Survey of Microbiota on Commercially Available Dry-Aged Beef

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Authorization to Submit

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Abstract

Dry-aging is the process of holding meat for extended periods of time, in a refrigerated system, without protective packaging thus exposing the meat to all environmental factors within that system. One of those factors is the presence of bacterial and fungal organisms, which may contribute to development of unique flavors observed in dry-aged products. It is commonly believed among meat specialist and butchers that mold growth contributes to the many unique flavors associated with dry-aged beef. The objective of this thesis is to identify and describe the microbial populations, bacterial and fungal, found on wet and dry-aged strips loins from commercial dry-aging facilities. Beef bone-in strip loins (N = 60) were dry-aged at 10 commercial dry-aging facilities for 45 days. Bacterial and fungal membership of strip loins were identified using culture-independent 16S Ribosomal Ribonucleic Acid (rRNA) and Internal Transcribed Spacer 1 (ITS1) sequencing, respectively. Relative abundances of taxa were calculated to characterize the respective microbial communities by dry-aging facility locations. Microbial DNA was found at all locations, including locations using ultraviolet light (UV) light. Relative abundances were calculated and used to characterize the bacterial and fungal communities. Fungal and bacterial communities did not show clear clustering by aging location, suggesting that communities were not unique to each aging location. Alpha diversities were not different between aging locations. Core microbiota included: *Mucor flavus, Pseudomonas, Lactobacillus algidus,* and *Pseudomonas* fragi. The mold *Mucor flavus* was present across dry-aging facilities and may lead to important sensory profiles observed in dry-aged beef. Further investigation is needed to identify what contributions these organisms may have on product sensory attributes of dry-aged beef products.

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Dedication

I want to dedicate this thesis to my family and friends. Most importantly my parents, for their love and support as I experience life. My brothers, for the late-night phone calls resulting in trips down “memory lane”. Thank you all for everything, words cannot fully express my appreciation.

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CHAPTER 1

Review of the Literature

Introduction

Following the publication of two scientific articles in the 1970s stating that vacuum sealing beef products was beneficial to the industry because of less shrinkage and yield loss, the process of dry-aging began to see a decrease while wet-aging became the new method used for storage and transportation of beef products (Savell, 2008). Wet-aging is the process of aging meat products in a vacuum sealed bag, decreasing product exposure to the external environment (Dashdorj et al., 2016). While meat is wet-aging, natural enzymatic breakdown of the muscle helps to create a more tender product. Dry-aging is the process in which whole muscle is aged in an open refrigeration system and exposed to environmental factors (air flow, humidity, temperature, and microorganisms) present within that system. Similar to meat during the wet-aging process, meat during dry-aging also experiences enzymatic breakdown leading to increased tenderness. Throughout the dry-aging process, the aging products experience evaporation that leads to a higher concentration of flavor compounds resulting in a stronger flavor profile observed in dry-aged beef (Dashdorj et al., 2016). Along with the concentration of the beefy flavor, unique flavors have also been observed in dry-aged products. Some describe these flavors as nutty, earthy, cheesy, and/or roasted (Laster et al., 2008). Potential contributors to the observed flavors in dry-aged meat could be certain microorganisms that colonized the surface of the meat which break down components of the muscle and fat resulting in unique flavor compounds (free amino acids and free fatty acids; Ryu et al., 2018). There has been a growing interest in dry-aged beef

products, and the increasing demand has started to create niche markets for dry-aged products in upscale restaurants and grocery stores (Dashdorj et al., 2016; USMEF, 2019). However, there is limited scientific literature on the contribution of certain microorganisms, and other environmental factors, that may have an influence on the development of unique sensory attributes.

Grading System

Dry-aging is a value-added process that demands a higher price from consumers compared to wet-aging; resulting in equally high expectations during consumption (Dashdorj et al., 2016). Typically, carcasses of high-quality grades are used for dry-aging to assure optimal quality (tenderness, flavor, and juiciness) of the finished aged product (Dashdorj et al., 2016). However, some research has been performed with attempts to add value to lower quality carcasses by allowing them to dry-age and become more tender (Bernardo et al., 2020). As shown in research performed at Iowa State university, wet- and dry-aged Prime carcasses resulted in quality attributes being scored lower, than Choice and Select carcasses, by consumer panelists (Parrish et al., 1991). Prime beef carcasses have the highest amount of marbling, or intramuscular fat, dispersed throughout the lean muscle (Hale et al., 2013). An increased amount of marbling has been shown to be associated with a more tender product, and tenderness is surveyed to be the most important sensory attribute taken into consideration when consumers are evaluating their eating experience of meat products (Miller et al., 2001). Select carcasses have a lower amount of marbling throughout the lean muscle, which result in products that are less desired by consumers (Miller et al., 2001).

