THESIS

GROUND BEEF DEGRADATION: EVOLUTION OF CHEMICAL AND MICROBIAL PROPERTIES AND SENSORY CHARACTERISTICS DURING SHELF-LIFE

Submitted by

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ABSTRACT

GROUND BEEF DEGRADATION: EVOLUTION OF CHEMICAL AND MICROBIAL PROPERTIES AND SENSORY CHARACTERISTICS DURING SHELF-LIFE

Two separate experiments were conducted on the same lots of ground beef. The first experiment aimed to examine changes in culture-dependent microbes and the changes in sensory characteristics and chemical properties in aerobically stored ground beef after 16/17d and 23/24d dark storage periods in anaerobic chub packaging. Three lots of ground beef one lot from the West (n=30) and two lots from the Midwest (n=100)) were treated as three separate replications. The three lots were stored in 4.54kg chub packages for 16 or 17 days and 23 or 24 days dark storage prior to regrinding and packaging into PVC overwrapped trays. The overwrapped trays were placed into a retail case under fluorescent lights for 5d. Subjective odor score (off-odor intensity; 1 = no off-odor to 5 = extreme off-odor), traditional culture bacterial counts (*Pseudomonas* spp., *Enterobacteriacae*, Lactic Acid Bacteria, and psychrotrophic plate counts), and lipid oxidation indicators were analyzed as a split-plot design, whereas subjective (1= Very bright red to 6= Very dark red or brown) and objective color (Hunter CIE L*, a*, and b* values) were analyzed as a repeated measures design. All bacterial counts increased ($P \leq 0.05$) during retail case display following both dark storage periods. Overall, off-odor intensity increased ($P \leq 0.05$) over both retail case display periods; however, the off-odor intensity score after 16/17d dark storage was lesser than the off-odor intensity score after 23/24d dark storage. Subjective color panel scores for ground beef redness decreased ($P \leq 0.05$) over both retail case display periods; however, a more rapid decrease ($P \leq 0.05$) in ground beef color during the retail case

display period was observed after 23/24d dark storage comparative to 16/17 day dark storage. Similarly, the redness (a^* value) decreased ($P \leq 0.05$) more rapidly following 23/24d dark storage comparative to after 16/17d dark storage. An increase ($P \leq 0.05$) in TBARS values was observed for both retail case display periods.

For the second experiment, instead of using culture-dependent microbiological methods, culture-independent methods of investigating microbial diversity were employed in conjunction with a GC/MS analysis of the volatile organic acids (VOCs) produced during storage. 16S rRNA amplicon sequencing was utilized to analyze the diversity and microbial constituents of the microbial community during the retail case display period after both 16/17d and 23/24d dark storage, and a targeted analysis utilizing GC/MS was used to evaluate the changes of VOCs production. The relative peak areas of the VOCs were analyzed as a split-plot design, where replication was the main plot, dark storage period was the sub-plot, and retail case storage day was the sub-subplot. Differences ($P \leq 0.05$) in Faith Phenotypic Diversity Index were observed during retail case display; however, the range of diversity was (1.02 to 1.28) was not large enough to be biologically relevant. The taxonomic analysis resulted in bacteria previously identified to contribute to beef spoilage. *Lactobacillales*, *Enterobacteriales,* and *Pseudomonadales* were in the top ten bacteria orders present across all samples throughout retail case display. Eighteen different VOCs were identified through the targeted analysis. The compounds identified via targeted analysis included aldehydes, ketones, volatile fatty acids, sulfones, and alcohols. Hexanal, an indicator of spoilage, increased ($P \leq 0.05$) during both retail case display periods. Moreover, acetoin and acetic acid also increased ($P \leq 0.05$) during both retail case display periods.

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Review of Literature

1.1. Ground Beef Consumption and Importance of Shelf-life

With increasing global population, demand for beef products is growing (OECD/FAO, 2016). Demand for beef in developing countries is projected to grow 21% by 2025, and surprisingly, demand for beef is expected to grow by 6% in developed countries in the same time span (OECD/FAO, 2016). Consumers are sensitive to changes in price of ground beef as a 53% increase in retail prices of beef occurred between 2006-2016, corresponding with an approximate 15% decrease in the consumption of beef transpired over the last decade in the United States (AMI/FMI, 2018; Badau, 2016; Westcott & Hansen, 2016). However, 2025 projections show the total supply in million pounds of beef production increasing by 11.7% and an expected decrease in price is projected to increase per capita consumption of beef in the United States from 55.3lbs (2016) to 56.8lbs (2025) (Westcott & Hansen, 2016). An estimated 62% of the beef consumed in the United States is in the form of ground beef and ground beef accounted for $9.57 billion in sales in 2017 (AMI/FMI, 2018; Close, 2014). Beef, specifically ground beef, remains a protein staple in the United States, and efforts to maintain and improve quality and safety of the product remain a priority.

Meat is a perishable product susceptible to deterioration of overall appearance (flavor, color, texture) due to both lipid oxidation and microbial processes (Fernández-López, Zhi, Aleson-Carbonell, Pérez-Alvarez, & Kuri, 2005). In the retail case, price per pound dominates consumer meat purchasing decisions, where appearance/quality of the meat product is the second driving factor in consumer decisions at the meat case (AMI/FMI, 2018). Therefore, increased

shelf-life remains a top priority of retailers, resulting in increased profits and sustainability (Xiao & Yang, 2017).

1.11. Definition of Shelf-life

Shelf-life is the duration of storage time where a food product maintains expected sensory characteristics, safety standards, and nutrients; shelf-life ends when the products becomes "unfit for human consumption" (Brooks et al., 2008; Giménez, Ares, & Ares, 2012). After safety of a product is met, sensory degradation becomes the limiting shelf-life factor (Hough, 2010). In fresh beef, consumers believe color other than bright cherry-red suggests decreased quality and safety (Faustman & Cassens, 1990). Factors affecting consumer's purchasing decisions for meat depend on intrinsic values such as meat color and packaging, yet Carpenter et al. (2001) found those intrinsic values had no effect on consumer eating satisfaction (Carpenter, Cornforth, & Whittier, 2001). Nonetheless, consumers base their purchasing decisions on sensory characteristics, so the importance of maintaining sensory characteristics throughout shelf-life is imperative.

1.12. Sustainability of Ground Beef

As a highly perishable item, meat accounts for a large portion of food waste and dollars lost in the United States each year. Food losses, as defined by Gustavsson et al. (2011), are a loss of an edible food through the food supply chain (Gustavsson, Cederberg, Sonesson, van Otterdijk, & Meybeck, 2011). Food loss of all food products is a major concern in the context of population growth and food security. At the end of shelf-life, food is considered "unfit for human consumption;" therefore, the food is discarded as waste. Globally, 263 million tons of meat is produced per year, yet 20% of the global supply is wasted (FAO, 2015). A better understanding and ability to monitor the mechanisms of degradation will enhance the control of supply chain

and decrease food loss over time. The FAO has a goal of eliminating hunger by 2030 and reduction of food waste will assist to reach the goal (FAO, 2015). Decreasing food loss will lead to a more efficient supply chain to feed the increasing world population and satisfy the need for a 60% increase in food production by 2050 (FAO, 2015).

In the United States, 430 billion pounds of food is available for human consumption, yet 31% of it is wasted (Buzby, Wells, & Hyman, 2014). Of the 31% of overall wasted food in the United States, 30% is meat waste, 19% is vegetables, and 17% is dairy products (Buzby et al., 2014). Wasted food in the retail sector is loss in profit. In the United States, an estimate by Buzby et al. (2014) suggests $3,737 million lost at the retail level and $27,911 million lost at the consumer level for meat products, excluding poultry and seafood, for the year 2008 (Buzby et al., 2014).

The likelihood of a consumer purchasing ground beef decreases as the pigment transforms from bright red (oxymyoglobin) to (brown) metmyoglobin. Carpenter et al. (2001) found that a correlation exists (r=0.09) between ground beef color and how likely a person is to buy the product, and Greene et al. (Greene, Hsin, & Zipser, 1971) found that consumers are unwilling to purchase ground beef that has 30-40% surface metmyoglobin, or brown color. Surface discoloration also causes retailers to discount beef products, resulting in a loss of profits. Around 20% of the ground chuck in a study completed by Sherbeck et al. (Sherbeck et al., 1995) was discounted due to discoloration in the retail case. Increasing the shelf-life of ground beef and other animal protein products coupled with a better ability to monitor shelf-life could dramatically increase profitability for the industry.

1.13. Properties of Ground Beef

The management of several properties of ground beef are important in maintaining and extending shelf-life. The properties are either intrinsic or extrinsic properties. A combination of factors play a role in the degradation of the ground beef product. Ways to control these properties include packaging, production environment, and temperature control (K. Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006). Consumers perceive quality via intrinsic and extrinsic cues displayed by the ground beef product and consumers' purchasing decisions are based on the expected quality (Issanchou, 1996).

1.2 Intrinsic Properties of Ground Beef

The intrinsic properties of meat are contained within the meat matrix itself, i.e. chemical make-up, physical properties such as texture, appearance, color, taste, flavor, and odor. Degradation of intrinsic properties decreases quality of the product; therefore, directly impacting consumer satisfaction (Issanchou, 1996). Repercussions from mismanagement of ground beef exploit the opportunity for rapid degradation of intrinsic properties. As such, any compromise that exists during the shelf-life of beef will impact the speed of degradation. The microbial and chemical spoilage processes can be slowed down and managed through extrinsic properties (K. Koutsoumanis et al., 2006).

1.21 Color

After the price per pound of ground beef, the second most important contributor of consumer purchasing decision is appearance (AMI/FMI, 2018; Mancini & Hunt, 2005). Of the characteristics that affect appearance, color is the most important factor for consumer purchasing decisions (AMI/FMI, 2018; Mancini & Hunt, 2005). Discoloration of meat products directly impacts revenue. Consumers use color as a gauge of quality and product safety, and are more

likely to buy the product based off color (Carpenter et al., 2001; Faustman & Cassens, 1990; Mancini & Hunt, 2005).

Myoglobin Reactions

Reactions and interactions of molecules with myoglobin (Mb), the water-soluble protein responsible for color, determine the chemical state of Mb, and consequentially the color of meat (Livingston & Brown, 1981). The molecule is globular, single-chained, and composed of eight α-helixes. In the hydrophobic part of myoglobin, there is a non-protein group, called the prosthetic group, that contains a porphyrin ring with iron contained in the center of the ring. The iron within the heme ring has 6 coordinate bonding sites with four of the sites coordinated with pyrrole nitrogens that make up the ring, one bond with histidine-93 attaching the heme ring to the globin chain, leaving the 6^{th} coordination site open to bonding with ligands (Mancini & Hunt, 2005; Suman & Joseph, 2013).

The different redox states of Mb determine the exhibiting color of the meat, and Mb can occur as oxymyoglobin (OxyMb), carboxymyoglobin (COMb), deoxymyoglobin (DeoxyMb), or metmyoglobin (MetMb). Consumers associate bright-red cherry color of beef with freshness and wholesomeness, the bright-red color is formed when Mb is either in OxyMb or COMb states (Cornforth & Hunt, 2008; Faustman & Cassens, 1990). The iron is in a ferrous state (Fe^{2+}) for both OxyMb and COMb. Diatomic oxygen binds to the 6^{th} coordination site of the myoglobin molecule to produce the bright-red cherry color of OxyMb (Mancini & Hunt, 2005). The OxyMb layer thickness and depth are conditional upon the intrinsic, i.e. pH and extrinsic properties, i.e. available atmospheric oxygen (Mancini & Hunt, 2005). The ligand bonded to the 6^{th} coordination site depends on the affinity the molecule has to myoglobin. Carbon monoxide has a

higher affinity for myoglobin compared to diatomic oxygen, so COMb forms when exposed to myoglobin in modified atmosphere packaging (MAP) (Mancini & Hunt, 2005).

When the meat is in an environment void of ligands, such as vaccum-packaging, DeoxyMb is formed and is purplish-red in color (Mancini & Hunt, 2005). Although the DeoxyMb is in an environment void of ligands, the iron is still in a ferrous state. MetMb is an irreversible color state where water bound at the 6^{th} coordination site (Livingston & Brown, 1981; Mancini & Hunt, 2005). The iron is in an irreversible ferric (Fe^{3+}) state that results in a brown color (Livingston & Brown, 1981). Oxidation of the iron begins below the surface of the meat where the layer of deoxygenated and oxygenated myoglobin meet (Mancini & Hunt, 2005). Manipulating the extrinsic factors, ie packaging, processing environment and method, work to prolong the oxidation of the Mb molecule, and enhance shelf-life.

Measurements of Color

Consumers rely on color to determine acceptability of a meat product; thus, there is an importance to measuring color or the perceived visual stimulation from the reflectance of light from an object during shelf-life studies (Mancini & Hunt, 2005). There are generally two ways color is measured during shelf-life studies: objectively and subjectively. Both utilizing a detector (eye (subjective) or instrument (objective)) to observe light reflectance off of a surface, which the stimulation is translated to a color with a processor (brain (subjective) or microprocessor (objective)) (AMSA, Revised 2012; 1991). Although there are plenty of objective measurement tools, concurrent use of both objective and subjective color measurements is suggested (AMSA, Revised 2012; 1991).

When discussing color, three separate properties are described: saturation, lightness and hue (AMSA, Revised 2012; 1991). Terminology utilized when describing the attribute of a color

linguistically, or the reflected light-spectrum wavelength off a specific surface is a hue, and hue is independent of lightness and saturation (AMSA, Revised 2012; 1991). Saturation is a descriptive word which takes into consideration the vividness or purity of a specific hue, where lightness refers to the value of color brightness or darkness (AMSA, Revised 2012; 1991). These properties are taken into consideration when measuring and describing color.

Objective meat color is generally measured in the CIE *L*a*b** color space (AMSA, Revised 2012; 1991; Mancini & Hunt, 2005). The CIE color space represents color in a three-dimensional model, contrary to the previous two-dimensional model, where lightness is accounted for within the model; the 3-D model was developed in 1976 by the Commision Internationale de l'Eclairage (CIE) (AMSA, Revised 2012; 1991; Tapp III, Yancey, & Apple, 2011). In the CIE *L*a*b** color space, colors are signified in a spherical space along three axes: Z-axis (*L**: 0 black – 100 white; brightness), X-axis (*a**: +red – (-)green), Y-axis (*b**: +yellow – (-) blue) (AMSA, Revised 2012; 1991).

A colorimeter and spectrophotometer are two instruments that operate to interpret within the CIE color space. The CIE *L* a* b** values are together are a representation of color, or relative color, and the values correlate with visual color (AMSA, Revised 2012; 1991). They are used to identify the hue by deviations of the incident angle of the measurement from the X-axis, lightness from the *L** value, and saturation by the distance *a** and *b** from the origin of the 3-D matrix (AMSA, Revised 2012; 1991).

Revised AMSA Meat Color Measurement Guidelines (2012) reviewed the considerations to take prior to conducting an experiment including instrumental meat color measurements, as instrument selection, illuminant selection, degree of observer, aperture size, instrument standardization, sample properties, sample collection should be given attention. Instrument

selection is important because different instruments have different settings of illuminant and observers (AMSA, Revised 2012; 1991). Red wavelengths, emphasized by Illuminant A, are better correlated with the relative color of meat; thus, Illuminant A is used more frequently in meat shelf-life projects, whereas Illuminant C and Illuminant D_{65} are more applicable for evaluating relative color of other food products (AMSA, Revised 2012; 1991).

Visual assessments of color by humans interrelated are the "fundamental standard" by which instrumental evaluations are based on (AMSA, Revised 2012; 1991). There are both trained and consumer panels that pertain to meat color evaluation depending on the objectives of the study (AMSA, Revised 2012; 1991). Consumer panels provide qualitative data using a hedonic scale, whereas trained descriptive panels are thoroughly trained on an anchored scale for a more quantitative evaluation (AMSA, Revised 2012; 1991). Trained panels need at least 5 participants to reduce variance in evaluations between panelists as recommended by the ASTM-434 (1968), but the revised AMSA Meat Color Measurement Guidelines (2012) suggests the minimum number of panelists to be eight (E-18, 1968). Although daily repeatability of a human's judgement is not probable, proper training and panel question selection can reduce bias and personal preference in resulting evaluation (AMSA, Revised 2012; 1991).

1.22 Lipid Oxidation

Lipid oxidation is a factor in degradation of the structural components within the meat matrix. Affected by a multitude of different environmental factors, including light, degree of unsaturation, temperature, pH, mechanical application and antioxidant availability, lipid oxidation varies between products and plays a role in shelf-life (Oswell, Thippareddi, & Pegg, 2018). Tolerance and acceptance of the amount of lipid oxidation differs between consumers; however, an abundance of oxidized lipid compounds in raw beef products mars the expected

quality characteristics of meat flavor (Campo et al., 2006). Oxidation is one of the three main mechanisms for meat spoilage, along with microbial growth and enzymatic autolysis, and understanding of the process of deterioration of the structural components within the meat matrix through oxidative processes is imperative in advancing shelf-life technologies (Dave & Ghaley, 2011).

Control of lipid oxidation increases shelf-life. Light and temperature accelerate propagation of autoxidation (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). However, the current consumer preferences for PVC overwrap packaging creates a packaging technique antagonistic to managing lipid oxidation. To maintain the PVC packaging that the consumer wants and increase shelf-life, the industry utilizes anaerobic or MAP storage prior to display of the product to slow down microbiological propagation of lipid oxidation processes (Borch, Kant-Muermans, & Blixt, 1996).

Free radicals are generally found in smaller concentrations due to the reactivity of the molecules, and are naturally forming during oxidative metabolic processes (Bekhit et al., 2013). Electrons naturally want to be in the lowest energy state, so when a free radical forms, the radical will try to interact with nearby compounds to lower the energy state. This can lead to changes in the structure of compounds or a cascade of exchanges of electrons between molecules, resulting in oxidation of meat tissue compounds (Bekhit et al., 2013). In the mitochondria of live tissue, the electron-transport chain creates hydrogen peroxide through a reaction that involves superoxide anion radicals, and the process ultimately produces the most reactive radical species, the hydroxyl radical (Bekhit et al., 2013; Candenas & Davies, 2000). Oxidative stress in live animals causes issues in metabolism and disease, but in meat tissue, it causes undesirable color, flavor, and quality properties (Bekhit et al., 2013).

The oxidative stress defense mechanisms muscle tissues possess are reduced postmortem, making the muscle tissue susceptible to oxidation (Bekhit et al., 2013). Reactive species (RS), or the compounds that include free radicals, are the primary contributing factor to the oxidation of lipids (Bekhit et al., 2013). A multitude of different RS are created through natural metabolic processes in the body, including $HO^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, and $HO_2^{\bullet}$ (Bekhit et al., 2013). The most reactive of the RS is hydroxyl radical $HO^{\bullet}$ that is created via the Fenton reaction, and is thought to be instrumental in significantly advancing meat quality attribute decline (Bekhit et al., 2013).

Off-odor related compounds, including ketones, aldehydes, alcohols, fatty acids, and malondialdehyde are produced from lipid oxidation reactions, such as the Fenton reaction (Bekhit et al., 2013; Min & Ahn, 2005). Certain types of lipids are more susceptible to lipid oxidation, such as polyunsaturated fatty acids (PUFAs) (Bekhit et al., 2013; Min & Ahn, 2005). Further processing of meat, such as grinding, reveals more PUFAs as the cells are damaged in the process of grinding (Min & Ahn, 2005). PUFAs generally contain bis-allylic carbons, and the hydrogen at the bis-allylic position are vulnerable to abstraction (Min & Ahn, 2005). The result of abstraction by a RS of the hydrogen of the PUFAs is secondary lipid peroxidation products and generally associated with rancid and off-odor related compounds (Bekhit et al., 2013; Min & Ahn, 2005).

Malondialdehyde (MDA) is one of the secondary products of PUFA lipid oxidation (Fernandez, Perez-Alvarez, & Fernandez-Lopez, 1997). Quantification of MDA concentration is used as an indicator of lipid oxidation (Fernandez et al., 1997). A popular method for measuring MDA concentration is using the thiobarbituric acid reactive substances (TBARS) test (Guillén-Sans & Guzmán-Chozas, 1998). Acid extraction of the thiobarbituric acid (TBA) reactive substrates, using trichloroacetic acid, results in a pink chromatin when the extraction filtrate is

reacted with TBA (Fernandez et al., 1997). MDA is a TBA reactive substrate; however, there are other substances that react with MDA, so TBARS is an indicator of lipid oxidation products (Fernandez et al., 1997; Guillén-Sans & Guzmán-Chozas, 1998).

