THESIS

INVESTIGATING THE IMPACT OF THE MICROBIOME ON BEEF STEAK COLOR

STABILITY

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ABSTRACT

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Meat color is the most influential characteristic for consumer purchasing decisions. In fact, consumer discrimination of discolored beef results in approximately $3.73 billion/year lost in revenue in the US. Interestingly, most often these products are not yet microbially spoiled, leading to unnecessary food waste. Complicating matters, different muscles originating from the same carcass discolor at different rates. Several studies have investigated the physiochemical, enzymatic, and intrinsic muscle properties of muscles with differing color stabilities such as color stabile beef longissimus lumborum (LL) and color labile psoas major (PM). However, the impact of microbial growth on the meat color stability has not been investigated yet. Therefore, the objective of this study was to characterize the microbial populations and their biochemical parameters of color labile and color stabile beef muscle cuts during aerobic retail display. Paired USDA Select LL and PM (n = 5) were collected from a local abattoir and aged for 14 days in darkness under vacuum at 3°C. After aging, the muscles were fabricated into 2.54-cm thick steaks and packaged aerobically in a foam tray wrapped with polyvinyl chloride film. Steaks were then placed into an open faced multi-decked retail display case for 7 days at 4°C ± 1°C. Each day, beginning day of fabrication, steaks were evaluated for visual color, percentage discoloration, instrumental color, water activity, pH, metmyoglobin reducing activity, microbial levels   as

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determined by using culture-dependent methods (aerobic plate counts, lactic acid bacteria plate counts, *Pseudomonas* spp. plate counts and Enterobacteriaceae plate counts), and 16S rRNA bacterial gene sequencing (microbiome). Visual color was darker ($P < 0.05$) for PM than LL for all days, and percentage discoloration was greater ($P < 0.05$) for PM than LL from the second daif retail display. Color stability (determined by MRA) was greater ($P > 0.05$) in LL compared to PM for all days. The pH was greater ($P < 0.05$) for PM for the first 5 days of display compared to LL. However, water activity was the same ($P > 0.05$) for both muscles across all display days. Microbiological analyses revealed that aerobic plate counts, and lactic acid bacteria plate count were greater ($P < 0.05$) for PM starting on day 1 of display compared to LL. The *Pseudomonas* spp. plate counts were similar ($P > 0.05$) until day 2, after which PM was greater ($P < 0.05$) than LL and remained greater for the remaining days. Moreover, the 16S rRNA gene sequencing showed no differences ($P > 0.05$) in the alpha or beta diversities of the microbial communities between muscles. The results indicated that PM has less color stability and a greater amount of microbial growth than LL during retail display. Despite the increased number of bacteria on PM earlier during display, the microbiome analyses showed no major differences in the microbial communities between the muscles on the same display day. These data may suggest that microbial metabolic pathways, evidenced by faster microbial growth on PM compared to LL, may be a bigger contributor to color stability differences than the microbial community composition. Further work establishing these metabolic differences is needed to understand the biochemical interaction between the microbiota and the beef steaks.

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CHAPTER 1

REVIEW OF LITERATURE

The single most important factor for beef sales is the color of the meat. Beef consumers select their product based on its bright-cherry red color. Other factors that have an impact on the palatability of the steak such as intramuscular fat and lean texture for whole muscle cuts and fat percentage or primal origin of ground beef, which all can impact the flavor, tenderness and juiciness of the product are largely ignored while purchasing decisions are made. The role of meat color stability cannot be overstated and has profound economic impacts on the beef industry. Additionally, the ability of meat to remain at the initial bright cherry-red color varies greatly between muscles of the same carcass. Although the causes behind this have been investigated from many aspects, the role of the natural microflora present on the meat during the retail display period, to our knowledge, has not been reported. Therefore, the objective of this literature review is to provide an overview of meat color chemistry, meat color stability and the factors impacting it, as well as the review meat spoilage during retail display, and the application of 16S rRNA gene sequencing as it relates to beef shelf-life.

1.1 Importance of Meat Color

Beef is the second most produced and consumed protein in the US behind poultry, with 54.6 pounds produced per US citizen in 2018 (ERS, USDA, 2021). It is well documented that meat color is the primary driver in consumer meat purchasing decisions, regardless of type of cut, ground or whole muscle (Forbes et al., 1974; Killinger et al., 2004; Mwashiuya et al., 2018).

Additionally, consumers begin to detect surface discoloration at low percentage coverage and begin to completely reject the product at twenty percent coverage creating an extremely large economic impact on the beef industry and contributing to waste (O'Keeffe and Hood, 1982; Feuz et al., 2020). It is worth noting, the surface color of beef is not indicative of the wholesomeness, or spoilage, although that is the commonly held concern for many meat purchasers (Mancini and Hunt, 2005). Moreover, consumers are less likely to discern differences in the actual redness and lightness of meat, until it has been in retail display for several days. Further, several attempts have been made to extend the color shelf life of beef. Despite these efforts, attempts to change to more preservative packaging types such as vacuum packaging and rigid tray gas flush modified atmospheric packaging (MAP) has not been successful on a large retail scale for red meat. This is primarily because when beef has not been exposed to oxygen it is a deep purple color, not the bright cherry-red that consumers are familiar with. Even though rigid tray MAP packaging has been able to provide a bright cherry-red color, the packaging takes specialized equipment, uses expensive elemental gasses, and provides logistical challenges because of its increased display case footprint and may mask microbial spoilage.

1.2 Meat Color Chemistry

1.2.1 Introduction to Myoglobin and Meat Color Chemistry

Meat color is mainly determined by the redox state of the protein, myoglobin, present within the muscle. This protein consisting of a heme ring, and globin polypeptide chain of 153 amino acids, is responsible for the transport and storage of oxygen in the live tissue to be used in aerobic cellular respiration (Ramanathan and Mancini, 2018). Specifically, myoglobin is monomeric tertiary structured protein with a heme ring. The iron atom contained within the

heme ring has six available electrons for binding, and four are in strong covalent bonds with nitrogen molecules, while the fifth is connected to the distal histidine (Suman and Joseph, 2013). The ordination of those bonds determines which light spectra it will absorb, thus the color observed with the eye (Suman and Nair, 2017). These can change in resonance dependent upon the ligand that is bound to the iron. In meat, the role of myoglobin is essentially physiologically defunct, however, it still performs the innate task of binding ligands such as oxygen, carbon dioxide, carbon monoxide, water, and other small molecules, and it is those bound ligands that determine the color of meat (Mancini and Hunt, 2005). Of note, in the current US meat production system, the blood oxygen transport protein hemoglobin is almost completely removed from the muscles during the exsanguination process, and therefore, contributes very little to overall color of meat (Livingston and Brown, 1981). Another protein, cytochrome C, a protein essential to the functioning of the electron transport chain also can contribute to the color of meat, although its impact is minimal (Suman and Joseph, 2013).

1.2.2 Myoglobin Reduction-Oxidation Reaction

Understanding the role of myoglobin in the meat matrix is essential to understanding meat color. A heme iron is centrally located within the myoglobin (Mancini and Hunt, 2005; Suman and Joseph, 2013). This structure contains an iron atom, that in conjunction with the distal histidine amino acid, has the ability to bind with ligands, and these bound ligands are the determinant of the color of meat (Mancini and Hunt, 2005). Myoglobin that is not exposed to oxygen or bound with another ligand is in the deoxymyoglobin form, which produces a purplish color. When postmortem muscle is first exposed to oxygen, the myoglobin begins the process of oxygenation. Prior to this, the myoglobin located in the postmortem muscle is in an anaerobic environment, at the presence of oxygen, the iron atom, is in the ferrous state, and remains so

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after the myoglobin molecule has bound oxygen. During this process, the conformation of the conjugated bonds of iron to the pyrrole ring of myoglobin changes, and the distal histidine interacts with the diatomic oxygen resulting in a change of the absorbance of light (Suman and Joseph, 2013). The process produces the bright cherry-red color known as oxymyoglobin. However, endogenous biochemical reactions remove the diatomic oxygen from the myoglobin molecule (Mancini and Hunt, 2005). When this occurs, the iron atom is oxidized, and the myoglobin absorbs a brownish color known as the metmyoglobin pigment. Next, through a series of enzymatic processes, the iron atom in the metmyoglobin is reduced, returning to the ferrous state. At this stage, the iron will be reduced and return to deoxymyoglobin, then may be oxygenated, returning to oxymyoglobin which absorbs purple light spectra. This process is continually ongoing in postmortem muscle. However, after a period of time, the ability of the endogenous systems to reduce the ferric iron becomes defunct, leading to the permanent formation of metmyoglobin in the muscle responsible for meat discoloration.

1.3 Intrinsic Factors Influencing Meat Color Stability

1.3.1 Impacts of pH on Meat Color and Color Stability

Living muscle has a pH value of 7.2, and through the early postmortem period, the absence of the circulatory system results in a switch from aerobic cellular respiration to anaerobic energy production (England et al., 2017). Production of hydrogen ions (H^+), by hydrolysis of adenosine triphosphate (ATP) by ATPase activity, lowers the postmortem muscle pH to a range of 5.4 – 5.8 in normal conditions (England et al., 2017). The meat within these parameters tends to be of normal meat color. However, a pH greater than 5.8 most often causes the Dark, Firm, and Dry (DFD) condition resulting in a darker colored lean because the meat absorbs a greater amount of light spectra instead of reflecting (Ijaz et al., 2020). Moreover, this

higher pH is more suitable for microbial growth, and the microbial shelf-life of these products is shorter compared to Red, Firm, and Non-exudative (RFN) meat, which is at an average meat pH range (Newton and Gill, 1981). Dark, Firm and Dry meat may also have increased color stability. Zhu and Brewer (1998) reported that in porcine longissimus dorsi chops with DFD had greater metmyoglobin reducing activity, than either RFN or pale, soft, and exudative (PSE) meat. This could likely be attributed to the increased pH resulting in less slowing of endogenous enzymatic systems responsible for the metmyoglobin reducing activity (MRA). Contrarily, when pH becomes too low, such as less than 5.4, the water holding capacity of the meat is significantly reduced. This results in a higher amount of free water on the surface of meat, which scatters more light wavelengths, and results in a paler, or lighter color than meat found within the normal pH ranges, often referred to as Pale, Soft, Exudative (PSE) meat. Additionally, lower pH meat has slower endogenous enzymatic systems. This is due to the less than optimal pH for the muscle proteins, resulting in decreased the color stability, which is readily observed in porcine (Zhu and Brewer, 1998).

1.3.2 Mitochondrial Functionality and its Impact on Meat Color and Stability

The mitochondria in meat are still functional in postmortem muscle, although they decrease in functionality as postmortem time increases (Tang et al., 2005). During this period, the mitochondria continue to utilize oxygen, albeit decreasing as time increases, leading to faster bloom times (Mancini and Ramanathan, 2014). The change from aerobic to anaerobic cellular respiration immediately postmortem results in a decrease in the innate antioxidant abilities within the cell, leading to an increase in free radicals or other pro-oxidants (Crimi and Esposti, 2011). In color labile muscles, the decrease in color stability may be partially attributed to the degradation of the mitochondria (Mancini and Ramanathan, 2014). Moreover, when the mitochondrial

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membranes are broken postmortem, the release of mitochondrial lipids, which are prone to lipid oxidation, increases the oxidation of myoglobin, which may result in greater metmyoglobin formation. Intact mitochondria compete with myoglobin for oxygen (Mancini and Ramanathan, 2014). When this occurs, the myoglobin is more likely to be reduced, thus, the color pigment is more stable over time (Ramanathan et al., 2010). However, too much mitochondrial activity postmortem, as seen in color labile muscles, also increases the rate of free radicals, increasing the oxidation rate of proteins and lipids (Joseph et al., 2012).

One mechanism by which mitochondria influence color stability is through mitochondria-mediated metmyoglobin reducing activity (M-MRA). During this process, enzymatic processes occurring in the mitochondria produce the reducing equivalent NADH and electrons, and both can reduce metmyoglobin (Echevarne et al., 1990; Joseph et al., 2012). Production of reducing equivalents and electrons will continue as long as mitochondria remain functional. Though, in color labile muscles, the duration of mitochondrial health is shorter (Yu et al., 2020). Without these, the intrinsic ability of muscles to reduce metmyoglobin is decreased significantly, engendering an increase in permanent metmyoglobin formation.