The grading of beef carcasses in the United States (US) is performed by the US Department of Agriculture (USDA) Agricultural Marketing Service and is a service offered to beef packing plants. In the US, carcasses are graded on two scales: yield grade and quality grade. Quality grade is assigned after assessing physiological maturity and marbling content of the *Longissimus dorsi* at the 12th and 13th rib interface to determine the palatability of the end products obtained from each individual carcass. Maturity assessment is conducted to determine the physiological age of the harvested animal (Holland and Loveday, 2013).

Historically, when assessing maturity of beef carcasses USDA graders would look at the ossification of the dorsal buttons on the thoracic vertebrae, rib color and shape, and lean color (Tatum, 2011). As the animal ages, the dorsal buttons begin to ossify or convert cartilage to bone, the rib bones become wider and flatter and the color of the lean becomes darker in color (Holland and Loveday, 2013). The texture of the muscle also becomes coarser as the animal ages (Holland and Loveday, 2013). However, some cattle experience premature ossification and are deemed over 30 months when they are in fact under 30 months of age; the majority of fed cattle in the US are less than 30 months of age. One reason for premature ossification is that feeder cattle are often implanted with steroidal growth promotants containing estrogen which promotes premature skeletal ossification in both steers and heifers (Tatum, 2011). Premature ossification occurring in fed steers and heifers has cost the beef industry an estimated annual loss of $42.5 million, as observed in study performed in May of 2014 (Radke, 2017).

In December 2017, the USDA announced the change in determining beef carcass maturity, allowing the division of carcasses under 30 months of age and carcasses over 30

months of age. This method of maturity assessment is now done most often by observing dentition or accurate records indicating the age of the animal (USDA, 2017). Dentition evaluation is done by observing the number of permanent erupted incisors (Graham and Price, 1982). Following the dentition assessment, any animals that have been determined to be over 30 months of age will also be graded by looking at skeletal ossification as described above and will not be grouped in the A maturity class (USDA, 2017). There are five maturity grades in the beef grading system: A, B, C, D, E. A maturity refers to carcasses less than thirty months of age through E maturity which defines carcasses over 96 months. Generally, the carcasses that achieve A maturity are 9-30 months of age and the carcasses in this group are most desirable for beef products of the highest quality (Hale et al., 2013).

Aside from beef carcass maturity assessment, a marbling score is determined for each individual carcass. Marbling is defined as small flecks of fat interspersed within the muscle and is associated with flavor, juiciness and tenderness of the final products (Hale et al., 2013). Larger amounts of marbling tend to be more desirable than lower amounts of marbling because more marbling produces a more flavorful, juicier, and tender product (Boleman et al., 1997). Graders evaluate the marbling and assign one of the nine scores to the carcass: abundant (most), moderately abundant, slightly abundant, moderate, modest, small, slight, traces and practically devoid (least). After determining maturity and marbling of the carcass, the two parameters are combined to establish the USDA quality grade. Prime, Choice, Select, and Standard are grades given to carcasses that have an A maturity grade or are under 30 months of age. Prime grades are given to A maturity carcass that have a marbling score of slightly abundant or higher. Choice grades are given to A maturity carcasses with marbling between moderate and small. A maturity carcasses that have a slight

marbling score are classified as Select grades. Carcasses that are deemed over 30 months of age are eligible for quality grades of USDA Commercial, Utility, Cutter, and Canner (Hale et al., 2013).

Following the assignment of a Quality Grade, carcasses are also assigned a Yield Grade. Yield grading is determining the amount of cutability, or lean salable meat, on a beef carcass. There are five USDA Yield Grades: USDA Yield Grade 1 through USDA Yield Grade 5. USDA Yield Grade 1 contains the carcasses with the highest amount of cutability. Though Yield Grade may not impact the eating quality of the carcass, it can decrease the value of the carcass for the producers and packers by decreasing the cutability of the carcass. When evaluating Yield Grade there are four factors that are taken into consideration: ribeye area, backfat thickness, kidney pelvic heart fat, and hot carcass weight. The following factors are put into the following equation: Yield Grade = 2.5 + (2.50 * adj. fat thickness, inches) + (0.20 * % kidney, pelvic and heart fat) + (0.0038 * hot carcass wt., pounds) - (0.32 * ribeye area, square inches) (Holland and Loveday, 2013). The calculated results are then taken without the decimals, and the decimals are not used while rounding down to the closest whole number. For example, a calculated Yield Grade of 2.45 or 2.81 will be a final Yield Grade 2.