1.23 Organic Volatile Acid Production

Since sensory characteristics, such as color and odor, drive consumer acceptance of raw ground beef in the supermarket, understanding the biochemical and physical changes in the product during display is the conation of shelf life. Literature suggests that microbial and chemical degradation of meat products occur synchronously, and separation of the products created by the two degradation pathways is not distinct (Khan, Jo, & Tariq, 2015; George-John E Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Degradation of raw meat begins at slaughter with enzyme activity breaking down sugars and peptides, but exposure to environmental (minerals, microbes, oxygen, etc) contaminates post-slaughter increases the production of products contributing to degradation (Casaburi, Piombino, Nychas, Vallani, & Ercolini, 2015; Kosowska, Majcher, & Fortuna, 2017). Knowledge of the volatile compounds and the predominant microorganisms present in raw ground beef, and simultaneous changes in sensory characteristics will further deepen the of fundamental understanding of shelf-life.

The succession of chemical and microbial processes and their production of volatile organic acids (VOCs) depends on both intrinsic and extrinsic properties of the meat matrix. Spoilage-associated catabolites production relies on the availability of nutrients to the microorganisms (George-John E Nychas et al., 2008). Aerobic and anaerobic/facultative anaerobic bacteria react to environmental changes by metabolizing the substrates available to the microbes, and interchanging metabolites under different conditions. The metabolites available to the microorganisms dictate the type of VOC produced (Casaburi et al., 2015).

An accumulation of VOCs cause off-odors in meat products, and certain microorganisms are known for producing VOCs that cause off-odors related to spoilage called “specific spoilage organisms” (SSO) (Casaburi et al., 2015; Huis in't Veld, 1996). The primary substrate utilized by microbes in meat products is glucose (Casaburi et al., 2015). As the concentration of glucose available decreases, the microbes utilize other substrates as nutrients such as lactate, pyruvate, amino acids, and ribose (Casaburi et al., 2015). The SSOs produce different compounds including, alcohols, organic acids, volatile aids, ketones, sulfur compounds, ethyl esters, aldehydes, ammonia, where the accumulation of these compounds produce different spoilage off-odors (Casaburi et al., 2015; George-John E Nychas et al., 2008). For example, lactic acid bacteria (LAB) will metabolize glucose into acetic acid, lactic acid, and ethanol, or under limited glucose, LAB use ribose to produce acetic acid or pyruvate to produce acetate (Casaburi et al., 2015). The acetate produces a vinegar odor, whereas acetic acid gives an acrid note (Casaburi et al., 2015).

Much of current literature describes VOCs and the impact on sensory implications of cooked product. The study of VOCs and raw beef are focused on shelf-life, but studies that focus on raw beef are studying the association of the VOCs produced during shelf-life and the cooked meat flavor (Casaburi et al., 2015). In the retail case, meat is discounted based on color defects, not odor (Sherbeck et al., 1995). However, food loss at the consumer level is higher than at the retail level (Buzby et al., 2014). Therefore, sensory characteristic degradation in meat products are a concern regarding food waste and shelf-life after retail.

1.3 Extrinsic Properties of Beef

The extrinsic properties of meat are properties that are the environment the meat matrix is exposed/subjected to; i.e., production environment, temperature, packaging, storage environment

and labeling. These attributes can be used to control the rate of spoilage and degradation of ground beef (Huis in't Veld, 1996; K. Koutsoumanis et al., 2006). The relationship between microorganisms that are on the ground beef product, introduced through production, and the chemical properties of the meat are managed through extrinsic properties (Huis in't Veld, 1996; K. Koutsoumanis et al., 2006).

1.31 Importance of Aerobic Packaging for Ground Beef

There are three predominant packaging types within the industry: 1) air-permeable, 2) modified atmosphere (MAP), 3) vacuum (McMillin, 2008, 2017). Air-permeable packaging is packaging, such as tray overwrapped in polyvinyl chloride (PVC), allows for oxygen transmission for the 'blooming' red color formation when O_2 binds to myoglobin (McMillin, 2017). Vacuum packaging voids the package of ambient air to maintain desired quality eating attributes for a longer time; however, it also promotes deoxymyoglobin, the deoxygenation of myoglobin, creating a purple color not desired in the retail case by consumers (McMillin, 2008, 2017). MAP packaging offers the ability to add mixtures of gases into a package for the purpose of maintaining color and microbial inhibition to increase shelf-life (McMillin, 2008, 2017).

A combination of packaging types can also be used to increase shelf-life. The industry has moved towards case-ready products which are manufactured and packaged for retail at a point of distribution and shipped to the retailers (McMillin, 2008, 2017). Overwrapped trays are generally packaged in a MAP motherbag at the distributor and shipped to the retailers, where the retailers can remove the overwrapped packages from the motherbag when they are ready for display (McMillin, 2017). Since consumers prefer the overwrap tray, this allows for the benefit of MAP packaging for increased shelf-life of about 23 days during storage, and for retailers to provide the preferred packaging for consumer display (AMI, 2012; McMillin, 2017). Retailers

also utilize a system of regrinding 5-10lb bulk chubs and packaging in overwrapped packages at the point of retail to maintain longer shelf-life of an approximate maximum of 23 days (AMI, 2012).

Although many packaging types are available for retail ground beef display, consumers still prefer the aesthetic of PVC film (Carpenter et al., 2001). Tray overwrapped packages, a technology from the 1950's, still prevail as the most predominant packaging type in the retail case (Carpenter et al., 2001; McMillin, 2008). One predicament facing retailers is the meat in overwrapped packages deteriorate in quality attributes important to consumers, ie color, within 3 days of display (McMillin, 2017). However, chub packaging is gaining momentum in popularity in the retail case, as a cost-saving product, but color does not apply to chub packaging (AMI/FMI, 2018; Mancini & Hunt, 2005).

The high oxygen transmissibility of the PVC packaging makes it susceptible to quicker quality deterioration comparative to other packaging. The shelf-life of ground beef stored in PVC packaging is 3 days. Currently in the realm of meat packaging, active and intelligent packaging are not widely utilized by the industry (McMillin, 2017). As defined by McMillin (2017), packages that contain devices that are able to indicate deviations in safety, quality or packaging of the product or environment are intelligent packaging, i.e. biosensors, barcode labels, volatile detectors (McMillin, 2017). Many factors, both intrinsic and extrinsic factor, concurrently affect the shelf-life of ground beef. Studying and understanding the interactions and relationships between all the factors will lead to better design of intelligent packaging. Thus, exploring the mechanisms of chemical and microbial degradation using aerobic shelf-life storage and current industry packaging practices is still relevant.

1.32 Microbial Degradation

Shelf-life is not only determined by chemical degradation, but is also influenced by microbial metabolic activities resulting in the biochemical deterioration of meat. Microorganisms collectively contributing to the biochemical degradation of meat culminating in spoilage are called specific spoilage organisms (SSO) (Gram et al., 2002; George-John E Nychas et al., 2008). Meat provides the nutrients utilized by microorganisms to cause production of metabolites related to off-odors, which directly influence the shelf-life; consumers will reject the meat product when enough of the off-odor compounds concentrate (Casaburi et al., 2015; Gram et al., 2002; George-John E Nychas et al., 2008). Casaburi et al. (2015) explained that microorganisms have "spoilage potential," defined as the ability to generate spoilage-related metabolites, where the environment and the initial introduction of the microorganisms to the meat and the storage environment affect the rate and types of metabolites produced (Casaburi et al., 2015; A. Doulgeraki, Paramithiotis, Kagkli, & Nychas, 2010; A. I. Doulgeraki, Ercolini, Villani, & Nychas, 2012; Ercolini et al., 2011; Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; George-John E Nychas et al., 2008; Pennacchia, Ercolini, & Villani, 2011). Consensus exists that meat is generally spoiled when traditional culture counts are 7 log cfu/g or above or the "potential spoilage level"; however, Brooks et al. (2008) mention that microbial count is not always a direct reflection of spoilage (Aryes, 1960; Brooks et al., 2008; K. P. Koutsoumanis, Stamatiou, Drosinos, & Nychas, 2008).

As the initial population of microbes within the meat matrix may vastly differ due to region and plant environment, specific SSOs, called Ephemeral Spoilage Organisms (ESO), will proliferate over the shelf-life of a meat product to out-compete other bacteria, producing "spoilage-associated metabolites" (A. I. Doulgeraki et al., 2012; Ercolini, Russo, Torrieri, Masi, & Villani, 2006; G-J.E. Nychas, Drosinos, & Board, 1998; George-John E Nychas et al., 2008;

Säde, Penttinen, Björkroth, & Hultman, 2017). The ESOs of concern for meat products are in the families of *Enterobacteriacae* and *Pseudomonadaceae,* the order of *Lactobacillales*, commonly called LAB, and the species *Brochothrix thermosphacta* (Casaburi et al., 2015; A. I. Doulgeraki et al., 2012; Gram et al., 2002; G-J.E. Nychas et al., 1998; George-John E Nychas et al., 2008). The ESOs catabolize precursors to volatile organic acids and other substrates within meat, releasing odors and visibly deteriorating quality traits that are associated with spoilage (Casaburi et al., 2015; George-John E Nychas et al., 2008). Even though microbes are not the only driver of meat degradation, their presence effectively increases the speed of degradation.

Consumers' preferred ground beef packaging, PVC overwrapped foam trays, is also the preferred type of packaging for microbes and accelerated spoilage with a shelf-life of 3 days (McMillin, 2017). Vacuum-packaging and MAP do inhibit some of the growth of and selects for different microbial communities than found in PVC tray overwrapped; thus, extending shelf-life (A. I. Doulgeraki et al., 2012; Ercolini et al., 2006). Moreover, ground beef is particularly perishable due to the susceptible nature of the product to microbes, and the stress the product endures through grinding. Modeling fluctuations in the microbial communities to better predict end of shelf-life will vastly improve the supply chain economics of ground beef, and new technologies to monitor the microbiome and its influence on the metabolites present in the meat matrix will assist in making the analysis of spoilage more robust (Casaburi et al., 2015; A. I. Doulgeraki et al., 2012; Säde et al., 2017).

There is a lot of research exploring and characterizing the microbes that are present throughout the shelf-life of meat; however, amount and presence of microbes at points of shelf-life do not necessarily reflect the organoleptic acceptability of the product (Brooks et al., 2008; A. I. Doulgeraki et al., 2012; Pennacchia et al., 2011). Spoilage, or end of shelf-life, is

determined by the consumer; therefore, measuring spoilage through objective methods is not precise in its methodology. As in traditional and current shelf-life research, organoleptic measures are needed in coordination with the objective methods to build the most applicable model to shelf-life (Brooks et al., 2008; Casaburi et al., 2015; A. I. Doulgeraki et al., 2012; Giménez et al., 2012). Sensory and consumer panels are still appropriate to incorporate in shelf-life research, and should continue to remain a part of shelf-life studies. All areas of ground beef shelf-life are fluid and dynamic (microbial, chemical, and organoleptic factors); therefore, all factors are considerations when assessing the shelf-life of ground beef.

As environment of the meat product plays a large role in selectivity and growth of microbes, continued exploration into the trends and patterns of the microbes utilizing meat nutrients and their catabolites will lead to a further productive shelf-life management and decline in overall food waste. Throughout the late 1900's, research focused on factors of chemical changes and microbial presence throughout shelf-life, as individual factors. Since the early 2000's, studies utilizing emerging technologies are starting to better connect the relationship between the primary drivers of shelf-life, developing better methods of detection, and modeling of spoilage (Casaburi et al., 2015; George-John E Nychas et al., 2008).

1.33. Exploring Culture-Independent Communities

Microbiome

Microbial communities within the meat matrix are vast and diverse, and influenced by many extrinsic factors. Historically, these microbes have been heavily studied through culture-dependent methods and the microbes that are associated with spoilage and end of shelf-life are well cataloged (A. I. Doulgeraki et al., 2012; Ercolini et al., 2009; Gram et al., 2002). These culture-dependent technologies exclude many of the community's constituents, leaving out

possible members of the microbial community that drive changes in degradation and intrinsic properties of ground beef over shelf-life when traditional shelf-life methodologies are utilized (Carpenter et al., 2001; Rappe & Giovannoni, 2003; Youssef , Couger, McCully, Criado, & Elshahed, 2015). However, recent technologies are allowing for further exploration of these microbial communities via culture-independent methods, giving a further and more in-depth understanding of the microbial communities throughout beef shelf-life.

The microbiome are the microbial communities that inhabit certain environments, and in the case of shelf-life, the ground beef environment (Ursell, Metcalf, Wegener Parfrey, & Knight, 2012). There is still much unknown about the different environments that microorganisms inhabit and why specific organisms are predominately found in particular environments (Fierer & Lennon, 2011). Culture-independent technologies allow analysis of microbes' natural environment without using culture media and enrichment that are inherently selective for certain microbial populations (A. I. Doulgeraki et al., 2012). Therefore, the study of the microbiome and the concept of using these culture-independent technologies in beef shelf-life are current.

Technologies Utilized in Spoilage Research

There are many culture-independent methodologies used in studying meat shelf-life. Technologies to examine microbial communities have been moving towards High-Throughput Sequencing technologies (HTS) to capture more of the microbial story. Current technologies employed in monitoring the changes in microbes during shelf-life of meat products are 1) polymerase chain reaction – denatured gradient gel electrophoresis (PCR-DGGE) 2) 16S rRNA amplicon sequencing using Roche's 454 pyrosequencing 3) 16S rRNA amplicon sequencing using bridge sequencing and 3) shotgun sequencing. Each of the technologies and methodologies

provide different strengths and weaknesses when contributing to the understanding of microbial communities within certain environments.

All the technologies mentioned, except shotgun, rely on PCR amplification of the genetic materials to sequence (Ercolini, 2004; Jovel et al., 2016). The primary difference in between the methodologies lies in the acquisition of the DNA/RNA sequences (A. I. Doulgeraki et al., 2012). PCR-DGGE separates different DNA sequences using the melting profiles in a denaturing gradient gel, whereas more current methods of sequencing the constituents of a community are pyrosequencing and bridge sequencing that have similar processes (Ercolini, 2004; Heather & Chain, 2016). Bridge sequencing modified the PCR step from the water-in-oil emulsion PCR (emPCR) to a solid phase PCR step (Heather & Chain, 2016).

Similarities and differences between the technologies present distinctive strengths and weaknesses. Overall, PCR-DGGE, pyrosequencing, and bridge sequencing utilize PCR amplification, which PCR primers inherently present a source of bias to certain members of the community (G. J. Caporaso et al., 2012). Moreover, the public databases used during sequence analysis apply bias due to erroneous annotation, differences in databases and lacking sequences (G. J. Caporaso et al., 2012). Even though PCR-DGGE is no longer a novel approach to investigating the members of a microbial community, Kraková et al. (2016) found that when comparing PCR-DGGE to Illumina MiSeq that both methods identified similar communities, but mentioned the technologies were complementary to each other as the technologies identified separate unique microbes that the other technology had not. When comparing Roche's 454 pyrosequencing to Illumina MiSeq, Luo et al. (2012) found that both sequencing technologies recovered similar diversity, as 90% of the contig sequences produced by both methods were similar. However, the study suggests that Illumina technology is better suited for microbiome

studies as Illumina is more cost effective (Luo, Tsementzi, Kyrpides, Read, & Konstantinidis, 2012).

To capture the biological and chemical changes that occur due to chemical and microbial deterioration during shelf-life, shelf-life studies began merging the use of analytical chemistry and microbial ecology to more precisely model meat and fish shelf-life (Ercolini, 2004; Ercolini et al., 2011; Ercolini et al., 2009). Shelf-life studies are continuously evolving and adding new technologies rapidly. Currently, PCR-DGGE seems the most widely used technology described in the literature to identify the diversity of the microbial community in the shelf-life of meat products when considering both microbial and organic volatile acid production changes (A. I. Doulgeraki et al., 2012; Ercolini, 2004; Ercolini et al., 2009). However, Next Generation Sequencing (NGS) techniques for 16S rRNA amplicon sequencing are gaining popularity in shelf-life studies (Säde et al., 2017). We have yet to find an article in the ground beef shelf-life literature that uses bridge sequencing using Illumina technology for 16S rRNA amplicon sequencing for identification of representatives in the microbial communities, instead many of the studies are using PCR-DGGE or Roche's 454 pyrosequencing for identification (A. I. Doulgeraki et al., 2012; Säde et al., 2017).

Microbiome and Meat

As meat is a perishable product, studying the microorganisms and their function within a food product provides understanding to the role they play in food spoilage and shelf-life. Shelf-life studies apply culture-independent methods for many applications. For ground beef and fresh meat products, the primary focus for shelf-life studies include known SSOs and identifying predominant culture-independent microorganisms found at different stages of shelf-life.

Ercolini et al. (2011) found that 16S pyrosequencing was more efficient at identifying changes in the microbial profile of meat over time under different storage conditions than PCR-DGGE. The 16S pyrosequencing technology allowed for the relative abundance of the bacteria within the samples to be monitored (Ercolini et al., 2011). Previous studies looked at whole muscle beef cuts and can represent a good idea of what the microbial changes are throughout the shelf-life of beef. However, the processing step of grinding is missing from the studies, and thus, contamination and stress placed on the beef during grinding as well as processing facility and lot could affect the initial microbial community (Stellato et al., 2016; Säde et al., 2017).

The role of the processing facility environmental microbiome plays part in the initial microbial community (Stellato et al., 2016). Although the study did not find differences ($P = 0.05$) between the distribution of the types of microbes found on the meat in either large-scale or small-scale processing facilities, the facilities did differ in their microbial communities (Stellato et al., 2016). The study suggests that differences in the environmental hygiene where the meat is processed could play a role in the contamination and subsequent shortening of shelf-life of meat products (Stellato et al., 2016). However, Sade et al. (2017) found initial microbial communities were more diverse between separate lots (produced on different days in the same facility), whereas later in shelf-life, the diversity of the microbial communities decreased. These studies highlight a culmination of utilizing the microbial communities to help better predict end-of-shelf by monitoring changes within the microbial community, as the studies suggest decreased diversity at the end of shelf-life and higher microbial contamination may lead to shorter shelf-life. These discoveries provide evidence that more microbiome shelf-life studies should occur to continue to map out microbes over time, and their subsequent effects on the quality and safety of beef, specifically ground beef, is needed.

CHAPTER 2

Culture-Dependent Bacteria and Subsequent Changes in Sensory Characteristics of Aerobically Stored Ground Beef

Introduction

Consumer expectations drive the shelf-life of ground beef. The end of shelf-life is the point where consumers find a food product unacceptable for consumption; therefore, maintaining shelf-life and understanding the influencing biological changes within ground beef and subsequent quality changes are pertinent (A. I. Doulgeraki et al., 2012; George-John E Nychas et al., 2008). Current literature cites specific-spoilage organisms (SSO) as the presence of specific microbes that predominate at certain points of shelf-life and are associated to driving quality characteristic deterioration in meat products (Gram et al., 2002; George-John E Nychas et al., 2008). The SSOs utilize nutrients within the meat matrix, and subsequently produce metabolites that are perceived by consumers as off-odors (A. I. Doulgeraki et al., 2012; George-John E Nychas et al., 2008). However, the amount and presence of microbes during shelf-life does not always reflect in organoleptic acceptability of the meat product (Brooks et al., 2008). The objective of this study was to characterize culture-dependent microbial fluctuations and the subsequent sensory changes over aerobic retail case display of ground beef. Five days (d) of retail case display occurred after both 16/17d and 23/24d refrigerated anaerobic storage. Further, the study explored the effect of two separate dark refrigerated storage periods and the difference of the rate of culture-dependent microbial growth during retail case display after each dark storage period.

Materials and Methods

Ground Beef Procurement

Three separate lots of finely ground beef chubs (80% lean, 20% fat, chuck, 10 pounds) were procured from one ground beef processing facility in the West and two lots from one ground beef processing facility in the Midwestern United States. The lot from the West was acquired at a supermarket, where storage of chubs was in dark refrigerated storage prior to transportation to Colorado State University Meat Lab. Lots from the Midwestern United States were obtained from the processing plant and transported to the Colorado State University Meat Lab directly. The lots were transported to the Colorado State University Meat Lab in Fort Collins, CO where they were stored in the dark at between 2-4°C for 16/17d and 23/24d to emulate common industry practices.

Ground Beef Processing and Retail Display

After 16/17d and 23/24d dark storage at 2°C, five chubs from each lot were finely reground prior to packaging 453.6g (±90.7g) amounts into polyvinyl chloride film (MAPAC DBL-MP film, AEP Industries Inc., South Hackensack, NJ) overwrapped black polystyrene foam trays (#2 Supermarket Tray, Genpack, Charlotte, NC) using a single layer of overwrap on the product. The overwrapped trays were displayed in a retail case (Model No. M3X-GEP, Hussmann Corp., Bridgeton, MO), under fluorescent lights (Philips F32T8/HL735/ALTO 30PK, Koninklijke Philips N.V., Amsterdam, Netherlands) with a light intensity of 1281 lux (range of 310 lux to 2120 lux), set for 2-4°C for 5d. Trays were displayed horizontally on the same bottom shelf of the three-tier retail case for all three replications and four temperature logger (iLog data logger, Cryopak Industries Inc., Monticello, AR) were on the same shelves as the trays monitoring retail case temperature. Due to slight differences in light intensity and temperature within the retail case, packages were rotated to different position in the retail case every 24 hours. Three to five packages per lot were displayed for evaluation per retail case display day.