Mitochondrial organization also plays a vital role in meat color. Mitochondria are double membraned organelles with an outer membrane consisting of a phospholipid bilayer that is comprised of over 50% unsaturated fatty acids (Casares et al., 2019). As the mitochondria lose their functionality, or become degraded, unsaturated fatty acids are more prone to oxidation by free radicals or auto-oxidation, and produce aldehydes and ketones (Faustman et al., 2010). Unfavorable organic compounds such as aldehydes can bind to the distal histidine in myoglobin, inducing a conformational change, that prevents the iron atom from binding ligands, resulting in decreased color stability (Faustman et al., 2010). Additionally, oxidation can produce organic

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compounds which may prevent enzymes involved in metabolic pathways from properly producing reducing equivalents or electrons. This can also adversely affect the color stability of beef, by failing to provide necessary mechanisms to reduce myoglobin.

In the US, it is not unusual for wholesale cuts of beef to be vacuum packaged for 14 to 21 days (aging) before it is fabricated into retail cuts. Although this process increases the palatability traits such as flavor, tenderness, and juiciness, the aging period has deleterious effects on the color stability of meat (Mancini and Ramanathan, 2014). The time spent aging increases mitochondrial degradation and death, but initial meat color increases in color intensity with increased aging time (Mancini and Ramanathan, 2014). In fact, English et al. (2016) reported that aging meat for longer than 14 days significantly increased the redness of the bloomed color but decreased the color stability of beef steaks upon being stored in aerobic packaging for retail display.

1.3.3 Muscle Fiber Type Impact on Meat Color and Stability

Muscle fiber composition differences between muscles have been extensively investigated in relation to meat color and color stability during retail display. In general, the composition of muscle fibers has profound effects on the tenderness and juiciness of meat. Additionally, muscle fiber composition has an impact on the color stability of beef steaks. Muscle fibers are broken into two main categories, type I and IIX, and each have their own subcategories within. In the color labile muscles such as the beef masseter, psoas major, and diaphragm, muscle fiber composition is skewed heavily to the type I (red) fibers (Hunt and Hedrick, 1977). Type I fibers are characterized by having increased myoglobin, mitochondria, and lipid content, due to the physiological usage of these muscles (Picard and Gagaoua, 2020). Additionally, type I fibers have a higher a* (redness) value during initial retail display periods,

compared to type IIX fibers, but fail to maintain that redness, and exhibit more rapid permanent metmyoglobin formation (Wood et al., 2004). The decrease in color stability may be attributable to the increase in mitochondria and lipids. When comparing the color stable beef longissimus lumborum (LL) to the color labile psoas major (PM), Mancini et al. (2018) reported that the psoas major had greater lipid oxidation, a lower MRA, and greater percentage of metmyoglobin compared to longissimus lumborum, after 24 hours of simulated retail display. This finding further supports the co-linear relationship between lipid oxidation and myoglobin oxidation.

Color stabile muscles such as the beef longissimus dorsi contain a higher concentration of type IIb muscle fibers (Hunt and Hedrick, 1977). As a result, there are fewer mitochondria and less lipids present, and oxidation rates decrease. As evidenced by Mancini et al. (2018), although the PM has a greater mitochondria content, the mitochondria in the LL remain functional for a longer period of time. Potentially, the more precipitous decline in functional mitochondria in the type I fibers could be due to the increased oxygen consumption of these muscles. The increased oxygen consumption generates more free radicals in color labile postmortem muscles, resulting in greater oxidation. Increased functional mitochondria also contribute to the increase in MRA, which in turn increases the color stability of these muscles. These two theories are in contrast with one another because increased oxygen in meat can result in more oxymyoglobin formation and brighter red colors, but too much can greatly increase oxidation thus decreasing the color stability.

1.3.4 Animal Feeding Practices Impact on Meat Color Stability

Animal feeding practices also have significant impacts on the bloom color and the overall color stability in beef steaks. The two main methods of finishing cattle for slaughter in the US are concentrated grain feeding operations and grass finishing systems. Nearly all cattle

raised in the US spend the first several months of their lives on forage-based feeding systems, however, for the final 120 days of life, most beef cattle in the US are finished using a highly formulated grain-based diet in concentrated feeding operations (CAFOs), whereas a small number are finished in forage fed systems involving hay or access to pastureland (grass-finished). The differences in feeding practices have a significant impact on meat color. Crouse et al. (1984) reported that the longissimus muscle from grass-fed heifers bloomed less and remained a darker red color during the subsequent retail display period, compared to the longissimus of the grain-fed heifers. Similar results were reported by Yang et al. (2002). Furthermore, the color stability of the beef steaks resulting from grass-finished beef is higher (Yang et al., 2002), and the authors speculated that this is due to increased presence of antioxidants in the diet of these animals. Antioxidants such as α tocopherol (Vitamin E) are highly abundant in forage, and animals on a forage-based diet have increased poly-unsaturated fatty acids (Yang et al., 2002). The increase in poly-unsaturated fatty acids without antioxidant increase would lead to significant oxidative stress, therefore, the increased antioxidant in muscles is an adaptation of these cattle that also improves the color stability during retail display.

A plethora of research has been conducted to understand the impacts of α tocopherol supplementation on beef color and color stability. Alpha-tocopherol (vitamin E) is a natural antioxidant that works by electron scavenging, meaning that when a vitamin E comes in contact with a free radical, it will take the extra electron and oxidize the free radical. In beef, the supplementation of vitamin E in high levels during the final stages of feeding prior to harvest, has demonstrated its ability to improve color stability in beef steaks throughout display (Crouse et al., 1984; Sherbeck et al., 1995). Sherbeck et al. (1995) reported that beef T-bone steaks from crossbred steers fed high dosages of dl-alpha-tocopherol acetate had significantly less percentage

discoloration and greater visual lean color panel values at the beginning of display compared to the non-supplemented T-bone steaks, for both the longissimus lumborum and psoas major muscles. Also, thiobarbituric values (indicating lipid oxidation) from the same steaks were lower for the steaks from the vitamin E group, at day 7 of display, further demonstrating the ability of vitamin E to work as an antioxidant in when used as a feed additive. Despite this knowledge, feeding vitamin E in large quantities to feedlot cattle remains an uncommon practice due to the lack of economic incentive (Ramanathan et al., 2021).

1.4 Relationship Between Bacterial Growth and Meat Color

Cattle hides are a major source of the microorganisms associated with meat spoilage (Gill and Newton, 1978). These microorganisms are primarily mesophiles and psychrotropic bacteria, although the mesophiles contribute less to the spoilage of meat because meat is stored and displayed at low temperatures (2° - 5°C). Additionally, the conditions of storage have significant impacts on the growth of bacteria. Use of oxygen impermeable vacuum bags in the US beef supply chain is ubiquitous. Vacuum bags help to extend the shelf-life and the process of leaving meat stored in these bags helps to increase desirable quality attributes including flavor and tenderness through the aging process. However, as discussed earlier, long term storage (aging) can have deleterious impacts on meat color and color stability.

The usage of vacuum bags creates an anaerobic environment which benefits the growth of spoilage organisms belonging to the lactic acid bacteria group and *Enterobacteriaceae* family, as these are facultative anaerobes (Gill and Newton, 1978). The low partial oxygen pressures have a bacteriostatic effect on oxygen-dependent spoilage microflora, such as species of *Pseudomonas*. Moreover, using modified atmospheric packaging (MAP), also impacts growth of spoilage microflora. The leading form of MAP in the US is the use of carbon monoxide at 0.4%

inclusion with other inert gases, mostly nitrogen. CO MAP occurs predominantly in the form of "motherbagging" or where a traditional overwrap package is used and placed into a barrier bag and flushed with CO and other gases (Hunt et al., 2004). This packaging technique allows for a a traditional looking package to be displayed when removed from the motherbag. This packaging technique increases the color stability of meat because the iron atom in the heme ring of myoglobin has a strong affinity with CO and produces a bright red color (Hunt et al., 2004). Furthermore, CO packaging creates an anaerobic environment while it is still bagged, limiting the growth of *Pseudomonas* spp. and other strict aerobic bacteria, until they are displayed for sale (Hunt et al., 2004; Brooks et al., 2008).

The role of the microbial spoilage community as a whole ecosystem on beef color has not been identified, to the best of our knowledge. Similarly, few studies have been conducted to investigate the impact of single strains of microorganisms on fresh beef steak lean color. Bala et al. (1977) investigated the impact of *Pseudomonas fragi* ATCC 4973 on beef longissimus lumborum color. For this study, sterile meat was used, and only one inoculum was evlauated. The authors reported that after 10 days in aerobic packaging at 1°C ± 1°C, *Pseudomonas fragi* ATCC 4973 decreased the redness of steaks, and the inoculated steaks had greater concentration of the secondary oxidation product malondialdehyde, most likely due to the lipolytic activity of *Pseudomonas fragi* (Bala et al., 1977). Moreover, the authors concluded that the inoculated steaks had a greater amount of metmyoglobin beginning at day 10, and less oxymyoglobin beginning at day 10 of the 20-day study compared to the control (Bala et al., 1977). Interestingly, O'Keefe and Hood (1982), reported that the salable timeframe for beef longissimus lumborum is only 6.6 days, thus the impacts of this specific microorganism may not be seen during a typical 1–5-day retail display setting.

Another study, Leisner et al. (1995), investigating the impact of lactic acid bacteria on meat sensory attributes, reported that beef steaks stored aerobically had little or no impact on the visual color scores as indicated by a trained sensory color panel. Moreover, the authors further noted that there was more of an effect on color by how long the meat was aged in vacuum packaging prior to fabrication versus the species-specific strains the meat was initially inoculated with. However, these investigators did find that overall shelf-life was decreased most drastically in samples that were inoculated with *Carnobacterium maltaromicus* LV17 that were stored for six weeks in vacuum packaging then displayed aerobically. Finally, the authors noted that with three of the four strains on lactic acid bacteria used, surface greening occurred, but no objective or statistical methods were used to determine the significance (Leisner et al., 1995).

1.5 Basics of Bacterial Growth

Bacteria are single-celled, highly specialized organisms, that are ubiquitous in nature. The past 120 years have seen drastic improvements in meat processing hygiene and the safety of meat. Increased safety measures targeted at pathogenic bacteria have also been effective at improving the shelf-life of meat (Jamilah et al., 2008). Ubiquitous in the US beef supply chain is the use of organic acids to curb the growth of bacteria (Mani-Lopez at al., 2012). This works by decreasing the surface pH of meat products, thus creating a hostile environment for bacteria to grow on meat, essentially, extending the time before the bacteria enter a rapid growth phase (Mani-Lopez et al., 2012).

Bacterial growth is characterized into four phases. First is the lag phase, which is best described as having a growth rate of zero, and the stagnation is thought to be the result of bacteria adapting to new environmental conditions (Maier and Pepper, 2015). The length of the lag phase is variable and dependent upon bacterial species, nutritional density, temperature, and

specific growth requirements. It is the primary goal of food scientists to prolong the lag phase for both food safety and quality (Baranyi and Roberts, 1994). Once the bacteria in the lag phase have completed one full generation of doubling, they are into phase two of the theoretical growth curve, the exponential phase. In this phase, bacteria are doubling at their maximum rate, and reproducing in number in a logarithmic fashion (Maier and Pepper, 2015). During this phase, bacteria are consuming the most carbon from the growing medium and producing the most cell metabolites (Maier and Pepper, 2015). However, once the bacteria have utilized a majority of available nutrients, they begin to reach critical mass. This phase of critical mass is known as the stationary phase. Although some increase in numbers may still occur, bacteria are performing a greater amount of carbon recycling from dead bacterial cells (Maier and Pepper, 2015). Additionally, the cytotoxic compounds are beginning to accumulate to levels that are lethal to the bacteria, thus leading into the final phase of microbial growth, the death phase. A typical death phase sees a net decrease in viable cells. The lack of nutrients and other environmental factors decrease bacterial growth much below the exponential phase (Maier and Pepper, 2015). Bacterial cell growth in food matrices is less organoleptically apparent compared with technical bacterial growth mediums from which these growth phases are based on; therefore, capturing the entire growth pattern in food matrices takes additional time, and should be repeated multiple times in order to have representative data (Baranyi and Roberts, 1994).

