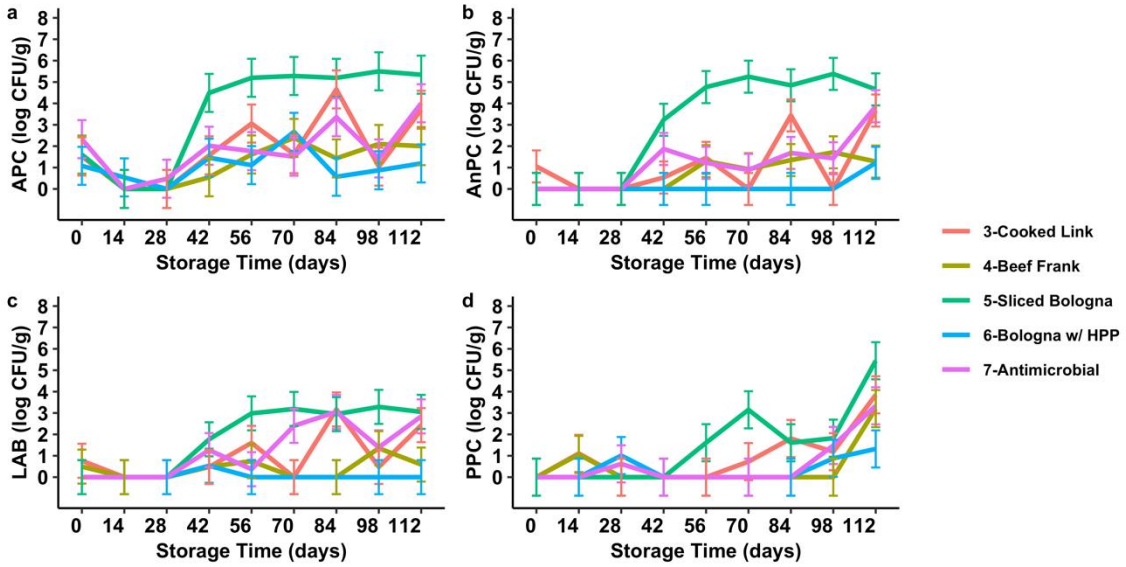

Figure 2. Mean plate counts for cooked samples (T3-T7) throughout storage time. Plating methods: a) aerobic plate count (APC); b) anaerobic plate count (AnPC); c) lactic acid bacteria count (LAB); d) psychrotrophic plate count (PPC).

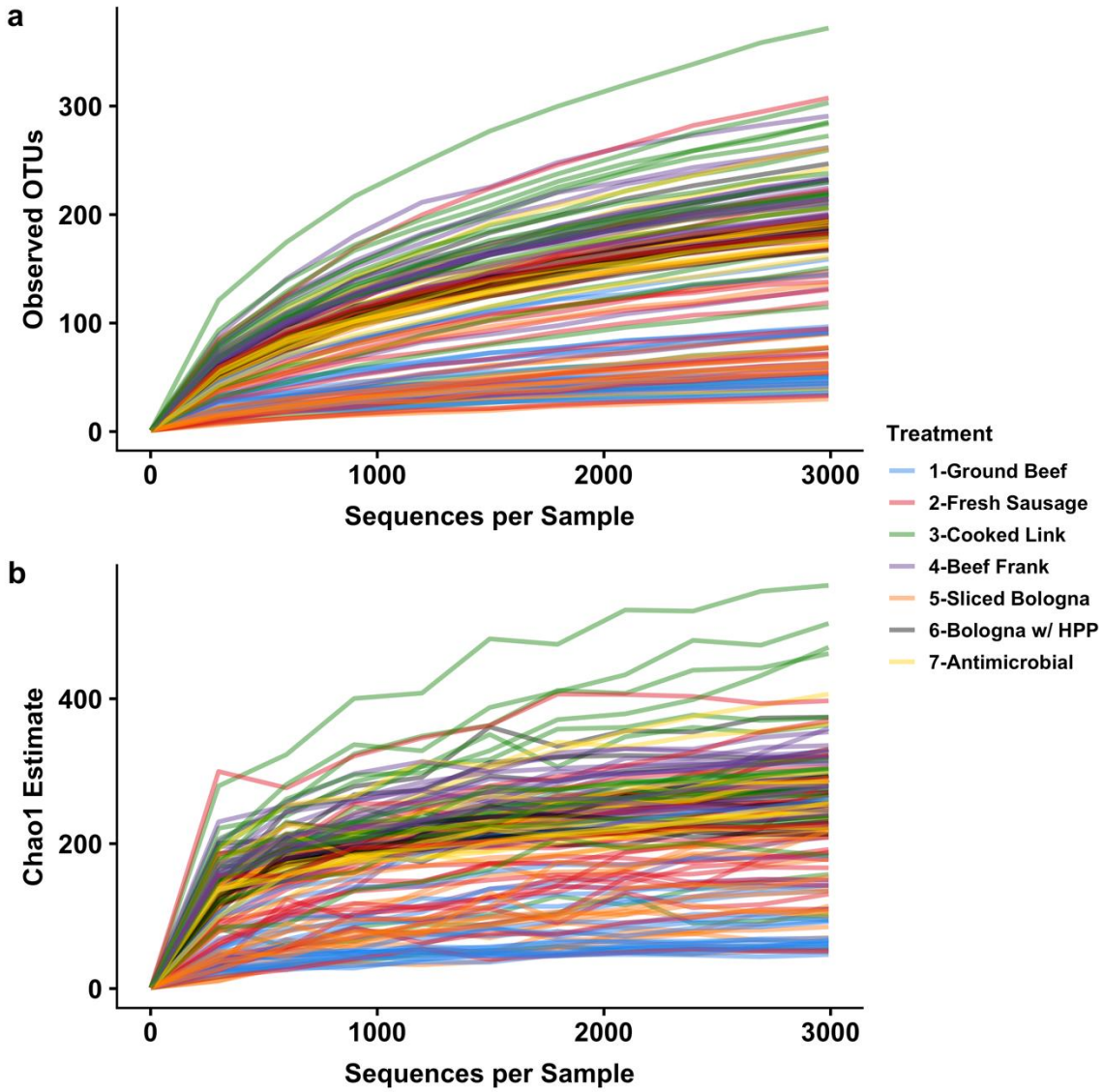

Figure 3. Alpha diversity estimates of observed OTUs (a) and Chao1 (b) across treatments.

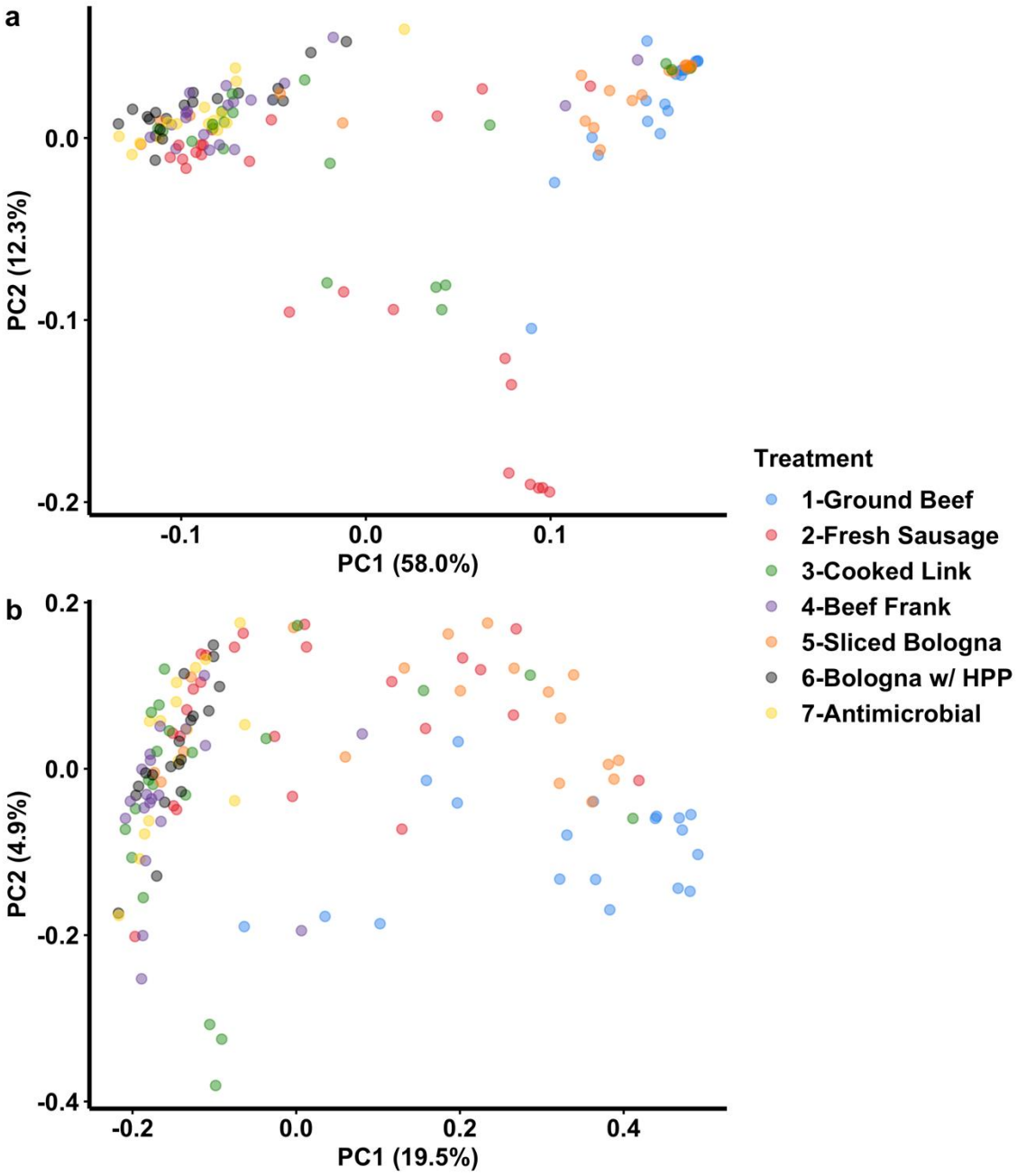

Figure 4. Beta diversity estimates of the weighted UniFrac (a) and unweighted UniFrac (b) distance matrices.

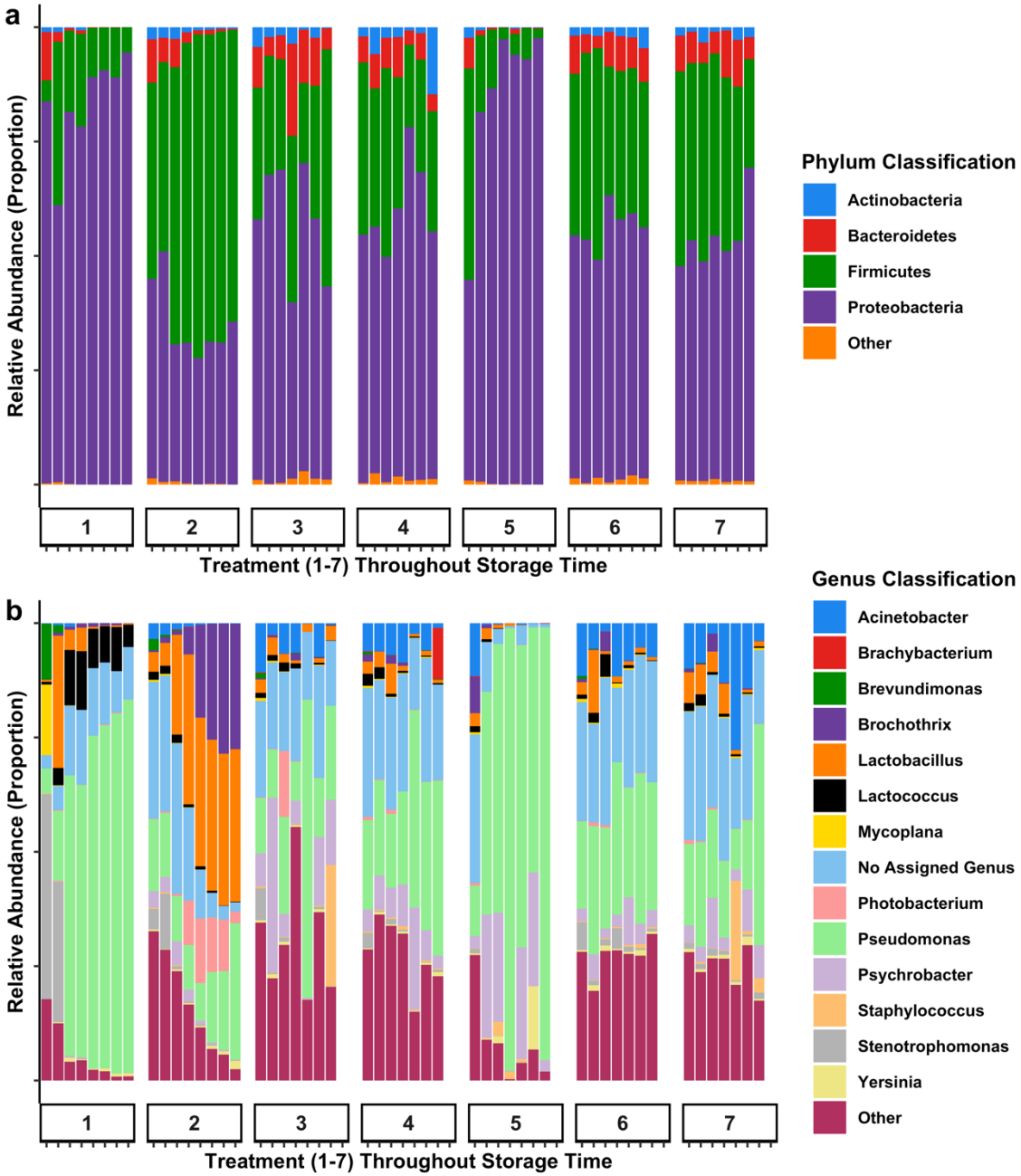

Figure 5. Relative abundances of most abundant OTUs grouped by phylum (a) and genus (b) classification throughout storage time. Treatments: 1-Ground Beef, 2-Fresh Beef Sausage, 3-Cooked Link, 4-Beef Frank, 5-Sliced Bologna, 6-Bologna w/HPP, 7-Bologna w/ antimicrobial. Ticks represent storage times of 3 day intervals for 21 days in T1 and T2, and 14 day intervals for a total of 114 days in T3-T7.