Tenderness

Tenderness has been determined to be the most influential factor when determining beef palatability, and consumers are willing to pay a premium for a more tender eating experience (Boleman et al., 1997; Platter et al., 2003). The impression of tenderness to the palate involves perforation of the meat by the teeth, in other words, the ease of fragmentation when chewing (Jeremiah and Phillips, 2000). Tenderness can be impacted by

breed of animal, amount of connective tissue, protein degradation, marbling content, and postmortem treatments to the meat such as aging (Houbak et al., 2008). A class of proteolytic enzymes known to naturally breakdown the aging muscle is the calcium-dependent calpain enzymes. When calcium is present it acts as a co-factor and allows calpains to bind to the z-discs of the sarcomere, resulting in multiple individual sarcomeres detached from one another. The act of breaking down the bundles of sarcomeres can also be thought of as tenderization, thus there are less areas of the muscle that need to be broken down when chewing of the muscle occurs. Other studies have also found that calves sired by bulls of Brahman lineage were less tender than calves of Angus origin after a 10-day aging period (Bidner et al., 2002). *Bos indicus* cattle tended to have a higher calpastatin activity than *Bos Taurus* cattle when compared in aging treatments. Calpastatin is an inhibitor protein on the calcium dependent proteases (calpains), which plays an important role in tenderization during the aging processes of fresh meat products. When calpastatin is in the presence of calpains it blocks the binding site for calpains to the muscle fibers which leads to less fragmentation of the meat and resulting in a tougher product (Bidner et al., 2002).

Wet-Aging

Wet-aging is an aging process, known to naturally tenderize meat products, where the meat product is aged in a vacuum sealed bag, preventing exposure to some environmental factors (Oreskovich et al., 1988). However, wet-aging products still need to be aged within a certain temperature range to prevent freezing or spoilage of the meat. The initial freezing point of meat begins when stored below 0°C, with a range from -0.9°C to -1.5°C and is dependent on mass, ash content, water, and other components within the product (Pham, 1996). Some studies have also demonstrated that the pH can impact the

initial freezing temperature of meat (Farouk et al., 2013). If the product is stored at temperatures below or close to freezing, the enzymatic activity will decrease, and enzymatic tenderization may be greatly decreased in rate or be completely stopped (Du et al., 2017). On the other hand, if meat is stored in elevated temperatures, above 4°C, spoilage of the product is likely to occur (Savell, 2008). Also, enzymatic activity will be elevated with increased storage temperature, and the product will begin to break down rapidly (Savell, 2008).

Dry-Aging

The process of dry-aging occurs in a somewhat different manner when compared to wet-aging as dry-aged meat products are exposed to additional environmental factors within the refrigerated aging system. Air flow, relative humidity, temperature, the use of ultraviolet light (UV), and the number of days of aging are important environmental factors to consider during the dry-aging process (Dashdorj et al., 2016). Another factor that culinary professionals commonly agree upon is the presence of molds and/or yeasts growing on the surface of dry-aging products, developing unique flavors observed in end products. Though scientific literature is sparse in the actual role microorganisms may play in the dry-aging process, some species which have been observed on dry-aged beef are also found in other food processes such as cheese production (Capouya et al., 2020; Dashdorj et al., 2016; Oh et al., 2019).

Though there are no strict guidelines on the number of days required for developing dry-aged beef, some research suggests that 14-40 days of aging is effective enough to create the desired traits (Savell et al., 2008. However, the US Meat Export Federation suggests that the preferred days of dry-aging is between 28 and 55 days (USMEF, 2019). Others believe

that allowing products to dry-age for a greater number of days will create a more tender and flavorful final product (Dashdorj et al., 2016). In contrast, it has been demonstrated that beef dry-aged for 120 days did not see the expected flavor development when compared to beef dry-aged for 35 and 80 days (Dashdorj et al., 2016). Some upscale restaurants still dry-age their beef products for more than 100 days in attempt to produce the most desired dry-aged steak, but steaks from dry-aged beef that were aged for more than 100 days seem to be a personal preference (Lam, 2013).

Relative humidity is a variable that is relatively undefined with regard to dry-aging; similar to that of days being aged. There is a wide range of relative humidity that can modify the final product. Humidity is important in dry-aging beef. Most studies agree that a relative humidity around 80% is the most beneficial, but some suggest a range of 60-80% (Dashdorj et al., 2016). One study dry-aged the beef in an area that had a relative humidity as low as 49% and steaks from these products exhibited better palatability compared to steaks that were aged at a higher humidity (Kim et al., 2016). Since there is limited information on the optimal level of relative humidity, it is recommended that dry-aging facilities record humidity levels daily (Dashdorj et al., 2016). Research has demonstrated that if the humidity in the dry-aging facility is too low, less than 80% relative humidity, the product will evaporate water too quickly and create a less juicy product (Perry, 2012). On the other hand, if humidity is too high, greater than 85% relative humidity, evaporation will not occur and the aging product will not get the concentration of flavors that is expected (Perry, 2012). Relative humidity above 85% may be advantageous to the growth of spoilage organisms and result in an unsaleable spoiled product (USMEF, 2019).