As previously mentioned, the initial bacterial community on fresh meat is quite diverse. Despite this, as time postmortem increases, the most adaptable bacteria most suited to thriving on meat during refrigerated storage begin to outcompete bacteria less suited for the environment. Other variables can also contribute to this growth but may affect the final microbial community population ratios (Koutsoumanis et al., 2006). For example, the temperature at which meat is

being stored has a significant impact on the growth of bacteria. *Pseudomonas*, which is a predominant spoilage bacterium of aerobically stored meat products, shows significant growth rate changes with each degree Celsius of temperature change. Giannuzzi et al. (1998) reported that aerobically stored meat inoculated with *Pseudomonas* spp. had a mathematically modeled growth curve lag phase duration of 11.961 days at 0°C, but drastically decreased to 5.232 days at 4°C, which is the storage temperature meat is normally held. This temperature interaction held constant for other bacterial species tested including lactic acid bacteria and members of the *Enterobacteriaceae* family (Giannuzzi et al., 1998). Zhang et al. (2011) reported similar results with naturally occurring *Pseudomonas* spp. found on beef steaks.

The packaging type used to store beef also has an impact on the microorganisms present. Vacuum packaging, which creates an anaerobic environment within the package, helps to reduce the growth of aerobic spoilage organisms (Pennacchia et al., 2011). Additionally, beef stored in vacuum packaging had an increase in lactic acid producing bacteria (Kaur, et al., 2017). In fact, after extended time in the anaerobic environment, Kaur et al. (2017) reported a predomination of *Carnobacterium*. This LAB constituted 29 to 97% of total bacteria detected using 16S rRNA gene sequencing in Australian beef that was vacuum packaged for 26 weeks (Kaur et al., 2017). Although, aerobic packaging methods are the traditional method for displaying beef at retail it results in much more rapid spoilage compared to modified atmospheric and vacuum packaging techniques (Pennacchia, et al., 2011). Specifically, species of *Pseudomonas* become predominant in aerobically packaged beef (Pennacchia, et al., 2011), which have deleterious effects on beef shelf-life including slime and putrid odor formation (Jackson et al., 1997). The use of modified atmospheric packaging (MAP), namely carbon monoxide gas, has been demonstrated to reduce

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the growth of bacteria during retail display and extend fresh beef shelf-life (Hunt et al., 2004; Brooks et al., 2008, Rogers et al., 2014).

Another determinant of bacterial growth rate is pH. A good example of this is the increased microbial growth on DFD beef, which has higher pH compared to normal beef. The higher pH allows for growth of bacteria typically inhibited by the lower pH in RFN meat, such as *Yersinia enterocolitica*, *Enterobacter* (now *Serratia) liquefaciens*, and *Aleromonas* (now *Shewanella*) *putrefaciens* (Gill and Newton, 1979). The latter two have deleterious effects on meat quality and can induce a green pigment on the surface of meat (Gill and Newton, 1979). Furthermore, in a meat medium matrix inoculated with several strains of *Pseudomonas* spp. isolated from meat, the modeled lag time at 7°C and pH value of 5.8 was substantially longer than the lag time of the *Pseudomonas* spp. at the same temperature but at pH 6.4 and 7.0 (Lebert et al., 1998). The lag times reported were 5.2, 14.7, and 47.4 hours for pH 7.0, 6.4, and 5.8, respectively (Lebert et al., 1998). By contrast, when ground beef was utilized, proliferation of lactic acid bacteria and *Enterobacteriaceae* was not affected by the increase of pH from 5.34 to 6.13 at 5°C (Koutsoumanis et al., 2006). Moreover, previous studies have indicated that although pH affects the growth rates of these bacteria, there is no evidence to support that pH affects the spoilage microbiota composition when meat is within average meat pH ranges (Gill and Newton, 1978).

Bacterial growth is also influenced by the nutrients available for the microorganisms to use for energy production (Lambert et al., 1991). The key to understanding bacterial cell metabolism begins at the gene expression level. Bacteria are capable of changing gene expression based upon the habitat they are occupying. This change in expression can change the compounds they are able to catabolize for energy production and influences the length of their

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lag phase of growth. Meat is a carbon dense substrate that allows for growth of multiple kinds of catabolizers, and some bacteria may have synergistic relationships with each other (Lambert et al., 1991). For example, *Pseudomonas* spp. favor gene expression for catabolizing organic acids and certain amino acids but will repress genes necessary to metabolize available carbohydrates such as glucose that is found in meat, as long as there are organic acids or amino acids present (Rojo, 2010). Conversely, two other prominent classes of spoilage organisms, lactic acid bacteria and members of the *Enterobacteriaceae* family suppress gene expression to catabolize organic acids and many amino acids, and only express these genes during times of duress (Rojo, 2010). Additionally, when *Pseudomonas* spp. are rapidly dividing and catabolizing the greatest amounts of nutrients, they are contributing heavily to muscle cell proteolysis, allowing access to the carbohydrates in the more interior portion of the meat (Signorini et al., 2003; Rojo, 2010). Other classes of bacteria are then potentially granted access to more of their genetically preferred catabolites.

Understanding the preferred catabolites of these spoilage bacteria may help explain differences in the differential growth rates of these bacteria in meat during simulated retail display periods. Abraham et al. (2017) reported that in the color stable beef longissimus lumborum there is an increased presence of glycolytic compounds including fructose, glucose-6-phosphate, and pyruvic acid, compared to psoas major for all days measured during an aerobic display period. However, greater mitochondrial metabolites were present in psoas major during the initial 5 days of display, including the organic acids, succinate and citric acid. Similar, results were reported by Yu et al. (2019) using Chinese Yellow cattle. The beef psoas major has increased content of organic acids, free amino acids, and a lower carbohydrate metabolic profile (Abraham et al., 2017).

1.6 Meat Microbial Spoilage

High water activity, neutral-acidic pH, and highly available protein, lipids, and organic acids provide a highly nutritious and hospitable environment for bacteria. Meat is generally considered spoiled at 7 logs of bacterial growth (Vieira et al., 2009). The internal surfaces of healthy muscles are considered sterile, and bacteria are introduced to these surfaces through processing. The microbial community present on meat during display is majorly affected by the season, climate, geographical region, processing facility hygiene, and worker hygiene (De Filippis et al., 2013). Therefore, the initial microbial community is quite diverse but culture-dependent methods such as plating onto bacteriological culture media are only capable of culturing a small percentage of the community present. Moreover, as the bacterial community continues to develop during spoilage few bacterial families begin to emerge as the predominant bacteria present, out competing other present bacteria.

1.7 Major Meat Spoilage Organisms

Use of next generation sequencing technology has identified thousands of bacterial species present on meat. However, using the same technology has determined that these species begin to quickly dwindle in number as time progresses and meat begins to spoil (De Filippis et al., 2013). In fact, meat spoilage has been determined to be most impacted by only a select few genera: *Pseudomonas* spp., lactic acid bacteria, members of the *Enterobacteriaceae* family, and *Brochothrix thermosphacta* (much more commonly found in European studies versus North American, mostly due to lack of low oxygen MAP usage in the US) (Nychas et al., 2008; Ercolini et al., 2010; Doulgeraki et al., 2012; De Filippis et al., 2013; Hanlon et al., 2021). Additionally, members of Moraxella and Acinetobacter are commonly reported, but are thought to contribute to spoilage to a lesser extent (Nychas et al., 2008). Each of these bacteria are most

prevalent in the end stages of spoilage and contribute to negative quality attributes associated with meat that impact the desirability of the products. The next three sections will be a more in-depth review of their contributions to meat spoilage.

1.7.1 Role of Pseudomonas in Meat Spoilage

Pseudomonas spp. are gram negative, bacilli-shaped aerobic bacteria, which grow in a variety of environments, temperatures, and pH ranges. However, specific to meat, *Pseudomonas* fragi and *Pseudomonas* fluorescens are the most common species and thrive well on meat because its iron-rich environment. Moreover, heme iron is a co-factor for *Pseudomonas'* endogenous enzymes (Ercolini et al., 2010). Very little research has been conducted to determine the strain-specific presence of these bacteria, or their strain-specific impact on meat during spoilage. However, the impact of *Pseudomonas* spp. on meat spoilage is well documented. First, *Pseudomonas* spp. found in meat may have lipolytic and proteolytic enzymes (Signorini et al., 2003; Ercolini et al., 2010; Wang et al., 2021). Certain strains of *P. fragi* have demonstrated ability to hydrolyze myofibrillar proteins up to 100 kDa in size (Ercolini et al., 2010). Further, spoilage by *Pseudomonas* spp. is characterized by the formation of slime, a floral off odor, and potential greening of the surface of meat (Jackson et al., 1997). These negative effects are reached when the bacteria are at approximately 8.4 log CFU and approaching the stationary phase of their growth cycle (Zhang et al., 2011).

1.7.2 Role of Lactic Acid Bacteria in Meat Spoilage

In food manufacturing, lactic acid producing bacteria (LABs) are frequently used as a commensal bacteria. The distinct flavor that LABs provide to fermented products including dairy and dried meat sausages is due to their heterofermentative metabolism, where they utilize

available hexoses or pentoses to produce organic acids such as lactic acid, acetate, and succinate (Gänzle, 2015). Despite this commensal relationship, LAB are also predominant spoilage bacteria of fresh meat. The LAB species predominant on meat utilize the pyruvate-formate-lyase pathway to catabolize hexoses (Rojo, 2010). After the available carbohydrates are used, LAB may begin to use lactate or tricarboxylic cycle derivatives, such as succinate and acetate, and under stress LAB may use amino acids to produce energy. Each one of these metabolic pathways produce various metabolites in the form of organic compounds including ethanol, acetate, succinate, and lactic acid. Meat spoilage produced by LAB is characterized by formation of strong off odors such as "chemical", "alcohol", and "sour", and "greening" of the meat surface, and previous research has shown this to occur at approximately nine days of aerobic retail display (Egan, 1983).

1.7.3 Enterobacteriaceae Role in Meat Spoilage

Members of the *Enterobacteriaceae* family including *Hafnia alvei*, *Serratia liquifaciens*, and *Pantoea agglomerans* are routinely found in association with meat spoilage (Säde et al., 2013; Tan et al., 2014). These bacteria are typically found in lower concentrations on fresh meat compared to *Pseudomonas* and LAB but are notorious for producing odorous volatile compounds after catabolizing amino acids for carbon (Dainty et al., 1985). Lastly, some species of *Enterobacteriaceae* also contribute to the "greening" of the surface of meat (Dainty et al., 1989).

1.8 16S rRNA Gene Sequencing

1.8.1 Basics of 16S rRNA Gene Sequencing

16S rRNA gene sequencing (microbiome) utilizes the V1-V8 subregions of the bacterial ribosomal DNA, which is highly conserved in bacterial species (Diaz et al., 2012). This technique uses databases to match DNA sequences collected to that of known genomes of microorganisms and can provide identification of prokaryotic organisms to the genus and species level. Interestingly, 16S rRNA gene sequencing is not limited to what can be cultured under culture-dependent methods but provides a much more holistic view of the microbial composition present. Microbiome sequencing is a useful tool to monitor changes in microbial communities over time and provides a detailed look at what kinds of bacteria are present. 16S rRNA sequencing has been applied to several research fields including cancer, forensics, botany, digestive health, reproductive physiology, and food research. Most often, when utilized in meat research studies, this technology is investigating the microbial population changes over time such as spoilage or investigating the impacts a specific treatment has on the microbial population.

1.8.2 16S rRNA Gene Sequencing Applied to Beef Steaks

De Filippis et al. (2013) investigated the sources of microbial contamination of beef steaks displayed aerobically by sampling meat contact surfaces, and the steaks at the beginning and end of retail display. These authors concluded that spoilage bacteria are introduced to steaks during processing, but that microbial diversity quickly dwindled during retail display. In fact, the authors reported that number of phyla decreased from the start of retail display from twelve to five phyla after five days of retail display, with Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria being predominant, by the end. Also, it was reported that different muscles from the same carcass as well as same muscles from different carcasses displayed different diversity metrics, suggesting muscle-specific spoilage communities. Furthermore, Hanlon et al. (2021) did

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not find differences in relative abundance of taxa between high oxygen MAP, CO MAP or vacuum packaging. In agreement with De Filippis et al. (2021), Hanlon et al. did discover differences in beta and alpha diversity metrics dependent on the originating carcass of the samples.