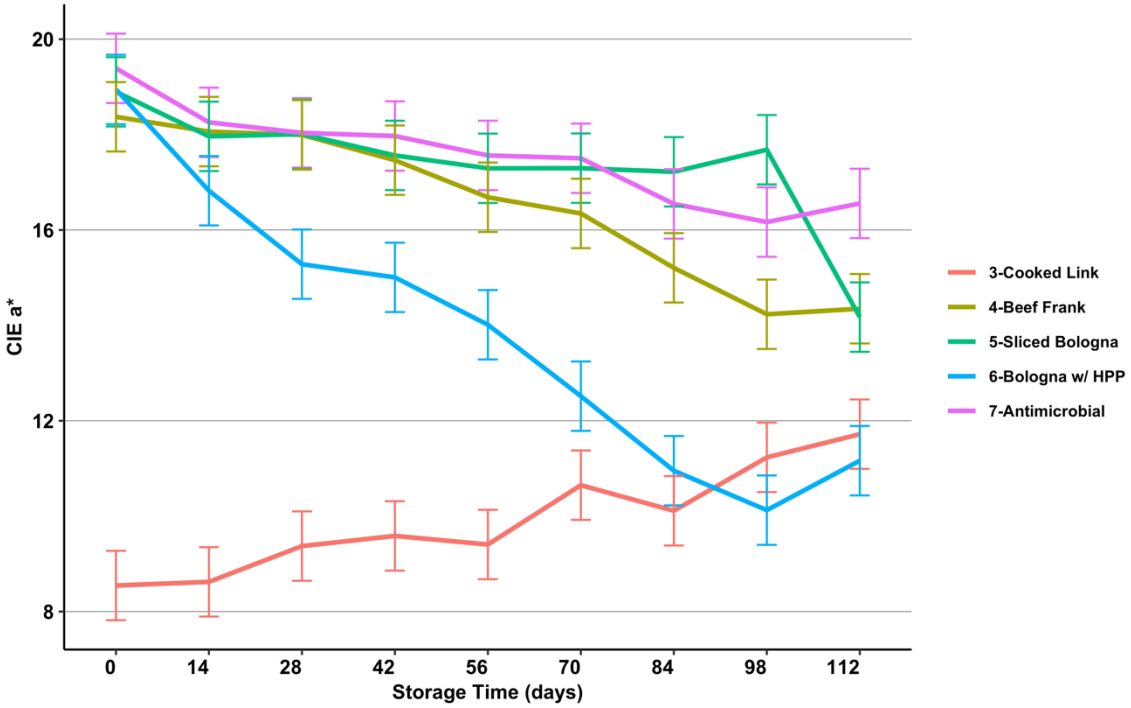

Figure 6. Mean CIE a* (redness) values for cooked samples (T3-T7) throughout storage time.

STUDY 3. EFFECTS OF TRADITIONAL AND CLEAN-LABEL ANTIMICROBIALS ON SPOILAGE COMMUNITIES ASSOCIATED WITH SLICED BOLOGNA

Abstract

The effects of traditional and clean-label organic acid antimicrobials on spoilage and the microbial community structure were evaluated. Potassium lactate/sodium diacetate (LD), propionic acid (P), buffered vinegar (BV), cultured sugar (CS), and control (C) with no antimicrobial were compared. For 14 weeks, traditional plating methods and 16S rRNA gene sequencing were evaluated in addition to cook yield, pH, objective color, and instrumental texture profile analysis. The control treatment had increased aerobic and psychrotrophic plate counts compared to all antimicrobial treatments ($P < 0.013$), as well as a different microbial community structure ($P < 0.001$) headed by a drastic increase in *Pseudomonas* in the control. Cooking yield increased in BV and decreased in LD and P when compared to the control ($P = 0.007$), and P and CS had a lower pH than other treatments ($P < 0.001$). Results from this study indicate that the organic acids used similarly reduced bacterial growth and did not alter the bacterial community differently from one another.

1. Introduction

Organic acids or their salts are commonly used as antimicrobial ingredients in meat products. Under the USDA FSIS *Listeria monocytogenes* guidelines for ready to eat meat and poultry products (USDA FSIS, 2014), various commercially available organic acids can constitute as antimicrobial agents to control *Listeria monocytogenes* growth. Mixtures of sodium or potassium lactate and diacetate have long been standard for organic acid antimicrobials in meat products, but others such as propionic acid are commercially available and commonly used. Recent food trends have led to the development of clean-label organic acids that can be included on food labels with consumer-friendly terminology such as vinegar and cultured sugar, rather than listing acetic, lactic, or propionic acids on the label. In theory, these clean-label organic acids are chemically similar to their traditional counterparts, however, they are made using a natural process and not purified. Thus, they meet different labeling requirements that are more attractive to consumers, and may also meet requirements for certain natural or organic claims. Both traditional and clean-label organic acids have been shown effective in reducing or eliminating the growth of *Listeria monocytogenes* in various cooked meats (Ahmed et al., 2015; Seman, Borger, Meyer, Hall, & Milkowski, 2002; Stekelenburg, 2003), however, most processers validate *Listeria monocytogenes* control for each product and formulation individually.

The main pathway for the antimicrobial action of organic acids is based on the pH of the food and the pKa of the organic acid. As described by Doyle, Beuchat, & Montville, (2001), when the food pH is near or below the pKa of a given acid, a portion of the organic acid present will be in the undissociated form. The undissociated acids

will penetrate the membrane of bacterial cells, and dissociate in the neutral pH of the cell cytoplasm, acidifying the cytoplasm. Once the acid becomes dissociated, it will be pumped out of the cell, and can again become undissociated in the meat matrix, and continue the process of acidifying the bacterial cell. Constant acidification of the cytoplasm will deplete cellular energy by driving the ATP pump attempting to remove protons from the cell to neutralize pH and the proton gradient (Doyle et al., 2001). This mode of action is similar between different organic acids, however, their pKa may vary, determining their effectiveness in foods with different pH.

As stated above, the inhibitory effect of organic acids on *Listeria monocytogenes* is well defined, however, with the recent development of tools used in microbial ecology, the effect of different organic acids on the microbial communities involved with meat spoilage is of great interest to the meat industry and the scientific community. Using 16S rRNA sequencing on the 454 platform, Benson et al., (2014) reported a shift in the microbiota of fresh pork sausage when lactate/diacetate was added, where the multiple complex waves of microbial growth seen in untreated sausage were mitigated and reduced to the growth of a single species of *Lactobacillus graminis* in sausage with sodium lactate and sodium diacetate added. Similarly, 16S sequencing revealed a shift from *Brochothrix* and *Pseudomonas* in untreated fresh pork sausage toward various lactic acid bacteria (LAB) species with the addition of lactate/diacetate (Bouju-Albert, Pilet, & Guillou, 2018). With the development of the Illumina MiSeq platform, longer reads and greater sequencing coverage can be achieved while maintaining adequate sequencing depth, and the cost of sequencing is more attainable for individual researchers and smaller projects (Caporaso et al., 2012).

To our knowledge, this is the first study to utilize the MiSeq platform to compare various clean-label and traditional antimicrobials and their effects on the microbial communities associated with spoilage of sliced deli meats. The aim of this study is to identify key differences in the microbiota of sliced deli meat made with a traditional organic acid (potassium lactate/sodium diacetate or propionic acid) or a clean label organic acid (buffered vinegar or cultured sugar) compared to a control with no organic acid added. These findings will be beneficial to the scientific community and the meat industry alike, allowing for a better understanding of microbial community dynamics in processed meats, as well as addressing specific spoilage concerns with the use of various organic acids.

2. Methods and Materials

2.1 Treatments & Experimental Design

Beef bologna was formulated and produced to include one of five common food grade antimicrobial organic acids: lactate/diacetate (LD), propionic acid (P), buffered vinegar (BV), and cultured sugar (CS), as well as a control (C) containing no antimicrobial. Due to various concentration and composition between antimicrobials, each treatment was formulated based on the supplier's recommendation for 90 days of *Listeria monocytogenes* inhibition. Each treatment was produced in three replications, on three separate days of processing. Each treatment was evaluated every 2 weeks for a total of 14 weeks, with day 0 being the day of slicing and packaging. Details on production and storage parameters are outlined below.

2.2 Bologna Production

Boneless beef clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen at -20 °C until use. On three separate processing days, shoulder clods were thawed for approximately 3 days at 4 °C, tempered clods were hand cut into pieces, placed into 11.34 kg batches, and randomly assigned to one of 5 treatments. Each treatment was mixed with 2.0% salt, 0.5% dextrose, 0.15% garlic powder, 0.30% white pepper, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, 10% water, and 10% ice (on a meat block basis). Meat and ingredients were chopped in a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany) to approximately 18 °C, and antimicrobial was added to the chopper near the end of chopping. Antimicrobial (on a meat block basis) inclusions were as follows: LD: 3.81% Optiform PD4 (Corbion, Lenexa, KS); P: 0.5% BactoCEASE 6 (Kemin Industries, Des Moines, IA) BV: 0.93% BactoCEASE NV (Kemin Industries); CS: 2.51% Verdad N70 (Corbion). Batter was stuffed using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA) into fibrous casings (90 mm x 24" pre-stuck, Kalle, Gurnee, IL), casings were pulled and clipped using a Tipper Clipper (Model PR465L; Tipper Tie, Inc., Apex, NC), cooked to an internal temperature of 71 °C in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI), followed by a 30 min cold shower, and chilled overnight at 0 °C. The following day, bologna logs were sliced into 2 mm slices (Model SE12, Bizerba USA Inc., Joppa, MD), 10 slices were stacked, placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), and vacuum sealed with approximately 1.4 kPa vacuum (Multivac Model C500; Multivac Inc., Kansas City, MO). Two, 13 mm slices were cut from each treatment to be used for texture profile analysis (TPA). All samples were stored covered

at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

2.3 Microbial Analyses

From each sample package, 5 slices (100-120 g) were aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate were collected for microbial community analysis and was stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB) and psychrotrophic aerobe plate counts (PPC) were performed using the homogenized samples. Brain Heart Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. For APC and LAB, plates were incubated at 37 °C for 48 h, AnPC were incubated at 37 °C for 48 h in an anaerobic box containing BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ), and PPC were incubated at 4 °C for 96 h. All plates were enumerated manually following instructions from the Eddy Jet manual. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

Bacterial community analysis using high throughput sequencing of the 16s rRNA

gene was performed on each sample collection using the MiSeq Illumina Sequencing Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Microbial DNA extraction from homogenized meat samples was performed using a modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed, and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR), amplification in a 25 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc.), approximately 20 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich et al. (2013). The PCR was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 58 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Due to low DNA concentration, purified DNA was subject to additional PCR using the same process listed

above with 5 amplification cycles. Following secondary amplification, DNA was applied to a 1.5% agarose gel, and the target band was manually excised and recovered using the MinElute PCR Purification kit (Qiagen, Inc., Germantown, MD). Final size and concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and library concentration was confirmed using a DeNovix QFX Fluorometer with the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc, Wilmington, DE).

The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller, Kononoff, & Fernando, 2016) using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed to 251bp. Sequences were binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

2.4 Physicochemical Analyses

The remaining half of each sample package (100-120 g) was used for physicochemical analyses. Salt concentration, water activity, and proximate composition

were measured on day 0 only, while objective color and pH were measured throughout storage time.

Cooking yield was determined by weighing each treatment batch prior to cooking, and again after chilling overnight prior to slicing. Cooking yield was calculated as cooked weight as a percentage of raw weight:

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to manufacturer's specifications. Salt concentration was measured as described by Sebranek et al. (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining water to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO). In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of sample in 90ml of double distilled water. Objective color (*L*, a*, b**) of was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from dirtying the colorimeter lens. Six readings were averaged from the surface of two slices.

Texture profile analysis (TPA) was performed by cutting a 13mm slice into a 4.0 cm × 4.0 cm square and measured using a 2500 kg load cell on an Instron (Model number 1123; Instron Worldwide, Norwood, MA) with a 140mm plate. Each slice was

compressed two times to 75% of its original thickness with a head speed of 30 mm/min, and the characteristics of hardness, springiness, cohesiveness, and chewiness were determined according to Bourne (1978). Briefly, hardness is the maximum force (N) during the first compression cycle. Springiness is the ratio of the duration (s) of the second compression cycle compared to the first cycle, measuring the elastic recovery of the product. Cohesiveness is the ratio of the positive force area under the curve of the second compression cycle compared to that of the first cycle. Chewiness is the product of hardness, springiness, and cohesiveness multiplied.

2.5 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For cook yield, salt concentration, water activity, and proximate composition (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Data were analyzed as a 5 (treatment) by 8 (storage time) interaction with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot2 and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 5,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All

statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Good's coverage test was performed to ensure adequate sampling depth was achieved. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2017) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the emmeans package in R (function lsmeans and cld; Lenth, 2016). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analyses. The weighted and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. The UniFrac distance matrix calculates sample dissimilarity based on a phylogenetic tree created from all sample sequences and calculates dissimilarity as a ratio of shared to unshared branches on the phylogenetic tree. The weighted UniFrac is adjusted for relative abundance of each OTU. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for a permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al.,

2017) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

3. Results & Discussion

3.1 Microbial Analyses

In addition to 16S rRNA genetic sequencing, four traditional plating methods were used to quantify microbial growth throughout storage time. There were no treatment by storage time interactions for APC, AnPC, LAB, or PPC ($P > 0.331$). There was a main effect of treatment on APC and PPC ($P < 0.013$). Averaged over all storage times, the control treatment had greater APC and PPC than all other treatments. There was a main effect of storage time on APC, AnPC, LAB, and PPC ($P < 0.002$). Aerobic counts remained steady around 2.2 to 2.6 log CFU/g from week 0 to week 8, decreased slightly to 1.6 log CFU/g on week 10, and increased on week 12 and 14, to a maximum of 4.7 on week 14. Anaerobic counts varied between 1.0 and 2.4 log CFU/g from week 0 until week 10, and increased on week 12 and week 14 to a maximum of 4.1 log CFU/g. Lactic acid bacteria counts were 2.3 log CFU/g at week 0, decreased to between 1.5 and 1.7 log CFU/g until week 8, decreased to 0.3 log CFU/g at week 10, and increased to 2.7 log CFU/g at week 14. Finally, PPC increased from 0.0 log CFU/g at week 0 to 1.0 log CFU/g at week 6, decreased to 0.3 log CFU/g at week 8, and steadily increased to 2.0 log CFU/g at week 14. Detailed growth for each treatment throughout storage time is shown in Figure 1.

Alpha diversity was analyzed in order to determine an appropriate even depth for rarefaction. Samples were rarefied to an even depth of 5,000 reads. Good's Coverage

index indicated at this depth, $\geq$ 95.5% of the bacterial community was identified. Furthermore, as sampling depth increased, all treatments began to plateau well before 5,000 reads, indicating adequate sequencing depth (Figure 2). Observed OTUs and Chao1 diversity estimates were used to determined differences in species richness, or the number of different species in a sample. Chao1 is used as nonparametric estimator which adjusts for singleton and doubleton OTUs in order to estimate species richness without skew from single or double counts. There was a main effect of storage time for both observed OTUs ($P < 0.001$) and Chao1 ($P = 0.001$) where species richness generally decreased throughout storage time according to both measures, with week 0 having the greatest observed OTUs and Chao1 estimates, and week 14 having the lowest. To determine differences in the overall bacterial community structure, the weighted and unweighted UniFrac distance matrices were calculated. There were main effects of both treatment and storage time for both the weighted and unweighted UniFrac ($P < 0.001$). As shown in the PCoA plots in Figure 3, there was no clear independent clustering between treatments, however C samples were somewhat grouped in both PCoA plots, and CS, LD, and P treatments were more spread throughout the plot compared to BV samples (Figure 3). Furthermore, samples from increased storage times were somewhat more spread away from the main grouping of samples than were the early storage times, meaning a greater difference in the bacterial community structure at later storage times compared to early storage times. Figure 4 shows the differences in relative abundance of OTUs grouped by phylum and genus based on treatment and storage times. From a Phyla perspective, all treatments were fairly similar throughout storage, characterized by a slight increase in *Proteobacteria* over time, which displaced *Firmicutes, Bacteroidetes,*

and *Actinobacteria*. At genus classification, the most obvious difference is the increased proportion of *Pseudomonas* in treatment C, especially later in storage time. This drastic increase was not observed in any of the antimicrobial treatments, which were characterized by a much more diverse community structure. The LD treatment had a higher prevalence of *Photobacterium* throughout storage than the other treatements, while all antimicrobial treatments saw an increase in *Stenotrophomonas* later in storage.