Proper air flow should also be present within a dry-aging facility. The US Meat Export Federation recommends the air flow range of 0.5-2.0 meters/second when dry-aging products (USMEF, 2019). Dry-aging products should be placed in a manner that prevents dead spots in air movement and should also be evenly spaced on racks or hung, either by hook or netting. If a product is placed in an area of little to no air flow, the meat will not dry as effectively in that area and could allow the growth of unwanted microorganisms resulting in spoilage (USMEF, 2019). Also, if the meat is not surrounded by a consistent air flow, the final product may be inconsistent in sensory attributes because of inconsistent evaporation. On the other hand, if the air flow velocity is too high the aging product may dry too quickly and cause a higher-than-normal yield loss when trimming the exterior crust (Dashdorj et al., 2016). One study also found that different air flow speeds impacted the growth of two organisms that have been observed in cheese making (Lee et al., 2019). This suggests that air flow could indirectly impact flavor formation in dry-aging products by increasing or decreasing beneficial microbial growth (Lee et al., 2019).

As the environmental factors in dry-aging seem to have a broad range for suggested guidelines, a factor that is not well defined is the growth of microorganisms on the product itself. Some dry-aging facilities try to limit or inhibit the growth of microorganisms through the use of UV light either by directly applying UV light to the products aging or filtering the air through a UV lit chamber (Dashdorj et al., 2016). Others attempt to, in fact, allow the growth of molds that may develop key flavor components when utilizing the surface of the meat as a food source (Oh et al., 2019). Limited research has been conducted on the microorganisms, or microbiomes, associated with dry-aging beef and the contribution they may have on sensory characteristics of the final dry-aged products. However, some studies

have been performed targeting the presence of certain molds and bacteria (Hanagasaki and Asato, 2018; Oh et al., 2019).

Microorganisms observed on dry-aged beef

Pilaira anomala and *Debaryomyces hansenii* have been observed on dry-aged beef and may play important roles in the development of unique flavors that are not observed in wet-aged beef products (Oh et al., 2019). *D. hansenii* is known for its lipolytic activity and is used in sausage and cheese fermentation (Martorell et al., 2005). *P. anomala* and *D. hansenii* were further investigated to better understand their contribution in developing dry-aged beef flavor and tenderness (Oh et al., 2019). Both organisms had proteolytic and lipolytic properties when cultured with skim milk and tributryn, meaning the two can actively degrade proteins and lipids (Oh et al., 2019). However, the yeast *D. hansenii* grew quicker up to day 14 than the mold *P. anomala*. Myofibril ultrastructure images were also taken for the dry-aging meat with the 2 different organisms and *P. anomala* had caused more myofibrillar degradation than *D. hansenii*. Both organisms caused more myofibrillar breakdown than the experimental wet-aging control leading to a more tender product (Oh et al., 2019).

Free fatty acid and free amino acid analyses were also conducted in the study by Oh and coworkers. Both organisms were expected to produce free fatty acids due to their lipolytic activity (Alapont et al., 2015). Though both *P. anomala* and *D. hansenii* have lipolytic activity, *P. anomala* increased the concentration of free fatty acids in the dry-aged meat more than *D. hansenii*. Both organisms, *P. anomala* and *D. hansenii*, increased the free fatty acid and free amino acid concentration which led to aroma compounds that provide the unique flavor observed in dry-aged beef (Oh et al., 2019). Another study also reported that

dry-aged beef had higher concentrations in flavor compounds when compared to wet-aged control groups (Kim et al., 2016). In contrast to the study by Oh and coworkers, this study did not take into consideration any microbial factors that could be responsible for the production of free amino acids and free fatty acids. Instead, they suggested the reason for the increased concentration of metabolites in dry-aged beef was due to water evaporation.

Though the previous studies focused primarily on two organisms that have shown to be beneficial to the dry-aging process, other studies have observed a multitude of different microorganisms present on the surface of dry-aged beef. One study performed at Ohio State University observed microbial communities associated with dry-aged beef at different commercial dry-aging facilities across the US (Capouya et al., 2020). The results showed significant differences in the microbial profiles between aging facilities, which could explain why product inconsistencies are seen within the dry-aging industry. The study also evaluated the diversity of the microbiome within each loin. This was done by sampling from different positions on the loin's surface and analyzing samples that were up to a depth of 1 centimeter from the surface crust. The results suggested that microbial communities were not different across the exterior surface and up to a depth of 1 centimeter for each loin (Capouya et al., 2020). Another study focused on pathogenic organisms, and the sequencing results suggested that the abundance of pathogenic organisms decreases as the dry-aging process prolongs and all pathogens completely disappeared from the surface after 60 days of dry-aging (Ryu et al., 2018).

Microbes used in food production

The use of microorganisms in food production has been practiced for centuries, creating products such as: cheese, beer, bread, yogurt and cured meats. The most historical

and wide use of microbes in food production involves the processes of cheese production. There are thousands of types of cheeses that require a unique input from microorganisms at each step of production in order to produce characteristic flavor, texture and appearance (Kindstedt, 2010). Most cheeses harbor a complex microbiota that is characterized by a succession of microbial groups during milk fermentation, curd maturation and storage (Irlinger and Mounier, 2009). The three stages of cheese production mentioned by Irlinger and Mournier are the key to better understanding the functions of different microbial groups used in cheese making.