1.8.3 Bacterial DNA Extraction Methodology for Meat Samples

Collecting bacteria from beef steaks for DNA extraction presents a unique challenge. First, for longitudinal shelf-life studies, samples collected during the initial days tend to have low biomass, meaning that there are not large quantities of bacteria present. In traditional culture-dependent bacterial enumeration methodologies, bacterial cells are detached from the meat surface using diluent-soaked sponges, excision of the surface, rinses, and tissue pulverization. Rossvoll et al. (2017) demonstrated that surface excision followed by tissue pulverization using stomaching or mechanical action resulted in the greatest bacterial detachment, when compared to swabbing or rinsates. However, this method results in a large quantity of host debris in the resulting liquid, which does not present an issue for traditional bacterial enumeration methods. However, beef is a high protein, high lipid substrate, and when suspended with the detached bacterial cells, both of these components interfere with microbial DNA extraction (Sajali et al., 2018; Cheema et al., 2021). Therefore, larger quantities of diluent/rinsate (30 – 100 mL) are used to help mitigate the low biomass as well as a centrifugation step. The centrifugation step separates cells from the diluent used to collect the sample, creating a cell pellet, and the supernatant may be discarded.

The use of centrifugation allows for the concentration of cells, including the host cells, and tissues from the pulverization step. Moreover, just utilizing a rinsate or swab method will not yield as many bacterial cells but will prevent excess host materials. To maximize bacterial DNA

output from low biomass samples, such as beef steaks, additional steps may be taken. First, to maximize bacterial detachment from the surface of meat, a gentle massaging technique combined with forceful shaking of excised material will reduce the amount of host material, but still allow for detachment of bacteria. This can be followed by centrifugation, creating a cell pellet that consists largely of bacteria cells, and not host organic material.

Two types of centrifugation are routinely employed in concentrating bacterial cells from high lipid products. First, is high-speed centrifugation at greater than 10,000 x g, and the other is slow speed centrifugation at 2,000 to 5,000 x g (Sun et al., 2019; Cheema et al., 2021; Stinson et al., 2021). In samples with high lipid content, such as meat or dairy, high-speed centrifugation has been the preferred method because it separates the lipids from the bacterial cells. Increased lipids present during DNA extraction can reduce extraction yield by up to 40 percent (Sajali et al., 2018). However, the increased DNA yield may be biased. Cheema et al. (2021) reported that in human breast milk, high speed centrifugation to remove fat also removes a higher abundance of gram-negative bacteria, ultimately impacting the diversity metrics of the 16S rRNA gene sequencing. Further, using inoculum in bovine milk, species of *Pseudomonas*, *Acinetobacter*, and *Psychrobacter*, were found in much lower abundance in skim milk compared to whole milk, following the same DNA extraction methods (Sun et al., 2019; Stinson et al., 2021). With low-speed centrifugation, DNA extraction is much less biased, however, with low biomass samples certain steps may need to be taken to ensure thorough extraction, including prevention of extraction inhibitors (lipids and proteins), and polymerase chain reaction inhibitors including proteins and detergents to maximize sequenced DNA output.

CHAPTER 2

UNDERSTANDING MUSCLE-SPECIFIC DISCOLORATION AND MICROBIAL SPOILAGE OF BEEF STEAKS USING 16S rRNA SEQUENCING

2.1 Introduction

In North America, about 16% of meat is lost at the distribution and consumer levels each year, primarily due to spoilage concerns, which equates to a loss of 18.6 kg per person (Gustavsson et al., 2011). The color of beef is the single most important criterion for fresh beef consumers (Forbes et al., 1974; Killinger et al., 2004; Mwashiuya et al., 2018). In fact, once beef has reached a redness (a* value) below 14.5 or has more than 20% discoloration, consumers regard this product as undesirable (O'Keeffe and Hood, 1982; Holman et al., 2017) regardless of the microbial quality. Although the color of fresh beef is not an indicator of microbial spoilage, this color discrimination is responsible for $3.73 billion lost annually (Ramanathan et al., 2022). Numerous studies have been conducted to understand the interactions between myoglobin chemistry and meat color (Mancini and Hunt, 2005). Despite this knowledge, there is still a fundamental lack of understanding as to why certain muscle cuts discolor at a more rapid pace than other muscle cuts from the same carcass. For example, the beef longissimus lumborum (LL) remains in a good color condition for more than six days in retail display, but the psoas major (PM) only remains in a color stable condition for little more than 24 h (O'Keefe & Hood, 1982). Between these two muscles, a plethora of work has been done to determine intrinsic properties of these muscles, but none have been able to define the cause of the shelf-life differential with

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certainty. Previous works have investigated the pH differences, the muscle fiber composition, and mitochondrial functionality (Hunt and Hedrick, 1977; Wood et al., 2004; Mancini and Ramanathan, 2014; Ijaz et al., 2020).

The pH of muscle has long been known to play an important role in meat color. Specifically, pH is of concern for dark cutting beef (Ashmore et al., 1972; Wulf et al., 2002). Higher pH can result in a darker colored lean, as well as provide a more conducive environment for spoilage microflora growth (Newton and Gill, 1981). Another key difference between the LL and PM is the muscle fiber composition. The LL has a greater proportion of glycolytic muscle fibers compared to PM, which has a greater proportion of oxidative muscle fibers (Hunt & Hedrick, 1977).

Another factor that determines shelf life of meat is the microbial quality. Meat provides an extremely suitable environment for bacteria because of its available nutrients and high water activity. Specifically, during the exponential phase of growth, microbial metabolites result meat in surface degradation and can result in the formation of slimes, green color pigments, and putrid odors (Nychas et al., 2008). However, meat does not typically reach these defects until bacterial levels have reached approximately 7-8 log CFUs (Vieira et al., 2009; Kamenik, 2012). Previous studies investigating the interaction of bacteria and beef color have concluded that the shelf-life of meat is limited by surface discoloration before the meat is microbially spoiled in aerobic packaging (Hunt et al., 2004; Beggan et al., 2005; Yang et al., 2016). Li et. al. (2015) reported that significant surface discoloration and microbial spoilage did not occur until after ten days of retail display on beef longissimus lumborum steaks, suggesting that there may be a correlation between beef steak color and microbial growth, due to the association of color metrics and microbial growth rates.

Utilizing culture-based methods, researchers have determined that species of Firmicutes, Proteobacteria, and Actinobacteria are the most predominant spoilage microorganisms (Nychas et al., 2008). However, 16S rRNA gene sequencing (microbiome) has allowed researchers to determine that there is a much more diverse population of bacteria on beef steaks during retail display, although their contribution to spoilage may be limited. De Fillipis et al. (2013) reported that carcasses originating from the same abattoir may have differences in their microbiome dependent upon the sampling location on the carcass. However, using 16S rRNA gene sequencing to determine if there are muscle-specific differences in the microbiome has not been undertaken before to the authors' knowledge. Further, previous work has indicated that microbial action may be impacting meat color (Bala et al., 1977; Li et al., 2015). Therefore, the objective of this study was to use a color stable muscle beef longissimus lumborum (LL) and a color labile muscle beef psoas major (PM) to evaluate the microbial community differences between steaks of these two muscles over seven days of retail display, and to determine if the microbiome is significantly impacting the color stability of beef steaks of these two muscles.

2.2 Materials and Methods

2.2.1 Meat Selection and Processing

Paired USDA Select beef striploins [*longissimus lumborum* (LL), $n = 5$] and tenderloins [*psoas major* (PM), $n = 5$], were collected from a commercial beef harvesting facility in northern Colorado. The muscles were collected from animals of similar backgrounds and age before any antimicrobial application (standard industry practice) to avoid any potential confounding factors. Samples were vacuumed packaged, transported on ice to the Colorado State University Global Food Innovation Center (Department of Animal Sciences, Fort Collins, CO), and aged at 2°C for 14 d in darkness. After aging, both muscles were removed from the bags and placed onto the

same sanitized cutting surface and were deliberately brought into contact with each other to ensure an initial homogenous microbial population on each loin. To simulate retail practices, the samples were cut without sanitation between the wholesale cuts, alternating between LL and PM loins. All wholesale cuts were fabricated into 2.54-cm thick steaks and placed onto foam trays lined with absorbent pads. Trays were subsequently wrapped in a polyvinyl chloride overwrap film (O_2 transmission = 23,250 mL x m^2 x d^{-1}, 72 gauge: Resinite Packaging Films, Borden, Inc., North Andover, MA). Wrapped packages were placed on the bottom deck of a multi-level retail display case with continuous lighting at 3°C ± 1°C (2800 lx, 1810LX4000 LED fixture; Kason, Newnan, GA; color rendering index = 84, color temperature = 4,500 K) and allowed to bloom for 1 h prior to initial analysis. Steaks were rotated every 12 h to ensure minimal differences from temperature and light intensity variations. In a pre-determined random order, one steak per loin was used each day for 7 d to assess instrumental color and physiochemical properties, and to conduct a trained panelist visual color evaluation, culture-dependent enumerations of microbial populations, and 16S rRNA DNA sequencing.

2.2.2 Instrumental Color Evaluation

Instrumental color measuring CIE lightness (L*), redness (a*) and yellowness (b*) was performed using a HunterLab MiniScan LabScan EZ4500 colorimeter (Hunter Associates Laboratory, Reston, VA), using a 2.54 cm diameter aperture, illuminant A, and 10° standard observer (AMSA, 2012). Three random locations on the light-exposed lean surface of each sample were scanned. The instrument was calibrated with white and black tiles prior to use.

2.2.3 Visual Color Evaluation

Six to eight trained panelists evaluated meat color and percent surface discoloration. A continuous lean color lexicon was adapted from AMSA, 2012, with values ranging from 1 to 8 (e.g., 1 = Extremely bright cherry red, 8 = Extremely dark red). Percent discoloration was on a continuous scale from 0 – 100%. Data were collected using order randomized surveys generated with an online survey software (Qualtrics, Provo, UT). Results for both are reported as the least square means of panelist scores per loin per day.

2.2.4 Metmyoglobin Reducing Activity

Metmyoglobin reducing activity (MRA) was performed using methods from (Zhai et al., 2019), A 5 x 5 x 1.5 cm section of each sample was removed from the interior portion of each steak and submerged for 20 min in a 0.3% sodium nitrite solution (Sigma-Aldrich, St. Louis, MO) at room temperature to produce metmyoglobin. Samples were be blotted dry, vacuum packaged, and the reflectance spectra from 400 to 700 nm was recorded immediately. The vacuum-packaged samples were incubated at 30°C for 2 h to induce the reduction of metmyoglobin, and the reflectance data were collected again. The percentage of surface metmyoglobin (pre-incubation as well as post-incubation) was calculated based on K/S ratios and according to established formulas (AMSA, 2012) and used to calculate MRA.

2.2.5 Meat pH and Water Activity

To measure pH and water activity, approximately 20 g of lean, avoiding connective tissue and external fat, from each sample were homogenized using a blender (Waring Laboratory Science, Stamford, CT). Duplicates 2.5 g portions of the meat homogenate were homogenized with 15 mL of distilled water using an immersion blender (Pro Scientific, Oxford, CT). An Orion Star A211 pH-meter (Fisher Scientific, Pittsburg, PA) was used for measuring pH. With the

remaining sample homogenate, water activity was evaluated, in duplicates, using an AquaLab 4TE water activity meter (Meter, Pullman, WA).