It would be expected that the most drastic difference in microbial growth and community structure would be between the control and all other treatments, as the antimicrobial mode of action between organic acids is similar. In foods, the undissociated form of an organic acid can enter the bacterial cell, and once in the neutral pH cytoplasm, will dissociate, acidifying the cytoplasm (Doyle et al., 2001). Thus, the main difference between the antimicrobial action of different organic acids is related to their pKa, or the pH at which the acid is 50% dissociated and 50% undissociated. In the case of the clean-label antimicrobials in this study, buffered vinegar is mostly comprised of acetic acid and cultured sugar is a mixture of lactic and acetic acids, with some residual sugars and other by-products of fermentation. The current study showed an increase in aerobic and psychrotrophic counts in the control treatment, and this correlated with a drastic increase in *Pseudomonas* in the control based on sequencing results. The aerobic psychrotroph *Pseudomonas* spp. commonly dominate refrigerated spoilage, and can be detrimental to product quality (Dainty & Mackey, 1992; Molin & Ternström, 1982). Saarela, (2005) demonstrated the methods by which organic acids suppress the growth of gram negatives such as *Pseudomonas*. It would make sense, then, that in the

presence of organic acids that *Pseudomonas* growth would be decreased and likely replaced by the more acid tolerant LAB.

3.2 Physicochemical Analyses

Cooking yield, water activity, and proximate composition were measured on day 0 only, while measures for pH, *L**, *a**, and *b** were collected throughout storage time. Least squared means of main effects of treatment are displayed in Table 1. Cooking yield was affected by treatment ($P = 0.007$), where BV had the highest cook yield, LD and P had the lowest cook yields, and CS and C were intermediate. Water activity also varied by treatment ($P = 0.023$). Water activity ranged from 0.9750 in CS to 0.9811 in the control, however any statistical differences within this range are likely of little practical importance. Despite differences in cook yield, there were no treatment effects on percent moisture, fat, or protein ($P \geq 0.190$). There was a treatment effect on percent ash, where LD and CS had the greatest ash, C and P had the least, and BV was intermediate. There were main effects of both treatment and storage time on pH ($P < 0.001$). Treatments C, LD, and BV had a greater pH than did P and CS. Furthermore, pH across all treatments was 6.00 at day 0, decreased to between 5.94 and 5.96 from day 14 to day 56, increased to 6.05 and 6.04 at days 70 and 84, respectively, and was 5.92 at day 98. While these values are statistically significant, the difference in pH values throughout storage time in this range are likely of little practical importance to the current study.

Cooking yield and pH are typically closely related, where salt and acids act antagonistically toward water holding capacity. Salt lowers the isoelectric point of meat, and an increased pH causes increased water holding capacity due to an increase in

negative charges. Medyński, Pospiech, & Kniat (2000) demonstrated the antagonistic effect of salt and lactic acid where lactic acid decreased water holding in salted products, but increased water holding capacity in products with no added salt. Results from this study are consistent with this theory, with the exception of CS, which had the lowest pH but the second greatest cooking yield. This increase in water holding despite a low pH may be due to the residual sugars and fermentation by-products in the cultured sugar that would not be present in the buffered vinegar and traditional antimicrobials, as these are filtered or distilled to remove other components. It is well established that soluble sugars bind water in food products (Gharsallaoui, Rogé, Génotelle, & Mathlouthi, 2008) and therefore the residual sugars in cultured sugar likely aid in water retention.

With the differences in cooking yield, it could be expected to see differences in moisture and fat as well. Typically, moisture and fat are inversely related in meat products, and a decrease in cooking yield would cause a decrease in moisture, however, that was not the case in this study, as there were no treatment differences in moisture or fat. Protein was not affected by treatment, as all products were made from a similar starting meat block. The treatment effects on ash can be attributed to the antimicrobial ingredients used. Treatments C and P had lower ash content compared to LD, CS, and to a certain degree BV, which is likely caused by the additional sodium and/or potassium used to make the acid salts potassium lactate and sodium diacetate, as well as the buffering agents added to the cultured sugar and buffered vinegar.

There was no storage time by treatment interaction for pH, L^*, a^*, nor b^* ($P > 0.108$). There was a storage time main effect on L^* ($P < 0.001$) where lightness generally decreased over time. The effect of treatment on L^* approached significance, where all

organic acid treatments were slightly darker than the control, however, these differences were not found to be statistically significant ($P = 0.051$). There was a storage time effect on a^* ($P < 0.001$) where redness steadily decreased throughout storage time. There were main effects of treatment ($P = 0.018$) and storage time ($P < 0.001$) on b^*. Yellowness was the greatest in the control treatment, lowest in LD, and intermediate in P, BV, and CS. Furthermore, b^* decreased from day 0 to day 14, and remained fairly steady for the remainder of storage.

Texture profile analysis measures of hardness, chewiness, springiness, and cohesiveness were collected and calculated to determine any textural differences caused by antimicrobial treatment. There were no statistical differences between treatments for hardness ($P = 0.600$), chewiness ($P = 0.268$), springiness ($P = 0.970$), and cohesiveness ($P = 0.485$). Texture profile analysis results are listed in table 2, and although the mean values for hardness and chewiness are greater in treatment CS, there were no statistically significant differences between treatments for either the overall F test nor mean separation using Tukey's HSD adjustment.

4. Conclusion

The spoilage patterns of sliced bologna treated with various clean-label and traditional antimicrobials were similar, regardless of the organic acid or source. The antimicrobial mode of action of organic acids is similar, however, the pKa of different acids varies, and could, in theory, determine their effectiveness at reducing the growth of certain bacteria. Furthermore, the clean-label antimicrobials evaluated, buffered vinegar and cultured sugar, could alter spoilage due to the minor chemicals, by-products, and

residual sugars that remain from their production. The main difference was in the control versus all other treatments, where microbial growth was increased and dominated by *Pseudomonas* in the control. There were minimal differences between the four antimicrobial treatments, indicating that the lactate/diacetate, propionic acid, buffered vinegar, and cultured sugar each have similar effectiveness in reducing microbial growth and suppressing the growth of spoilage *Pseudomonas.* Also of interest was the increase in cooking yield observed in the two clean-label treatments, BV and CS compared to the traditional ingredients LD and P. One of the major fallbacks of using organic acids as antimicrobials is the perceived decrease in cooking yield and water holding capacity, however, the current results indicated the two clean-label antimicrobials increased cooking yield. These results further develop the knowledge base surrounding the microbiota of meat products, especially with regard to how the microbial community is affected by organic acids.

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Tables

Table 1. Least squared means for main effect of treatment on cook yield, water activity, pH and objective color (CIE *L**, *a**, *b**).

	Treatment[1]						
	C	LD	P	BV	CS	SEM[2]	*P* value
Cook Yield (%)	92.53[ab]	91.59[b]	90.55[b]	94.26[a]	92.55[ab]	0.54	0.007
Water Activity	0.9811[b]	0.9765[ab]	0.9789[ab]	0.9789[ab]	0.9750[a]	0.0011	0.023
pH	6.08[b]	6.04[b]	5.85[a]	6.12[b]	5.79[a]	0.04	< 0.001
L*	64.20	61.61	61.22	62.70	62.28	0.62	0.051
a*	16.46	16.82	16.38	16.64	16.35	0.23	0.594
b*	8.24[b]	7.70[a]	7.89[ab]	8.00[ab]	7.91[ab]	0.09	0.018
Moisture (%)	65.0	67.8	66.0	66.7	67.9	1.1	0.336
Fat (%)	17.0	12.2	14.3	13.9	12.2	1.45	0.190
Protein (%)	16.3	17.2	17.9	16.9	17.1	0.47	0.318
Ash (%)	2.50[b]	3.43[a]	2.55[b]	2.94[ab]	3.35[a]	0.12	< 0.001

[1] C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

[2] SEM: Standard error of the overall mean.

[a,b] Means in the same row lacking a common superscript are significantly different ($P < 0.05$) after Tukey's HSD adjustment.

Table 2. Least squared means for treatment effect on texture profile analysis (TPA).

	Treatment[1]						
	C	LD	P	BV	CS	SEM[2]	P value
Hardness	661	754	714	724	1010	162	0.600
Chewiness	51.75	59.64	49.89	63.41	85.19	11.44	0.268
Springiness	0.4069	0.3979	0.3762	0.4209	0.4232	0.0542	0.970
Cohesiveness	0.1870	0.2037	0.1899	0.2076	0.2204	0.0141	0.485

[1] C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

[2] SEM: Standard error of the overall mean.

Figures

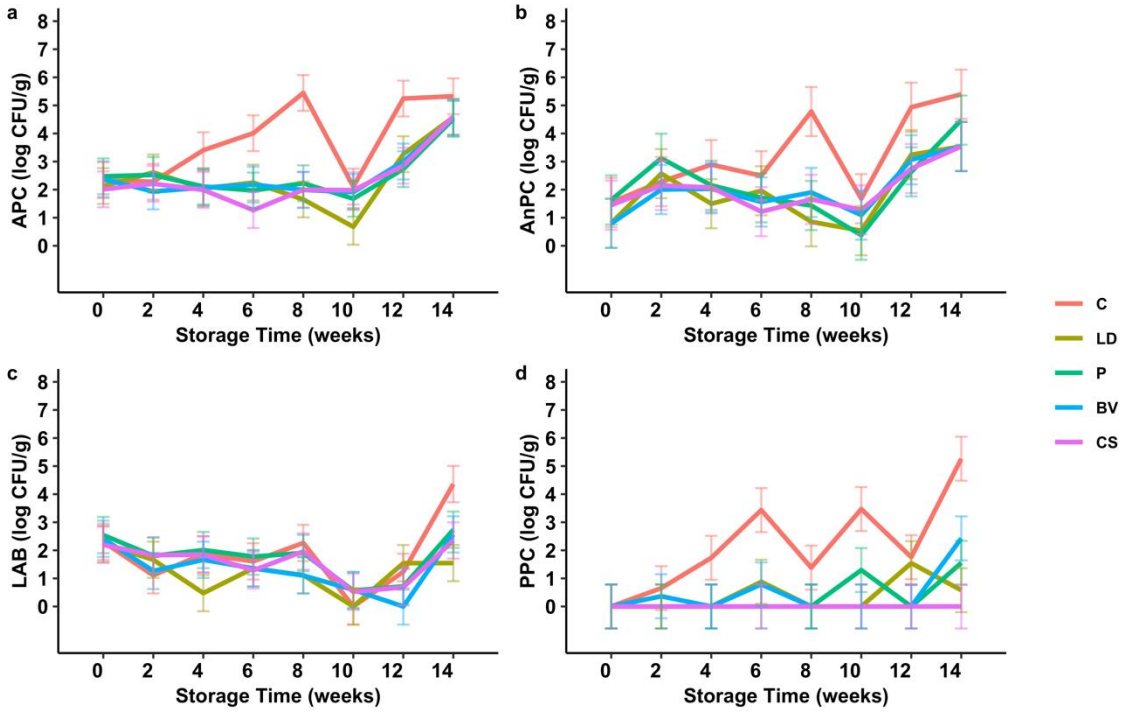

Figure 1. Mean plate counts throughout storage time. Plating methods: a) aerobic plate count (APC); b) anaerobic plate count (AnPC); c) lactic acid bacteria count (LAB); d) psychrotrophic plate count (PPC). Treatments: C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

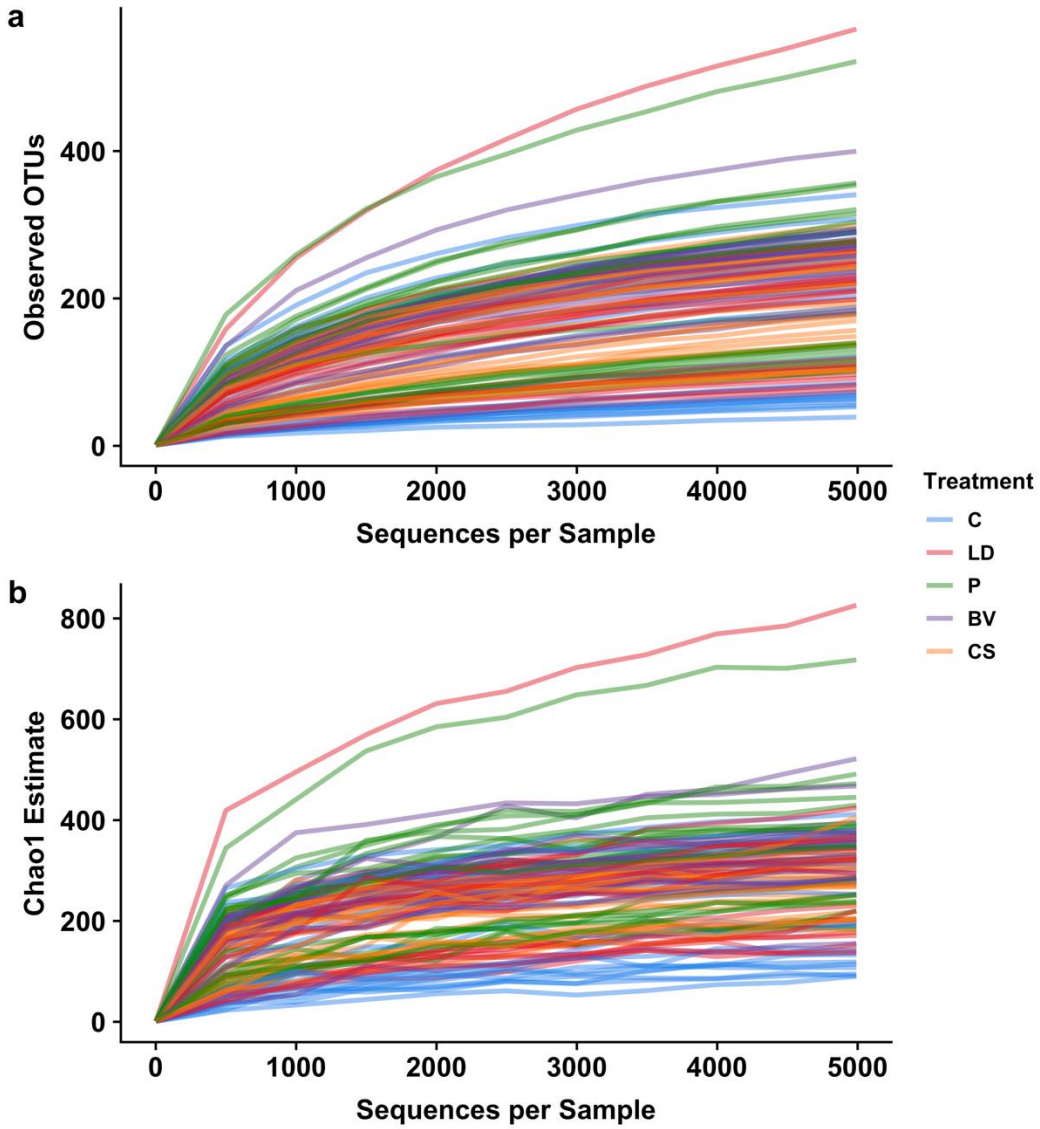

Figure 2. Alpha diversity estimates of observed OTUs (a) and Chao1 (b) across treatments. Treatments: C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