During the first stage of cheese production, milk fermentation, lactic acid bacteria grow quickly, resulting in an acidic environment. The resulting acidic environment establishes a pH level which impacts some microbial growth. Donnelly reported that this high pH level paired with a small input of salt results in an optimal environment for the growth of *Propionibacterium freudenreichii* (Donnelly, 2013). This microorganism facilitates the fermentation of lactate, producing carbon dioxide (CO_2), acetate, and propionate. These three products give Alpine cheeses their traditional traits: eyes in the cheese curd and a sweet, and nutty, flavor (Donnelly, 2013).

Another factor that plays a role in microbial growth during cheese making is moisture, because these microorganisms require water to grow. During the cheesemaking process it is easy to manipulate microbial growth by either reducing or adding moisture through water soluble ingredients (Brennan et al., 2002). It is important to control moisture in a cheese because an increase in moisture leads to spoilage, similarly to the process of dry-aging beef. In the production of Taleggio cheese, low moisture and slow acidification help

Brevibacterium linens, *Geotrichium candidum*, and *Debaryomyces hansenii* grow. These microorganisms lead to Taleggio's creamy texture and unique odor.

Different enzymes are also known to affect flavor profiles in cheese. In a study performed in the UK in 1997, researchers found that it was starter enzymes that were mainly responsible for producing the unique amino acids in Cheddar cheese making (Williams and Banks, 1997). However, the study did find some evidence that enzymes from lactobacilli may contribute to the presence of some of the other amino acids such as glutamate, leucine, proline and ornithine observed in the process. The produced amino acids are important because they form the unique flavor compounds which lead to flavor profiles of the Cheddar cheese during the ripening stage.

Similar to dry-aged beef, people have been eating dry cured pork since ancient Roman times. In 2006, over 9 million dry cured thighs were produced for consumption (Pugliese et al., 2010). Because these muscles are not only dry-aged but cured, they require an extra step: salting. Just like with cheese, salt inhibits microbial growth by decreasing water content. Salting along with dry-aging and resting the thighs leads to a rapid increase in the lactobacilli present, who are responsible for the final sensory markers of the prosciutto (Sanchez-Molinero and Arnau, 2008). Dry curing ham shares characteristics with both dry-aged beef and cheese making.

Microbial Identification

Identifying microorganisms in a biological system can be a difficult and time-consuming task, depending on the method used for identification. Historically, morphological observations were made at the microscopic level to identify individual

microorganisms (Reller et al., 2007). Identifying microorganisms through morphological or phenotypic characteristics is challenging and requires expertise, which can be lacking within a laboratory system. Also, when studying microorganisms through phenotypic characteristics, most organisms would need to be cultured *in vitro*. This can be a difficult task to complete while attempting to observe organisms that may not be able to grow in culture. Some organisms may also change their phenotype depending on the culturing environment, which could lead to false identifications (Sandle, 2011). However, through the development of sequencing technologies the task of microbial identification has shifted to a genotypic approach. Next generation sequencing technologies allow for more information, such as relative abundances of unculturable microbial communities, or microbiomes, within a biological system of interest.

Ribosomal RNA

Most microorganisms have ribosomes that play a crucial role during protein synthesis. These organisms have specific genes within their genome coding for their ribosomal make up. Ribosomal RNA (rRNA) is the major structural component of the ribosome and has complementary sequences which interact with messenger-RNA to carry out the process of protein synthesis (Fredrick and Ibba, 2009).

For prokaryotic organisms, bacteria and archaea, there are 3 rRNA molecules within the 50S and 30S subunits of the ribosome: 5S, 16S, and 23S. The 16S rRNA gene has been a popular target for identifying bacteria due to its abundance of conserved regions within the gene (Woese and Fox, 1977). These conserved regions are regions that are slow to evolve and are used as primer target regions when performing polymerase chain reaction (PCR) procedures on extracted DNA from a given sample (Fuks et al., 2018). Within the 16S

rRNA gene lies hypervariable regions that are unique to most bacterial taxa (Fuks et al., 2018). Following PCR amplification of prokaryotic genomic DNA using primers targeting the conserved regions, PCR amplicons can be sequenced, and the sequencing results of the hypervariable regions used for identification of microbes. Though the 16S rRNA gene is heavily used in identification through sequencing technologies, some research suggests that these regions may not be as highly conserved as thought, meaning target-specific primers may not bind as well as expected (Porchas et al., 2017).

Identifying fungal organisms is done in a similar manner, but the internal transcribed spacer (ITS) regions are used for sequence, or genotypic, identification (White et al., 1990). Between ITS1 and ITS2 lies the conserved gene for the 5.8S rRNA subunit which is used as a primer target region. The ITS region has been heavily used in studying fungal systems and has led to over two hundred thousand fungal ITS sequences in the international nucleotide sequence database (Mizrachi et al., 2012).