2.2.6 Culture Based Analysis of Microbial Population Levels

One steak per loin was evaluated for microbial populations levels at 24 h ± 1 h intervals for 7 d ($n = 10$ per d). A section of steak was aseptically excised using a sterile 16 cm^2 template and scalpel, avoiding external fat and connective tissues. The excised section of meat was placed into a filter separated sterile 24-oz bag (Whirl-Pak, Nasco, Modesta, CA) containing 50 mL of maximum recovery diluent (MRD; Neogen, Lansing, MI). Samples were gently hand massaged for 60 s followed by manual shaking 60 times (for approximately 60 s) to detach bacterial cells. A 35-mL aliquot of the rinsate was collected for 16S rRNA (microbiome) analysis. Aliquots were centrifuged (Sorvall Legend X1R, ThermoFisher Scientific, Germany) at 4,280 x g for 20 min at 4°C, the supernatant was discarded and the remaining cell pellet – consisting of both bacterial and bovine cells, – was frozen at -80°C for approximately 7 d. The remaining sample rinsate was used for enumeration of aerobic microbial populations (aerobic plate counts; APC), and populations of *Pseudomonas* spp., lactic acid bacteria, and *Enterobacteriaceae*. Specifically, sample rinsates were tenfold serially diluted in MRD, and appropriate dilutions were spread-plated, in duplicate, onto tryptic soy agar (TSA; Neogen), and *Pseudomonas* agar base (Oxoid Ltd., Wake Road, Basingstoke, Hants, U.K.) supplemented with *Pseudomonas* CFC supplement (comprised of cetrimide, fucidin, and cephalosporin; Oxoid Ltd.). A pour plate overlay method was used with Lactobacilli MRS Agar (MRS; Difco^{TM}; Becton, Dickinson and Company, Sparks, MD) for enumeration of lactic acid bacteria. For enumeration of *Enterobacteriaceae* populations, *Enterobacteriaceae* Petrifilm plates (ENT; 3M^{TM} *Enterobacteriaceae* Count Plates, St. Paul, MN) were used. Colonies were counted after incubation of plates at 25°C for 72 h (TSA

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and *Pseudomonas* agar), 25°C for 96 h (MRS agar) or 35°C for 24 h (ENT). Colony counts were converted to log CFU/cm^2.

The culture-dependent microbial enumeration data were modeled as a function of time using the Baranyi and Roberts (1994) equation, with the Microsoft Excel predictor plug-in, DMFit available from ComBase (https://www.combase.cc/index.php/en/). The prediction models characterize growth kinetics based on lag time (λ), specific growth rate (μ_{max}), beginning population size (N_0), and maximum population density (N_{max}).

2.2.7 16S rRNA Gene Sequencing

Sample preparation: Stored sample bacteria pellets were resuspended in 4 mL of phosphate buffered saline (PBS; Sigma Life Science, St. Louis, MO). Half (2 mL) was used for DNA extraction, and the remain portion was preserved by centrifuging at 13,000 x g for 3 minutes and decanting the supernatant before being placed in -80°C for long-term storage.

Bacterial DNA Extraction, Library Preparation and Sequencing

Bacterial DNA was extracted using the DNeasy PowerFood Microbial Kit (Qiagen, Germantown, MD), following the manufacturer's instructions, with minor modifications. An additional 200 µL of lysis buffer was added per sample, and samples were heated in a water bath at 65°C for 10 min, to facilitate complete cell lysis. Following mechanical cell lysis, samples were centrifuged for 15 min to ensure proper separation of cell debris and DNA. Lastly, DNA was eluted into 50 µL of elution buffer. Extracted DNA was quantified using a NanoDrop Lite UV spectrophotometer (Thermo Scientific, Pittsburg, PA). The DNA concentration was standardized in all samples to 15 – 90 ng/µL, prior to amplification. Amplification and sequencing preparation were performed according to the Earth Microbiome Project

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protocol. The DNA sequences were demultiplexed using QIIME2 and were denoised using the DADA2 pipeline. After denoising, forward reads were trimmed at 13 base pairs, and all sequences were truncated at 250 base pairs to ensure quality. QIIME2 with the SILVA taxonomical database was used to create a relative abundance feature table with taxonomy and phylogenetic tree. Chloroplasts, eukaryota, and mitochondria were removed prior to analysis. Additionally, the negative extraction controls contained less than 5,000 reads, and the positive controls did not have unexpected taxa present. Without evidence of severe contamination, the controls were removed from the data set prior to analysis. Samples with less than 4,000 sequences were also excluded, leaving 64 samples for analysis. Sample sequences ranged from 4,125 to 52,576 with an average of 16,074 sequences and 18,024 amplicon sequence variants (ASVs). Alpha diversity metrics were analyzed using a pairwise Wilcox test, and beta diversity was analyzed using the cumulative sum squared with a PERMANOVA with 9,999 permuations in the ADONIS package in R. Microbiota dispersion was analyzed using PERMDISP function in the ADONIS package in R. Finally, relative abundance was analyzed using the pairwise Wilcox test. For both diversity and relative abundance, the Benjamin Hochberg multiple comparisons test adjustment was used. For all microbiome analyses, significance was set at $\alpha = 0.05$.

2.2.8 Statistical Analysis

Water activity, pH, MRA, visual color evaluation, and culture dependent microbiology were analyzed in a split plot design. Muscles, day, and their interaction were the fixed effects. Carcass was treated as a blocking factor. Results are reported as least square means, and an analysis of variance with Tukey's correction was used to determine significance at $\alpha = 0.05$.

2.3 Results

2.3.1 Instrumental and Trained Panelist Color Evaluation

Instrumental and Trained Panelist Color Evaluation

There were no differences ($P > 0.05$) for L^* (lightness) between muscles throughout all days of display. Conversely, a^* (redness) was higher ($P < 0.05$) in PM than LL for the first two days of retail display, after which, LL was higher ($P < 0.05$) than PM. The b^* value for PM was greater ($P < 0.05$) at the beginning of display, but by day 2, the two muscles were similar. Color panelists indicated that PM was darker ($P < 0.05$; Table 2) than LL, beginning at day 0; however, lean darkness of PM did not increase throughout display. Additionally, LL did not become darker over the seven-day display period. After 24 h of simulated retail display, PM exhibited discernable surface discoloration, but was not greater ($P > 0.05$) than LL until day 3. By day 3, surface discoloration for PM was greater ($P < 0.05$) than LL with PM displaying over 15 % surface discoloration compared to less than 1 % for LL. Surface discoloration remained greater ($P < 0.05$) for PM versus LL for the remainder of the display period. Additionally, LL did not show an increase ($P > 0.05$) in percent surface discoloration throughout the study.

2.3.2 MRA, pH, Water Activity

The data for MRA, pH and water activity are presented in Table 1. The pH was greater ($P < 0.05$) in PM for the first 5 days of retail display compared to LL, after which PM and LL were similar ($P > 0.05$). On the other hand, MRA was higher ($P < 0.05$; Figure 1.) for LL than PM across all days. Overall, PM had a sharp decrease in MRA after the first day of retail display with a 33.9 % decrease from 62.7 % on day 0 to 28.8% 24 h later, and MRA remained low for PM for the next 5 days of display (Table 2). The MRA for PM remained at or below 30% from display day 1 to the end of the display duration. Water activity was greater than 0.98 for both muscles

across all days, and there were no differences ($P > 0.05$) in water activity for either muscle across all display days (Table 2).

2.3.3 Culture-Based Analysis of Microbial Population Levels

The analyses of culture-based microbial populations showed that for both APC and LAB both muscles were similar ($P > 0.05$) with less than 2.0 CFU/cm^2. With both count types, LL showed less ($P < 0.05$) microbial growth compared to PM after 1 day, and PM had higher ($P < 0.05$) APC and LAB populations than LL for days 1-7. At the conclusion of the 7 d display period APC populations were 6.6 and 5.2 log CFU/cm^2 for PM and LL, respectively, and LAB growth was slightly higher than APC with 6.9 log CFU/cm^2 for PM compared to LL at 5.6 log CFU/cm^2. Enumeration of *Pseudomonas* spp. revealed that both LL and PM were below the 0.2 log CFU/cm^2 detection limit on day 0. After 24 h of display, *Pseudomonas* spp. were detectable for PM, but for LL *Pseudomonas* spp. remained below detection limits until day 3. Further, PM had greater ($P < 0.05$) *Pseudomonas* spp. levels beginning on day 2 of display, and was greater ($P < 0.05$) until the end of the study compared with LL. On the final day, PM had 1.6 greater log CFU/cm^2 of *Pseudomonas* spp. than LL with 5.4 and 3.8 log CFU/cm^2, respectively. A majority of LL and PM samples analyzed had ENT populations that were below the microbial detection limit (< 0.2 log CFU/cm^2). Out of the 70 total samples evaluated for ENT growth, only 13 resulted in colony forming units, and at day 7, ENT counts for LL and PM were < 0.2 and 1.3 log CFU/cm^2, respectively.

Baranyi and Roberts (1994) growth kinetics were applied to the bacteria count data to determine estimated duration of the lag phase and estimated maximum rate of microbial growth during the display period (Figure 2; Table 4). From the APC, LAB count, and *Pseudomonas* count growth kinetics data, it was evident that LL had a longer lag phase duration and a greater

maximum growth rate compared to PM. Overall, PM achieved higher bacterial concentrations in less days than the LL, for APC, LAB, and *Pseudomonas* spp. counts.

2.3.4 16S rRNA Gene Sequencing

There were 1,111,678 total sequences, with 18,071 ASVs and a mean of 16,014 sequences per sample collected from 65 total samples. The most predominant phyla identified were Firmicutes and Proteobacteria. The most abundant taxon was in the family Lactobacilliceae followed by Pseudomonales.

2.3.5 Microbial Community Differences

There were no differences ($P < 0.05$; Figure 5.) for Faith's PD, Shannon's Index, or Simpson's Index between muscles on the same day. However, there were differences in display day in Faith's PD and Shannon's Index, as well as differences between the muscles, across all display days. Additionally, there were no differences ($P > 0.05$; Figure 3) in weighted UniFrac, unweighted UniFrac, or generalized UniFrac (beta diversity) using PERMANOVA between muscles on the same display day. Moreover, there were no differences ($P > 0.05$) in beta diversity in display day or muscle type when analyzed with PERMANOVA. However, PERMDISP showed differences in microbial dispersion between muscles ($P < 0.05$) and between day ($P < 0.05$) for generalized UniFrac. Still, PERMDISP analysis showed there were no differences ($P < 0.05$) in microbiota dispersion between muscles on the same day for weighted, unweighted and generalized UniFrac analyses.

2.3.6 Relative Abundance of Microbial Populations

Ten phyla were identified to make up greater than 95% of total ASVs. Of these ten phyla, there were no differences ($P > 0.05$) between muscles on the same day as shown in Figure 4.

Also, there were no differences ($P > 0.05$) in the relative abundances of microbial composition between muscles across all days, nor between display day, regardless of muscle. 36 orders constituted greater than 95 percent of the total ASVs, and between muscles on the same day.

2.4 Discussion

2.4.1 Instrumental Color and Trained Color

Holman et al. (2017) reported that consumers are significantly less likely to purchase beef with an $a*$ value of 14.5 or less. Previously, Seyfert et al. (2006) reported that it took 24 h of retail display for the PM to have a lower $a*$ value than LL; however, the authors also reported that the $a*$ value for PM did not decrease below 15 for the 8 days of retail display. In the same study, PM also had discernable discoloration at 24 h of retail display, whereas the LL did not until the fourth day. In the present study, $a*$ was greater for PM than LL initially, and similar findings have been reported by Joseph et al. (2012) and Nair et al. (2018), but similar to Seyfert et al. (2006), these other authors did not have redness values below 14.5 for any days of retail display. Although, the PM in the current experiment did not decrease to levels below the 14.5 threshold until the 5th day of simulated retail display, the PM did experience discernable discoloration within 24 d of being on display. Further, PM was at the greater than 20% discolored consumer threshold within 3 days of display meaning that by the third day, the redness, measured as $a*$ value of the steak, alone may not be as important because the meat was too discolored to be accepted by consumers. In contrast, LL in all aforementioned studies maintained relatively steady $a*$ values throughout the display periods, similar to findings in this study.