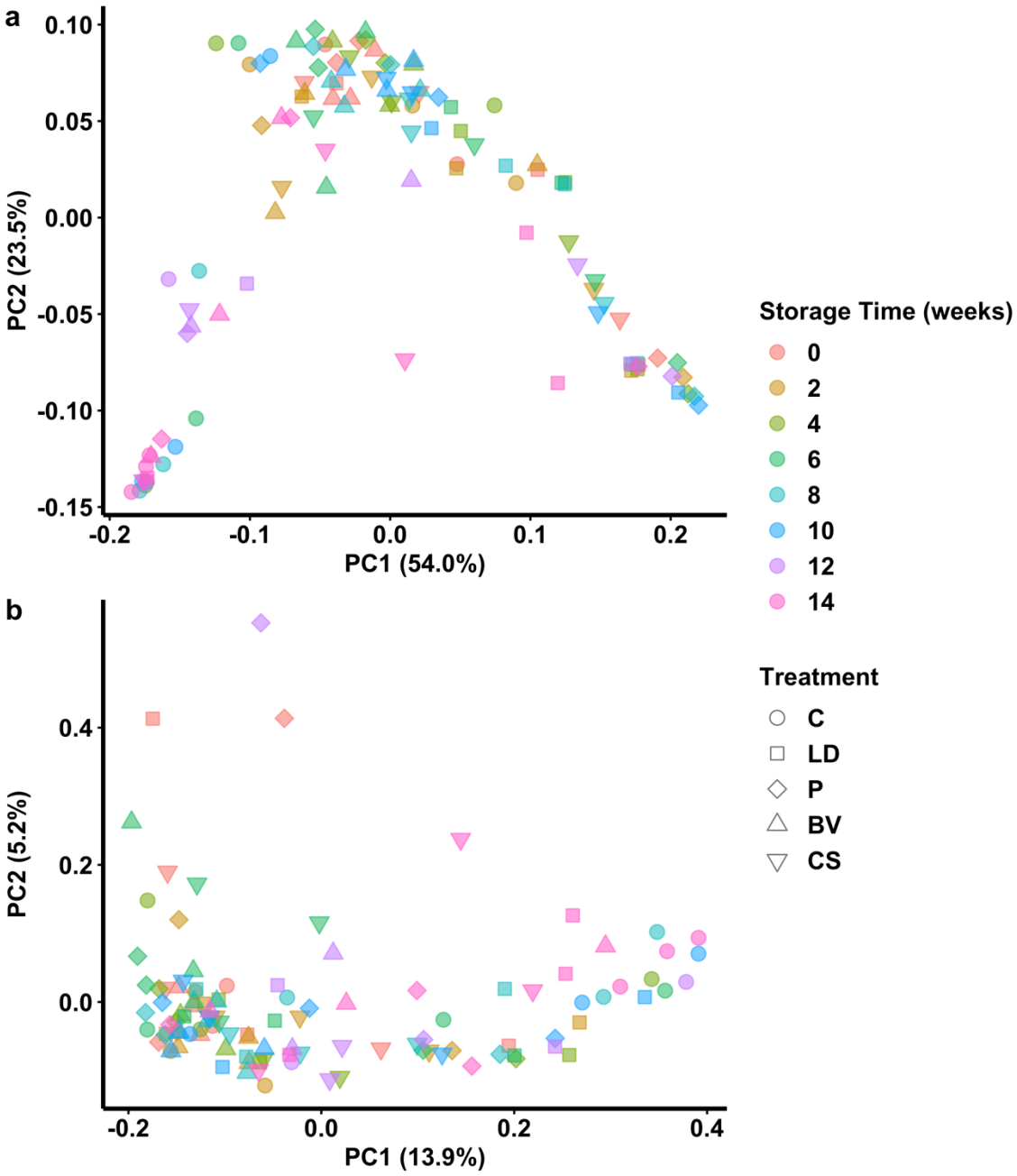

Figure 3. Beta diversity estimates of the weighted UniFrac (a) and unweighted UniFrac (b) distance matrices. Treatments: C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

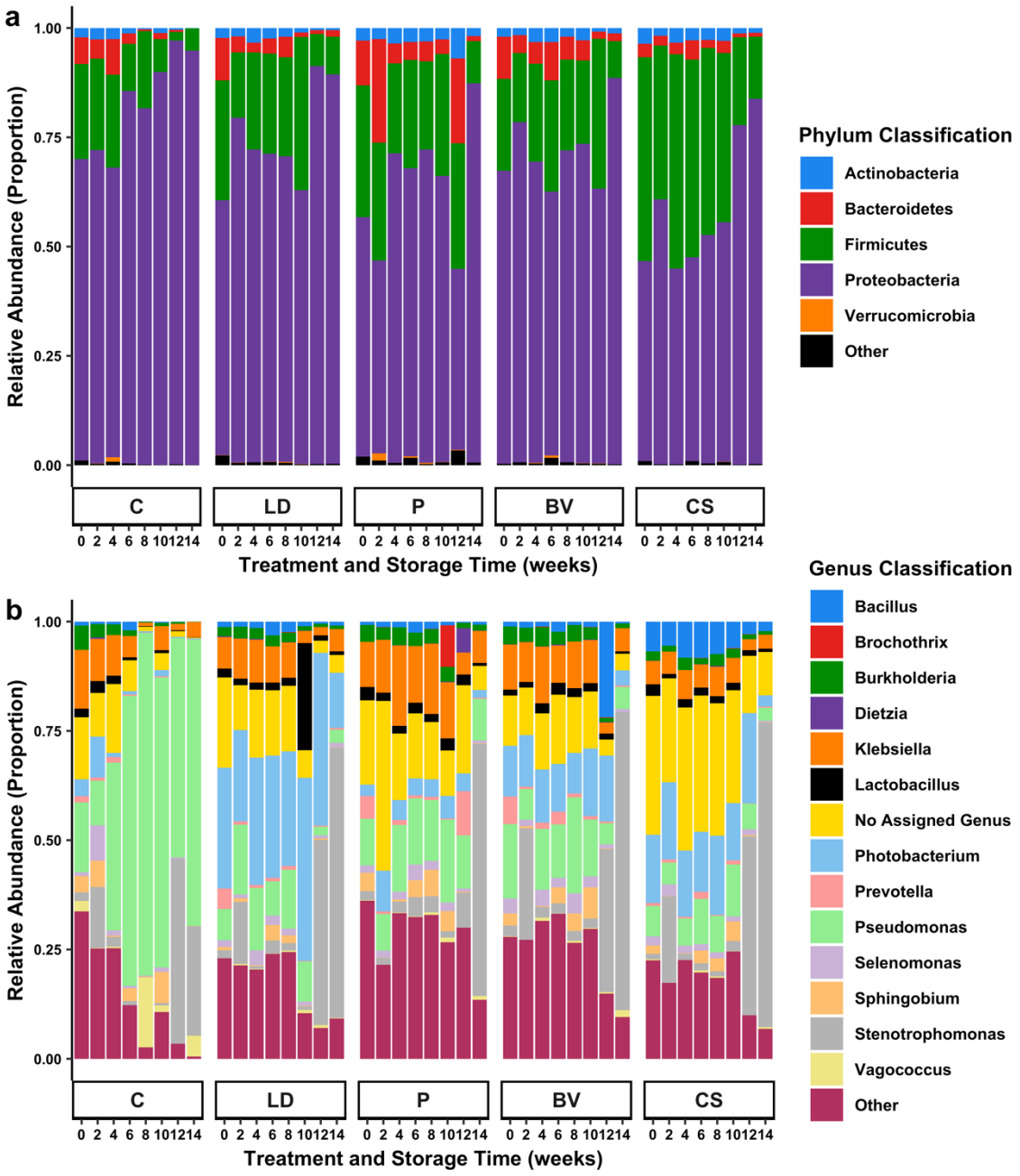

Figure 4. Relative abundances of most abundant OTUs grouped by phylum (a) and genus (b) classification throughout storage time. Treatments: C: control, no antimicrobial; LD: potassium lactate/ sodium diacetate; P: propionic acid; BV: buffered vinegar; CS: cultured sugar.

STUDY 4. EFFECTS OF NATURAL HARDWOOD SMOKE AND LIQUID SMOKE ON SPOILAGE COMMUNITIES ASSOCIATED WITH BEEF FRANKFURTERS

Abstract

The effects of smoking method on the spoilage patterns of beef frankfurters were evaluated. Beef franks were cooked with natural wood smoke applied (NS), dipped in liquid smoke prior to cooking (LS), or unsmoked (US). Throughout 14 weeks of refrigerated storage, traditional plating methods and 16S rRNA gene sequencing were used to evaluate spoilage patterns of beef franks. There were no differences between treatments for bacterial growth ($P > 0.05$), and slight difference in their bacterial community structure according to the unweighted UniFrac ($P = 0.034$), where the unsmoked franks had a different overall structure. Spoilage was characterized by a relatively large proportion of unnamed genera, followed by a sharp increase in *Stenotrophomonas* between weeks 10 and 14, as well as a slight increase in *Vagococcus* in LS and NS samples. Results indicate that smoking method has no effect on overall bacterial growth of cooked franks, but may alter the bacterial community composition.

1. Introduction

Smoking meat is one of the oldest known preservation methods, however, it has not often been a subject of microbiological studies in meat products. While smoking is commonly paired with cooking which inactivates pathogenic bacteria, smoke itself has some antimicrobial function as well (Lingbeck et al., 2014). In modern meat products, smoking is generally done to add flavor and color characteristics to a product rather than purely for preservation. Smoked meats have a characteristic flavor, aroma, and color that cannot easily be replicated by other methods. Furthermore, smoking is an inexpensive process that can differentiate a given product from others on the market, and some common products, such as frankfurters, are traditionally known to be smoked or have smoke flavor added. Some examples of commonly smoked meats that are commercially produced include frankfurters, bologna, summer sausage, jerky, ham, bacon, pastrami, and smoked sausage. The sensory characteristics associated with smoked meats are well understood and accepted as common knowledge, however, an in depth understanding on the role smoking plays in shelf-life and microbial community dynamics has not been reached.

Smoke has an acidic pH, but it is likely a combination of acids, formaldehydes, and phenols that are produced during smoking that contribute to its preservative effects on meat products (Hui, Nip, Rogers, & Young, 2001). In reality, smoke is made of a mixture of thousands of compounds, many of which are due to the pyrolysis of cellulose, hemicellulose, and lignin, and include, but are not limited to, hydrocarbons, alcohols, aldehydes, ketones, acids, and phenols (Tóth & Potthast, 1984). Most of these antibacterial compounds are water-soluble and are readily absorbed on the surface of

meats. However, both the flavor and preservative effects of smoke are basically limited to surface treatment only.

Smoke can be applied to meat products either by the burning of wood in the smoke chamber or in a standalone smoke generator which forces smoke into the chamber. The development of liquid smoke has added another method in which meats can have smoke flavor added, either by coating the surface of the product or by including liquid smoke in the brine or seasoning blend. Liquid smoke is produced by condensation and fractional distillation after the burning of sawdust or wood chips (Aberle, Forrest, Gerrard, & Mills, 2012) and is readily available in the commercial market in various flavors and application methods. It is widely thought that proper application of liquid smoke delivers a more uniform coating of the product surface, as well as a greater concentration of antimicrobial compounds interacting with the meat surface. Regardless of perceived differences, natural wood smoke and liquid smoke are both commonly used in the meat industry.

The antimicrobial effects of smoking have been studied, mostly in evaluating the ability of smoke to eliminate or reduce pathogens in food products. A greater than 99.9% reduction of *Listeria monocytogenes* was observed in vacuum packaged beef franks dipped in liquid smoke (Messina, Ahmad, Marchello, Gerba, & Paquette, 1988), as well as inhibition of *L. monocytogenes* growth in smoked salmon (Niedziela, MacRae, Ogden, & Nesvadba, 1998). Furthermore, neither salt nor smoke phenols prevented *L. monocytogenes* growth, however, formaldehyde from wood smoke suppressed growth for two weeks (Niedziela et al., 1998). The effects of general spoilage patterns of smoked meats, however, are not well studied.

To our knowledge, this is the first study utilizing high throughput genetic sequencing to evaluate the spoilage microbiota of meat products produced with different smoking methods. The aim of this study is to identify key differences in bacterial growth and microbiological communities associated with beef frankfurters based on surface treatment of natural hardwood smoke and liquid smoke compared to no smoke treatment. The genomic methods used in the current study allow for a broad, but in-depth evaluation of the spoilage microbiota associated with cooked frankfurters and how various smoke application methods effect shelf-life and microbial activity. Results from this study are of great practical value to the scientific community and the meat industry in order to better understand spoilage characteristics and microbial community dynamics of processed meats, as well as understanding the antimicrobial effects of smoke application.

2. Methods and Materials

2.1 Treatments & Experimental Design

Beef frankfurters were produced using one of three smoking methods: liquid smoke (LS), natural smoke (NS), and unsmoked (US). Each treatment was produced in three replications, on three separate days of processing. Each treatment was evaluated every 2 weeks for a total of 14 weeks, with day 0 being the day of peeling and packaging. Details on production and storage parameters are outlined below.

2.2 Frankfurter Production

Boneless beef clods (IMPS – 114; USDA, 2014) were procured from a local abattoir and frozen at -20 °C until use. On three separate processing days, clods were

tempered at 4 °C, and thawed clods were hand cut into pieces. One 34 kg batch mixed with 2.0% salt 0.5% dextrose, 0.15% garlic powder, 0.30% white pepper, 156 ppm sodium nitrite, 550 ppm sodium erythorbate, 10% water, and 10% ice, on a meat block basis. Meat and ingredients were chopped in a bowl chopper (Seydelmann Model K 64, Stuttgart, Germany) and batter was stuffed using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA) into 24.5 mm cellulose casings (27 Caliber USA, Viscofan USA, Montgomery, AL) into approximately 70 g links. Stuffed links were split into three approximately equal batches to be cooked independently, and each treatment was cooked to an internal temperature of 71 °C using the same smokehouse program in an Alkar smokehouse (Alkar-RapidPak Inc., Lodi, WI), followed by a 30 min cold shower, and chilled overnight at 0 °C. Unsmoked (US) franks were placed directly in the smokehouse and cooked using no smoke application; LS franks were dipped in a 20% liquid smoke mixture (CharSol Select 24P, Red Arrow, Manitowoc, WI) for one minute prior to entering the smokehouse; NS franks were smoked using natural wood smoke from an Alkar smoke generator (Alkar-RapidPak Inc., Lodi, WI) set to a temperature of 246 °C with Frantz Hickory Sawdust (Frantz Company Inc., Butler, WI) for approximately 45 minutes during the cook cycle. The same cooking cycle was used for each treatment, with the exception of the addition of natural smoke to the NS franks during the appropriate steps. The following day, franks were peeled and four franks were placed into a 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), and vacuum sealed (Multivac Model C500; Multivac Inc., Kansas City, MO). All samples were stored covered at 0 °C (+/- 3 °C) for the entirety of storage time, and a new sample package was used for each sampling period.