Sequencing

The ability to obtain genomic sequence information has been available since the 1970s. A method developed by Frederick Sanger and colleagues, known as the Sanger sequencing method, has been the most widely used sequencing program for the last 40 years (Schoales, 2015). Though a method with acceptable accuracy, the Sanger method is time consuming and labor intensive. This process involves the use of chain terminating dideoxynucleic acids that are labeled with a fluorescent probe, each nucleic acid labeled with a specific color. Fluorescently labeled nucleic acids are then added to the extension products at random during the PCR process while using fragmented genomic DNA as a template. The extension process is then stopped, and the resulting products are separated

through capillary electrophoresis. As extension products move through the capillary of the gel they are separated by size, shorter DNA fragments move through the gel at a quicker pace than longer fragments. A light source then excites each fluorescent probe to determine which nucleic acid is present at that given location. The fragmented sequencing results are then pieced together computationally to identify overlaps and sequences of interest (Sanger et al, 1977).

Following the completion of the human genome project, which used the Sanger sequencing method, the National Human Genome Research Institute started a funding program to find ways to decrease the time and cost to obtain sequence information (Schloss, 2008). This led to the development of next generation sequencing (NGS) technologies that could give sequence results in a shorter time frame with the ability for clustered parallel sequencing and be more cost effective. The new sequencing technologies also allowed for sequencing by synthesis on certain platforms, whereas the Sanger method involved sequencing with chain-terminating nucleic acids that involved the need for capillary electrophoresis for sequence identification. However, NGS faced a drawback with the fact that the technology was only able to give information for short read lengths (Dijk et al., 2014).

Through years of development, the technology involved with sequencing platforms has improved tremendously, one of which is the Illumina MiSeq. Illumina has been the dominating sequencing technology because of the capability for high throughput, accuracy, and low cost (Mavromatis et al., 2012). When beginning the process to obtain sequencing results of interest, genomic DNA is first fragmented, or specific regions are targeted with

well-designed primers. When sequencing bacterial and fungal taxa, common targeting regions are the 16S rRNA gene (bacterial) and the ITS region (fungal), respectively (Fuks et al., 2018; Mizrachi et al., 2012). As previously mentioned, following amplification of target regions, flow cell adapter sequences are ligated to each end of the amplicons. Index sequences may also be ligated to the amplicons for sample identification (Illumina, 2017). Amplicons are then hybridized to complementary adapters that are chemically bound to the surface of a flow cell. Once amplicons are bound to the flow cell, they undergo a series of bridge amplifications in order to create a cluster of cloned sequences from the original template amplicon. Fluorescently labeled nucleotides are then added to the flow cell, bases are incorporated into the extending sequence, and the excess nucleotides are washed away. A light source then excites the flow cell and the emission wavelength is recorded to determine which labeled nucleotide was present in each cluster of amplicons. The cycle then repeats itself for up to 300 cycles (Illumina, 2017).

Sequencing results are then trimmed of primer and adapter sequences and clustered with a 97% similarity in order to group into operational taxonomic units. After grouping, a sequence in each cluster is chosen to define the operational taxonomic unit and searched for a match in a database (Kuczynski et al., 2012). In an ideal environment each operational taxonomic unit should define a species, but the presence of every species complete genomic data is limited (Kuczynski et al., 2012). Some sequence data will only be able to match the resulting sequences to the kingdom, family, order, or class level (Huse et al., 2010).

Statistical calculations are then performed to analyze the obtained sequencing information and decipher microbiome information. Some statistical methods used in

microbiome research include Goods coverage, alpha diversity, and beta diversity. Goods coverage is a calculation developed to test the completeness of amplicon sequencing. Whereas alpha and beta diversity are used to calculate diversity within biological systems and exploring diversity between systems (Willis and Martin, 2020). Common alpha diversity measures include Shannon and Simpson. Shannon diversity measures the difficulty to predict a randomly select individual within a sample. Whereas Simpson diversity measures the probability the two randomly selected individuals are the same. In other words, alpha diversity measures predict the probability of randomly selecting an individual from within a sample. Common beta diversity measures include Bray-Curtis and Weighted Unifrac. Bray-Curtis measures take into consideration the sum of species with a lower abundance and is divided by the sum of all species counts in the communities being compared. Weighted Unifrac measures the amounts of phylogenetic tree branches that are shared between two communities.

Summary

Though the process of dry-aging beef has been around for decades, the science behind the development of these unique products is yet to be completely defined. After observing the benefits that wet-aging can have for product yields, dry-aging began to lose interest and the method began to see a decrease in practice. However, there has been a recent surge in interest for dry-aged beef and the understanding of the science of the complete process in order to create a more consistent product for consumers. Dry-aging not only adds value to high quality cuts of beef, but the process could potentially add value to carcasses of lower quality. With the well-studied food production systems such as cheese production, and other food production processes that utilize microorganisms, understanding the dry-aging

process and the potential benefits of microorganism could increase demand as products become more consistent and well known. Along with the development of next generation sequencing technologies, microbial systems are becoming more assessable for investigation which could also benefit the field of study observing microbial communities that could lead to a more thorough understanding of dry-aged beef products.