There were 30 packages from one of the replications (three packages on display per retail case day), and 50 packages (five packages on display per retail case day) for two of the replications, for a total of 130 packages.

Microbial Analyses

Bacterial plate counts for psychrotrophic, lactic acid bacteria (LAB), *Pseudomonas* spp., and *Enterobacteriacae* were enumerated on three to five individual packages of ground beef every 24 hours for a total of five retail case display days. A 50g composite ground beef sample from various locations within one sample were aseptically excised from each package and placed into a sterile 710ml filtered Whirl-Pak bag (Nasco, Fort Atkinson, WI). 100ml of Maximum Recovery Diluent (MRD) (Acumedia Maximum Recovery Diluent (7658), Neogen Corporation, Lansing, MI) was added to each sample bag. Each sample was mechanically pummeled (Stomacher® 400 Circulator, Seward Laboratory Systems Inc, Bohemia, NY) for 2 mins at 230 RPM. The samples were serially 10-fold diluted using 0.1% buffered peptone water (BPW) (Difco Laboratories, Sparks, MD). The dilutions were plated for enumeration counts on four agars in duplicate, where duplicates were averaged when recorded. For psychrotrophic bacteria, dilutions were plated on tryptic soy agar (TSA; Acumedia Tryptic Soy Agar (7100B), Neogen Corporation, Lansing, MI), and incubated at 7°C for 10d. Dilutions of LAB were plated on Lactobacilli MRS (de Man, Rogosa and Sharpe) agar (BD Difco™ Dehydrated Culture Media: Lactobacilli MRS Agar, Difco Laboratories, Sparks, MD) in two layers, where 10ml of 50°C MRS agar was poured onto the plate over the diluted BPW and another 10ml layer was poured 20min later onto the first layer to create an anaerobic environment and incubated at 25°C for 144hr (De Man, Rogosa, & Sharpe, 1960; *Difco & BBL Manual - Manual of Microbiological Culture Media*, 2009). For *Pseudomonas* spp., dilutions plated on *Pseudomonas* selective agar

(*Pseudomonas* Agar CFC Selective Agar; Oxoid Ltd, Basingstoke, UK), and incubated for 72hr at 25°C. For *Enterobacteriacae*, dilutions were plated on 3M™ Petrifilm™ Enterobacteriaceae Count Plates (3M Food Safety, St. Paul, MN) and incubated for 24hr at 35°C.

pH

A pH sample was taken from each of the three to five individual packages of ground beef. One gram (+/- 0.1g) samples of ground beef from each package were weighed out, and 9ml of deionized water were added to a 207ml sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI), and mechanically pummeled (Masticator Silver 400ml 110/120 V 50/60 Hz 50 bags, IUL S.A., Barcelona, Spain) for 2 mins at 230RPM. Sample pHs were measured with a pH meter (Denver Instruments, Arvada, CO), and calibrated using buffered solutions of a 4.0 and 7.0 pH.

Odor Panel

Once a day for 0d to 4d of retail case display, 10g samples were taken from the 3-5 trays to be sampled for the sampling collection time. Three to five samples (N=130) were evaluated on each retail display day by a trained sensory panel with each panel which included no less than 6 qualified panelists per session. The collected odor samples were placed into glass jars (Ball® Regular Mouth 4 Oz. Baby Food Jars with Storage Caps, Hearthmark, LLC, Fishers, IN), and presented to panelists with an assigned three-digit random number. Each panelist was trained quantify off-odor on a Likert scale, anchored from 1 = no off-odor to 5 = extreme off-odor, and to describe what they perceive as the predominant off-odor. Distribution of ballots occurred electronically via Qualtrics Survey Software (Qualtrics, LLC., Provo, UT). Panelists' scores were averaged over the individual package and the average of the panelists' scores were reported as one panel rating for each package.

Color Measurements

Objective Color Measurements

Color measurements were taken every 12 hours during 5d of retail case display using a spectrophotometer (Hunter MiniScan EZ spectrophotometer; Model 4500 S; Hunter Associates Laboratory Inc., Reston, VA) with a standard observer angle of 10º and a 5mm aperture size on the three to five packages for each lot. The Hunter MiniScan EZ spectrophotometer used an Illuminant A light source. Calibration of the spectrophotometer occurred using a black glass tile and white tile prior to measurements. Like methods described in the revised AMSA Meat Color Measurement Guidelines (2012), data were documented as an average of the triplicate measures taken on each of the overwrapped packages. The data recorded were the Hunter CIE L*, a*, and b* values.

Color Panel

Three to five samples (N=130) were evaluated every 12 hours, over a 5-day retail display period, by a minimum 6 panelists who were trained to evaluate ground beef color and surface discoloration (AMSA, Revised 2012; 1991). Panelists used a 6-point whole number scale to evaluate ground beef color anchored at 1= Very bright red, 2= Bright red, 3= Dull red, 4= Slightly dark red or brown, 5= Moderately dark red or brown, and 6= Very dark red or brown. Panelists evaluated surface discoloration by evaluating the overall percentage of discoloration of lean surface of 0% indicating no discoloration to 100% indicating severe discoloration. Ballots were distributed electronically via Qualtrics Survey Software (Qualtrics, LLC., Provo, UT). Panelists' scores were averaged over individual samples and the average of the panelists' scores were reported as one panel rating for each package.

Thiobarbituric Acid Reactive Substances (TBARS)

TBARS procedure occurred using similar methodology to Witte et al. (1970) (Witte, Krause, & Bailey, 1970). Five grams (+/- 0.01g) of frozen ground beef homogenate was weighed out in duplicate, and placed into a blender (Waring Commercial, Torrington, CT) where 22.5ml of 11% trichloroacetic acid (TCA) (Tricholoracetic Acid (T6399), Sigma-Aldrich Co., Saint Louis, MO) was added. The meat homogenate and TCA were homogenized together for 30 seconds. The homogenate was then filtered through Whatman No. 1 filter paper (Whatman™ 1001185) into a clean 50ml beaker. 1ml of thiobarbituric acid (20mM) (Thiobarbituric Acid (T5500-25G), Sigma-Aldrich Co., Saint Louis, MO) and 1ml of the filtrate were added to a test-tube for a 20hr incubation period, in a dark location, at room temperature prior to reading on a spectrophotometer. Absorbance values were obtained at 532nm on a spectrophotometer (Shimadzu Inc., Columbia, MD, USA) in a 10mm polystyrene cuvette (Fisherbrand™ Disposable Cuvettes, Standard, Polystyrene; Thermo Fisher Scientific, Waltham, MA). Values were recorded in duplicate and reported as an average of absorbance values of the duplicates per individual package.

Statistical Analysis

The experimental design is a split-plot design with the replication as the main plot, the two dark storage periods as the subplot, and the retail case day as the sub-subplot. Data were analyzed in R (version 3.4.3) (Team, 2017). Data for subjective odor scores, bacterial populations and pH were evaluated in the lme4, lmerTest, and lsmeans packages in R (version 3.4.3) (Bates, Maechler, Bolker, & Walker, 2015; Kuznetsova, PB, & RHB, 2017; R. V. Lenth, 2016). Data for microbial populations were presented as log CFU/cm^2. Data for subjective odor scores were log transformed to achieve normality. Least squares means for subjective odor

scores were reported as back transformed means. All data were conveyed as least squares means and differences reported with an alpha value of 0.05.

The experimental design for color evaluation, both subjective and objective, was a longitudinal repeated measure and analyzed in the R packages nlme, plyr, and emmeans in R (version 3.4.3) (R. Lenth, 2018; R. V. Lenth, 2016; Pinheiro, Bates, DebRoy, Sarkar, & Team, 2017; Team, 2017; Wickham, 2011). Lsmeans for subjective color panel scores were reported as back transformed means. Both subjective color panel scores and hunter spectrophotometer values were conveyed as least squares means and pairwise comparison with a Tukey's adjustment, where differences were reported with an alpha value of 0.05.

Results and Discussion

Microbiological Results

There is a strongly significant interaction between dark storage and retail case display day, for *Enterobacteriacae* (P=0.02), psychrotrophic bacteria (P<0.0001), *pseudomonas* (P<0.0001), and LAB (P<0.0001). The emmeans function allowed for comparison of log CFU/cm^2 during each of the retail case display days (Table 2.1). Populations of *Pseudomonas* changed over retail display days; however, the populations of microbes also differed between dark storage periods. *Pseudomonas* counts during 0d of retail case display were higher (P<0.0001) after 23/24d dark storage than 16/17d dark storage. A larger increase in *Pseudomonas* occurred during retail case display after 16/17d dark storage comparative to retail case display after 23/24d dark storage. The population of *Pseudomonas* on 4d of retail case display after 16/17d dark storage were higher (P<0.0001) than on 4d of retail case display after 23/24d dark storage. Surprisingly, populations of psychrotrophic APC and LAB started above 7.0 log CFU/cm^2 after 16/17d dark storage in chub packaging. However, an increase in

populations of psychrotrophic APC, LAB, and *Enterobacteriaceae* occurred over both retail display case periods ($P \leq 0.05$), but no difference was observed between the dark storage periods (Table 2.1).

Major spoilage organisms of concern in fresh meat, identified through culture-dependent methods, are *Enterobacteriaceae, Pseudomonas, Carnobacterium* spp., *Lactobacillus* spp., *Pseudomonas* spp., and *Brochothrix thermosphacta (Borch et al., 1996; A. I. Doulgeraki et al., 2012)*. Our results for starting bacterial loads after a 16/17d dark storage period in chub packaging are consistent with results from a study conducted by Peters et al. (1998) where LAB reached 7 log CFU/g by day 18 of storage. Similarly, Pennacchia et al. (2011) found LAB to predominate the bacterial community on vacuum packaged beef samples after 7 days of storage.

Previous research has revealed that LAB predominate in anaerobically packaged meat, whereas *Pseudomonas* spp. dominate in aerobically packaged meat (Ercolini et al., 2009; Jay, Vilai, & Hughes, 2003). *Pseudomonas* spp. is genus of a gram-negative, generally aerobic, non-spore forming organisms that thrive in proteinaceous environments between a pH range of 5.3-7.8 (McMeekin & Ross, 1996). Koutsoumanis et al. (2006) found that *Pseudomonas* spp. were not only the dominant bacteria in aerobically stored ground beef and pork, but were consistent in activity and growth which allowed creation of a statistically validated model, based off the Arrhenius equation for meat spoilage, accounting for temperature, sensory, and pH. An experiment conducted by Blixt and Borch (2002) compared the shelf-life of vacuum packaged beef and pork. Their study found that even through LAB were the dominant microorganism, *Pseduomonas* spp. increased during storage from ~10^2 log CFU/g to over 10^5 CFU/g between week 3 and 4 of vacuum packaged storage. In our experiment, *Pseudomonas* spp. persisted between 10^3 log CFU/g and 10^4 log CFU/g during dark storage in chub packaging for both dark

storage periods. Since the LAB and psychrotropic APC were similar in count, presumably, LAB were the predominate culture-dependent organism, outcompeting *Pseudomonas* spp. during the anaerobic dark storage periods in chub packaging.

As mentioned by Brooks et al. (2008) showed different ideas in the scientific literature as to when the amount of microbial growth reaches a level to deem meat spoiled. Generally, a level of 10^7 CFU/g of microbial growth is considered spoiled. However, Brooks et al. (2008) found microbiological testing is not always reflective of the point of spoilage, and a consumer may still accept the product at a high level of bacterial growth. In this study, LAB and psychrotrophic APC were above 10^7 CFU/g after both 16/17d and 23/24d dark storage, but the 16/17d dark storage period was within the industry's 21d shelf-life of chub packaged ground beef.

pH Results

For the variable of pH, there is a significant interaction (P<0.0001) between dark storage and retail case display days. The evaluation of the pH during each of the retail case display days was done using the emmeans function (Table 2.2). Differences in pH were found between 0d, 1d and 2d of 16/17d and 23/24d dark storage (P < 0.001); however, no differences (P>0.05) were found between 3d and 4d of retail case display between 16/17d and 23/24d dark storage. Generally, there was a decrease (P<0.05) in pH during retail case display after 16/17d dark storage. There was a slight decrease in pH after 0d of retail case display but a slight increase in pH over 1d, 2d, 3d and 4d of retail case display after 23/24d dark storage.

The intrinsic property of pH plays a role in a microbe's ability to adapt and thrive in the proteinaceous meat environment. *Pseudomonas* spp. and LAB are tolerant to changes in pH, especially within the normal pH range of meat (Borch et al., 1996; Gill & Newton, 1982). LAB cause a decrease in the overall pH of the meat product, a defect that contributes to a sour flavor,

and is inhibitory to other spoilage bacteria such as *Enterobacteriaceae and B. thermosphacta* (Borch et al., 1996). *Pseudomonas* are tolerant to a pH between 5.3-7.8; however, growth rates of *Pseudomonas* spp. are slowed when pH is between 5.1-5.3 (McMeekin & Ross, 1996). *Pseudomonas* spp. growth in this study grew a total of ~1.73 log CFU/g throughout the retail case display period after 16/17d dark storage as pH remained above 5.49 for the entire week. Interestingly, *Pseudomonas* spp. growth followed a similar trend to the pH after 23/24d dark storage. As pH declined below 5.3 for retail case display for 1d and 2d, *Pseudomonas* spp. growth also decreased, and subsequently grew once pH was above 5.3.

Subjective Odor Panel Results

A significant interaction resulted between dark storage and retail case display regarding subjective odor panel. Comparison of the subjective odor scores were evaluated using the emmeans function during each of the retail case display days and values were back-transformed to the original scale (Table 2.3). Overall, there was an increase in off-odor intensity score over the retail case display periods after both dark storage periods ($P < 0.05$). The off-odor intensity score started at a lower value ($P<0.0001$), 1.28, on 0d after 16/17d dark storage when compared to 0d after 23/24d dark storage, 1.45. After 23/24d dark storage, a quicker increase in off-odor intensity was observed over 1d, 2d, 3d retail case display comparative to the same period after 16/17d ($P \leq 0.05$).

A study conducted by Sørheim et al. (1999) compared high oxygen packaging, a $CO/CO_2/N_2$ mixture and chub packaging. The chub packages of ground beef performed as well as the $CO/CO_2/N_2$ mixture in extending the time to detection of off odors by 2-3 days compared to the high oxygen packaging at 4° C (Sørheim, Nissen, & Nesbakken, 1999). Brooks et al. (2008) observed quicker development of off-odor in overwrapped packages comparative to low-

oxygen packages. Similarly, minimal to no off-odors were detected on day zero of retail case display after both dark storage periods in chub packaging. Off-odors developed during the time that the retail packages were in overwrapped packages that have higher oxygen transmission rates.

Subjective and Objective Color Results

An interaction between dark storage period and retail case display occurred for both ground beef color and percent surface discoloration. A comparison of the results was evaluated through the emmeans function (Table 2.4; Table 2.5). Overall, ground beef color (6-point scale; very bright red to very dark red or brown) deteriorated over both retail case display periods ($P \leq 0.05$). During retail case display, the redness decreased faster after 23/24d dark storage than 16/17d dark storage ($P \leq 0.05$). Increased percentages of surface discoloration of the lean tissue followed a similar trend to the increase in the color score over both retail case display periods. The percent surface discoloration did not progress past 75% discoloration until hour 84 after 16/17d dark storage, whereas the percent surface discoloration after 23/24d dark storage progressed past 75% discoloration by hour 48 of retail case display.

For all measures of Hunter Color, L*, *a** and *b**, comparison of the color scores were evaluated using the emmeans function. For both *a** and *b**, there was a significant interaction between dark storage period and retail case display.

Observationally, a faster decrease in *a** (red to green) occurred during retail case display after 23/24 dark storage compared to 16/17d dark storage (Table 2.6). The *a** value started at 26.82 at hour 0 during retail case display after 16/17d dark storage and decreased ($P \leq 0.05$) until hour 72 of retail case display with a color score of 7.58. Whereas, at hour 0 of retail case display after 23/24d dark storage, the *a** value started at 25.91 and decreased ($P \leq 0.05$) to 7.13 at hour

48. The decline of the a* value, or redness, to a stagnated value occurred within the first 48 hours of retail case display after 23/24d dark storage, a much faster decline comparative to the decline of the *a** value after 72 hours of retail case display after 16/17d dark storage.

For *L** values, the further along in hours of display, the lighter ($P \leq 0.05$) the samples, until around 60 hours where the change in lightness stagnated. There were differences ($P \leq 0.05$) between *b** values during hour 36 and 48 of the dark storage periods. Also, *b** values decreased during retail case display after both dark storage periods, a potential indicator of discoloration during retail case display.

The *L**, *a** and *b** values followed a similar pattern in reduction in redness as the color panel. Both the color panel and instrumental color indicated a more rapid progression of loss of redness occurred after 23/24d dark storage compared to 16/17d dark storage. As color is one of the primary indicators of freshness to consumers, a decrease in redness over retail case display leads to consumer discrimination (Carpenter et al., 2001). The faster the decrease in redness over retail case display leads to a shorter shelf-life and discounts due to discoloration (Mancini & Hunt, 2005). The decrease in redness over retail case display can be attributed to the oxidation of the ferric pigment from free radicals produced through lipid oxidation (Greene et al., 1971).

TBAR Results

A significant interaction between dark storage period and retail case display resulted for the TBARS absorbance values. Comparison of the TBARs absorbance values were evaluated using the emmeans function (Table 2.7). There were no differences ($P > 0.05$) between TBARS absorbance values for retail case display 0d and 1d between dark storage periods; however, there was a difference ($P \leq 0.05$) for retail case display 2d, 3d and 4d between the two dark storage periods. On 4d of retail case display, the TBARS absorbance value for 16/17d dark storage was

0.355, whereas the TBARS absorbance value for 23/24d dark storage was 0.586, a higher amount of lipid oxidation indicators. The oxidation of myoglobin and lipid oxidation are associated (AMSA, Revised 2012; 1991). A study conducted by Alderton et al. (Alderton, Faustman, Liefler, & Hill, 2003) found an increase in lipid oxidation product leads to myoglobin oxidation, or browning (AMSA, Revised 2012; 1991). The acceleration in lipid oxidation may explain why a decrease in redness observed over retail case display, as lipid oxidation indicators increased.

Conclusion

A study examining culture-dependent microbial growth and changes in organoleptic and chemical quality of the beef during aerobic shelf-life was conducted. An increase ($P \leq 0.05$) in LAB, *Enterobacteriacae, Pseudomonas* spp., and psychrotrophic bacteria during retail case display was observed after both 16/17d and 23/24d dark storage. Decreased ($P \leq 0.05$) redness, both as measured objectively and subjectively during retail case display were observed, as well increased ($P \leq 0.05$) intensity of off-odor of the ground beef.

Results from this study are similar to previous results in the literature for microbial growth and changes in quality attributes of ground beef during aerobic shelf-life. Further studies are necessary to continue to understand the succession of culture-dependent microbial fluctuations and the subsequent sensory changes over aerobic retail case display of ground beef. Moreover, studies focusing on predictive models for shelf-life should occur.

Table 2.1. Adjusted least squares (LS) mean of microbial populations (log CFU/cm^2) for ground beef during retail case display following dark storage for 16/17d and 23/24d days.

	Retail Case Display (Days)					
	0	1	2	3	4	SEM[3]
Pseudomonas						
16/17d	3.71^{ay}	4.00^{ay}	4.52^{by}	4.99^{cy}	5.44^{dy}	1.08
23/24d	4.39^{az}	4.36^{abz}	4.33^{aby}	4.66^{bcz}	4.71^{cz}	1.08
Lactic Acid Bacteria						
16/17d	7.45^{ay}	8.39^{by}	8.08^{cy}	8.59^{cy}	8.54^{cy}	0.24
23/24d	8.15^{ay}	8.46^{ay}	8.13^{aby}	8.71^{bcy}	8.38^{cy}	0.24
Enterobacteriaceae						
16/17d	3.18^{ay}	3.49^{abx}	3.42^{aby}	3.73^{by}	4.19^{cy}	0.64
23/24d	3.4^{ay}	3.65^{aby}	3.81^{by}	4.02^{by}	3.95^{by}	0.64
Psychrotrophic						
16/17d	7.94^{ay}	$^{*}8.25^{by}$	8.27^{by}	8.69^{cy}	8.68^{cy}	0.2
23/24d	8.59^{az}	8.66^{ay}	8.71^{aby}	8.82^{aby}	8.61^{by}	0.2

^{a,b,c} LSMeans exhibiting different superscript letters within the rows ($P<0.05$).
^{y,z} LSMeans exhibiting different superscript letters within columns ($P<0.05$).
LSMeans and Pooled Standard Error (SE) are based off a sample size of nine.
*LSMeans and SE calculated based off a sample size of three.