2.3 Microbial Analyses

Two links from each sample package (approximately 110-130 g) was aseptically transferred to a 400 ml BagFilter (Interscience USA, Woburn, MA), weighed, mixed with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and placed in a bag blender (bioMerieux Inc., Durham, NC) for 3 minutes to homogenize the sample. Two, 2 ml samples of homogenate were collected for microbial community analysis and stored at -20 °C until used for DNA extraction. Additionally, aerobic plate counts (APC), anaerobic plate counts (AnPC), lactic acid bacteria plate counts (LAB) and psychrotrophic aerobic plate counts were performed using the homogenized samples. An Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain) was used to plate 50µl of homogenate, in duplicate, onto the respective agar. Brain Heart Infusion agar (BHI) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) were used for APC, AnPC, and PPC, and Difco Lactobacilli MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for LAB. For APC and LAB, plates were incubated at 37 °C for 48 h and enumerated manually following Eddy Jet directions. The AnPC plates were incubated at 37 °C for 48 h in an anaerobic box containing BD GasPak EZ sachets to create an anaerobic environment (Becton, Dickinson and Company, Franklin Lakes, NJ) and enumerated manually following Eddy Jet Directions. The PPC plates were incubated at 4 °C for 96 h and enumerated manually following Eddy Jet Directions. Bacterial counts were converted to log10 colony forming units (CFU)/gram of sample.

Bacterial community analysis using high throughput sequencing of the 16s rRNA gene was performed on each sample collection using the MiSeq Illumina Sequencing

Platform as outlined by Kozich, Westcott, Baxter, Highlander, & Schloss (2013). Microbial DNA extraction from homogenized meat samples was performed using a modified protocol of the Epicentre QuickExtract DNA extraction kit. Briefly, a 1 ml sample was centrifuged at 10,000xg for 10 minutes at 20 °C, supernatant was removed, and 500 µl of QuickExtract solution (Epicentre, Madison, WI) was added to the pellet. Following addition of lysis solution, samples were vortexed, incubated at 65 °C for 10 minutes, vortexed again, and incubated at 98 °C for 2 minutes. The resulting DNA was used for Polymerase chain reaction (PCR), amplification in a 25 µl reaction that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA), approximately 20 ng of extracted DNA, and 0.5 µM barcoded universal primers as described by Kozich et al. (2013). PCR reaction was performed in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), where samples were subjected to the following PCR cycle: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 58 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min.

Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. Products were normalized using an Invitrogen Sequal Prep Normalization Kit (Thermo Fisher Scientific, Walther, MA) according to the manufacturer's protocol for binding, washing, and elution steps to yield ~25ng DNA per well. Barcoded PCR products were pooled and purified using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD), and further gel purified using the Pippin Prep system (Sage Science, Inc., Beverly, MA). Due to low DNA

concentration, purified DNA was subject to additional PCR using the same process listed above with 5 amplification cycles. Following secondary amplification, DNA was applied to a 1.5% agarose gel, and the target band was manually excised and recovered using the MinElute PCR Purification kit (Qiagen, Inc., Germantown, MD). Final size and concentration of the 16S rRNA libraries was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and library concentration was confirmed using a DeNovix QFX Fluorometer with the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc, Wilmington, DE).

The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the V2 500 cycle kit. Analysis of sequencing data was performed as described previously (Paz, Anderson, Muller, Kononoff, & Fernando, 2016), using the bioinformatics pipeline Quantitative Insights Into Microbiological Ecology (QIIME; Caporaso et al., 2010). Briefly, sequences shorter than 245bp and longer than 275bp were removed and remaining sequences were trimmed to 251bp. Sequences were binned into operational taxonomic units (OTUs) at 97% similarity using the UPARSE pipeline (USEARCH v8.1). Representative sequences from each OTU were assigned taxonomy using the UCLUST consensus taxonomy assigner (QIIME default) method using Greengenes database release 119 as reference sequences. Reads identified as Archaea, Mitochondria, and *Cyanobacteria* were removed from analysis.

2.4 Physicochemical Analyses

The remaining two links of each sample package (110-130 g) were used for physicochemical analyses. Measures of salt, water activity, and proximate composition

were measured on day 0 only, while objective color and pH were measured throughout storage time.

Samples used for water activity and salt concentration were homogenized using a food processor (Black & Decker Handy Chopper, Black & Decker Inc., Baltimore, MD). Water activity was measured using an Aqualab 4TE dew point water activity meter (Decagon Devices, Inc., Pullman, WA) according to the manufacturer's specifications. Salt concentration was measured as described by Sebranek et al. (2001) by adding 90ml of boiling water to 10g of ground sample, stirring, and straining water to measure using Quantab high range chloride titration strips (Hach Company, Loveland, CO).

In duplicate, pH was measured using an Orion 410A+ pH meter (Thermo Electron Corporation, Waltham, MA) on a slurry of 10g of sample in 90ml of double distilled water. Objective color (*L*, a*, b**) of was measured using a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer with an 8mm aperture and a D65 illuminant. The calibration plate and samples were read through Saran polyethylene wrap (S.C. Johnson & Son, Racine, WI) to keep from dirtying the colorimeter lens. Six readings were averaged from both the interior and exterior of two split links.

2.5 Statistical Analyses

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2019). For salt, water activity, and proximate composition (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). For pH, color, and

plate counts, data were analyzed as a 3 by 8 interaction with storage time as a repeated measure with an independent covariance structure using the nlme package (lme function; Pinheiro, Bates, DebRoy, & Sarkar, 2017). Means were separated using the emmeans package in R (lsmeans and cld functions; Lenth, 2019). Figures were made using the ggplot and cowplot packages in R (Wickham, 2016; Wilke, 2017). Significance was declared at $\alpha = 0.05$ throughout the study.

The OTU table was rarefied across samples to a depth of 6,000 reads/sample using QIIME, and samples under this threshold were removed from analysis. All statistical analyses were performed at an even depth. Chao1 estimates and observed OTUs were calculated for the entire community using QIIME *alpha _diversity.py* command. Chao1 is a nonparametric estimator of richness calculated after removing singleton and doubleton OTUs. Good's coverage test was performed to ensure adequate sampling depth was achieved. Interactions and main effects on mean alpha diversity were calculated using the ANOVA function in R (R Core Team, 2017) with storage time as a repeated measure. Pairwise comparisons on significant ($P < 0.05$) interactions and main effects of Chao1 and observed OTUs were performed using the emmeans package in R (function lsmeans and cld; Lenth, 2016). To reduce variation between replications the OTU table was filtered to include only OTUs present in all three replications. This filtered OTU table was used for subsequent analyses. The weighted and unweighted UniFrac distance matrices were calculated on the bacterial community using QIIME *beta_diversity.py* command. Both UniFrac distance matrices compute the dissimilarity between each pair of samples by determining the ratio of shared to unshared branches in the phylogenetic tree created from sample sequences, and the weighted UniFrac adjusts

for relative abundance of species. The unweighted UniFrac is more sensitive to detecting lineage and founder effects, while the weighted UniFrac is more suited for studying transient changes in microbial communities caused by nutrient availability or other growth parameters (Lozupone & Knight, 2005). Bacterial community composition differences were estimated using the weighted and unweighted UniFrac distance matrices as input for a permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R (function adonis; Oksanen et al., 2017) to analyze interactions and main effects. Significance was declared at $P \leq 0.05$ throughout the study.

3. Results & Discussion

3.1 Microbial Analyses

Four traditional plating methods were used to evaluate bacterial growth across smoking treatments throughout storage time (Figure 1). There were no treatment by storage time interactions ($P > 0.268$), nor were there any treatment main effects ($P > 0.531$) for APC, AnPC, LAB, and PPC. There was a main effect of storage time for APC ($P < 0.001$), where, APC remained fairly stable between 1.5 and 2.2 log CFU/g from week 0 until week 10, and APC increased to 2.8 log CFU/g on week 12 and 4.6 log CFU/g on week 14.

Good's coverage index was used to determine adequate sequencing depth. At an even depth of 6,000 reads, Good's coverage indicated $\geq$ 97.2% of the bacterial community was identified. Furthermore, as sampling depth increased, all treatments reached a plateau well before 6,000 reads. Observed OTUs and Chao1 diversity estimates were used to determine species richness of samples (Figure 2). There were no

interactions or main effects for either observed OTUs ($P > 0.094$) or Chao1 ($P > 0.091$), indicating that there was no difference in species richness between samples from different treatments or storage times. To determine differences in the overall bacterial community, the weighted and unweighted UniFrac distance matrices were used. There was a main effect of storage time on the weighted UniFrac ($P = 0.001$) and main effects of both smoke treatment ($P = 0.034$) and storage time ($P = 0.001$) on the unweighted UniFrac (Figure 3). Samples with increased storage time were more peripheral and spread from the main cluster, compared to earlier storage times. Furthermore, the unweighted UniFrac shows US samples spreading from the main cluster differently than LS and NS samples (Figure 3). This indicates there may be differences in the bacterial community structure between US compared to LS and NS. Figure 4 shows the differences in relative abundance of OTUs grouped by phylum and genus classification based on treatment and storage time. Similar to the UniFrac results, there seems to be very little difference in bacterial community structure between treatments. Communities for all three treatments were characterized by a high proportion of *Proteobacteria* with a smaller amount of *Firmicutes* present as well. All three treatments had a highly diverse population characterized by relatively large amounts of *Photobacterium*, *Pseudomonas*, and *Stenotrophomonas*, with a sharp increase in *Stenotrophomonas* between weeks 10 and 14. A slight increase in *Vagococcus* during the later weeks of storage was seen in LS and NS samples.

The effects of smoking on cooked meat spoilage has not been a common topic of research in recent years, however, some researchers have evaluated smoking and its inhibition of *Listeria monocytogenes* in cooked meats. While smoking is typically used

in combination with cooking, smoke itself has some antibacterial properties. The current study demonstrated that there is little difference between liquid smoke and natural smoke in terms of altering the spoilage microbiota, and frankly very little difference between smoked and unsmoked franks. Plating methods revealed no difference between treatments in terms of overall growth, and 16S sequencing revealed very little difference between treatments as well. The unweighted UniFrac indicated a slight shift in the overall bacterial community structure, however, these differences were not recognizable when evaluating relative abundances of prominent genera. Considering the UniFrac uses a multivariate approach to evaluate the entire community, it is likely that there was a combination of differences in lower-abundance OTUs that comprise the differences revealed by PERMANOVA. Furthermore, while there was some clustering of the control in the PCoA plot (Figure 3b), the principal component analysis revealed that the first two principal components (PCs) account for merely 12.4% and 5.8%, respectively, and therefore do not carry much weight in characterizing the variance of the data. While these minute statistical differences were identified, it is likely that they are of little practical value in the current study.

3.2 Physicochemical Analyses

Water activity and proximate composition were measured on day 0 only, while pH and objective color were measured throughout storage time. There was no treatment effect on water activity ($P = 0.324$). There were no treatment effects for percent moisture, fat, protein or ash ($P \geq 0.231$). There was no smoking treatment by storage time interaction for pH, internal color nor external color ($P > 0.367$). The only treatment

main effect was for external L^* ($P = 0.017$) where unsmoked was the lightest, liquid smoke was the darkest, and natural smoke was intermediate, but not statistically different from either treatment (Table 1). There was a main effect of storage time on both internal and external L^* and a^* ($P < 0.012$), as well as external b^* ($P < 0.001$; Table 2). Internal L^* (lightness) was 61.82 at week 0, and slightly decreased for weeks 2 to 12, and decreased again at week 14 to 57.29. Internal a^* (redness) decreased throughout storage time from week 0 until week 12 and slightly increased again at week 14. External lightness increased steadily throughout storage. External redness remained similar from week 0 to week 6, decreased slightly from week 6 to week 12, and decreased more drastically on week 14. External b^* (yellowness) values were similar from week 0 to week 10 and then decreased from week 10 until week 14.

Water activity and proximate composition were not affected by smoke treatment, which would be expected as all products were made from a similar meat block and subject to a similar cooking process. It is not likely that a surface smoking treatment would have an effect on water activity or any measures of proximate composition of the homogenized product.

Smoke has an acidic pH, and liquid smoke is typically quite acidic since it is simply a condensed form of hardwood smoke. The current study revealed little to no difference between treatments for pH measurements, indicating that a simple surface treatment of liquid or wood smoke does not significantly alter the pH of frankfurters. The only physicochemical measure which was affected by smoking was external L^*, where the liquid smoke treatment was darker. This would make sense, as liquid smoke

dipping inherently gives more consistent surface coverage than wood smoking, and would thus create a darker overall surface on the franks.

4. Conclusion

Results from this study indicated little to no difference in the spoilage patterns of beef franks made with liquid smoke, wood smoke, or no smoke. Due to the chemical composition of smoke, it was hypothesized that smoking method would have spoilage implications. In the current study, however, there was little bacterial growth, even in the unsmoked franks, and thus, yielded no differences in overall growth and minimal differences the bacterial community composition through 14 weeks of storage. Further research should evaluate franks for longer storage time, to allow for greater levels of spoilage bacteria growth, or inoculate franks with pathogens of interest or with a cocktail of spoilage organisms, in order to stimulate spoilage growth.

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Tables

Table 1. Least squared means for main effect of treatment on water activity, pH, and objective color (internal and external CIE *L**, *a**, *b**).

	Treatment[1]				
	US	LS	NS	SEM[2]	P value
Water Activity	0.9765	0.9723	0.9744	0.0018	0.324
pH	6.06	6.00	6.02	0.05	0.676
L* Internal	60.01	59.90	59.99	0.91	0.996
a* Internal	17.13	16.75	16.90	0.55	0.891
b* Internal	8.47	8.40	8.27	0.21	0.801
L* External	53.48[b]	50.97[a]	52.01[ab]	0.43	0.017
a* External	21.20	21.47	20.85	0.33	0.460
b* External	11.64	12.57	12.68	0.47	0.293
Moisture (%)	62.1	61.6	62.2	0.24	0.231
Fat (%)	17.2	16.4	16.4	0.90	0.742
Protein (%)	18.7	20.0	19.3	0.50	0.264
Ash (%)	3.01	2.93	2.92	0.09	0.781

[1] US: unsmoked, LS: liquid smoke, NS: natural smoke.
[2] SEM: Standard error of the overall mean.