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CHAPTER 2

Survey of Microbiota on Commercially Available Dry-Aged Beef

Abstract

Microbial communities on the surface of dry-aged beef may be a key factor when targeting specific flavor development in fresh beef products. Previous research has demonstrated that microbial communities vary significantly by aging location which may be the cause for inconsistencies observed in flavor profiles of dry-aged beef. The objective of this study was to survey microbial communities found on wet and dry-aged strip loins from commercial dry-aging facilities with a goal of assessing potential quality attributes microbial populations may have on end products. Beef bone-in strip loins (N = 60) were dry-aged at 10 commercial dry-aging facilities for 45 days. Six strip loins were also wet-aged as a control at the same time. DNA was extracted from surface crust samples of each strip loin, amplified using bacterial- and fungal-specific primers, and sequenced using an Illumina MiSeq. Resulting sequences were filtered for quality and assigned taxonomy. Relative abundances were calculated and used to characterize the bacterial and fungal communities. Microbial communities were complex for each aging location. Fungal and bacteria communities did not show clear clustering by aging location, suggesting that communities were not unique to each aging location. Alpha diversities were not different between aging locations. Core microbiota included: *Mucor flavus, Pseudomonas, Lactobacillus algidus,* and *Pseudomonas fragi*. Further investigation is needed to identify what contributions these organisms may have on product sensory attributes of dry-aged beef products.

Materials and Methods

Beef Loins

66 *Certified Angus Beef*® bone-in strip loins (NAMI #175) were sourced from a single commercial beef packing operation. Loins were transported to Vandal Brand Meats on the University of Idaho campus under refrigerated conditions (≤ 4ºC) and randomly assigned to one of ten commercial dry-aging facilities. A total of six loins were assigned to each dry-aging facility (N = 60; 6 strip loins, 10 dry-aging locations). The remaining six loins were wet-aged as a control group at Vandal Brand Meats, Moscow, ID. Once assigned and sorted into respective aging locations, loins were shipped overnight to their assigned aging facility. The loins were then dry-aged for 45-days under the unique environmental conditions found within each individual commercial dry-aging facility. The use of ultraviolet (UV) light was recorded by dry-aging facility.

Microbiome Sampling

Following the 45-day dry-aging period, loins were vacuum sealed and shipped in styrofoam coolers, with ice packs, overnight to Vandal Brand Meats at the University of Idaho. Loins were handled in a manner that prevented cross-contamination and surface contact to the face of each loin. After each loin was weighed, a 2.54-cm^2 segment, no more than a depth of 1-cm, was taken from the anterior face of each loin (Appendix A). Prior to removing each segment of each loin, new sterile scalpels (VWR #82029-858) and forceps (VWR #97001-199) were used to reduce contamination. Gloves were changed between each loin. Samples were then placed into an 18 oz. whirl-pack bag (Nasco #B01365) and stored at -20°C until further analyses.

Subsampling

Samples were thawed on ice for approximately one hour. Prior to subsampling, the biosafety cabinet was thoroughly disinfected with 70% ethanol. Sterile scalpels, forceps, petri dishes, and bead bashing tubes were placed into the biosafety cabinet and treated with UV light for no less than 15 minutes. Following UV treatment, 200 mg (± 10 mg) of each surface sample segment was removed from the exterior crust and placed into a prelabeled bead bashing tube. New scalpels, forceps, petri dishes and gloves were used for each sample to avoid cross contamination between samples (Appendix B). Once weighed, samples were stored on ice or frozen at -20°C until DNA extraction. Subsampling was performed in groups of 10 random samples.

DNA Extraction

DNA extractions were performed in groups consisting of 10 random subsamples, one ZymoBIOMICS Microbial Community Standard (positive control; Zymo Research, Irvine, CA) and one blank (negative control), for a total of 12 samples. The ZymoBIOMICS Microbial Community Standard (Catalog No. D6300) consisted of eight bacterial organisms (*Pseudomonas aeruginosa, Escherichia coli, Salmonella enterica, Lactobacillus fermentum, Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes, and Bacillus subtilis*) and 2 yeast organisms (*Saccharomyces cerevisiae* and *Cryptococcus neoformans*). Of the eight bacteria, three were easy-to-lyse Gram-negative bacteria and five were tough-to-lyse Gram-positive bacteria. Both yeast strains were classified as tough-to-lyse organisms. A blank/negative, consisting of nuclease-free water, was included with each extraction group to investigate if cross contamination was occurring between samples during the DNA extraction process. DNA extraction was performed using the ZymoBIOMICS

DNA Miniprep Kit (Zymo Research Catalog No. D4300), with minor modifications. Extracted DNA was eluted in a total volume of 50 µL of DNase/RNase free water (Zymo Research, Irvine, CA). Once extracted, group positive and blank/negative samples were checked using a target-specific PCR to ensure DNA was being extracted (positive control) and to ensure cross contamination was not occurring between samples during the DNA extraction process (blank). Positive and negative extraction amplicons were then separated by gel electrophoresis using a 1% agarose gel and visualized using a Bio-Rad ChemiDoc MP Imaging System (Bio-rad Laboratories, Hercules, CA).