2.4.2 Metmyoglobin Reducing Activity

Metmyoglobin reducing activity is one of the primary ways that meat maintains a cherry-red color during retail display. This is accomplished by the reduction of metmyoglobin to deoxymyoglobin that can then be oxygenated to oxymyoglobin and is achieved through the production of reducing equivalents NADH produced by the mitochondria and free electrons by muscle intrinsic biochemical systems (Echevarne et al., 1990; Joseph et al., 2012). In color labile muscles, the MRA declines earlier, and the ability of the meat to return from brown to red is greatly diminished, significantly reducing the shelf-life. Conversely, in color stabile muscles the MRA remains higher for a longer duration resulting in meat that remains brighter red for longer. For this study, the color labile PM had a sharp decline in MRA after 24 hours in retail display. This finding is similar to multiple previous studies, further solidifying that PM is color labile (Joseph et al., 2012; Canto et al., 2016; Mancini et al., 2018; Ramanathan et al., 2021). In contrast to findings reported by Hood and O'Keefe (1982), the MRA for LL did not decrease steadily throughout the display period in this study but maintained its color stability until day 4 of display. This may be due to the difference in temperature of retail display conditions between the two studies with Hood and O'Keefe (1982) using slightly higher temperatures (5°C) compared to the current study (3°C). Moreover, in a study investigating the differences in the mitochondrial activity between these same two muscles, the MRA of the PM and LL behaved in a similar fashion to the current study with PM demonstrating a sharp decline in MRA after 24 h (Ramanathan et al., 2021). Joseph et al. (2012) also reported MRA was lower for PM at day 0, 5, and 9 of retail display, however, this study only collected measurements at day 0, 5, and 9, thus determining when the MRA decreased to low levels is not distinguishable.

2.4.3 Culture-Dependent Microbial Enumeration and Predicted Bacterial Growth Kinetics

Results of the culture-dependent analysis of microbial population levels during retail display showed that both the PM and LL started day 0 of display at similar levels. However, it took less than 24 h for the bacteria on the PM to begin to enter the exponential phase of the bacterial growth curve, whereas the LL experienced a much longer lag phase which was greater than 48 h, for all bacterial counts measured. Using this modeling technique helps to confirm that PM, the color labile muscle, experienced virtually no lag phase. Moreover, previous microbial modeling on beef LL has shown a lag time of only 26 h for naturally occurring *Pseudomonas* spp. at 4°C, but a 104 h lag time at 0°C (Zhang et al., 2011). The samples for the current study were held at 3°C, which re-iterates the importance of temperature parameters on bacteria growth.

Hunt et al. (2004) reported that carbon monoxide packaging, PM had nearly two times the levels of aerobic bacteria compared to LL after 7 days of simulated retail display with 1.3 and 0.7 log CFU/g, respectively. When evaluating the impacts of direct fed vitamin E on microbial growth of subsequent beef LL steaks, Chan et al. (1995) reported aerobic plate counts to be 3.5 log CFU/cm^2 at the beginning of display and reached approximately 6 log CFU/cm^2 by the 7th day of retail display. Although the authors in the latter study previously mentioned reported higher initial levels of aerobic bacteria present than in this study, by the end of their retail display period, aerobic bacteria levels on the LL were similar to the current study. Pennacchia et al. (2011) reported that beef LL had between up to 5.8 log CFU/g of *Pseudomonas* spp. before aerobic storage, but only increased by 2 logs over a 7 day aerobic storage time compared to the current study which saw an increase of nearly 4 log CFU/cm^2 over the retail display period of the same duration. Further, the authors reported that lactic acid bacteria had up to 4.5 log CFU/g of bacteria at 0 day of retail display and increased on average by 2 log CFU/g after 7 days of aerobic storage. Again, the initial bacterial counts begin much higher than the current study, but

by the 7th day of display, bacteria populations levels are similar between previous findings and this study.

Overall, PM exhibited much faster microbial spoilage which warrants more investigations into why the PM is a more suitable substrate for microbial growth. It is reasonable to expect that confounding factors including pH, glucose content, and protein isomers are influencing the faster rate of growth on the PM versus the LL (Nychas et al., 2008). Further, taxonomical families in the *Enterobacteriaceae* family have been reported to be common meat spoilage organisms, such as species of *Serratia* or *Hafnia* (Nychas et al., 2008). The results of this investigation did not indicate a contribution from this family of bacteria as they remained below detection limits for the first six days of the study. Although the literature is conflicted on the contribution of these bacteria to spoilage, they are routinely observed as part of the ephemeral spoilage organisms because they are able to produce volatile organic compounds and produce surface greening in meat Chaves-Lopez et al., 2006; Stellato et al., 2016). Li et al. (2012) reported that *Enterobacteriaceae* may have a strong correlation with color stability due to the catalysis of amino acids, although that was not observed in the present study.

2.4.4 16S rRNA Gene Sequencing

In the current study, the spoilage organism community is potentially beginning to form at day 3 of display for PM, while a similar decrease in diversity is not observed in LL (Figure 2). As characterized by the trend of lower diversity measures, and the increase in abundance of the taxa *Lactobacillales* and *Pseudomonales* from day 3 until day 6 in where they comprise close to 95% of total taxa in PM at day 6, whereas this trend is not observed in LL.

Although there are no differences in microbial diversity between muscles on the same display day, this might be attributed to the fact that these two muscles are extremely similar matrices. However, there is ample evidence supporting that PM and LL provide different forms of nutrients for the microbes (Lambert et al., 1991; Abraham et al., 2017; Yu et al., 2019; Yang et al., 2022). The data from the microbiome may not be specific enough to determine differences that are occurring at the species and strain levels because the genomes of these species and strains are nearly identical (Gwak & Rho, 2020).

The data in this study indicated that the two most abundant taxa were Firmicutes, and more specifically of the family *Lactobacillaceae*. These bacteria are heterofermenters that prefer carbohydrates as their carbon sources (Rojo, 2010). Just in this family, there are 15 genera, with some having greater than 150 species each (Salvetti et al., 2012). The third most abundant taxon present in this study was in the genus of *Pseudomonas*, which has hundreds of strains, and a myriad of metabolic pathways. For instance, *Pseudomonas aeruginosa* cannot readily utilize carbohydrates for energy requirements, but utilizes organic acids and proteins as the preferred carbon source (Rojo, 2010). This preference by *Pseudomonas* spp. results in more proteolysis, and more access to nutrients for other bacteria, which could explain why the growth rates in the PM were more rapid (Yang et al., 2022). Yu et al. (2019) reported that PM has a higher concentration of citrate, aconitate, and fumarate at 24 h postmortem, whereas LL did not exhibit any higher concentrations of aerobic respiration metabolites. Additionally, when evaluating the metabolites between the same two muscles over a seven-day simulated retail display, Abraham et. al. (2017), reported similar results. The PM had a greater amount of carbohydrate metabolites in the form of organic acids than LL, while the LL had a greater concentration of pyruvate.

The 16S rRNA gene sequencing is a well-rounded tool for investigating the microbial ecology of bacterial species during meat spoilage. However, 16S rRNA gene sequencing lacks the resolution to identify most individual organisms to the species and or strain levels (Doulgeraki et al., 2012). By further understanding the species-specific strains dominating in the spoilage process and understanding their metabolic needs in combination with understanding the differences in the habitat provided by different muscles may help to further explain the microbial impact on meat color stability.

Conclusions

This results of this study agreed with previous studies that examined the color stability differences between the beef LL and PM, with PM being less color stable and discoloring much faster than LL. Additionally, LL exhibited a slower initial bacterial growth for a greater amount of time (longer lag phase) during retail display than PM. Even though pH for PM was higher for the first five days of display, LL had a higher maximum bacterial growth rate at a lower pH than PM, suggesting that pH may not be majorly contributing to this phenomenon. Further, no major differences were observed in the microbiome between PM and LL suggesting that the microbiome difference alone may not be responsible for rapid discoloration of the PM muscle in beef. Therefore, further studies examining the intrinsic biochemical differences and its interaction with the microbiome is required to understand the muscle-specific discoloration of beef muscles.

Table 1. Least Square means with standard error of panelist lean color and percentage discoloration, CIE $L*$ (lightness), $a*$ (redness), and $b*$ (yellowness) of beef LL and PM during 7 d simulated retail display period.

	Muscle	Display Day 0	1	2	3	4	5	6
Lean Color	LL	3.19 ± 0.19^{az}	3.35 ± 0.07^{az}	3.34 ± 0.08^{az}	3.35 ± 0.14^{az}	3.62 ± 0.17^{az}	3.55 ± 0.06^{az}	3.55 ± 0.10^{az}
	PM	5.04 ± 0.28^{abx}	4.44 ± 0.26^{ax}	5.13 ± 0.25^{abx}	5.44 ± 0.30^{bx}	5.67 ± 0.27^{bx}	5.77 ± 0.18^{bx}	5.59 ± 0.22^{bx}
Percentage Discoloration	LL	0.17 ± 0.12^{az}	0.36 ± 0.22^{az}	0.66 ± 0.14^{az}	2.88 ± 1.07^{az}	2.22 ± 0.37^{az}	2.22 ± 1.11^{az}	2.41 ± 0.30^{az}
	PM	0.13 ± 0.13^{az}	2.03 ± 0.71^{az}	15.78 ± 3.87^{bx}	25.50 ± 4.13^{bx}	49.99 ± 3.02^{cx}	60.45 ± 6.50^{dx}	48.67 ± 3.40^{cx}
$L*$ value	LL	33.83 ± 0.43^{az}	33.94 ± 1.37^{az}	34.46 ± 0.42^{az}	34.38 ± 0.58^{az}	34.72 ± 1.38^{az}	34.47 ± 0.87^{az}	34.51 ± 0.62^{az}
	PM	33.14 ± 1.41^{az}	34.73 ± 1.56^{az}	33.78 ± 1.18^{az}	34.11 ± 1.48^{az}	33.35 ± 2.21^{az}	36.37 ± 1.3^{az}	34.19 ± 0.65^{az}
$a*$ value	LL	19.28 ± 0.71^{az}	19.47 ± 1.08^{az}	19.20 ± 0.27^{az}	20.21 ± 0.47^{az}	19.14 ± 0.70^{az}	19.50 ± 0.13^{az}	19.17 ± 0.27^{az}
	PM	23.68 ± 0.68^{ax}	21.33 ± 0.56^{ax}	17.18 ± 0.71^{bx}	17.54 ± 0.68^{bx}	16.34 ± 0.47^{bx}	15.23 ± 1.08^{bcx}	13.41 ± 0.62^{cx}
$b*$ value	LL	19.28 ± 0.71^{az}	19.47 ± 1.08^{az}	19.20 ± 0.27^{az}	20.21 ± 0.47^{az}	19.14 ± 0.70^{az}	19.50 ± 0.13^{az}	19.17 ± 0.27^{az}
	PM	23.68 ± 0.68^{ax}	21.33 ± 0.56^{ax}	17.18 ± 0.71^{bx}	17.54 ± 0.68^{-x}	16.34 ± 0.47^{bx}	15.23 ± 1.08^{bcx}	13.41 ± 0.62^{cx}

^{ad}Within each row, means without a common superscript letter are different ($P < 0.05$).
^{xz}Within the same measurement without a common superscript letter are different ($P < 0.05$).

Table 2. Mean values with standard error for pH, water activity (A_w), and metmyoglobin reducing activity (MRA) for beef longissimus lumborum (LL) and psoas major (PM) muscles (n = 5) over a 7 d simulated retail display (3°C).

	Muscle	Display Day						
		0	1	2	3	4	5	6
pH	LL	5.59 ± 0.05^{az}	5.59 ± 0.03^{az}	5.54 ± 0.01^{az}	5.52 ± 0.01^{az}	5.56 ± 0.02^{az}	5.59 ± 0.01^{az}	5.55 ± 0.02^{az}
	PM	5.89 ± 0.05^{ax}	5.83 ± 0.07^{abx}	5.80 ± 0.04^{abx}	5.72 ± 0.03^{abcx}	5.76 ± 0.11^{abcx}	5.60 ± 0.01^{abcz}	5.66 ± 0.05^{abcz}
A_w	LL	0.994 ± 0.001^{az}	0.993 ± 0.001^{az}	0.995 ± 0.002^{az}	0.995 ± 0.000^{az}	0.993 ± 0.000^{az}	0.994 ± 0.001^{az}	0.994 ± 0.001^{az}
	PM	0.995 ± 0.000^{az}	0.995 ± 0.001^{az}	0.996 ± 0.001^{az}	0.995 ± 0.000^{az}	0.994 ± 0.001^{az}	0.995 ± 0.000^{az}	0.994 ± 0.000^{az}
MRA	LL	87.19 ± 1.15^{abz}	93.60 ± 3.29^{az}	86.26 ± 3.16^{abz}	83.95 ± 2.64^{abz}	65.94 ± 6.07^{bz}	70.04 ± 7.25^{abz}	63.74 ± 4.68^{bz}
	PM	62.72 ± 13.65^{ax}	28.80 ± 5.98^{bx}	21.49 ± 3.09^{bx}	30.71 ± 6.55^{bx}	22.96 ± 7.92^{bx}	14.49 ± 2.66^{bx}	17.84 ± 4.23^{bx}

[a-d]Within each row, means without a common superscript letter are different ($P < 0.05$).
[x-z]Within the same measurement without a common superscript letter are different ($P < 0.05$).