Table 2.2. Adjusted least square (LS) means of pH of ground beef during retail case display following dark storage for 16/17d and 23/24d.

	Retail Case Display (Days)					
	0	1	2	3	4	SEM[3]
pH						
16/17d	5.70^{ay}	5.59^{bcy}	5.61^{aby}	5.50^{cy}	5.49^{cy}	0.04
23/24d	5.48^{az}	5.25^{bz}	5.35^{bcz}	5.40^{acy}	5.46^{acy}	0.04

^{a,b,c} LSMeans differ within rows without common superscript differ *(P<0.05).*
^{y,z} LSMeans differ within column without common superscript differ *(P<0.05).*
LSMeans and Pooled Standard Error (SEM[3]) are based off a sample size of nine.

Table 2.3. Back-transformed adjusted least square (LS) means of odor panel off-odor intensity score (1 = no off-odor to 5 = extreme off-odor) during retail case display following dark storage for 16/17d and 23/24d.

Retail Case Display (Days)	Days Dark Storage					
	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
0	1.28^{ay}	1.14	1.43	1.45^{az}	1.30	1.62
1	1.59^{by}	1.42	1.77	2.21^{bz}	1.98	2.47
2	2.21^{cy}	1.98	2.47	3.65^{cz}	3.27	4.08
3	3.10^{dy}	2.78	3.46	3.60^{cz}	3.23	4.03
4	3.84^{ey}	3.43	4.29	4.04^{cy}	3.61	4.51

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).
^{y,z} LSMeans differ within row without common superscript differ (P<0.05).
LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit are based off a sample size of nine.
Odor Intensity Score is based off a 5-point scale (No off-odor to extreme off-odor).

Table 2.4. Back-transformed adjusted least square (LS) means color panel values (1=Very bright red to 6=Very dark red or brown) of ground beef for hours of retail case display following 16/17d and 23/24d dark storage.

Retail Case Display (Hour)	Days Dark Storage					
	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
0	1.07^{ay}	0.90	1.28	1.07^{ay}	0.91	1.25
12	1.29^{by}	1.11	1.51	1.55^{bz}	1.33	1.81
24	1.51^{cy}	1.30	1.77	2.41^{cz}	2.07	2.82
36	2.12^{dy}	1.82	2.48	3.99^{dz}	3.42	4.66
48	2.56^{ey}	2.20	2.99	5.69^{ez}	4.88	6.65
60	3.26^{fy}	2.80	3.81	5.81^{ez}	4.98	6.78
72	4.92^{gy}	4.22	5.75	6.00^{ez}	5.14	7.01
84	5.84^{hy}	5.01	6.82	6.00^{ey}	5.14	7.01
96	6.00^{hy}	5.14	7.01	5.95^{ey}	5.10	6.94

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).
^{y,z} LSMeans differ within row without common superscript differ (P<0.05).
LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit are based off a sample size of nine.
Ground Beef Color Score is based off a 6-point scale (1=Very bright red to 6=Very dark red or brown).

Table 2.5. Back-transformed adjusted least square (LS) means of percent surface discoloration of ground beef evaluated by a color panel for hours of retail case display following 16/17d and 23/24d dark storage.

Retail Case Display	Days Dark Storage					
(Hour)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
0	0.03^{ay}	-0.49	1.07	0.00^{ay}	-0.47	0.85
12	1.38^{by}	0.27	3.47	0.06^{az}	-0.44	0.98
24	0.99^{bcy}	0.06	2.73	3.45^{bz}	1.37	7.34
36	2.62^{cdy}	0.93	5.81	34.21^{cz}	17.78	65.06
48	3.64^{dy}	1.48	7.71	91.80^{dz}	48.48	173.08
60	19.12^{ey}	9.73	36.74	98.42^{dz}	52.00	185.47
72	70.17^{fy}	36.94	132.49	98.58^{dy}	52.09	185.78
84	93.84^{fy}	49.56	176.89	98.56^{dy}	52.08	185.74
96	98.58^{fy}	52.09	185.78	98.57^{dy}	52.08	185.76

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).
^{y,z} LSMeans differ within row without common superscript differ (P<0.05).
LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit are based off a sample size of nine.

Table 2.6. Adjusted least square (LS) means of instrumental *L*, a*, b** values of ground beef (80% lean/20% fat) for hours of retail case display following 16/17d and 23/24d dark storage.

Retail Case Display (Hour)	Days Dark Storage					
	*L**		*a**		*b**	
	16/17d	23/24d	16/17d	23/24d	16/17d	23/24d
0	*48.59^{ab}	50.53^{ab}	*26.82^{ay}	25.91^{ay}	*21.66^{ay}	21.27^{ay}
12	48.58^{a}	50.03^{a}	23.75^{by}	21.57^{by}	19.42^{by}	18.25^{by}
24	50.44^{abcd}	52.02^{abc}	22.63^{by}	19.02^{cz}	18.84^{bcy}	17.68^{by}
36	49.33^{abc}	50.60^{abc}	21.06^{cy}	11.35^{dz}	18.08^{cy}	15.00^{cz}
48	50.60^{bcd}	51.59^{abc}	16.92^{dy}	7.13^{fz}	16.43^{dy}	14.42^{cz}
60	51.11^{cd}	52.40^{bc}	14.16^{ey}	6.69^{fz}	16.05^{dy}	14.99^{cy}
72	51.52^{d}	52.49^{c}	7.58^{fy}	6.23^{fy}	14.84^{ey}	14.40^{cy}
84	51.22^{cd}	52.33^{bc}	7.31^{fy}	6.53^{fy}	14.81^{ey}	15.10^{cy}
96	51.50^{d}	52.05^{bc}	7.01^{fy}	6.31^{fy}	15.00^{ey}	14.90^{cy}
SEM[3]	0.64	0.64	0.46	0.46	0.38	0.38

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).
^{y,z} LSMeans differ within row without common superscript differ (P<0.05).
*Pooled Standard Mean (SEM[3]) of 0.52 based off 9 aggregate samples

Table 2.7. Adjusted least square (LS) means of Thiobarbituric Acid Reactive Substances absorbance value (Abs) of ground beef during retail case display following dark storage for 16/17d and 23/24d.

	Retail Case Display (Days)					
	0	1	2	3	4	SEM[3]
Abs						
16/17d	0.043^{ay}	0.132^{aby}	0.191^{by}	0.206^{by}	0.355^{cy}	0.04
23/24d	0.038^{ay}	0.137^{by}	0.271^{cz}	0.521^{dz}	0.586^{dz}	0.04

^{a,b,c} LSMeans differ within rows without common superscript differ *(P<0.05)*.
^{y,z} LSMeans differ within column without common superscript differ *(P<0.05)*.
LSMeans and Pooled Standard Error are based off a sample size of nine.

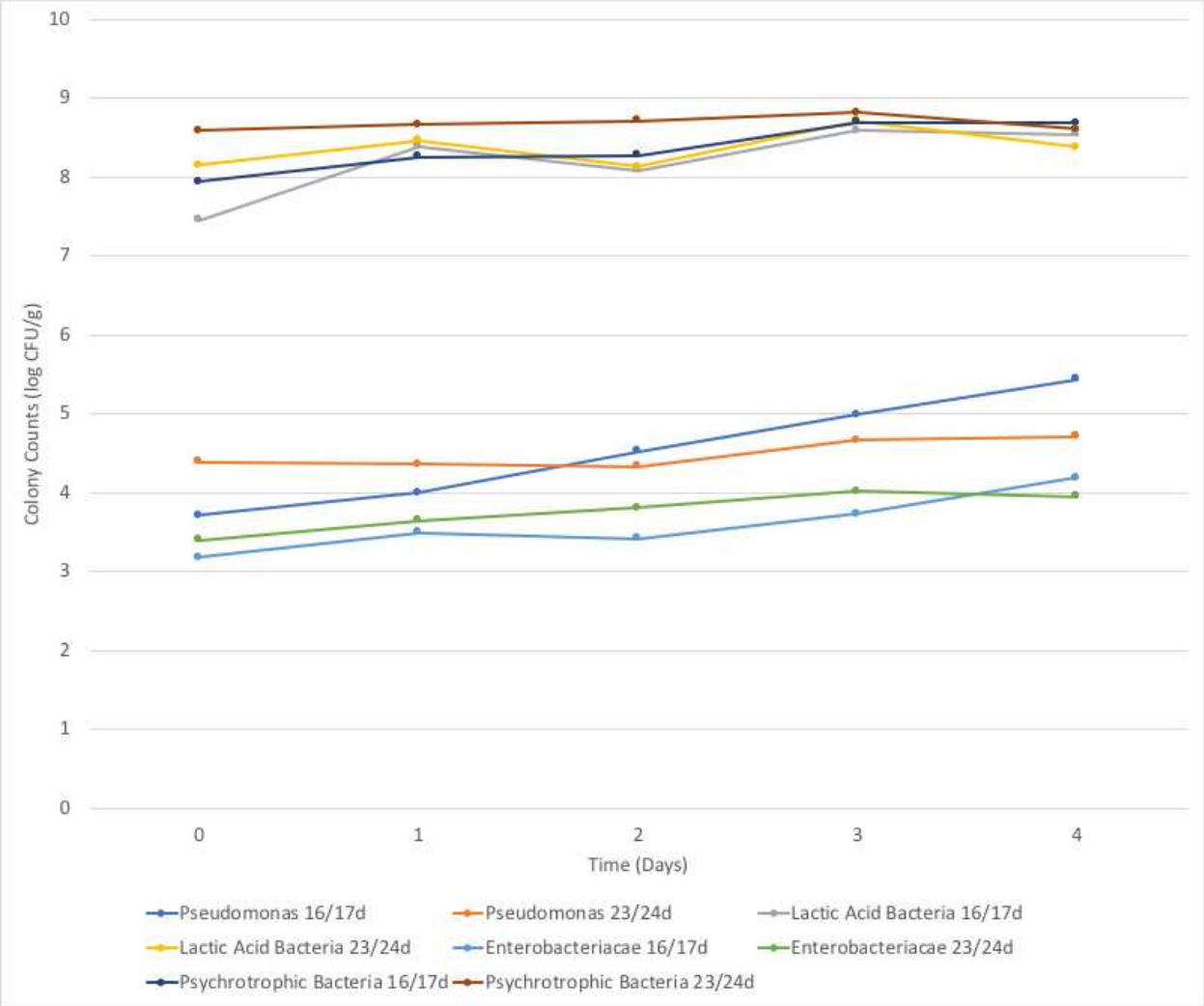

Figure 2.1. The growth curves based off the adjusted least squares means of microbial populations (log CFU/cm^2) of psychrotrophic APC, *Enterobacteriaceae*, lactic acid bacteria and *Pseudomonas*. The x-axis is retail case display days (0d-4d), where individual straight line segments represent either 16/17d or 23/24d for each bacterial count type.

CHAPTER 3

Dynamic Changes in the Diversity of the Microbial Community Found on Ground Beef and the Volatile Organic Acids Produced Throughout Aerobic Shelf-Life

Introduction

Ground beef accounts for 62% of the beef consumed in the United States (Close, 2014). Demand for beef products are projected to increase 6% by 2025 in developed countries, and 21% in developing countries (OECD/FAO, 2016). Ground beef is perishable product, and a concern with the growing demand in beef is the production of food waste. Currently, 20% of the global meat supply is lost or wasted, resulting in loss of profits and reduced efficiency and sustainability (FAO, 2015). Better management of shelf-life would decrease food waste and increase sustainability of beef products.

"Specific spoilage organisms," (SSOs) are organisms that contribute to the degradation of meat quality attributes (Casaburi et al., 2015; Ercolini et al., 2009). Common SSOs found in meat are Lactic Acid Bacteria (LAB), *Enterobacteriaceae*, *Pseudomonas* spp., and *Brochothrix thermosphacta* (Borch et al., 1996; Casaburi et al., 2015; A. I. Doulgeraki et al., 2012; Ercolini et al., 2009; Pennacchia et al., 2011). Microbes utilize the nutrients within the meat matrix for metabolism, and the volatile organic acids (VOCs), including alcohols, ketones, sulfur compounds, ethyl esters, aldehydes, produced during metabolic processes are associated with spoilage-related off-odors (Casaburi et al., 2015; Ercolini et al., 2011).

The shifts in the microbial community and population are associated with changes in the amount and types of VOCs present (Casaburi et al., 2015; Ercolini et al., 2011). Exploring the dynamics of the microbial communities found in ground beef, and the changes that occur throughout the shelf-life of the product are crucial to better shelf-life management. Historically,

shelf-life studies utilized culture-dependent techniques to monitor the fluctuations in microbial communities; however, only a small portion of the vast species of microbes can be cultured, leaving gaps in knowledge (Pace, 2009; Rappe & Giovannoni, 2003; Stewart, 2012). Recently, shelf-life studies have started using High-Throughput Sequencing (HTS), a culture-independent methodology to look at microbial diversity fluctuations and consider the vast and diverse microbial population that are unable to be cultured (Ercolini et al., 2011; Säde et al., 2017). To our knowledge, most of the shelf-life studies to date on ground beef utilizing HTS technology have either used polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) or pyrosequencing, and have yet to publish utilizing 16S rRNA amplicon sequencing via Illumina MiSeq methods.

The aims of this study were to explore the diversity and fluctuations in the microbial communities, utilizing 16S rRNA amplicon sequencing, and production of VOCs throughout retail case display time. Furthermore, to determine what spoilage related taxa are present across different storage periods.

Materials and Methods

Ground Beef Procurement

10lb ground chuck chubs (80% lean, 20%fat) were obtained from two beef processing facilities in the United States (Midwest and West). Lots of ground beef were separated into three separate replications (1 lot from the West and 2 lots from the Midwest). The chubs from the West were procured from a grocery retailer, and the chubs from the Midwest were procured from the processing plant. The chubs were transported to Colorado State University (Fort Collins, CO) for study. The chub packages were held in dark storage at 2°C for 16/17d and 23/24d prior to regrinding and packaging.

Ground Beef Processing and Retail Display

Five chub packages were reground at Colorado State University's meat lab (Fort Collins, CO) after 16/17d and 23/24d dark storage at 2°C. The meat was ground into 453.6g (±90.7g) "fluff" packages in polyvinyl chloride film (MAPAC DBL-MP film, AEP Industries Inc., South Hackensack, NJ) overwrapped foam trays (#2 Supermarket Tray, Genpack, Charlotte, NC). Packages were displayed for five days in a fluorescent lit (Philips F32T8/HL735/ALTO 30PK, Koninklijke Philips N.V., Amsterdam, Netherlands) retail case (Model No. M3X-GEP, Hussmann Corp., Bridgeton, MO) where the light intensity was 1281 lux (range of 310 lux to 2120 lux), and retail case temperature was monitored (iLog data logger, Cryopak Industries Inc., Monticello, AR) at 2-4°C.

Sample Collection for 16S

Five tray samples were taken at 0d, 2d, 4d every 24 hours for the replication from the Western United States, and 5 trays were sampled at 0d, 1d, 2d, 3d, 4d, every 24 hours, for the two replications from the Midwestern United States. Samples were placed in liquid nitrogen until frozen and powdered in a blender (Oster® Precise Blend™ 200 Blender, Sunbeam Products, Inc, Boca Raton, FL) (Figure 3.1). Each individual tray was blended in its own individual glass jar, with its own blade, sealing ring, and bottom cap. Glass jars, blades, sealing rings, and bottom cap were soaked for 10 mins in a 10% bleach solution for 10 mins. The blender jar parts were then rinsed in deionized water and set to dry in a container that was also bleached and rinsed. Samples were placed into a -80 ºC freezer until extraction.

Extraction and Library Preparation

Extraction of DNA from the samples occurred using a DNeasy PowerSoil Kit (Qiagen, Inc, Germantown, MD), where samples were extracted on a 96 well plate (Qiagen, Inc,

Germantown, MD). Sequencing library preparation used 515f/926r primer set, using methods by Walters et al. (Walters et al., 2015).

16S amplicon sequencing

Amplicons were sequenced using the Illumina MiSeq platform (300+300 bp) (Illumina Inc., San Diego, CA) at the Delhousie University Integrated Microbiome Resource (Halifax, Nova Scotia).

Sequence Analysis

The samples were demultiplexed using QIIME2 (version 2018.4) (J. G. Caporaso et al., 2010). The DADA2 was used for assignment of reads into sequence variants (Callahan et al., 2016). Sequences were truncated at 260 for the forward reads and 240 for the reverse reads bases using DADA2 to preserve quality reads. The data were rarified at a depth of 1,207 reads due to decrease in quality reads, and 113 samples remained after samples were rarified. A phylogenetic tree was constructed using FastTree (Price, Dehal, & Arkin, 2010). Taxonomy was assigned using the greengenes database, where mitochondria and chloroplasts were filtered out (DeSantis et al., 2006). To examine alpha diversity, Faith's phylogenetic diversity index was utilized. Due to unbalance in sampling between the two replications (1st replication sampled on days 0, 2, and 4, where the 2nd and 3rd replication were sampled on all retail case display days), day 1 and 3 were removed from the analysis due to confounding results. Faith diversity was assessed via Kruskal-Wallis rank sum test and pairwise comparisons evaluated via Wilcoxon rank sum test in R packages car and emmeans (version 3.4.3) (Fox & Weisberg, 2011; R. Lenth, 2018; Team, 2017). Beta diversity was analyzed through weighted UniFrac metrics and EMPeror was used to create a PCoA plot to visualize the differences (Lozupone & Knight, 2005; Vazquez-Baeza,

Pirrung, Gonzalez, & Knight, 2013). To determine beta diversity of the treatments, a PERMANOVA test of significance was used (Anderson & Walsh, 2013).

Sample Collection for Volatile Identification via GC-MS

Every 24 hours, 3 tray samples were taken for the replication from the Western United States replications and 5 trays were sampled for the Midwestern United States replications. Following similar methods to studies previously done at Colorado State University, samples were frozen in liquid nitrogen prior to being powdered in a blender (NutriBullet Lean™, Capital Brands, LLC, Los Angeles, CA) until powdered (Figure 3.2). Five grams (±0.001g) of frozen, powdered meat samples were weighed (5 grams ±0.001g) of into 20mL headspace vials (20ml S/T CLR Headspace vial, Thermo Scientific, Waltham, MA). Samples were stored in a -80°C freezer until placement on autosampler.

Extraction using SPME fiber

A proprietary cooling system was built around the autosampler sample tray to maintain the meat sample below 10°C prior to analysis to reduce oxidation of the sample prior to incubation. Prior to headspace extraction, sample incubation occurred at 40°C for 30 min. After incubation, extraction of headspace volatiles was done for 40 min using methods described by Pérez et al. (Pérez, Rojo, González, & De Lorenzo, 2008) with a Carboxen/PDMS fiber (85µm, Stableflex, Sigma-Aldrich, St. Louis, MO).

Gas chromatography-mass spectrometry (GC-MS) for identification of Volatile Organic Acids

A Trace1310 GC (Thermo Fisher Scientific, Waltham, MA) and the attached ISQ-LT MS (Thermo Fisher Scientific, Waltham, MA) were used as the GC/MS system. The GC operated with a DB-WAXUI column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies, Santa Clara, CA), where the SPME fiber was injected after collection of the volatiles from the headspace.

Desorption was completed under the splitless mode of the GC inlet, and desorption from the SPME fiber to the injector port occurred for 3min (250°C). The fiber was then conditioned at the conditioning port for 10min (270°C). The oven of the GC ran at 35°C for 5 min before a rate of 8°C/min ramp up to 100°C, then another ramp up at a rate of 12°C/min to 240°C, where the temperature was held at 240°C for 5 min. The MS was operated in the electron impact mode, scan rate of 10 scans/second, full scan 35-350 amu, source temperature and transfer line temperature at 250°C. Chromeleon™ 7 Chromatography Data System (CDS) (Thermo Fisher Scientific, Waltham, MA) software was used for targeted analysis of volatiles. The NIST v12 EI spectral database was used for annotation of unknown spectra. Examples of the chromatograph (Figure 3.11) and mass spectrum (Figure 3.12) are typical output for the targeted analysis.

Volatile Data Analysis

Data were analyzed as a split-plot design in R (version 3.4.3) (Team, 2017). The replication is main plot, where the dark storage periods are represented as the subplot and the sub-subplot is retail case day. Relative peak areas were analyzed as a split-plot design using the packages lme4, lmerTest, and emmeans packages in R (version 3.4.3) (Bates et al., 2015; Kuznetsova et al., 2017; R. Lenth, 2018; R. V. Lenth, 2016). A log transformation of data was used to attain normality. For the compounds of octanal, iso-butyric and *3-methylbutanoic acid*, a square root transformation was used to achieve normality. The emmeans for relative peak areas are displayed as back transformed means. Data are presented as least squares means and differences are separated using an alpha value of 0.05.