Table 2. Least squared means for main effect of storage time on pH and objective color (internal and external CIE *L**, *a**, *b**).

	Storage Time (Weeks)									
	0	2	4	6	8	10	12	14	SEM[1]	P value
pH	6.07	5.99	6.00	5.95	6.05	6.07	6.07	5.99	0.05	0.387
L* Internal	61.82^{b}	58.97^{ab}	59.99^{ab}	60.73^{ab}	59.96^{ab}	60.18^{ab}	60.77^{ab}	57.29^{a}	0.90	0.012
a* Internal	18.10^{c}	18.05^{bc}	17.75^{bc}	17.31^{bc}	16.50^{abc}	16.39^{abc}	15.26^{a}	16.06^{ab}	0.52	< 0.001
b* Internal	8.46	8.57	8.34	8.22	8.11	8.22	7.89	9.22	0.34	0.237
L* External	51.04^{b}	51.20^{b}	51.41^{b}	52.20^{ab}	52.09^{ab}	51.70^{ab}	52.83^{ab}	54.75^{a}	0.70	0.012
a* External	22.75^{c}	22.83^{c}	22.45^{c}	22.21^{c}	21.49^{bc}	21.02^{bc}	19.80^{b}	16.85^{a}	0.43	< 0.001
b* External	12.42^{b}	12.99^{b}	12.85^{b}	12.65^{b}	12.25^{b}	12.74^{b}	11.80^{ab}	10.67^{a}	0.39	< 0.001

[1] SEM: Standard error of the overall mean.

[a,b,c] Means in the same row lacking a common superscript are significantly different ($P < 0.05$) after Tukey's HSD adjustment.

Figures

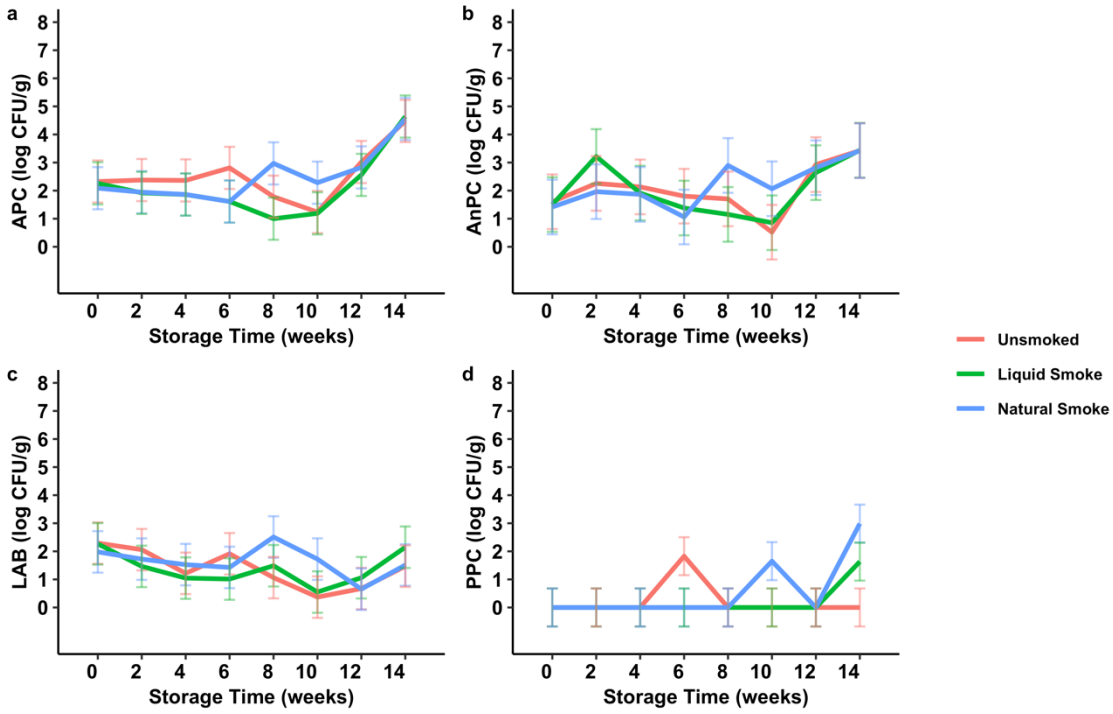


Figure 1. Mean plate counts throughout storage time. Plating methods: a) aerobic plate count (APC); b) anaerobic plate count (AnPC); c) lactic acid bacteria count (LAB); d) psychrotrophic plate count (PPC).

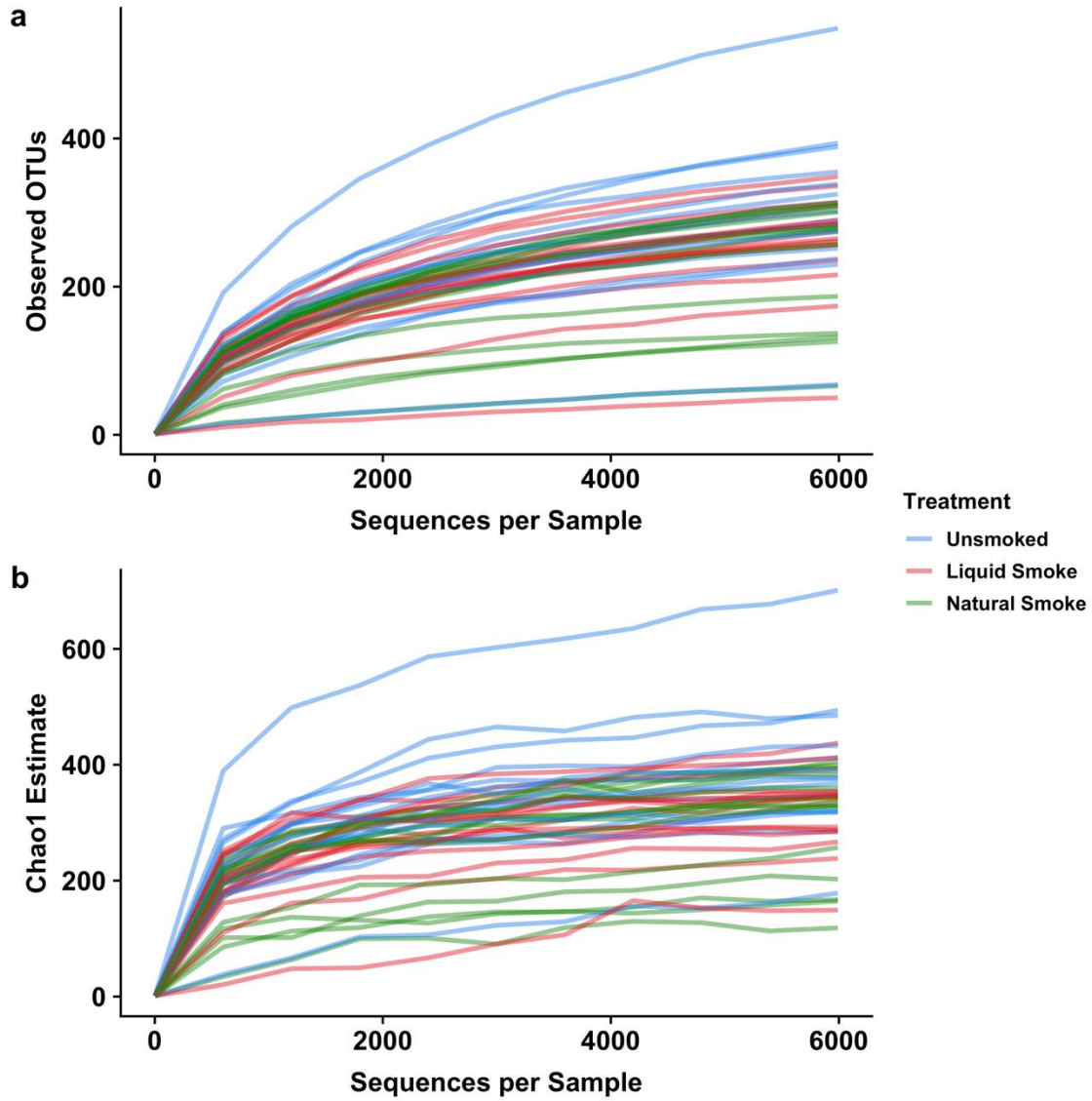


Figure 2. Alpha diversity estimates of observed OTUs (a) and Chao1 (b) across treatments.

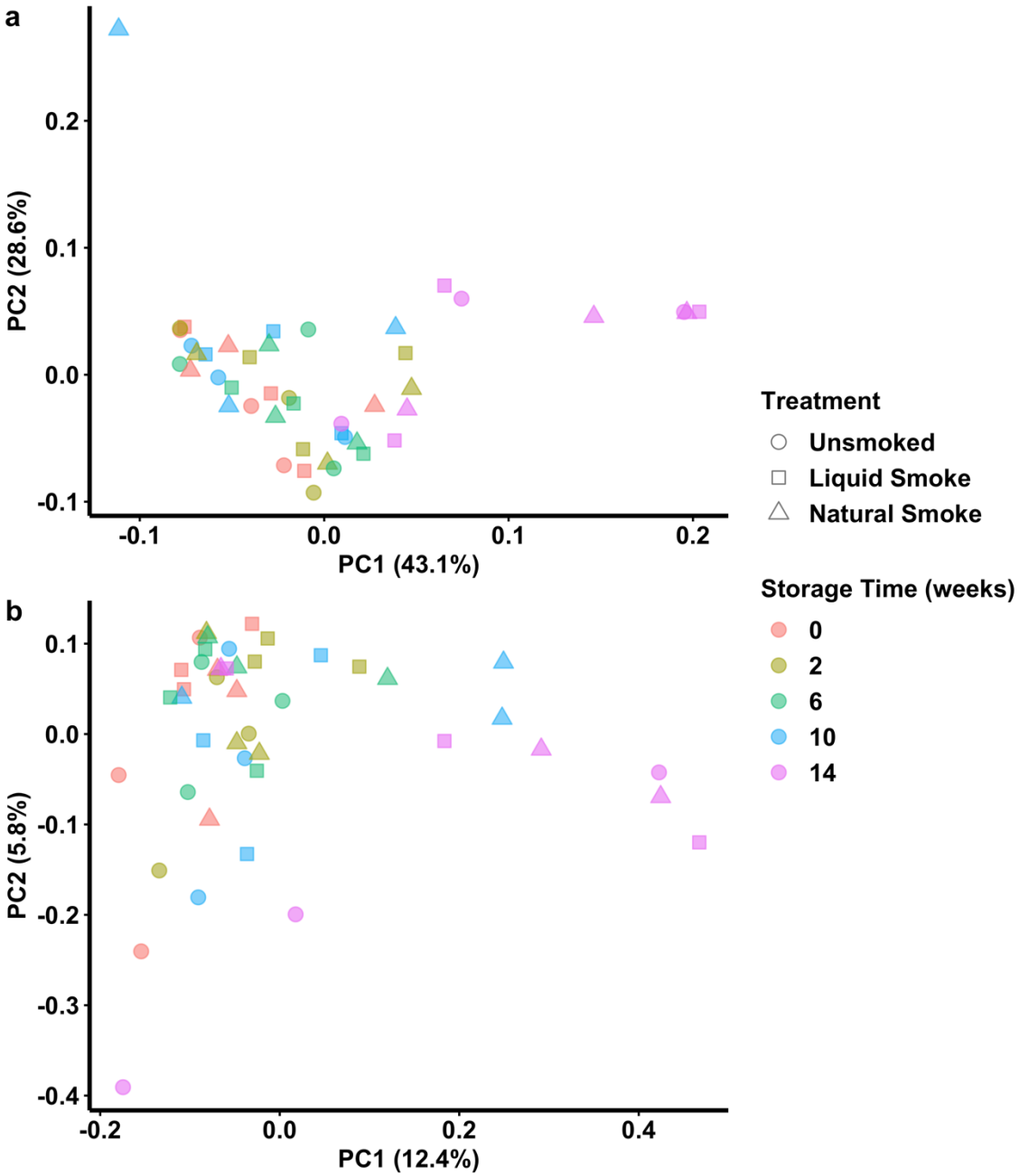


Figure 3. Beta diversity estimates of the weighted UniFrac (a) and unweighted UniFrac (b) distance matrices.

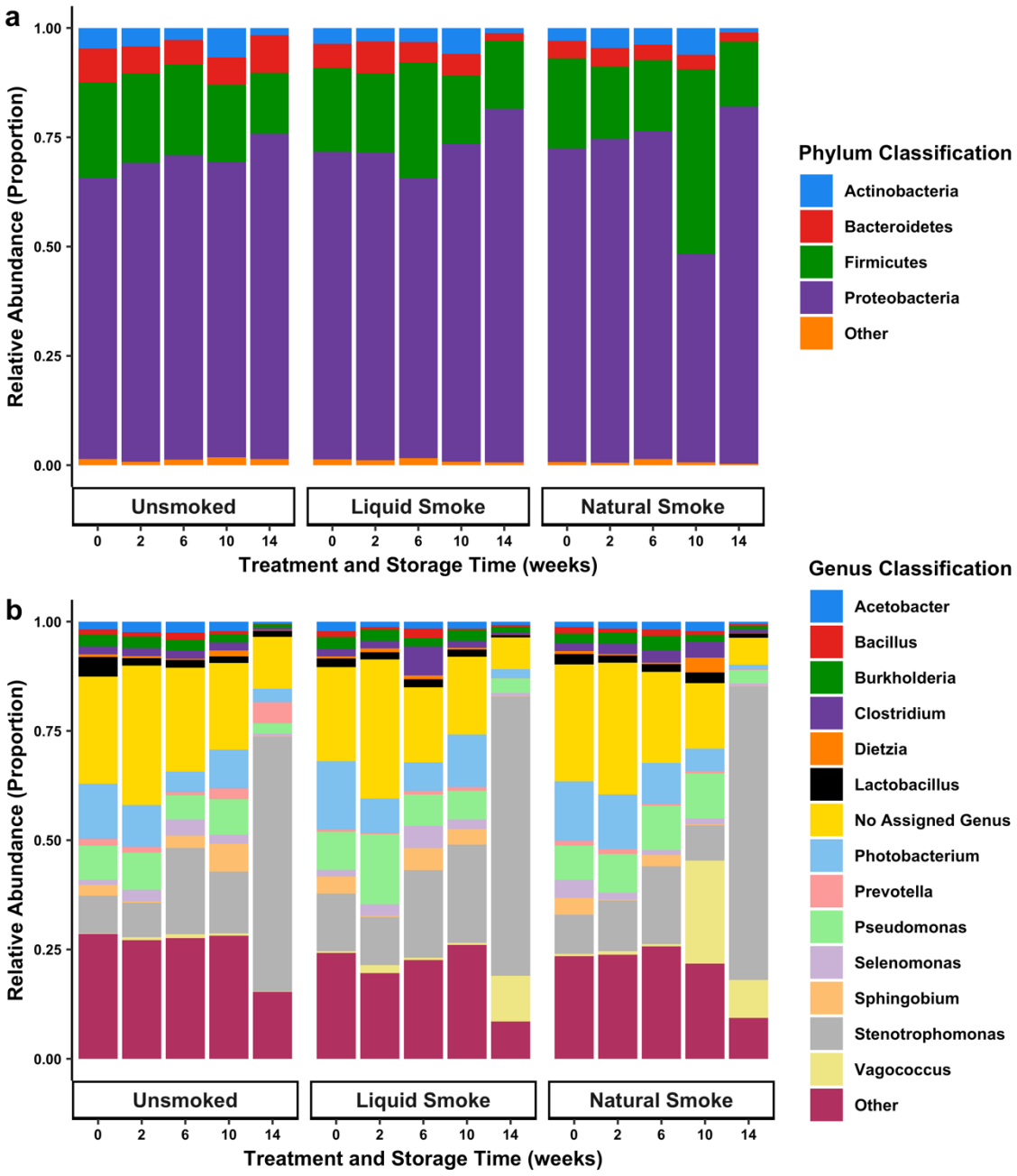


Figure 4. Relative abundances most abundant OTUs grouped by phylum (a) and genus (b) classification throughout storage time.