Table 3. Mean (log CFU/cm^2 ± standard deviation) aerobic counts (APC), lactic acid bacteria counts (LAB), *Pseudomonas* spp. counts (Pseudo), and *Enterobacteriaceae* counts (ENT) for beef longissimus (LL) and psoas major (PM) over a 7-d simulated retail display period.

Count Type	Muscle	Display Day 0	1	2	3	4	5	6
APC	LL	1.6 ± 0.5^{az}	1.7 ± 0.3^{az}	1.8 ± 0.2^{az}	2.4 ± 0.6^{az}	3.4 ± 0.7^{bz}	4.1 ± 0.8^{bz}	5.2 ± 0.3^{cz}
	PM	2.0 ± 0.3^{az}	2.4 ± 0.3^{abx}	3.2 ± 0.6^{bx}	4.3 ± 0.9^{cx}	4.7 ± 0.6^{cdx}	5.2 ± 0.4^{dx}	6.6 ± 0.4^{ex}
LAB	LL	1.8 ± 0.3^{az}	1.7 ± 0.3^{az}	2.2 ± 0.4^{abz}	2.7 ± 0.7^{bz}	3.8 ± 0.5^{cz}	4.3 ± 0.8^{cz}	5.6 ± 0.3^{dz}
	PM	2.0 ± 0.4^{az}	2.5 ± 0.2^{abx}	3.3 ± 0.5^{bx}	4.4 ± 0.8^{cx}	4.9 ± 0.6^{cdx}	5.4 ± 0.6^{dx}	6.9 ± 0.5^{ex}
Pseudo	LL	< 0.5 ± 0.3^{az1}	< 0.8 ± 0.3^{az1}	< 0.7 ± 0.3^{az1}	0.7 ± 0.7^{az}	1.8 ± 0.4^{bz}	2.8 ± 0.4^{cz}	3.8 ± 0.3^{dz}
	PM	< 0.7 ± 0.3^{az1}	0.9 ± 0.3^{az}	1.4 ± 0.5^{ax}	2.4 ± 0.6^{bx}	3.0 ± 0.7^{bcx}	3.8 ± 0.4^{cx}	5.4 ± 0.4^{dx}
ENT	LL	< 0.2 ± 0.0^{az2}	< 0.2 ± 0.0^{az2}	< 0.2 ± 0.0^{az2}	< 0.2 ± 0.0^{az2}	< 0.2 ± 0.0^{az2}	< 0.3 ± 0.1^{az3}	< 0.2 ± 0.0^{az2}
	PM	< 0.2 ± 0.0^{az2}	< 0.2 ± 0.0^{az2}	< 0.3 ± 0.2^{az3}	< 0.6 ± 0.6^{ax4}	< 0.5 ± 0.6^{az4}	< 0.3 ± 0.1^{az3}	1.3 ± 0.3^{bx}

[a-e]Within each row, means without a common superscript letter are different ($P < 0.05$).

[x-z]Within the same measurement without a common superscript letter are different ($P < 0.05$).

[1]At least one of the five samples analyzed had a *Pseudomonas* count that was below the microbial analysis detection limit of 0.2 CFU/cm^2; therefore, the mean is reported as < (less than) the mean.

[2]All five samples analyzed had an *Enterobacteriaceae* count that was below the microbial analysis detection limit of 0.2 CFU/cm^2; therefore, the mean is reported as < (less than) the mean.

[3]Four of five samples analyzed had an *Enterobacteriaceae* count that was below the microbial analysis detection limit of 0.2 CFU/cm^2; therefore, the mean is reported as < (less than) the mean.

[4]Three of five samples analyzed had an *Enterobacteriaceae* count that was below the microbial analysis detection limit of 0.2 CFU/cm^2; therefore, the mean is reported as < (less than) the mean.

Table 4. Growth kinetic parameters using predictive modeling with DMFit from ComBase utilizing the Baranyi and Roberts model applied to beef steaks from two muscles [longissimus lumborum (LL) and psoas major (PM)] over a 7-d simulated retail display (3°C). Lag (λ) represents lag time in days, rate (μmax) represents the maximum growth rate in log CFU/cm2 per day, N0 is the initial microbial population, Nmax represents the predicted microbial density, and R2 shows the goodness of fit for the model.

Count Type		Growth Parameters				
		Lag (λ)	Rate (μ_{max})	N_0	N_{max}	R^2
	Muscle					
APC	LL	2.19	0.92	0.89	5.40	0.87
	PM	< 1[1]	0.73	2.12	6.93	0.89
LAB	LL	2.07	0.95	1.65	6.08	0.88
	PM	<1[1]	0.79	2.32	7.39	0.89
Pseudo	LL	3.07	1.11	0.19	4.19	0.90
	PM	1.57	1.00	0.97	5.99	0.91

[1]Modeled lag phase was less than minimum 1 day, thus the confidence interval contained zero, and the lag phase parameter is less than (<) 1 day.
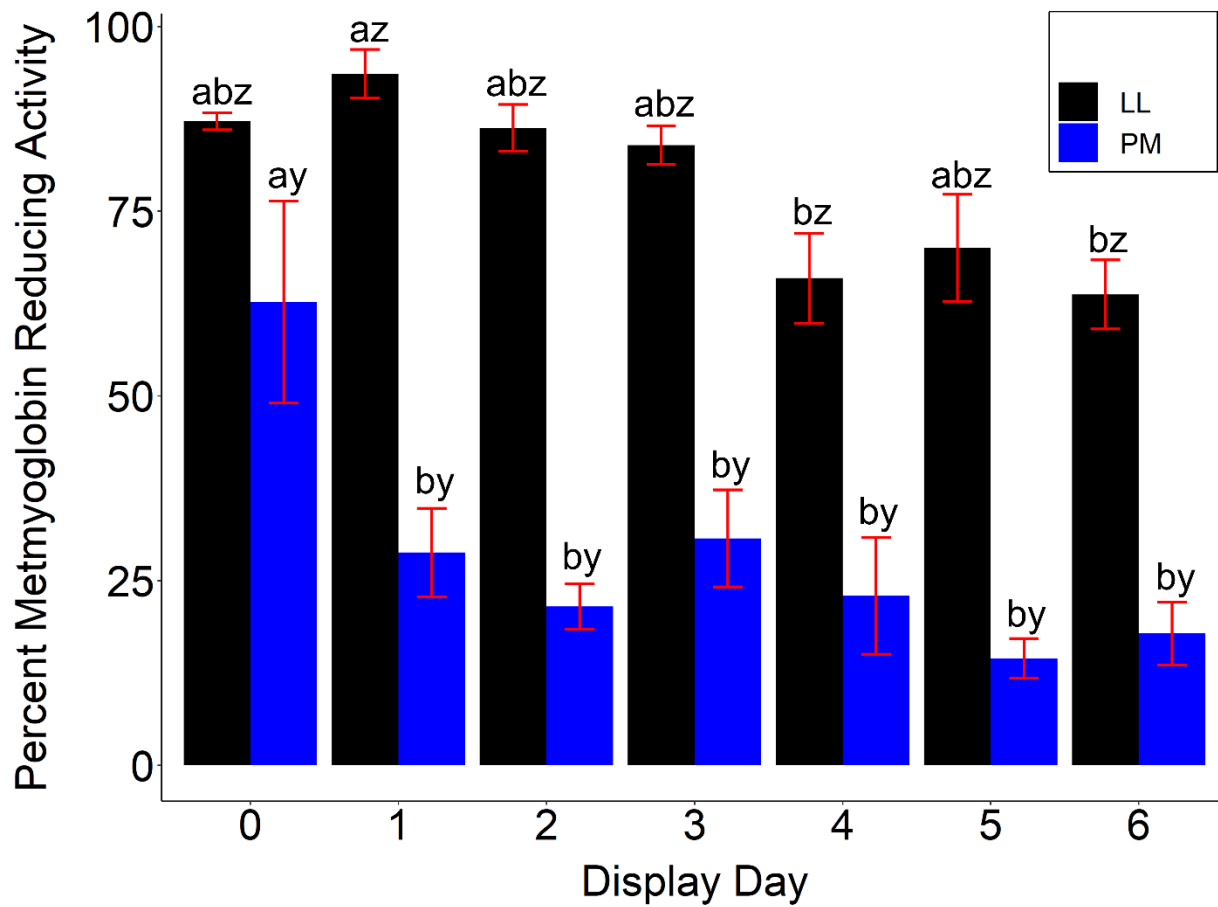
^{ab}Bars within same muscle without a common letter superscript are different ($P < 0.05$).

^{zy}Bars within the same display day without a common letter superscript are different ($P < 0.05$)