Results and Discussion

Alpha and Beta Diversity of the Microbial Community

A low range of alpha diversity, evaluated using Faith phylogenetic diversity index, was observed for both retail case display days and region (Faith, 1992). Differences were not found (P=0.08) using the Kruskal-Wallis rank sum test (Figure 3.3). The small range of alpha diversity indicates a dominance of an organism due to the lack of phylogenetic differences observed within the samples. Another point of interest is differences in alpha diversity between regions. The observed alpha diversity in region (Midwest vs West) was relatively low. The mean of alpha diversity in the West was 1.52 (±0.06 SE) and in the Midwest was 1.09 (±0.03 SE). A difference was observed in alpha diversity of regions (P<0.001) using the Kruskal-Wallis rank sum test (Figure 3.4); however, the narrow range and low alpha diversity index indicates a lack of alpha diversity. An explanation to the relatively low alpha diversity is likely by result of a dominant species persisting throughout the periods of shelf-life.

The beta diversity metrics, assessed using the weighted UniFrac metrics, supported differences in the composition between the microbial communities of the regions (Figure 3.5). Region had a significant outcome on the composition of the microbial communities (P=0.001) when compared using a PERMANOVA across all dark storage periods and retail case display day (Figure 3.5). Further exploration into differences in the composition of the microbiome from different processing facilities should occur to assess the impact the initial microbial population has on the dynamics of spoilage-related microbial species. A difference (P=0.001) was observed during retail case display when days were compared within dark storage period using a PERMANOVA, was observed when compared within dark storage period, suggesting difference in the complexity of the microbial community throughout shelf-life (Figure 3.6).

De Filippis et al (De Filippis, La Storia, Villani, & Ercolini, 2013) analyzed bacterial communities from beefsteaks sampled across two separate abattoirs on day 0 of aerobic storage

and day 7 of aerobic storage. The study found complexity of the microbial communities, beta diversity, to decrease (P=0.001) during aerobic storage. Comparatively, the current study found difference in the beta diversity between regions (P=0.001), but a difference in storage prior to aerobic retail display may impact the differences observed. Further study into difference of storage conditions on the complexity of microbial communities between separate facilities is necessary to understand the facilities impact on the composition of the microbiome. Säde et al. (Säde et al., 2017) explored lot-to-lot differences of microbial community composition in high-O_2 modified atmosphere packaging. The mentioned study found similar patterns of microbial changes throughout the shelf-life of the meat, where distinct separate microbial community structures existed at the beginning of shelf-life (Säde et al., 2017). Both studies highlight the decrease in diversity of microbial communities throughout shelf-life, and the succession of the dominant spoilage-related microbial species. The studies indicate a decrease in diversity over shelf-life, where the lack of diversity in the current study may be a result of the 16/17d and 23/24d storage period prior to sampling. The complexity of the microbial community likely decreased within the dark storage period, lending to a less diverse population shift during retail case display.

Taxonomic Classification of the Microbiome

Taxonomic structures were evaluated for dark storage period and retail case display at the order level (Figure 3.7 and Figure 3.8). Unsurprisingly, the dominate organism was from the order of *Lactobacillales*. Within the top 10 taxa present throughout all retail case display days, organisms from the orders of commonly associated spoilage bacteria were present: *Lactobacillales*, *Enterobacteriales,* and *Pseudomonadales.*

Lactic acid bacteria (LAB) are the predominant spoilage bacteria in vacuum packaged products, whereas *Pseudomonas* spp. are the predominant aerobic spoilage organism (Borch et al., 1996). In the current study, the ground beef was packaged in anaerobically stored chub packaging for 16/17d or 23/24d before regrinding and packaging in PVC overwrapped packages, where the environment favored growth of LAB (Borch et al., 1996; A. I. Doulgeraki et al., 2012). As *Pseudomonas* spp. are associated with rapid degradation in quality of meat products during aerobic storage, anaerobic storage prior to display is used to extend shelf-life of the product (Borch et al., 1996). Presence of LAB does not indicate spoilage, as some species do not cause degradation of meat quality; however, there is a strong relationship between the presence of *Pseudomonas* and spoilage (A. I. Doulgeraki et al., 2012). In the current study, there is a greater proportion of LAB present than *Pseudomonadales*, suggesting the storage in anaerobic type environment allowed LAB to dominate.

On the family level, Streptococcaceae, Lactobacilliaceae, Leuconostocaceae, Enterobacteriaceae, Pseudomonadaceae, Neisseriaceae, Listeriacae, and Moraxellaceae were among the top 10 organisms identified. Within the families of Lactobacilliaceae, Leuconostocaceae, Enterobacteriaceae, Listeriacae and Pseudomonadaceae are genera commonly classified as spoilage organisms (A. I. Doulgeraki et al., 2012).

Limitations

A high abundance of bovine DNA was sequenced, on average 89.4% of reads in each sample (range of 72.1% to 98.6%) were of bovine origin prior to quality filtering (Figure 3.9). After removal of bovine DNA, *Lactobacillales* was the dominate order (Figure 3.10). This highlights sampling method concerns as the primary contaminate was host related, not environmental, which could be a result of a few different factors.

A similar study by Kaur et al. (Kaur, Shang, Tamplin, Ross, & Bowman, 2017) evaluated the lamb steak microbiome using culture-independent methods with a different method for DNA extraction than the current study. Kaur and coauthors (2017) used a rinsate of the meat instead of freezing and homogenizing as was done in this study, resulting in samples over 10,000 reads (as suggested by them rarifying to 10,000 reads). This contrasts with the current study which required the data to be rarefied at 1,207.

Primers used for PCR prior to sequencing can preference detection of sequences. The 515f/806r primer set is the primer set recommended by the Earth Microbiome Project ("16S Illumina Amplicon Protocol," 2018) for amplification of 16S rRNA for Bacteria and Archaea. The primer is considered a consistent and reproducible primer set that amplifies the variable region 4 of 16S rRNA and contains a blocking primer to reduce vertebrate DNA ("16S Illumina Amplicon Protocol," 2018). The current study used the 515f/926r primer set that amplifies both the variable region 4 and 5 of 16S rRNA. The 515f/926r primer set amplifies Eukaryotes, Bacteria and Archaea (Walters et al., 2015). The choice in primer set, unfortunately, possibly contributed to the unwanted amplification of bovine DNA. Both sampling technique and primer set should be examined further to understand the source of the host contamination.

Organic Volatile Acid Profile Results

There were 18 compounds identified that were related to ground beef spoilage (Casaburi et al., 2015). Of the 18 compounds identified, 4 were ketones (Table 3.1), 2 were aldehydes (Table 3.2), 7 were volatile fatty acids (Table 3.3), 4 were alcohols (Table 3.4), and 1 was a sulfone (Table 3.5). The volatile organic acids found in meat products are related to breakdown of different primary compounds into secondary compounds via microbial metabolism, lipid oxidation, and autolytic enzymatic reactions (Casaburi et al., 2015; A. I. Doulgeraki et al., 2012;

George-John E Nychas et al., 2008). Methodology utilized by researchers to capture volatiles using SPME is not standardized, so the methodology employed by researchers affects the compounds identified (Lyte et al., 2016; Pérez et al., 2008). However, in the current study, there are specific compounds identified that are related to microbial degradation or lipid oxidation of compounds found within the meat matrix (Casaburi et al., 2015; Lyte et al., 2016; Pérez et al., 2008).

Hexanal is known to cause rancid off odors and used as an spoilage indicator (Casaburi et al., 2015; Insausti, Beriain, Gorraiz, & Purroy, 2006). The production of hexanal can be associated with Bacteria, such as *Bronchothrix thermosphacta,*and *Pseudomonas fragi*; however, hexanal is most notably formed in meat products as a product of the oxidation of linoleic acid (Ajuyah, Fenton, Hardin, & Sim, 1993; Casaburi et al., 2015; Smit, Smit, & Engels, 2005). A strongly significant interaction exists between dark storage and retail case display day for Hexanal ($P<0.0001$). The emmeans function allowed for comparison of relative peak areas during each of the retail case display days (Table 3.2). Hexanal increased ($P\leq0.05$) over retail case display after both dark storage periods (Table 3.2). The relative peak area for hexanal on 2d, 3d, and 4d of retail case display after 23/24d dark storage is greater ($P\leq0.05$) than the relative peak area for hexanal for the same retail case display days after 16/17d dark storage. An accelerated increase in the formation of hexanal after 23/24d dark storage may be attributed to the longer dark storage period prior to retail case display. Moreover, the increase ($P\leq0.05$) in hexanal during both retail case display periods indicates an occurrence and acceleration of lipid oxidation during retail case display.

Acetic acid is one of the compounds identified in the current study (Table 3.3). Acetic acid is one of the compounds produced by LAB under reduced glucose presence (Borch et al.,

1996). A significant interaction exists between dark storage and retail case display day for acidic acid (P=0.01). The emmeans function was used for comparison of relative peak areas during each of the retail case display days (Table 3.3). Presence of *Lactobacillaceae* (Figure 3.7) and an increase ($P \leq 0.05$) in acetic acid (Table 3.3) was observed during retail case display. A likely explanation for the increase in acetic acid is the genera *Lactobacillus* spp. is a spoilage organism within the family of Lactobacillaceae that produces acetic acid from the metabolism of ribose when glucose availability is limited, and produces an acrid off-odor (Casaburi et al., 2015).

Acetoin is an interesting product of growth and metabolism of LAB (Jääskeläinen et al., 2013). Acetoin (3-hydroxybutan-2-one) is a ketone that has been identified in both aerobic and vaccum-package storage systems (Casaburi et al., 2015). The ketone is associated with creamy/buttery off flavor notes, and is a product of the metabolism of glucose (Jääskeläinen et al., 2013). The main effect of retail case display day was significant (P<0.0001). The emmeans function was utilized to compare relative peak areas during retail case display days (R. Lenth, 2018). There was an increase ($P \leq 0.05$) of acetoin during both retail case display periods. In a study conducted by Jääskeläinen et al. (2013), LAB metabolism, specifically *Leuconostoc gasicomitatum*, increased the production of acetoin under aerobic conditions, where under anaerobic conditions the LAB produced lactic acid. In the current study, *Leuconostoc* was found (Figure 3.7) and an increase ($P \leq 0.05$) in acetoin occurred over aerobic retail case display. This rapid increase in the production of acetoin during aerobic storage may be linked to the change in LAB respiration (Jääskeläinen et al., 2013). The species within the *Leuconostoc* family found during retail case display may contribute to acetoin production (Jääskeläinen et al., 2013).

Conclusion

A study investigating the changes in microbial communities using 16S rRNA amplicon sequencing technologies and fluctuations in VOC release during the shelf-life of ground beef was conducted. The 16S rRNA amplicon sequencing provided another tool to explore the microbial community; however, more research to enhance the sampling methodology should be explored. Taxonomic analysis provided information useful to improve the comprehension of the VOCs present.

Differences ($P \leq 0.05$) in alpha diversity were not large enough in range to be biologically relevant. The lack of alpha diversity may be due to sampling, as alpha diversity decreases over the shelf-life of meat. *Lactobacillales*, *Enterobacteriales,* and *Pseudomonadales* were all identified within the study, and are commonly associated as spoilage organisms in beef.

Eighteen different compounds associated with beef spoilage were identified. Compounds included ketones, aldehydes, volatile fatty acids, alcohols (Table 3.4), and a sulfone. An increase ($P \leq 0.05$) in hexanal, a compound used as an identifier for spoilage, was observed over both retail case display periods. Some of the compounds identified are associated with different microbial metabolic processes in the literature.

More research is necessary to continue to build the understanding of the microbial community in ground beef, and the VOCs produced by the microorganisms. Use of 16S rRNA amplicon sequencing is another tool that can be used to continue to explore the microbiome of ground beef during shelf-life.

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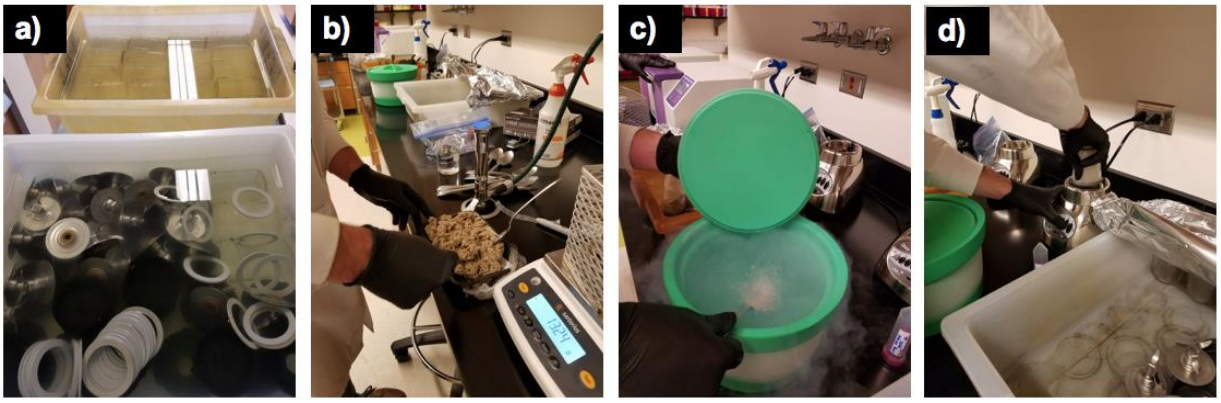

Figure 3.1. Sample collection methods for DNA extraction.
a) Blenders (Oster® Precise Blend™ 200 Blender, Sunbeam Products, Inc, Boca Raton, FL) and blender parts were placed into 10% bleach solution bath and soaked for 10 minutes prior to being rinsed with deionized water and left to air dry. b) Metal spoon doused in 100% ethanol was placed into flame and allowed to burn out and cool prior to meat removal. c) Meat is frozen in individual liquid nitrogen bath, where liquid nitrogen was changed between replications. d) Meat blended to a powder in individual blender prior to transfer into 50ml Falcon Tubes for storage in -80˚C until DNA extraction.

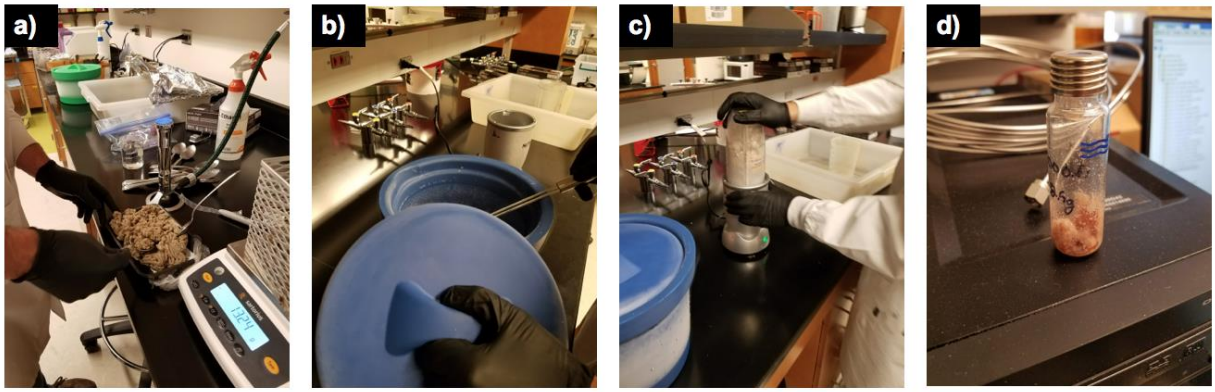

Figure 3.2. Sample collection for volatile extraction.
a) Samples were aseptically removed from tray using metal spoon that was doused in alcohol, flamed, and let cooled prior to removal of meat from tray. b) Meat was placed on metal colander and frozen in liquid nitrogen. c) Samples were blended in a clean blender cup (NutriBullet Lean™, Capital Brands, LLC, Los Angeles, CA) to a powder. d) Samples were stored in 20ml headspace vials (20ml S/T CLR Headspace vial, Thermo Scientific, Waltham, MA) until extraction and analysis done on GC/MS.

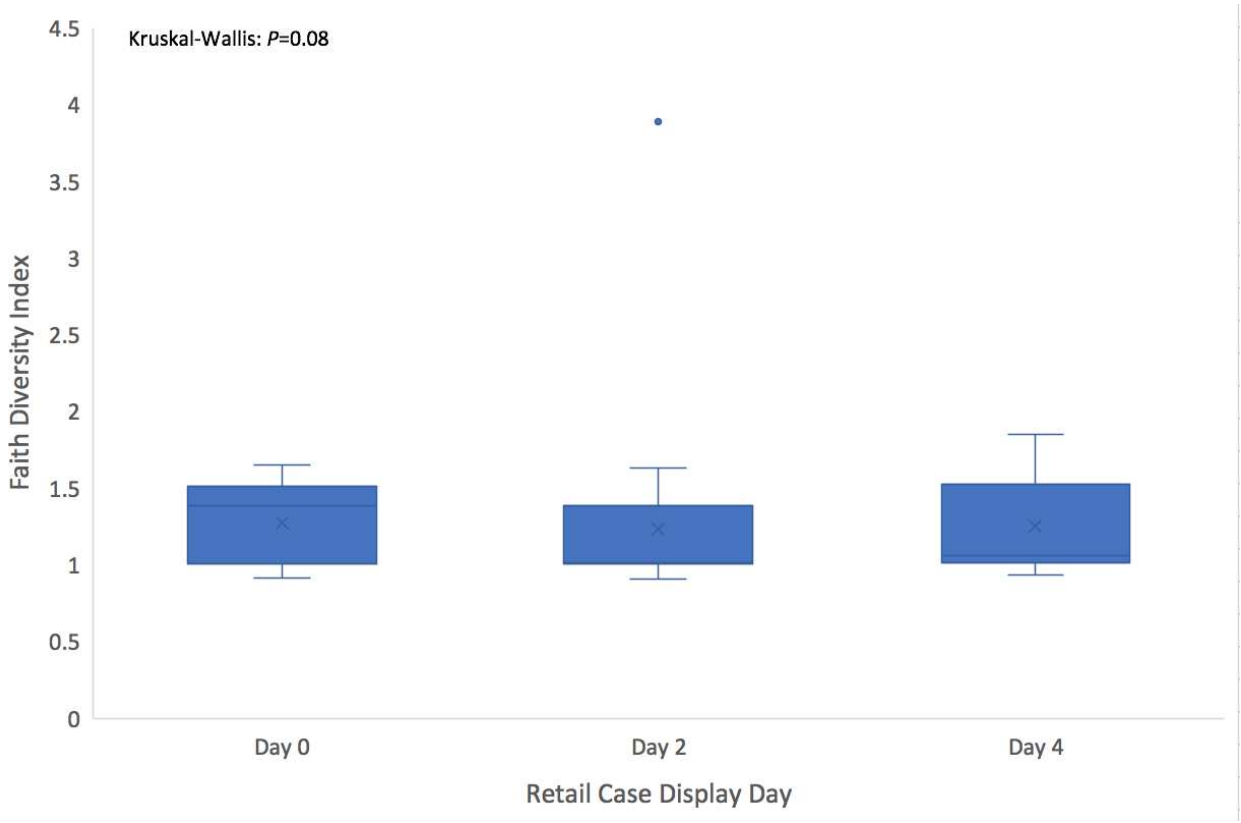

Figure 3.3. Alpha diversity of samples assessed using the Faith Phylogenetic Diversity index. Differences in retail case display day samples determined by Kruskal-Wallis test.