RECOMMENDATIONS FOR FUTURE RESEARCH

Meat spoilage is a serious concern not only to processors focused on optimizing the distribution of their products, but also to society as a whole in terms of food waste. Roughly one third of food produced in the world for human consumption gets lost or wasted, and nearly 20% of the meat produced worldwide is wasted (FAO, 2011). The development of strategies to improve the shelf-life of meat products is vital to improve food security worldwide, and to ensure high quality protein is accessible and affordable. The research presented in this study is relevant to the meat industry and academia alike, as it provides for a better understanding of the bacterial communities involved with meat spoilage, and also improves our knowledge of solving specific issues related to meat spoilage in processing environments.

The results from these studies are compelling and somewhat challenge the conventional wisdom surrounding the spoilage of processed meats. It has long been thought that as a broad generalization, cooking, salting, curing, and vacuum/modified atmosphere packaging select for lactic acid bacteria (LAB) and minimize the growth of aerobic psychrotrophs like *Pseudomonas*, that are commonly found in fresh meats. Clearly, the evidence from this study and others utilizing high throughput genetic sequencing to characterize spoilage communities points to the contrary, or at least that the former conclusion is not so cut and dry. Bower, Stanley, Fernando, & Sullivan, (2018b) and Bower, Stanley, Fernando, Burson, et al., (2018a) both reported a high prevalence of *Pseudomonas* growth in sliced deli meats, regardless of salt concentration or nitrite levels, and Mertz et al., (2014) reported that *Pseudomonas* was the most commonly recovered organism from the surface of meat slicers in commercial facilities. The current

study also found *Pseudomonas* in sliced deli meats, although some treatments were able to minimize their proportion. *Pseudomonas* was present in each of the retail brands evaluated as well as in the raw ground beef and sliced bologna in Study 2. The addition of antimicrobial ingredients and treatments, however, seemed to reduce *Pseudomonas* proportions in Study 2 and Study 3. There was also a much higher prevalence of *Pseudomonas* in the sliced products, than compared to any of the links or frankfurters, which raises the question of whether *Pseudomonas* proportions are simply due to the increased growth and contact surface area of these products, or if lethality of *Pseudomonas* is somehow altered in the large diameter sliced products. Clearly, there are still research questions to be solved regarding the role of *Pseudomonas* in processed meat spoilage, and the factors that either allow or suppress its growth.

Given the conclusions reached in the current study some topics of interest for further research include:

1. Identify by-products produced by certain bacteria during refrigerated storage which facilitate rapid spoilage as a method for species-specific spoilage.
2. Using genomic tools, identify genes or pathways unique to spoilage organisms that explain their ability to spoil products more quickly.
3. Identify mechanisms to shift the microbiome in order to increase shelf-life, either via genomic analysis or through practical trials.
4. Determine if the prevalence and growth of *Pseudomonas* in cooked meats is a function of post-lethality contamination, or if there are vegetative cells that may be sub-lethally injured during the cooking process on the interior of large diameter products.

References

Bower, C. G., Stanley, R. E., Fernando, S. C., Burson, D., & Sullivan, G. A. (2018a). *Effects of salt and nitrite on the spoilage microbiota of deli-style ham. 49.* Proceedings of the Reciprocal Meat Conference, Kansas City, MO, June 2018.

Bower, C. G., Stanley, R. E., Fernando, S. C., & Sullivan, G. A. (2018b). The effect of salt reduction on the microbial community structure and quality characteristics of sliced roast beef and turkey breast. *LWT - Food Science and Technology*, *90*(December 2017), 583–591.

FAO. (2011). *Global food losses and food waste - Extent, causes and prevention*. Rome, Italy.

Mertz, A. W., Koo, O. K., O'Bryan, C. A., Morawicki, R., Sirsat, S. A., Neal, J. A., Crandall, P. G., & Ricke, S. C. (2014). Microbial ecology of meat slicers as determined by denaturing gradient gel electrophoresis. *Food Control*, *42*, 242–247.

APPENDICES

Appendix A: Methodology Troubleshooting

For studies 2-4, some troubleshooting was required to develop a PCR protocol to prepare DNA from meat samples.

The initial PCR cycle was run as follows: initial denaturation at 98 °C for 2 min, followed by 30 cycles of 98 °C for 30s, 55 °C for 30s, and 68 °C for 45s, and a final extension of 68 °C for 4 min. Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size and amplification. After initial PCR amplification, two bands appeared in close proximity to one another, around 300-400 bp (Figure 1). After analyzing samples on the bioanalyzer, there were also two bands. It was proposed that the second smaller band may be a misbinding amplification of mitochondrial DNA from muscle cells remaining in the sample. Several techniques were tested to exclude this second band from final purified DNA.

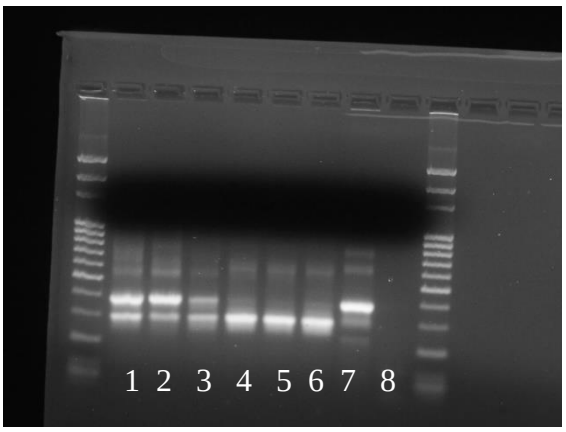


Figure 1. Sampling of PCR product run on 1.5 % agarose gel with and without problematic double bands. From L to R: 1-3, samples with double band; 4-6, samples with single band; 7, positive control, 8, negative control.

Prior to DNA extraction, samples were centrifuged at 3000 x g for 10 min, and supernatant was removed and used for DNA extraction. In order to ensure there was no bacterial DNA removed with the meat pellet, both the supernatant and the meat pellet were subject to DNA extraction and PCR amplification separately, as outlined above. The supernatant produced a clean single band at the appropriate size, but the meat pellet

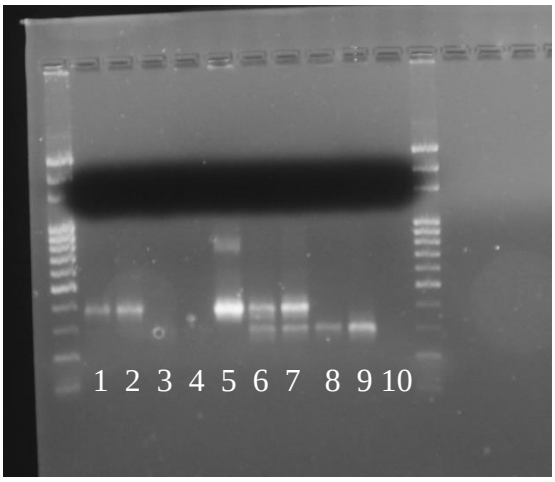


Figure 2. PCR product after altered DNA extraction. From L to R: 1-4, DNA pellet; 5, positive control; 6-9, meat pellet; 10, negative control.

produced a double band, indicating that there was likely some bacterial DNA remaining in the meat pellet which would be excluded from analysis if this method was used.

Annealing temperature was adjusted in the initial PCR to try to limit misbinding and decrease the concentration of the incorrect sized band. Temperatures of 55-58 °C, 60 °C, and 62 °C were tested on reactions of the same set of samples. Temperatures of 58-60 °C were determined to reduce the intensity of the incorrect band without compromising the intensity of the target band, and an annealing temperature of 58 °C was used for the remainder of the study.

After adjusting the annealing temperature, the incorrect band intensity was reduced, however there was still a double band present in some samples. It was attempted to remove the band through gel electrophoresis separation. Initially, samples were purified on the Pippin Prep system, however the DNA concentration was reduced to < 0.5 nM after separation.

An attempt to manually excise the target band was performed, using a 1.5% agarose gel, at 80 volts for approximately 2 hours. Samples were successfully separated, however both the target band and incorrect band were too faint to be able to manually excise. At this point, a second PCR was performed using the pooled and normalized DNA according to the same parameters as the initial PCR but with only 5 amplification

cycles rather than 30. Also, custom primers were used to amplify only the barcoded sequences produced by the initial amplification. Primers used were as follows: I5 adaptor (5'-AATGATACGGCGACCACCGAGATCTACAC-3') and I7 adaptor (5-'CAAGCAGAAGACGGCATACGAGAT -3'). After secondary amplification, samples were separated on a 1.5% agarose gel, manually excised, and recovered using the MinElute PCR Purification kit. Size of this DNA was determined using the Agilent 2100 Bioanalyzer, and library concentration was confirmed using a DeNovix QFX Fluorometer with the Denovix dsDNA High Sensitivity reagent kit. It was determined that this DNA was of the correct size and concentration, and was subsequently used to sequencing.

Appendix B: Formulations from Study 2

T2: Fresh Sausage

Product Name:	Fresh Sausage			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**96.99%**
Beef	25	11339.8	100.00%	96.99%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.775**	**351.5338**	**3.10%**	**3.01%**
Salt	0.5	226.796	2.00%	1.94%
Dextrose	0.125	56.699	0.50%	0.48%
garlic	0.075	34.0194	0.30%	0.29%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0	0.00	PPM
Sodium Erythorbate	0	0	0.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.775**	**11691.33**		

T3: Cooked Links

Product Name:	Cooked Sausage			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**96.99%**
Beef	25	11339.8	100.00%	96.99%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.775**	**351.5338**	**3.10%**	**3.01%**
Salt	0.5	226.796	2.00%	1.94%
Dextrose	0.125	56.699	0.50%	0.48%
garlic	0.075	34.0194	0.30%	0.29%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0	0.00	PPM
Sodium Erythorbate	0	0	0.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.775**	**11691.33**		

T4: Frankfurter

Product Name:	Frankfurter			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**96.71%**
Beef	25	11339.8	100.00%	96.71%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.775**	**351.5338**	**3.10%**	**3.00%**
Salt	0.5	226.796	2.00%	1.93%
Dextrose	0.125	56.699	0.50%	0.48%
garlic	0.075	34.0194	0.30%	0.29%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.29%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.85115**	**11725.87**		

T5: Sliced Bologna

Product Name:	Bologna			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**96.71%**
Beef	25	11339.8	100.00%	96.71%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.775**	**351.5338**	**3.10%**	**3.00%**
Salt	0.5	226.796	2.00%	1.93%
Dextrose	0.125	56.699	0.50%	0.48%
garlic	0.075	34.0194	0.30%	0.29%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.29%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.85115**	**11725.87**		

T6: Bologna with HPP

Product Name:	Bologna HPP			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**96.71%**
Beef	25	11339.8	100.00%	96.71%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.775**	**351.5338**	**3.10%**	**3.00%**
Salt	0.5	226.796	2.00%	1.93%
Dextrose	0.125	56.699	0.50%	0.48%
garlic	0.075	34.0194	0.30%	0.29%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.29%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.85115**	**11725.87**		

T7: Bologna with potassium lactate/ sodium diacetate

Product Name:	Bologna PD4			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**85.54%**
Beef	25	11339.8	100.00%	85.54%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**4.15**	**1882.407**	**16.60%**	**14.20%**
Salt	0.5	226.796	2.00%	1.71%
Dextrose	0.125	56.699	0.50%	0.43%
garlic	0.075	34.0194	0.30%	0.26%
black pepper	0.075	34.0194	0.30%	0.26%
	0	0		0.00%
PD4	0.875	396.893	3.50%	2.99%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	8.55%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.26%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**29.22615**	**13256.75**		

Appendix C: Formulations from Study 3

Control

Product Name:	bologna			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**81.13%**
Beef	25	11339.8	100.00%	81.13%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**5.7375**	**2602.484**	**22.95%**	**18.62%**
Salt	0.5	226.796	2.00%	1.62%
Dextrose	0.125	56.699	0.50%	0.41%
garlic	0.0375	17.0097	0.15%	0.12%
white pepper	0.075	34.0194	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	8.11%
ice	2.5	1133.98	10.00%	8.11%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.25%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**30.81365**	**13976.83**		

Cultured Sugar (CS)

Product Name:	bologna with n70			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**79.51%**
Beef	25	11339.8	100.00%	79.51%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**6.365**	**2887.113**	**25.46%**	**20.24%**
Salt	0.5	226.796	2.00%	1.59%
Dextrose	0.125	56.699	0.50%	0.40%
garlic	0.0375	17.0097	0.15%	0.12%
white pepper	0.075	34.0194	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	7.95%
ice	2.5	1133.98	10.00%	7.95%
	0	0		0.00%
	0	0		0.00%
verdad N70	0.6275	284.629	2.51%	2.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.24%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**31.44115**	**14261.45**		

Potassium Lactate/Sodium Diacetate (LD)

Product Name:	bologna with PD4			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**78.70%**
Beef	25	11339.8	100.00%	78.70%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**6.69**	**3034.53**	**26.76%**	**21.06%**
Salt	0.5	226.796	2.00%	1.57%
Dextrose	0.125	56.699	0.50%	0.39%
garlic	0.0375	17.0097	0.15%	0.12%
white pepper	0.075	34.0194	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	7.87%
ice	2.5	1133.98	10.00%	7.87%
	0	0		0.00%
	0	0		0.00%
optiform PD4	0.9525	432.0464	3.81%	3.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.24%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**31.76615**	**14408.87**		

Buffered Vinegar (BV)

Product Name:	bologna with NV			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**80.53%**
Beef	25	11339.8	100.00%	80.53%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**5.97**	**2707.944**	**23.88%**	**19.23%**
Salt	0.5	226.796	2.00%	1.61%
Dextrose	0.125	56.699	0.50%	0.40%
garlic	0.0375	17.0097	0.15%	0.12%
white pepper	0.075	34.0194	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	8.05%
ice	2.5	1133.98	10.00%	8.05%
	0	0		0.00%
	0	0		0.00%
optiform bactoCEASE NV	0.2325	105.4601	0.93%	0.75%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.25%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**31.04615**	**14082.29**		

Propionic acid (P)