Figure 1. Mean metmyoglobin reducing activity of beef longissimus lumborum (LL) and psoas major (PM; n = 5) measured over each day over a 7-d retail display (3°C).
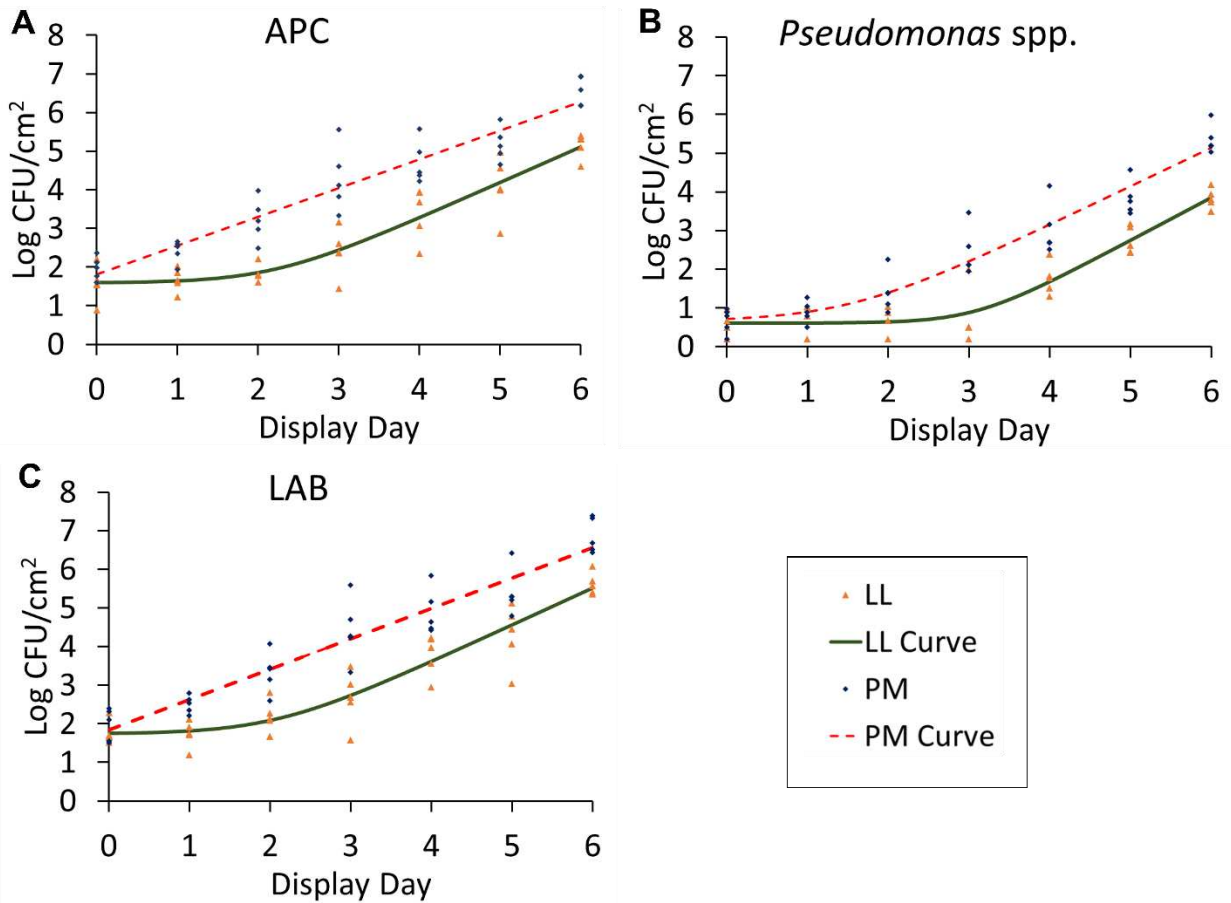
Figure 2. Baranyi and Roberts (1994) predicted growth curves for aerobic plate counts (APC), *Pseudomonas* spp., and lactic acid bacteria (LAB), with calculated lag phase and maximum growth rate, determined from the log CFU/cm² plate counts on beef longissimus lumborum (LL) and psoas major (PM) steaks from each day of a 7-d
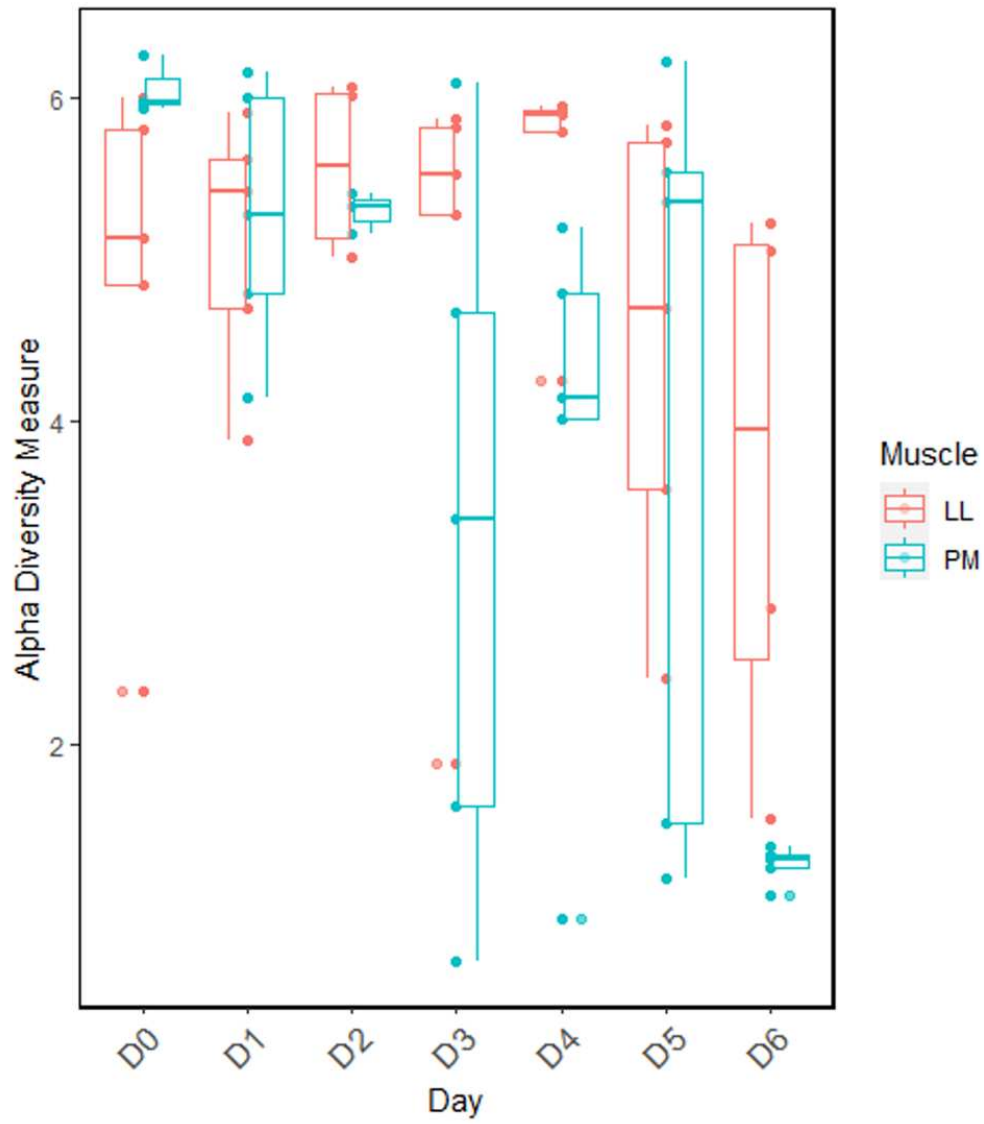
Figure 3. Alpha diversity by Shannon statistic for bacterial populations collected from beef longissimus lumborum (LL) and psoas major (PM) over a 7-d retail display period.
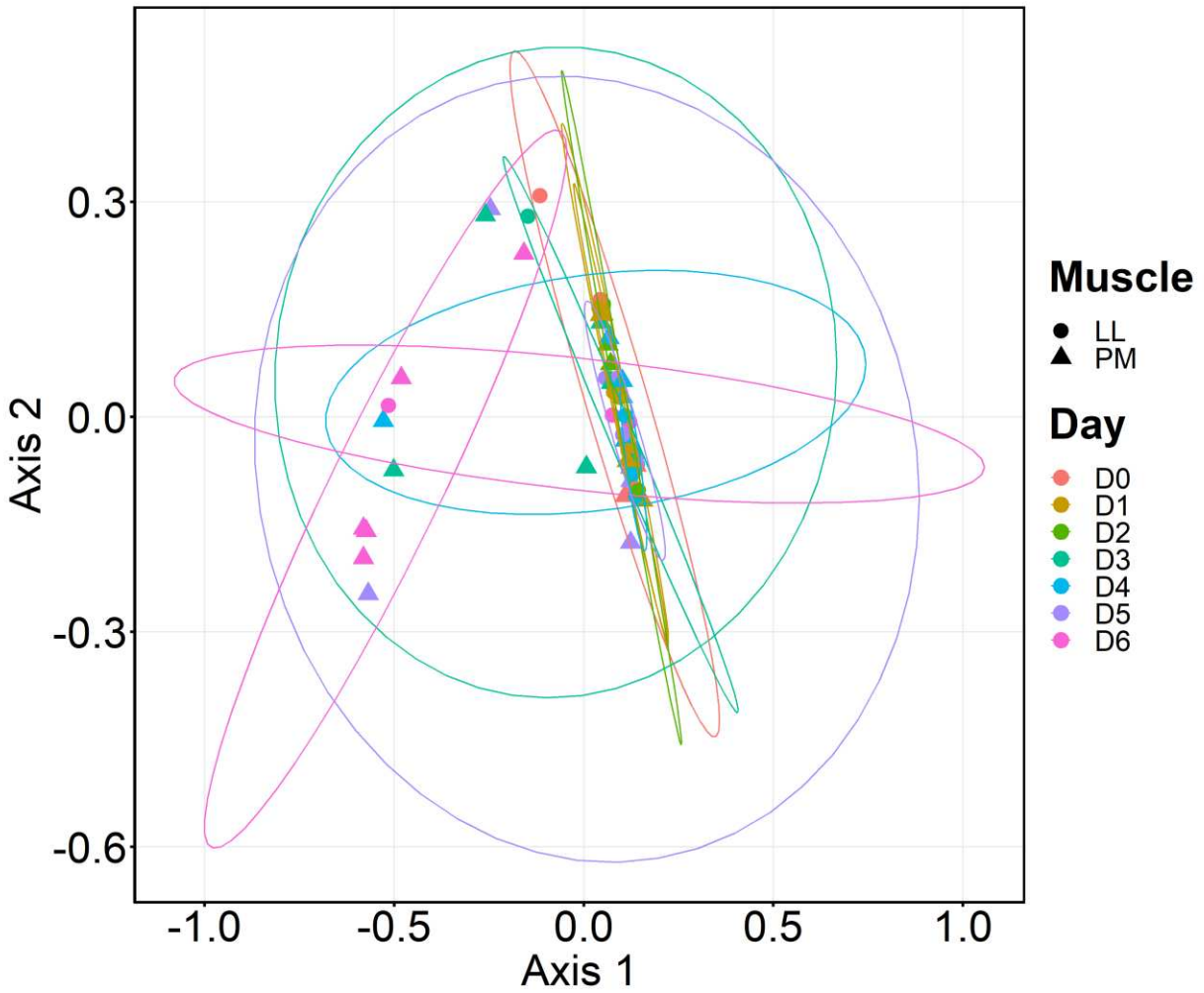
Figure 4. Unweighted uniFrac PCoA of beta diversity for microbial populations collected from beef longissimus lumborum (LL) and psoas major (PM) steaks over a 7-day simulated retail display period.
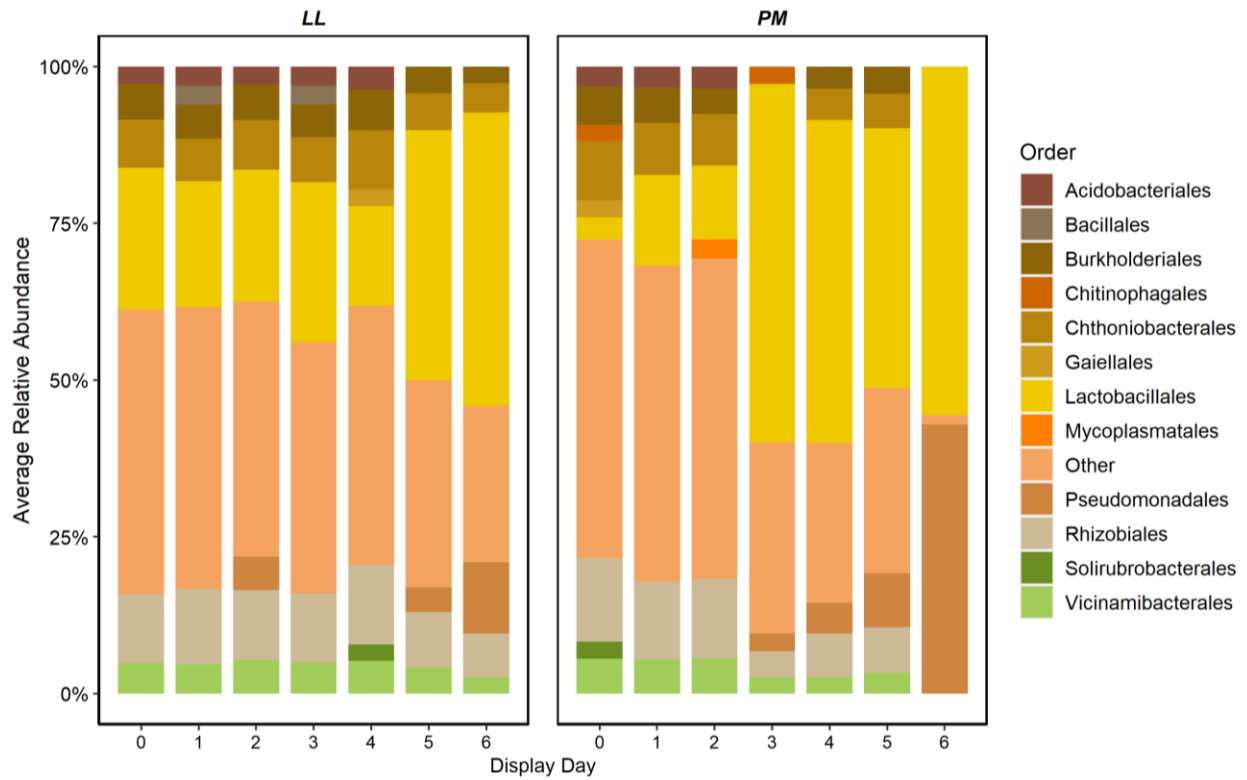
Figure 5. Relative abundance taxonomy plot grouped by either beef longissimus lumborum (LL) or psoas major (PM) organized at the order level over a 7-d simulated retail display period.

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