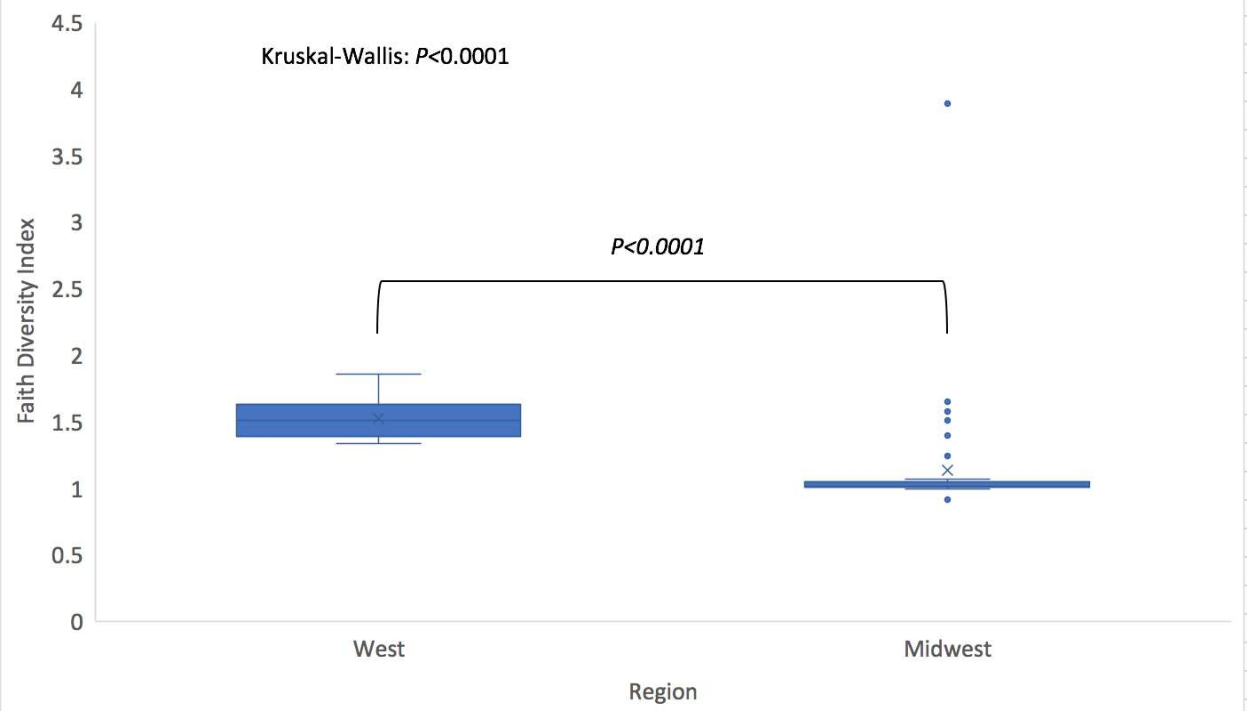

Figure 3.4. Differences in alpha diversity of samples observed using the Faith Phylogenetic Diversity index. Differences in region determined by pairwise comparison using the Wilcoxon rank sum test.

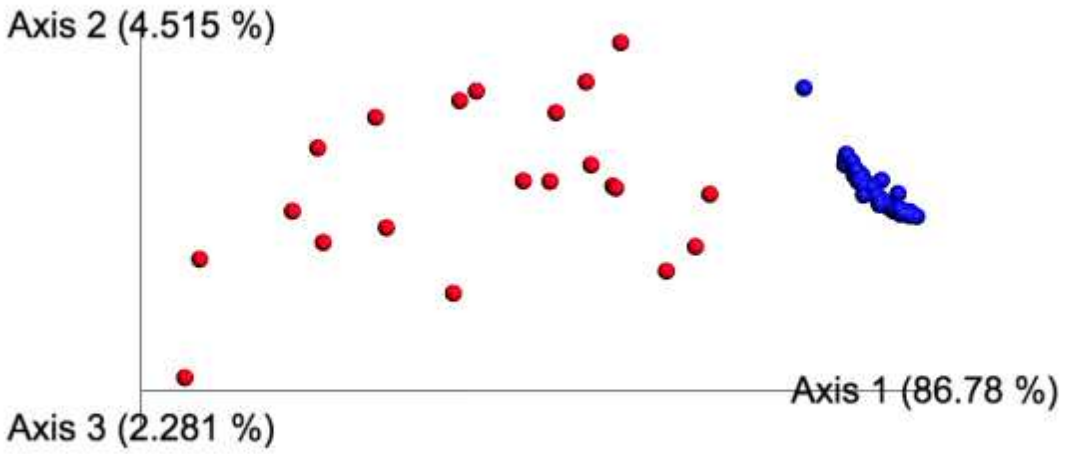

Figure 3.5. PCoA plot of beta-diversity, assessed using the weighted unifrac distance, of region, West (red) and Midwest (blue).

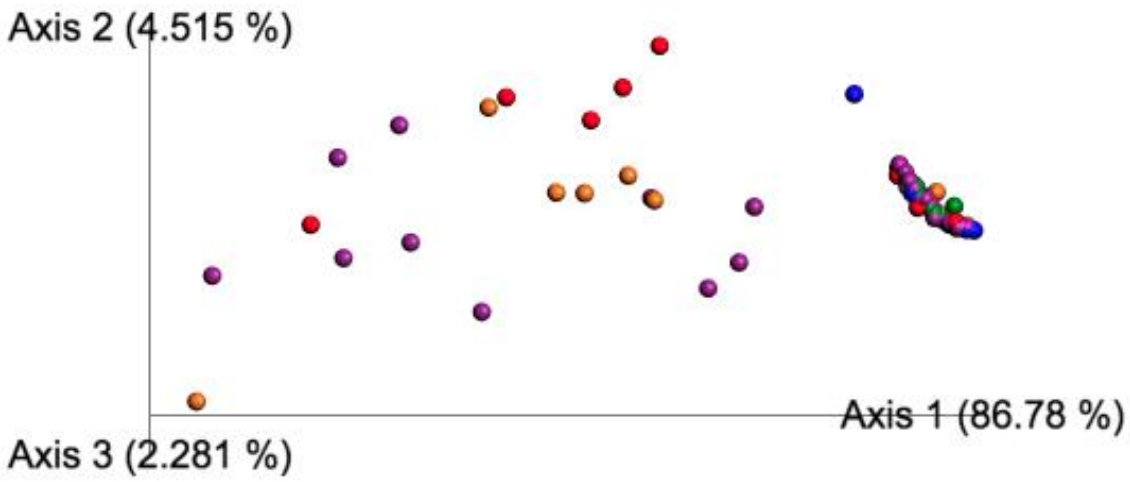

Figure 3.6. PCoA plot of beta-diversity, assessed using the weighted unifrac distance, of retail case display day, 0d (red), 1d (blue), 2d (orange), 3d (green), 4d (purple).

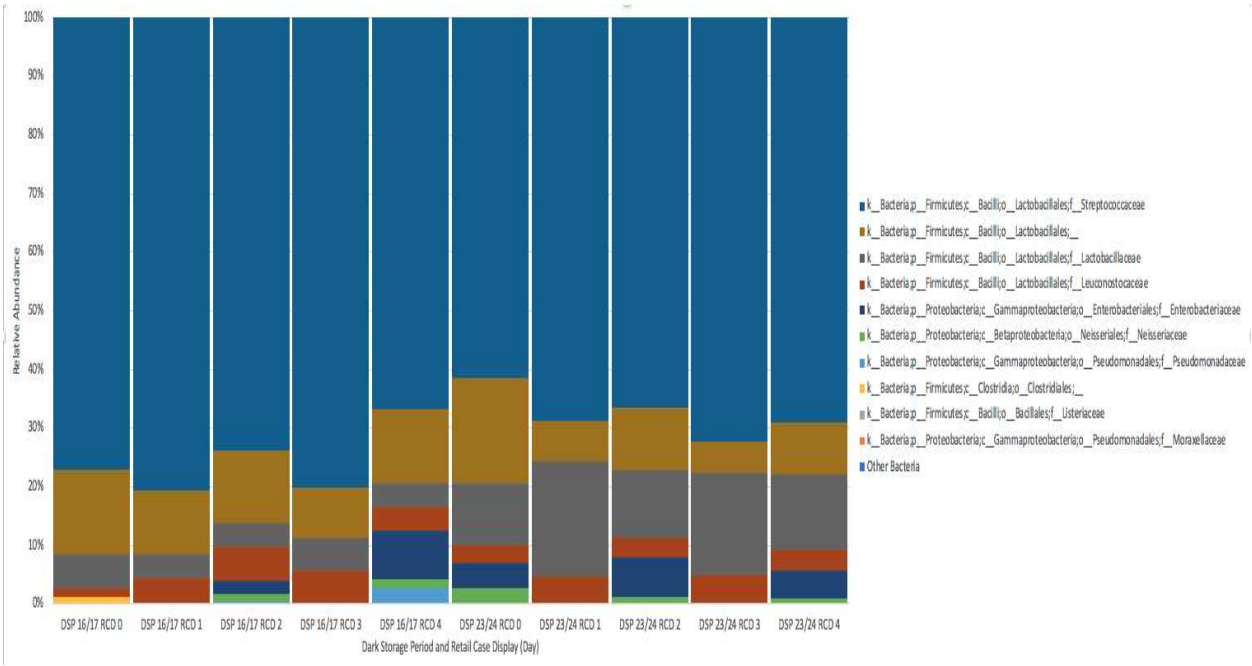

Figure 3.7. Relative abundance (%) of taxa from retail case display after either 16/17d or 23/24d dark storage. Relative abundances of samples are averaged over retail case display day (RCD) and dark storage period (DSP). Individual bars represent the average of sample relative abundance over retail case display (RCD) and dark storage period (DSP).

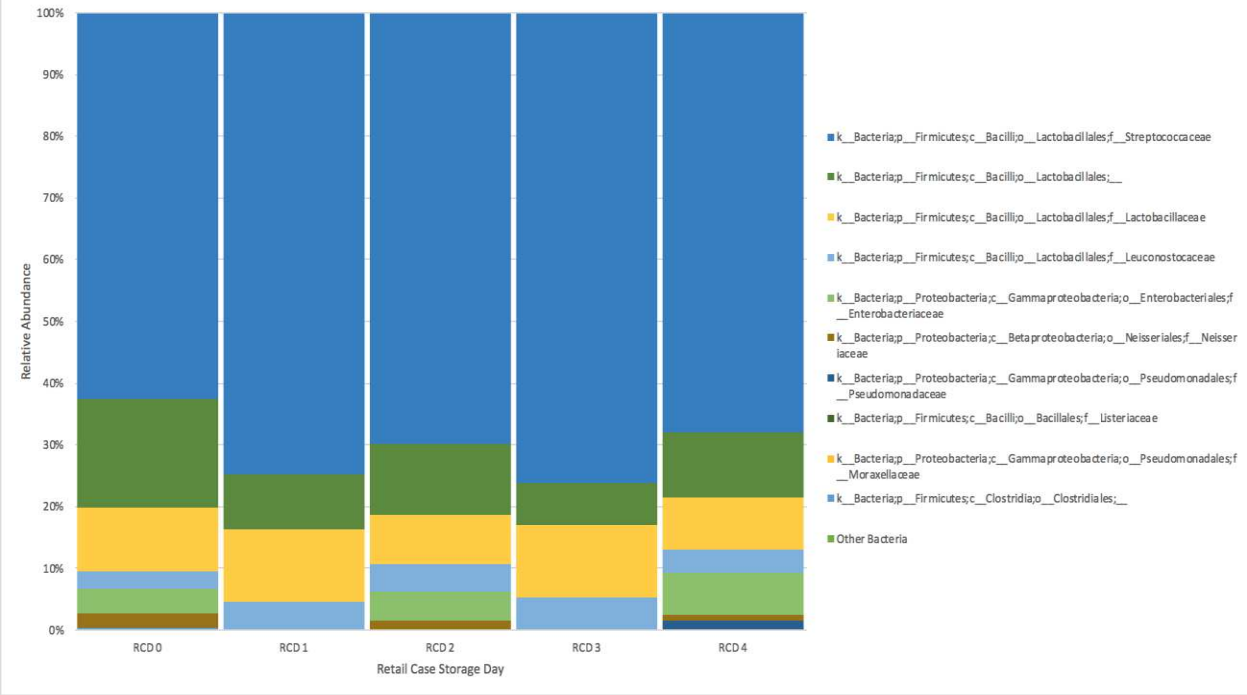

Figure 3.8. Relative abundance (%) of taxa from retail case display. Relative abundances of samples are averaged over retail case display. Individual bars represent the average of sample relative abundance over retail case display (RCD). After 4 days of retail case display, an increase in order *Pseudomonadales* from the family *Pseduomonadaceae* is noticeable.

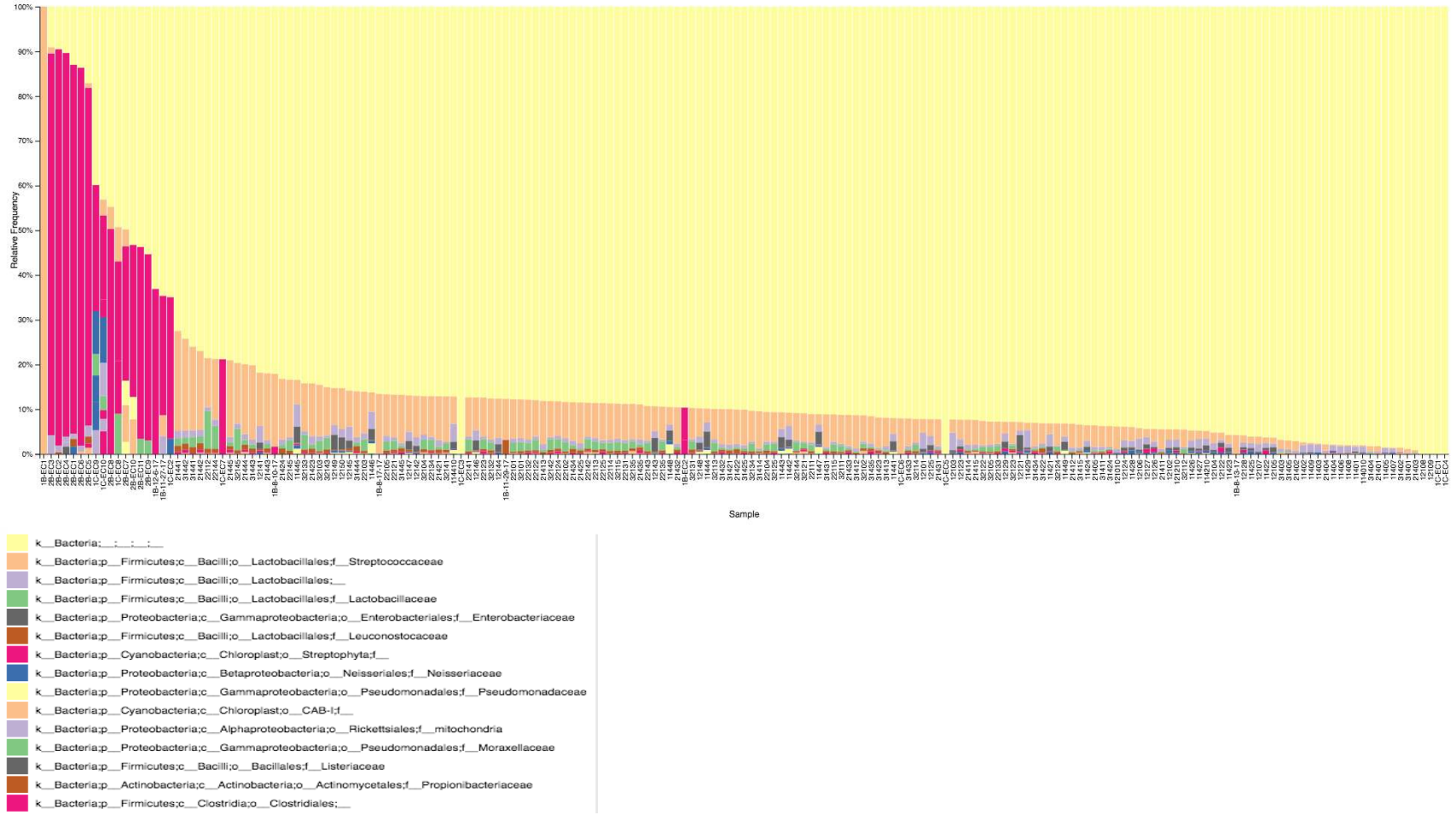

Figure 3.9. The relative abundance of taxa across prior to removal of bovine DNA from all samples. The taxa plot shown below is before rarefying these data; the first five samples were removed during rarefying. Each bar represents an individual sample. In each of the samples below, 89.4% (range of 72.1% to 98.6%) of reads, on average, were of bovine origin. The sample identifier number includes 5-digits. The first number in the sample identifier is the replication the sample is from (1, 2, or 3), the next two numbers represent the dark storage period (14= 16/17d or 21= 23/24d), the second to last number represents the retail display day (0, 1, 2, 3, 4), and the last number represents the sample number. The other samples listed are extraction controls and deionized water controls. The legend includes the top 15 taxa displayed in the taxa bar plot.

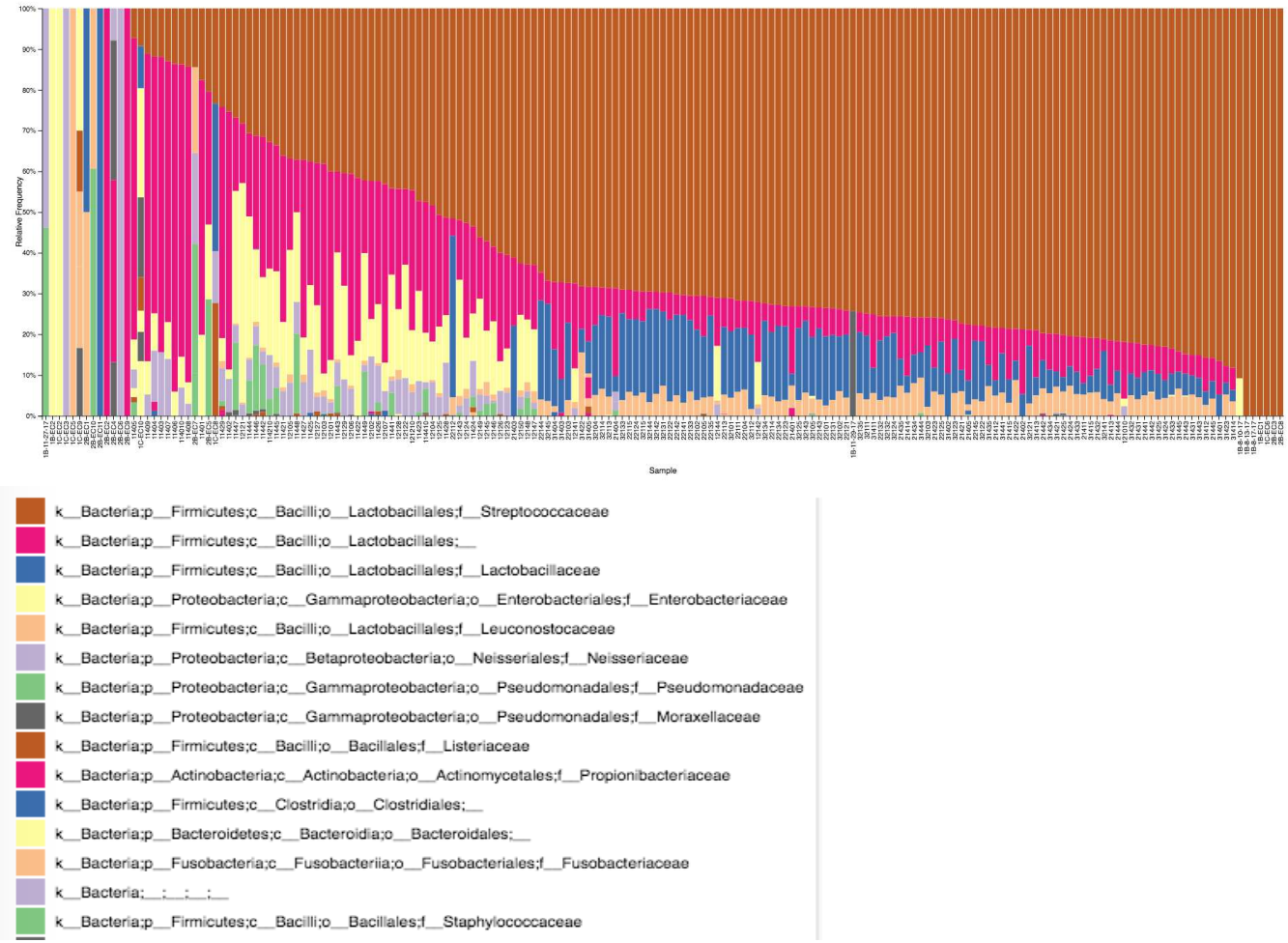

Figure 3.10. The relative abundance of taxa from all samples after removal of bovine DNA. The taxa plot shown below is before rarefying these data. Each bar represents an individual sample. The sample identifier number includes 5-digits. The first number in the sample identifier is the replication the sample is from (1, 2, or 3), the next two numbers represent the dark storage period (14= 16/17d or 21= 23/24d), the second to last number represents the retail display day (0, 1, 2, 3, 4), and the last number represents the sample number. The other samples listed are extraction controls and deionized water controls. The legend includes the top 15 taxa displayed in the taxa bar plot

Table 3.1. Back-transformed adjusted least square (LS) means of relative peak areas of ketones identified in aerobically stored ground beef for 5 days of retail case display following 16/17d and 23/24d dark storage.