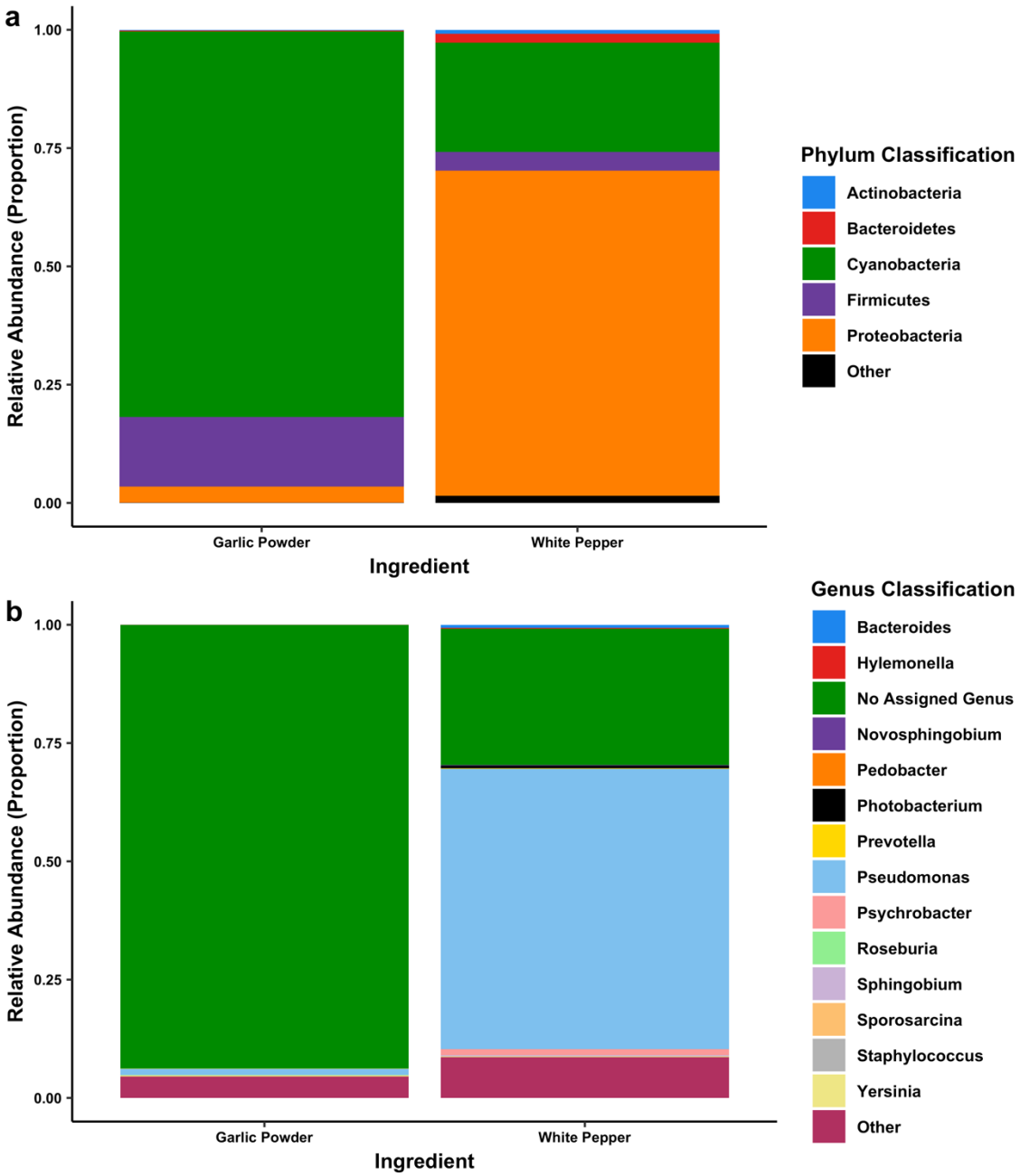
Product Name:	bologna with BC6			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**80.81%**
Beef	25	11339.8	100.00%	80.81%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**5.8625**	**2659.183**	**23.45%**	**18.95%**
Salt	0.5	226.796	2.00%	1.62%
Dextrose	0.125	56.699	0.50%	0.40%
garlic	0.0375	17.0097	0.15%	0.12%
white pepper	0.075	34.0194	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	2.5	1133.98	10.00%	8.08%
ice	2.5	1133.98	10.00%	8.08%
	0	0		0.00%
	0	0		0.00%
bactoCEASE 6	0.125	56.699	0.50%	0.40%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.25%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**30.93865**	**14033.52**		

Appendix D: Formulation from Study 4

Product Name:	Frankfurters			
Meat Block:	75			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**75**	**34019.4**	**100.00%**	**81.13%**
Beef	75	34019.4	100.00%	81.13%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**17.2125**	**7807.452**	**22.95%**	**18.62%**
Salt	1.5	680.388	2.00%	1.62%
Dextrose	0.375	170.097	0.50%	0.41%
garlic	0.1125	51.0291	0.15%	0.12%
white pepper	0.225	102.0582	0.30%	0.24%
	0	0		0.00%
	0	0		0.00%
water	7.5	3401.94	10.00%	8.11%
ice	7.5	3401.94	10.00%	8.11%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.22845	103.6231	0.30%	0.25%
Sodium Nitrite (6.25% curing salt)	0.1872	84.91242	156.00	PPM
Sodium Erythorbate	0.04125	18.71067	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**92.44095**	**41930.48**		

Appendix E: Bacteria in Spice Blend

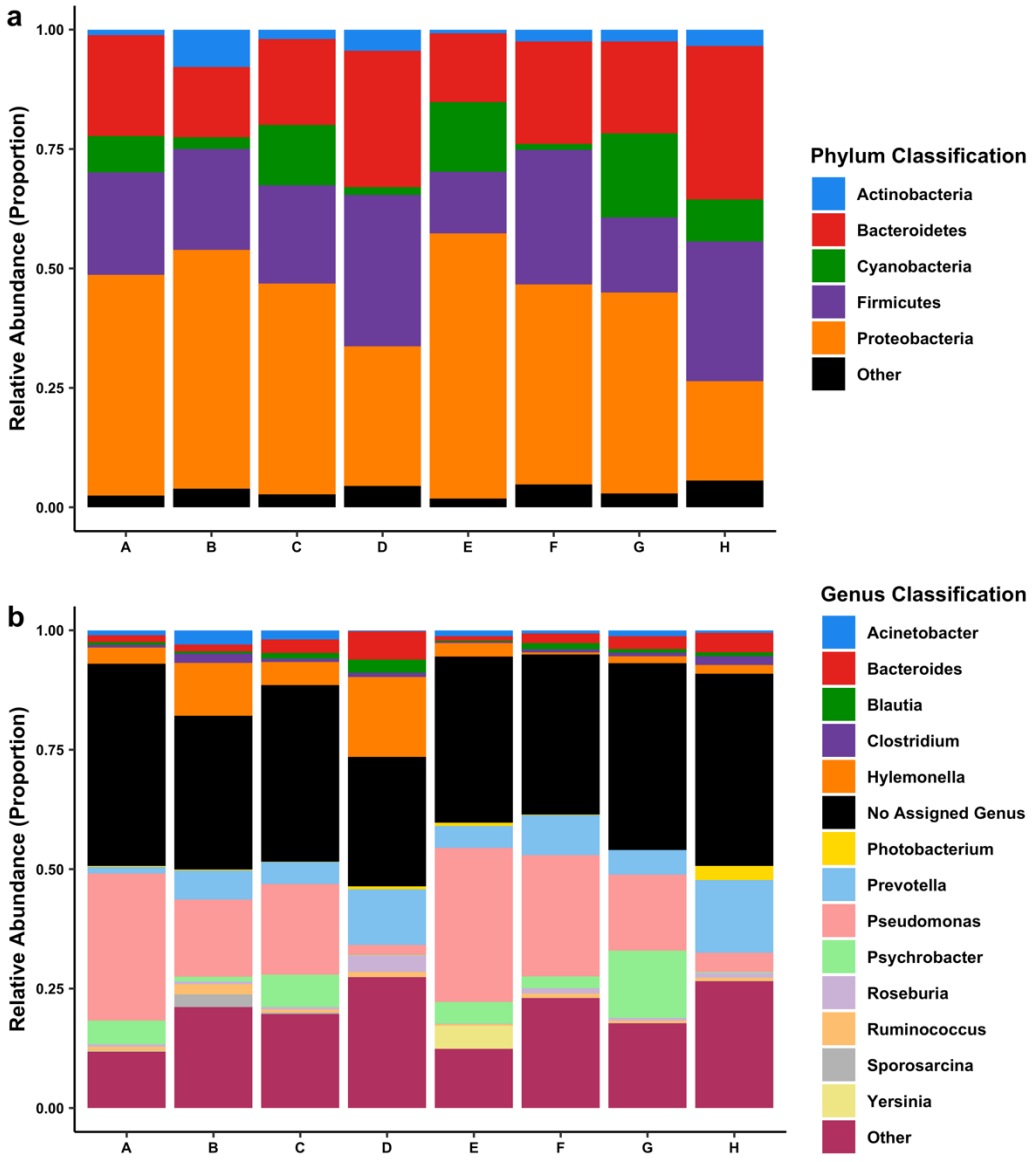
To identify bacteria present in the spice blend, garlic powder and white pepper used in projects 2, 3, and 4 were evaluated for bacterial community analysis. From each ingredient, 1 g was mixed with 10 mL of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) and vortexed for 30 s. Two 2 mL tubes were filled with mixed sample and stored at -80 °C until 16S sequencing, which followed the protocol of all other samples.



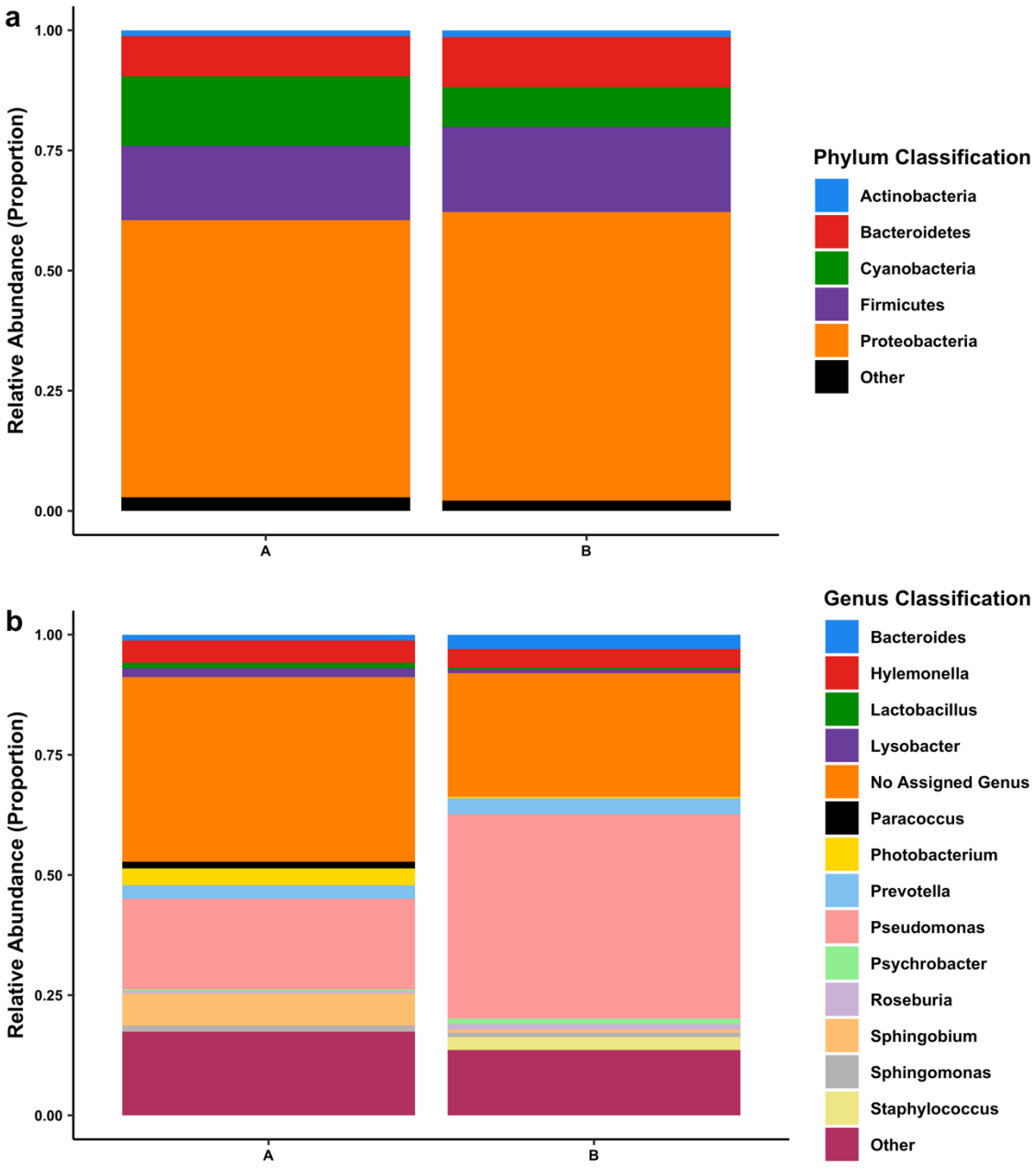
Phylum (a) and genus (b) classification of OTUs found in the garlic powder and white pepper used in the spice blend of study 2, study 3, and study 4.

Appendix F: Bacteria from Contact Surfaces

In studies 2, 3, and 4 contact surfaces in the ready to eat (RTE) packaging room were sampled to evaluate their role in the post-lethality microbiome of processed meats. Two sampling methods were used in an attempt to avoid any selective bias that may occur by using just traditional sponge swabbing methods. Sponge swabbing was performed using 1.5 x 3" cellulose sponges (EnviroSponge; Biotrace international, Bridgend, United Kingdom). Prior to sampling, sponges were moistened with 10 mL sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ). A 10 cm by 10 cm area was sponged on each surface, and an additional 10 mL peptone water was added to the bag and mixed with the sponge by hand. Swabbing was performed using Puritan Calgiswab calcium alginate tipped swabs (Puritan Medical Products, Guilford, ME). Swabs were dipped in 5 mL of sterile peptone water, and a 10 cm by 10 cm area was swabbed on each surface, and placed back in the bag with an additional 5 mL of 20% sodium citrate to create a 10% sodium citrate solution to dissolve the swabs. Swabs were mixed by hand until dissolved. For both sponge and swabs, 2 mL of the mixed sample was removed and stored at -80 °C until analyzed for 16S rRNA sequencing, according to the protocol outlined for all other samples.



Phylum (a) and genus (b) classification of OTUs found on contact surfaces from the RTE processing environment in study 2. A: table swab pre-processing; B: Table sponge pre-processing; C: Slicer swab pre-processing; D: slicer sponge pre-processing; E: Table swab post processing; F: Table sponge post processing; G: Slicer swab post processing; H: Slicer sponge post processing.



Phylum (a) and genus (b) classification of OTUs found on contact surfaces from the RTE processing environment in study 3 and study 4. A: Table and slicer swab post-processing; B: Table and slicer sponge post-processing.