	Days Dark Storage					
Retail Case Display (Days)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
Acetoin						
Day 0	156249.6[a]	44153.6	552937.4	121464.8[a]	34323.7	429836.4
Day 1	634460.3[b]	179286.4	2245233.3	285144.1[b]	81197.5	1001350.4
Day 2	1166012.1[bc]	332139.4	4093455.3	502293.6[b]	141938.6	1777502.8
Day 3	1748921.7[c]	498176.6	6139783.3	1322560.3[c]	376732.2	4643040.5
Day 4	1639464.1[c]	466853.1	5757362.3	1622761.7[c]	458561.6	5742642.3
2,3-butanedione						
Day 0	17652.3[ay]	6321.0	49296.6	16715.3[az]	5985.5	46679.5
Day 1	55946.2[by]	20033.5	156237.1	35224.6[bz]	12721.9	97529.5
Day 2	102315.1[cy]	36941.7	283376.0	102497.5[cy]	36703.0	286235.4
Day 3	237285.7[dy]	85673.9	657197.6	448441.3[dz]	161917.6	1241983.2
Day 4	403586.9[dy]	145762.1	1117450.1	555255.7[dy]	198829.5	1550611.6
2,3-pentanedione						
Day 0	1636.8[ay]	827.2	3239.0	1061.5[ay]	536.4	2100.5
Day 1	1519.7[ay]	768.0	3007.2	1033.5[ay]	523.4	2040.8
Day 2	1147.0[ay]	580.4	2266.7	1328.3[ay]	671.3	2628.4
Day 3	2054.5[ay]	1039.6	4060.1	9675.0[bz]	4895.8	19119.4
Day 4	4256.4[bz]	2155.6	8404.7	8557.9[bz]	4324.8	16934.3
Unknown Ketone (Possibly 2,5 octanedione)						
Day 0	3117.8[ay]	1528.0	6361.7	2171.5[ay]	1064.2	4430.8
Day 1	3168.1[ay]	1552.7	6464.4	2280.0[ay]	1119.3	4641.6
Day 2	2165.1[ay]	1062.8	4410.4	3391.9[az]	1956.4	8145.3
Day 3	3058.3[ay]	1501.3	6229.9	24456.7[bz]	12006.2	49818.5
Day 4	9392.8[by]	4613.8	19121.8	18338.7[bz]	8987.6	37419.1

[a,b,c] LSMeans differ within columns without common superscript differ (P<0.05).
[y,z] LSMeans differ within row without common superscript differ (P<0.05).
LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit are based off a sample size of nine.

Table 3.2. Back-transformed adjusted least square (LS) means of relative peak areas of aldehydes identified in aerobically stored ground beef for 5 days of retail case display following 16/17d and 23/24d dark storage.

	Days Dark Storage					
Retail Case Display (Days)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
Hexanal						
Day 0	63207.6^{ay}	27149.5	147156.2	113600.1^{az}	48794.6	264477.1
Day 1	51479.0^{ay}	22111.6	119850.5	65807.6^{aby}	28427.9	152338.5
Day 2	30271.7^{aby}	13073.1	70096.6	51511.5^{bz}	22125.7	119926.0
Day 3	29833.6^{by}	12883.8	12883.8	325536.5^{cz}	140588.3	753790.7
Day 4	86639.4^{by}	37426.9	200562.3	288286.5^{cz}	123827.7	671171.5
Octanal*						
Day 0	172.1^{ay}	76.2	306.3	204.2^{ay}	98.1	2094.9
Day 1	179.7^{ay}	81.4	316.5	252.4^{ay}	130.2	2363.2
Day 2	208.0^{ay}	98.6	357.8	207.9^{ay}	100.7	1791.3
Day 3	146.7^{ay}	58.0	275.7	684.2^{bz}	469.4	939.4
Day 4	248.7^{ay}	127.6	410.0	783.3^{bz}	557.1	1047.9

^{a,b,c} LSMeans differ within columns without common superscript differ ($P<0.05$).

^{y,z} LSMeans differ within row without common superscript differ ($P<0.05$).

*Square-root transformation of data

LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit

Table 3.3. Back-transformed adjusted least square (LS) means of relative peak areas of volatile fatty acids identified in aerobically stored ground beef for 5 days of retail case display following 16/17d and 23/24d dark storage.

	Days Dark Storage					
Retail Case Display (Days)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
Acetic Acid						
Day 0	362612.5^{ay}	121526.8	1081954.8	1108701.4^{az}	371576.5	3308151.1
Day 1	525728.4^{ay}	176193.8	1568672.1	1304134.3^{az}	440801.5	3858310.7
Day 2	973652.8^{by}	329117.5	2880429.6	1426507.9^{ay}	478083.2	4256381.7
Day 3	1594818^{by}	539085.8	4718068.2	1920181.0^{aby}	649072.7	5680557.9
Day 4	1381486^{by}	466951.2	4087156.3	2788209.0^{bz}	934456.2	8319477.4
Propionic Acid						
Day 0	7069.8^{ay}	5316.2	9402.0	8121.0^{ay}	6106.6	10799.9
Day 1	8668.8^{ay}	6518.5	11528.5	9266.2^{ay}	6923.6	12401.5
Day 2	10432.8^{ay}	7785.9	13979.7	9339.6^{ay}	7023.0	12420.5
Day 3	11742.5^{ay}	8763.2	15734.5	13287.4^{aby}	9916.4	17804.3
Day 4	10415.3^{ay}	7782.2	13939.4	17087.3^{bz}	12848.8	22723.8
Isobutyric Acid*						
Day 0	1054.4^{a}	777.6	1373.4	1307.6^{a}	996.9	1660.4
Day 1	1178.7^{ab}	884.7	1514.7	1574.3^{ab}	1221.9	1971.3
Day 2	1742.4^{ab}	1368.5	2161.5	1603.0^{ab}	1256.8	1991.4
Day 3	1688.9^{ab}	1321.1	2101.8	1976.8^{ab}	1577.0	2421.7
Day 4	1662.4^{b}	1299.7	2069.8	2118.9^{b}	1717.7	2562.3
Butyric Acid						
Day 0	30895.9^{ay}	24350.1	39201.9	43797.0^{az}	34517.4	55570.6
Day 1	37566.6^{aby}	29607.1	47665.8	50618.4^{ay}	39645.4	64627.8
Day 2	45235.2^{aby}	35389.8	57819.4	49678.6^{ay}	39152.9	63034.0
Day 3	48874.9^{aby}	38237.4	62471.8	62651.9^{ay}	49016.4	80079.9
Day 4	43457.1^{by}	34036.9	55485.1	64543.2^{az}	50868.0	81894.7
3-Methylbutanoic Acid*						
Day 0	471.5^{a}	20.1	1516.6	481.0^{a}	22.1	1533.6
Day 1	948.4^{ab}	184.1	2306.4	912.4^{ab}	170.4	2242.8
Day 2	1387.8^{bc}	404.2	2959.6	1096.8^{bc}	252.5	2534.8
Day 3	1951.6^{c}	730.5	3760.7	1336.0^{bc}	376.5	2883.6
Day 4	1757.0^{c}	613.3	3489.2	1603.3^{c}	520.4	3279.9
Valeric Acid						

Day 0	1245.8^{ay}	890.1	1743.7	1418.6^{ay}	1013.6	1985.6
Day 1	1665.5^{aby}	1189.9	2331.1	1485.1^{ay}	1061.0	2078.6
Day 2	1342.3^{aby}	958.8	1879.2	1722.1^{ay}	1230.4	2410.4
Day 3	1848.5^{bcy}	1320.4	2587.8	3244.5^{bz}	2317.6	4542.1
Day 4	2390.2^{cy}	1707.7	3345.5	3083.6^{by}	2203.1	4316.0
Hexanoic Acid						
Day 0	2473.2^{ay}	1722.2	3551.8	2234.2^{ay}	1555.7	3208.5
Day 1	3559.8^{aby}	2478.8	5112.2	2765.2^{aby}	1926.1	3969.8
Day 2	3091.7^{by}	2153.3	4439.0	3246.7^{by}	2260.7	4662.5
Day 3	3503.3^{by}	2440.0	5030.0	6783.7^{cz}	4724.7	9739.8
Day 4	5157.6^{cy}	3592.7	7404.3	6130.8^{cy}	4269.1	8804.5

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).

^{y,z} LSMeans differ within row without common superscript differ (P<0.05).

*Square-root transformation of data

LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit

Table 3.4. Back-transformed adjusted least square (LS) means of relative peak areas of alcohols identified in aerobically stored ground beef for 5 days of retail case display following 16/17d and 23/24d dark storage days.

	Days Dark Storage					
Retail Case Display (Days)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
1-butanol						
Day 0	2626.0^{a}	1642.2	4199.2	1933.3^{a}	1209.0	3091.5
Day 1	2239.1^{a}	1400.2	3580.5	1767.1^{a}	1090.0	2865.0
Day 2	2860.7^{a}	1759.5	4651.0	2345.8^{ab}	1467.0	3751.1
Day 3	2792.2^{a}	1717.3	4539.6	5955.8^{b}	3663.2	9683.2
Day 4	3974.4^{a}	2451.4	6443.5	4201.4^{ab}	2627.4	6718.4
1-penten-3-ol						
Day 0	35205.6^{ay}	21324.4	58123.2	35488.7^{ay}	21496.0	58590.6
Day 1	36632.1^{ay}	22188.4	60477.7	28981.4^{ay}	17570.1	47804.3
Day 2	31557.0^{ay}	19118.3	52088.5	27399.8^{ay}	16596.4	45235.6
Day 3	38646.8^{ay}	23413.6	63791.2	90912.3^{by}	55079.3	150056.9
Day 4	49740.3^{ay}	30155.1	82044.7	104031.6^{bz}	63013.1	171750.8
1-pentenol						
Day 0	37773.4^{ay}	25197.1	56626.9	42194.3^{ay}	28146.0	63253.7
Day 1	39600.6^{ay}	26415.8	59365.6	31226.4^{ay}	20813.9	46847.4
Day 2	31182.1^{ay}	20773.8	46805.7	27176.3^{ay}	18128.3	40740.6
Day 3	34332.6^{ay}	22872.7	51534.7	88322.5^{bz}	58841.9	132573.2
Day 4	43444.5^{ay}	28958.2	65178.2	92509.8^{bz}	61709.6	138682.2
1-hexanol						
Day 0	5118.7^{ay}	3410.8	7681.7	6409.2^{ay}	4270.8	9618.4
Day 1	6254.8^{ay}	4167.9	9386.7	7154.4^{aby}	4753.8	10767.4
Day 2	5961.8^{aby}	3959.0	8977.7	11471.9^{bz}	7644.3	17216.0
Day 3	10363.3^{by}	6881.9	15605.8	53507.2^{cz}	35532.9	80573.6
Day 4	23315.5^{cy}	15492.0	35089.8	83227.5^{cz}	55458.5	124900.8

^{a,b,c} LSMeans differ within columns without common superscript differ (P<0.05).
^{y,z} LSMeans differ within row without common superscript differ (P<0.05).
LSMeans and LCL= Lower Confidence Limit, UCL= Upper Confidence Limit are based off a sample size of nine.

Table 3.5. Back-transformed adjusted least square (LS) means of relative peak areas of sulfones identified in aerobically stored ground beef for 5 days of retail case display after 16/17d and 23/24d dark storage days. No differences were observed, and no significant interaction occurred.

	Days Dark Storage					
Retail Case Display (Days)	16/17d	95% LCL	95% UCL	23/24d	95% LCL	95% UCL
Dimethyl sulfone						
Day 0	1805.7	780.0	4180.3	1720.4	743.1	3982.9
Day 1	1957.2	845.4	4531.2	1968.3	853.8	4537.6
Day 2	2094.9	908.6	4829.9	2113.7	913.0	4893.5
Day 3	2363.2	1025.0	5448.4	3159.3	1370.3	7284.0
Day 4	1791.3	777.0	4129.6	2929.4	1265.4	6781.9

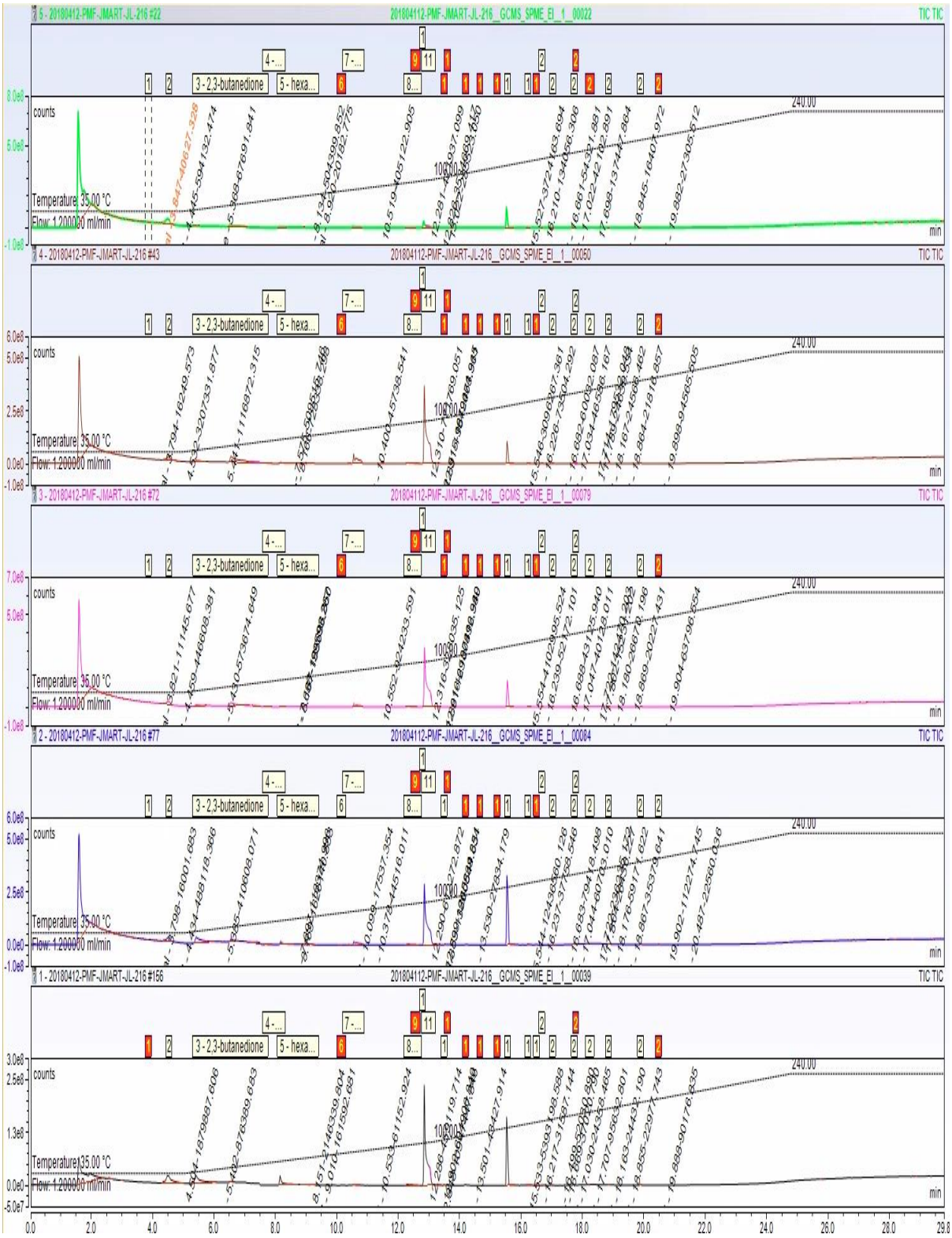

Figure 3.11 An example of chromatographs from samples during one week of retail case display.

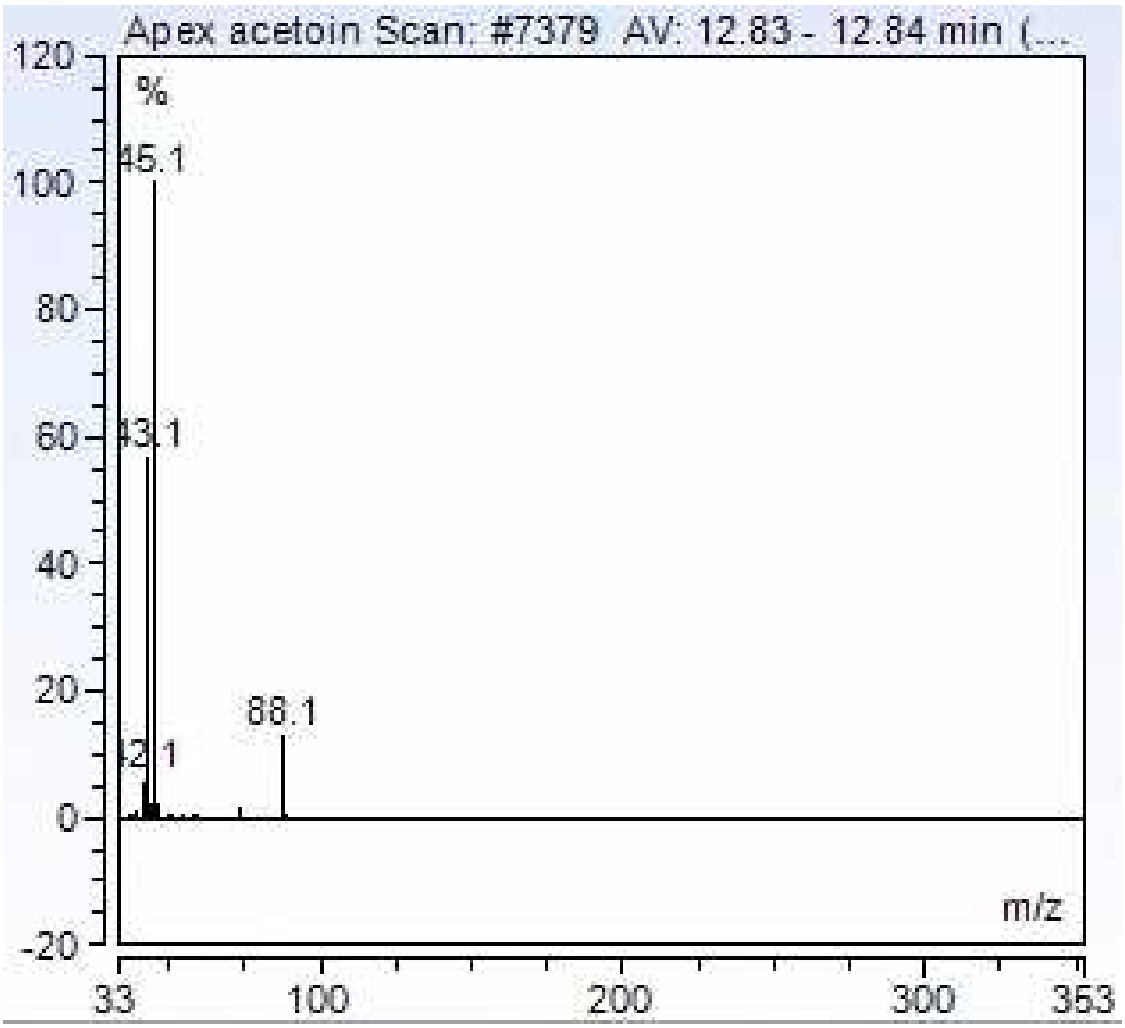

Figure 3.12 An example of a mass spectrum of *3-hydroxybutan-2-one* (Acetoin), where the mass spectrum from sample compound was compared to NIST v12 EI spectral library to identify the compound.

REFERENCES

16S Illumina Amplicon Protocol. (2018). Retrieved from http://www.earthmicrobiome.org/protocols-and-standards/16s/

Ajuyah, A. O., Fenton, T. W., Hardin, R. T., & Sim, J. S. (1993). Measuring Lipid Oxidation Volatiles in Meats. *Journal of Food Science, 58*(2), 270-273. doi:https://doi.org/10.1111/j.1365-2621.1993.tb04253.x

Alderton, A. L., Faustman, C., Liefler, D. C., & Hill, D. W. (2003). Induction of Redox Instability of Bovine Myoglobin by Adduction with 4-Hydroxy-2-nonenal. *Journal of Biochemistry, 42*(15), 4398-4405. doi:10.1021/bi0271695

AMI. (2012). Ground Beef Shelf Life Guide. In A. M. Institute (Ed.). Washington, D.C.: North American Meat Institute.

AMI/FMI. (2018). *Power of Meat: An In-Depth Look at Meat Through the Shoppers' Eyes*. Retrieved from Arlington, VA: http://www.meatconference.com/sites/default/files/books/Power_of_meat_2018.pdf

AMSA. (Revised 2012; 1991). *AMSA Meat Color Measurements Guidelines*. Retrieved from Champaign, Illinois: http://meatscience.org/docs/default-source/publications-resources/Hot-Topics/2012_12_meat_clr_guide.pdf?sfvrsn=d818b8b3_0

Anderson, M. J., & Walsh, D. C. I. (2013). PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecological Monographs, 83*(4), 557-574. doi:https://doi.org/10.1890/12-2010.1

Aryes, J. C. (1960). Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. *Journal of Food Science, 25*(1), 1-18. doi:https://doi.org/10.1111/j.1365-2621.1960.tb17930.x

Badau, F. (2016). *U.S. Beef and Pork Consumption Projected to Rebound*. Retrieved from https://www.ers.usda.gov/amber-waves/2016/september/us-beef-and-pork-consumption-projected-to-rebound/?__hstc=12316075.e72c280a7921bf0d7ab734f9822a9c39.1525219200089.1525219200090.1525219200091.1&__hssc=12316075.1.1525219200092&__hsfp=1773666937

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software, 67*(1), 1-48. doi:10.18637/jss/v067.i01

Bekhit, A. E.-D. A., Hopkins, D. L., Fahri, F. T., & Ponnampalam, E. N. (2013). Oxidative Processes in Muscle Systems and Fresh Meat: Sources, Markers, and Remedies. *Comprehensive Reviews in Food Science and Food Safety, 12*(5), 565-597. doi:https://doi.org/10.1111/1541-4337.12027

Borch, E., Kant-Muermans, M., & Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *International Journal of Food Microbiology, 33*(1), 103-120. doi:https://doi.org/10.1016/0168-1605(96)01135-X

Brooks, J. C., Alvarado, M., Stephens, T. P., Kellermeier, J. D., Tittor, A. W., Miller, M. F., & Brashears, M. M. (2008). Spoilage and safety characteristics of ground beef packaged in traditional and modified atmosphere packages. *Journal of Food Protection, 71*(2), 293-301. doi:https://doi.org/10.4315/0362-028X-71.2.293

Buzby, J. C., Wells, H. F., & Hyman, J. (2014). *The Estimated Amount, Value, and Calories of Postharvest Food Losses at the Retail and Consumer Levels in the United States*. Retrieved from https://www.ers.usda.gov/webdocs/publications/43833/43680_eib121.pdf

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. A., & Holmes, S. P. (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581-583. doi:10.1038/nmeth.3869

Campo, M. M., Nute, G. R., Hughes, S. I., Enser, M., Wood, J. D., & Richardson, R. I. (2006). Flavour perception of oxidation in beef. *Journal of Meat Science, 72*(2), 303-311. doi:https://doi.org/10.1016/j.meatsci.2005.07.015

Candenas, E., & Davies, K. J. A. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biology and Medicine, 29*(3-4), 222-230. doi:https://doi.org/10.1016/S0891-5849(00)00317-8

Caporaso, G. J., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., . . . Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Multidisciplinary Journal of Microbial Ecology, 6*(8), 1621-1624. doi:10.1038/ismej.2012.8

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., . . . Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*(5), 335-336. doi:10.1038/nmeth.f.303

Carpenter, C. E., Cornforth, D. P., & Whittier, D. (2001). Consumer preferences for beef color and packaging did not affect eating satisfaction. *Journal of Meat Science, 57*(4), 359-363. doi:https://doi.org/10.1016/S0309-1740(00)00111-X

Casaburi, A., Piombino, P., Nychas, G.-J., Vallani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. *Journal of Food Microbiology, 45*(A), 83-102. doi:https://doi.org/10.1016/j.fm.2014.02.002

Close, D. (2014). *Ground Beef Nation: The Effect of Changing Consumer Tastes and Preferences on the U.S. Cattle Industry*. Retrieved from https://web.extension.illinois.edu/oardc/downloads/52548.pdf

Cornforth, D., & Hunt, M. (2008). *Low-oxygen packaging of fresh meat with carbon monoxide: meat quality, microbiology and safety*. Retrieved from Savoy, IL: https://www.meatscience.org/docs/default-source/publications-resources/white-papers/wp_002_2008_co_map_packaging.pdf?sfvrsn=7233bbb3_4

Dave, D., & Ghaley, A. E. (2011). Meat Spoilage Mechanisms and Preservation Techniques: A Critical Review. *American Journal of Agricultural and Biological Sciences, 6*(4), 486-510. doi:10.3844/ajabssp.2011.486.510

De Filippis, F., La Storia, A., Villani, F., & Ercolini, D. (2013). Exploring the Sources of Bacterial Spoilers in Beefsteaks by Culture-Independent High-Throughput Sequencing. *PLoS One, 8*(7), 1-10. doi:10.1371/journal.pone.0070222

De Man, J., Rogosa, M., & Sharpe, E. (1960). A Medium for the Cultivation of Lactobacilli. *Journal of Applied Microbiology, 23*(1), 130-135. doi:https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

DeSantis, T., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E., Keller, K., . . . Andersen, G. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Journal of Applied and Environmental Microbiology, 72*(7), 5069-5072. doi:10.1128/AEM.03006-05

Difco & BBL Manual - Manual of Microbiological Culture Media. (2009). (2nd ed.). Sparks, MD: Becton, Dickinson and Company.

Doulgeraki, A., Paramithiotis, S., Kagkli, D., & Nychas, G. (2010). Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions. *Journal of Food Microbiology, 27*(8), 1028-1034. doi:10.1016/j.fm.2010.07.004

Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G.-J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. *International Journal of Food Microbiology, 157*(2), 130-141. doi:https://doi.org/10.1016/j.ijfoodmicro.2012.05.020

E-18, A. C. (1968). *Manual on Sensory Testing Methods*: American Society for Testing and Materials.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods, 56*(3), 297-314. doi:https://doi.org/10.1016/j.mimet.2003.11.006

Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A., . . . Villani, F. (2011). Monitoring of Microbial Metabolites and Bacterial Diversity in Beef Stored under Different Packaging Conditions. *Journal of Applied and Environmental Microbiology, 77*(20), 7372-7381. doi:10.1128/AEM.05521-11

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and Psychrotrophic Bacteria from Meat and Their Spoilage Potential In Vitro and in Beef. *Journal of Applied and Environmental Microbiology, 75*(7), 1990-2001. doi:10.1128/AEM.02762-08

Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the Spoilage-Related Microbiota of Beef during Refrigerated Storage under Different Packaging

Conditions. *Journal of Applied and Environmental Microbiology, 72*(7), 4663-4671. doi:10.1128/AEM.00468-06

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation, 61*(1), 1-10. doi:https://doi.org/10.1016/0006-3207(92)91201-3

FAO. (2015). *70 Years of FAO (1945-2015)*. Rome, Italy: FAO.

Faustman, C., & Cassens, R. G. (1990). The biochemical basis for discoloration in fresh meat: A review. *Journal of Muscle Foods, 1*(3), 217-243. doi:10.1111/j.1745-4573.1990.tb00366.x

Fernandez, J., Perez-Alvarez, J. A., & Fernandez-Lopez, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat. *Journal of Food Chemistry, 59*(3), 345-353. doi:https://doi.org/10.1016/S0308-8146(96)00114-8

Fernández-López, J., Zhi, N., Aleson-Carbonell, L., Pérez-Alvarez, J. A., & Kuri, V. (2005). Antioxidant and antibacterial activities of natural extracts: application in beef meatballs. *Journal of Meat Science, 69*(3), 371-380. doi: 10.1016/j.meatsci.2004.08.004

Fierer, N., & Lennon, J. T. (2011). The Generation and Maintenance of Diversity in Microbial Communities. *American Journal of Botany, 98*(3), 439-448. doi:10.3732/ajb.1000498

Fox, J., & Weisberg, S. (2011). An {R} Companion to Applied Regression, Second Edition. Thousand Oaks, CA: Sage. Retrieved from http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Gill, C. O., & Newton, K. G. (1982). Effect of Lactic Acid Concentration on Growth on Meat of Gram-Negative Psychrotrophs from a Meatworks. *Journal of Applied and Environmental Microbiology, 43*(2), 284-288. Retrieved from https://aem.asm.org/content/aem/43/2/284.full.pdf.

Giménez, A., Ares, F., & Ares, G. (2012). Sensory shelf-life estimation: A review of current methodological approaches. *Food Research International, 49*, 311-325. doi:10.1016/j.foodres.2012.07.008

Gram, L., Ravn, L., Rasch, M., Bartholin Bruhn, J., Christensen, A. B., & Givskov, M. (2002). Food spoilage - interactions between food spoilage bacteria. *International Journal of Food Microbiology, 78*(1-2), 79-97. doi:https://doi.org/10.1016/S0168-1605(02)00233-7

Greene, B. E., Hsin, I.-M., & Zipser, M. W. (1971). Retardation of Oxidative Color Changes in Raw Ground Beef. *Journal of Food Science, 36*, 940-942. doi:https://doi.org/10.1111/j.1365-2621.1971.tb15564.x

Guillén-Sans, R., & Guzmán-Chozas, M. (1998). The Thiobarbituric Acid (TBA) Reaction in Foods: A review. *Critical Reviews in Food Science and Nutrition, 38*(4), 315-350. doi:10.1080/10408699891274228

Gustavsson, J., Cederberg, C., Sonesson, U., van Otterdijk, R., & Meybeck, A. (2011). *Global Food Losses and Food Waste - Extent, causes and prevention*. Rome, Italy: FAO Retrieved from http://www.fao.org/docrep/014/mb060e/mb060e00.pdf

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing. *Genomics, 107*(1), 1-8. doi:https://doi.org/10.1016/j.ygeno.2015.11.003

Hough, G. (2010). *Sensory Shelf Life Estimation of Food Products*. Boca Raton, FL: CRC Press.

Huis in't Veld, J. H. J. (1996). Microbial and biochemical spoilage of foods: an overview. *International Journal of Food Microbiology, 33*(1), 1-18. doi:https://doi.org/10.1016/0168-1605(96)01139-7

Insausti, K., Beriain, M. J., Gorraiz, C., & Purroy, A. (2006). Volatile Compounds of Raw Beef from 5 Local Spanish Cattle Breeds Stored Under Modified Atmosphere. *Journal of Food Science, 67*(4), 1580-1589. doi:https://doi.org/10.1111/j.1365-2621.2002.tb10325.x

Issanchou, S. (1996). Consumer Expectations and Perceptions of Meat and Meat Product Quality. *Journal of Meat Science, 43*(1), 5-19. doi:https://doi.org/10.1016/0309-1740(96)00051-4

Jay, J. M., Vilai, J. P., & Hughes, M. E. (2003). Profile and activity of the bacterial biota of ground beef held from freshness to spoilage at 5-7 degrees Celcius. *International Journal of Food Microbiology, 81*(2), 105-111. doi:https://doi.org/10.1016/S0168-1605(02)00189-7

Jovel, J., Patterson, J., Wang, W., Hotte, N., O"Keefe, S., Mitchel, T., . . . Wong, G. K. S. (2016). Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. *Frontiers in Microbiology, 7*, 1-17. doi:10.3389/fmicb.2016.00459

Jääskeläinen, E., Johansson, P., Kostiainen, O., Nieminen, T., Schmidt, G., Somervuo, P., . . . Björkroth, J. (2013). Significance of Heme-Based Respiration in Meat Spoilage Caused by *Leuconostoc gasicomitatum Journal of Applied & Environmental Microbiology, 79*(4), 1078-1085. doi:10.1128/AEM.02943-12

Kaur, M., Shang, H., Tamplin, M., Ross, T., & Bowman, J. P. (2017). Culture-dependent and culture-independent assessment of spoilage community growth on VP lamb meat from packaging to past end of shelf-life. *Journal of Food Microbiology, 68*, 71-80. doi:https://doi.org/10.1016/j.fm.2017.06.015

Khan, M. I., Jo, C., & Tariq, M. R. (2015). Meat flavor precursors and factors influencing flavor precursors - A systematic review. *Journal of Meat Science, 110*, 278-284. doi:https://doi.org/10.1016/j.meatsci.2015.08.002

Kosowska, M., Majcher, M. A., & Fortuna, T. (2017). Volatile compounds in meat and meat products. *Journal of Food Science and Technology, 37*(1), 1-7. doi:http://dx.doi.org/10.1590/1678-457x.08416

Koutsoumanis, K., Stamatiou, A., Skandamis, P., & Nychas, G.-J. E. (2006). Development of a Microbial Model for the Combined Effect of Temperature and pH on Spoilage of Ground Meat, and Validation of the Model under Dynamic Temperature Control. *Journal of Applied and Environmental Microbiology, 72*(1), 124-134. doi:doi: 10.1128/AEM.72.1.124-134.2006

Koutsoumanis, K. P., Stamatiou, A. P., Drosinos, E. H., & Nychas, G.-J. E. (2008). Control of spoilage microorganisms in minced pork by a self-developed modified atmosphere induced by the respiratory activity of meat microflora. *Food Microbiology, 25*(7), 915-921. doi:https://doi.org/10.1016/j.fm.2008.05.006

Kuznetsova, A., PB, B., & RHB, C. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software, 82*(13), 1-26. Retrieved from http:// doi.org /10.18637/jss.v082.i13. doi:10.18637/jss.v082.i13

Lenth, R. (2018). emmeans: Estimated Marginal Means, aka Least-Squares Means. Retrieved from https://CRAN.R-project.org/package=emmeans

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software, 69*(1), 1-33. doi:10.18637/jss.v069.i01

Livingston, D., & Brown, W. (1981). The chemistry of myoglobin and its reaction. *Journal of Food Technology, 35*(5), 244-252.

Lozupone, C., & Knight, R. (2005). UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Journal of Applied and Environmental Microbiology, 71*(12), 8228-8235. doi:10.1128/AEM.71.12.8228–8235.2005

Luo, C., Tsementzi, D., Kyrpides, N., Read, T., & Konstantinidis, K. T. (2012). Direct Comparisons of Illumina vs. Roche 454 Sequencing Technologies on the Same Microbial Community DNA Samples. *PLOS One, 7*(2), 1-12. doi:https://doi.org/10.1371/journal.pone.0030087

Lyte, J. M., Legako, J. F., Martin, J. N., Thompson, L., Surowiec, K., & Brooks, J. C. (2016). Volatile compound characterization of modified atmosphere packaged ground beef held under temperature abuse. *Journal of Food Control, 59*, 1-6. doi:https://doi.org/10.1016/j.foodcont.2015.04.041

Mancini, R. A., & Hunt, M. C. (2005). Current research in meat color. *Journal of Meat Science, 71*(1), 100-121. doi:https://doi.org/10.1016/j.meatsci.2005.03.003

McMeekin, T. A., & Ross, T. (1996). Shelf life prediction: status and future possibilities. *International Journal of Food Microbiology, 33*(1), 65-83. doi:https://doi.org/10.1016/0168-1605(96)01138-5

McMillin, K. W. (2008). Where is MAP Going? A review and future potential of modified atmosphere packaging for meat. *Journal of Meat Science, 80*(1), 43-65. doi:https://doi.org/10.1016/j.meatsci.2008.05.028

McMillin, K. W. (2017). Advancements in meat packaging. *Journal of Meat Science, 132*, 153-162. doi:https://doi.org/10.1016/j.meatsci.2017.04.015

Min, B., & Ahn, D. U. (2005). Mechanism of Lipid Peroxidation in Meat and Meat Products- A review. *Food Science and Biotechnology, 14*(1). Retrieved from http://www.public.iastate.edu/~duahn/publication/pdf%20files/125_FSB-14-152.pdf.

Nychas, G.-J. E., Drosinos, E. H., & Board, R. G. (1998). *The Microbiology of Meat and Poultry* (A. R. Davies & R. J. Board Eds. 1 ed.). London; New York: Blackie Academic & Professional.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Journal of Meat Science, 78*(1-2), 77-89. doi:10.1016/j.meatsci.2007.06.020

OECD/FAO. (2016). *"Meat", in OECD-FAO Agricultural Outlook 2016-2025*. Paris: OECD Publishing.

Oswell, N. J., Thippareddi, H., & Pegg, R. B. (2018). Practical use of natural antioxidants in meat products in the U.S.: A review. *Journal of Meat Science, 145*, 469-479. doi:https://doi.org/10.1016/j.meatsci.2018.07.020

Pace, N. R. (2009). Mapping the Tree of Life: Progress and Prospects. *Microbiology and Molecular Biology Reviews, 73*(4), 565-576. doi:10.1128/MMBR.00033-09

Pennacchia, C., Ercolini, D., & Villani, F. (2011). Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack. *Journal of Food Microbiology, 28*(1), 84-93. doi:10.1016/j.fm.2010.08.010

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & Team, R. C. (2017). nlme: Linear and Nonlinear Mixed effects Models (Version R package version 3.1-131). Retrieved from https://CRAN.R-project.org/package=nlme

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 - Approximately Maximum-Likelihood Trees for Large Alignments. *PLos ONE, 5*(3), e9490. doi:https://doi.org/10.1371/journal.pone.0009490

Pérez, R., Rojo, M., González, G., & De Lorenzo, C. (2008). Solid-phase microextraction for the determination of volatile compounds in the spoilage of raw ground beef. *Journal of AOAC International, 91*(6), 1409-1415. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/19202802.

Rappe, M. S., & Giovannoni, S. J. (2003). The Uncultured Microbial Majority. *Annual Review of Microbiology, 57*, 369-394. doi:10.1146/annurev.micro.57.030502.090759

Sherbeck, J. A., Wulf, D. M., Morgan, J. B., Tatum, J. D., Smith, G. C., & Williams, S. N. (1995). Dietary Supplementation of Vitamin E to Feedlot Cattle Affects Beef Retail Display Properties. *Journal of Food Science, 60*(2). doi:https://doi.org/10.1111/j.1365-2621.1995.tb05648.x

Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*(3), 591-610. doi:https://doi.org/10.1016/j.fmrre.2005.04.002

Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., & Ercolini, D. (2016). Overlap of Spoilage-Associated Microbiota between Meat and the Meat Processing Environment in Small-Scale and Large-Scale Retail Distributions. *Journal of Applied and Environmental Microbiology, 82*(13). doi:10.1128/AEM.00793-16

Stewart, E. J. (2012). Growing Uncluturable Bacteria. *Journal of Bacteriology, 194*(16), 4151-4160. doi:10.1128/JB.00345-12

Suman, S. P., & Joseph, P. (2013). Myoglobin Chemistry and Meat Color. *Annual Review of Food Science and Technology, 4*, 79-99. doi:https://doi.org/10.1146/annurev-food-030212-182623

Säde, E., Penttinen, K., Björkroth, J., & Hultman, J. (2017). Exploring lot-to-lot variation in spoilage bacterial communities on commercial modifiedatmosphere packaged beef. *Journal of Food Microbiology, 62*, 147-152. doi:https://doi.org/10.1016/j.fm.2016.10.004

Sørheim, O., Nissen, H., & Nesbakken, T. (1999). The storage life of beef and pork packaged in an atmosphere with low carbon monoxide and high carbon dioxide. *Journal of Meat Science, 52*(2), 157-164. doi:https://doi.org/10.1016/S0309-1740(98)00163-6

Tapp III, W. N., Yancey, J. W. S., & Apple, J. K. (2011). How is the instrumental color of meat measured? *Journal of Meat Science, 89*(1), 1-5. doi:https://doi.org/10.1016/j.meatsci.2010.11.021

Team, R. C. (2017). R: A language and environment for statistical computing. (Version 3.4.3). Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/

Ursell, L. K., Metcalf, J. L., Wegener Parfrey, L., & Knight, R. (2012). Defining the Human Microbiome. *Nutrition Reviews, 70*(1), S38-S44. doi:10.1111/j.1753-4887.2012.00493.x

Vazquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience, 2*(1), 16. doi:10.1186/2047-217X-2-16

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., . . . Knight, R. (2015). Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal

Internal Transcribed Spacer Marker Gene primers for Microbial Community Surveys. *American Society for Microbiology, 1*(1), 1-10. doi:10.1128/mSystems.00009-15

Westcott, P., & Hansen, J. (2016). *USDA Agricultural Projections to 2025*. Retrieved from https://www.ers.usda.gov/publications/pub-details/?pubid=37818

Wickham, H. (2011). *The Split-Apply-Combine Strategy for Data Analysis*. Retrieved from http://www.jstatsoft.org/v40/i01/

Witte, V. C., Krause, G. F., & Bailey, M. E. (1970). A new extraction method for determining 2-thiobarbituric acid values of pork and beef during storage. *Journal of Food Science, 35*(5), 582-585. doi:10.1111/j.1365-2621.1970.tb04815.x

Xiao, Y., & Yang, S. (2017). The Retail Chain Design for Perishable Food: The Case of Price Strategy and Shelf Space Allocation. *Sustainability, 9*(1), 1-11. doi:10.3390/su9010012

Youssef , N. H., Couger, M. B., McCully, A. L., Criado, A. E. G., & Elshahed, M. S. (2015). Assessing the global phylum level diversity within the bacterial domain: A review. *Journal of Advanced Research, 6*(3), 269-282. doi:https://doi.org/10.1016/j.jare.2014.10.005