Polymerase Chain Reaction (PCR)

After DNA was extracted from all samples, target-specific PCR was performed using 2 µL of template DNA in a total reaction volume of 12.5 µL. The V1-V3 hypervariable region on the 16S rRNA gene of bacterial organisms was targeted using primer pair 27F/534R (Appendix D and E). In separate PCR reactions, the internal transcribed spacer 1 (ITS1) domain was targeted for fungal organisms using primer pair ITS1/ITS2 (Appendix D and E). After amplification of target regions in the first PCR reaction, unique sample barcodes and Illumina adapters were added to sample amplicons in a second PCR reaction with a total volume of 25 µL. Sample amplicons were then separated by gel electrophoresis utilizing a 1% agarose gel and visualized as described above.

PCR Cleanup

Following PCR and gel electrophoresis, sample amplicons were treated with ExoSAP-IT™ *Express* PCR Product Cleanup Reagent (ThermoFisher Scientific #75001.1.ML, Waltham, MA), following manufacturers protocol in order to remove

remaining single-stranded primers and dNTPs. ExoSAP-IT *Express* (8 µL) was added to 20 µL of sample amplicons for a total reaction volume of 28 µL.

DNA Quantitation/Pooling

Sample amplicons were quantified using the AccuClear High Sensitivity dsDNA Quantitation Kit (Biotium, Fremont, CA) (Appendix C) and 25 ng of dsDNA of each sample were pooled into six respective pools. Pool 1 and 2 consisted of bacterial samples that had over 25 ng of dsDNA. Pool 3 contained bacterial samples that did not have more than 25 ng dsDNA and were pooled at max volume. Fungal amplicons were pooled based on DNA concentration and gel band intensity. Pool 4 contained fungal samples that did not have a visible band in the gel; while pool 5 contained fungal samples with strong bands at the expected size. Pool 6 contained fungal samples that had a visible band at the expected size, but the band was smeared and/or faint after visualizing the gel.

DNA Sequencing/Data Analysis

Amplicon pools were submitted for sequencing to the University of Idaho Institute for Bioinformatics and Evolutionary Studies (IBEST) Genomics Resource Core facility where amplicons were size-selected using AMPure beads (Beckman Coulter, Indianapolis, IN) using bead ratios of XX and XX, respectively for bacterial and fungal amplicon pools; quality checked on a Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA); and quantified using the KAPA Biosciences Illumina library quantification kit and Applied Biosystems StepOne Plus real-time PCR system. Sequencing of bacterial and fungal amplicons was completed using an Illumina MiSeq (San Diego, CA) v3 paired-end protocol for 600 cycles. Sequence reads were then demultiplexed and trimmed of the primer sequences using a custom python application, dbcAmplicons

(https://github.com/msettles/dbcAmplicons). Sequences were then separated into R1 and R2 files for each sample using a custom python script (splitReadsBySample.py; https://github.com/msettles/dbcAmplicons/blob/master/scripts/python/splitReadsBySample.py). Sequence reads were evaluated for quality, trimmed, filtered, and merged using the DADA2 sequence process pipeline (vs 1.12.1 (Callahan et al., 2016). After modeling the errors from a subset of reads from the sequencing runs, amplicon sequence variants (ASV) are identified. ASV were assigned taxonomy using the SILVA 16S rRNA version 138 (Quast et al., 2013) and the UNITE version 8.2 (Abarenkov et al., 2020) databases for bacterial and fungal sequences, respectively. Sequence data were processed and analyzed in R version 3.6.1 (Vienna, Austria). Bacterial sequences were rarefied at 1000 reads. Fungal sequences were rarefied at 200 reads due to low read counts following sequencing. Stacked bar charts were created in Microsoft Excel (version 2101).

Statistics

Reported beta diversity was calculated using Bray-Curtis dissimilarity and compared with Principal Coordinate Analysis (PCoA). Differences between alpha diversities were calculated using the Kruskal-Wallis Test and grouped using Tukey's HSD.

Results and Discussion

Relative Abundance and Core Microbiota

The top fungal genera found across all aging locations include: *Mucor, Penicillium, Vishniacozyma, Cladosporium, Rhodotorula, Debaryomyces, Cadophora, Trichosporon, Irpex* and *Alternaria*. Top bacterial genera found at all aging locations include: *Pseudomonas, Lactobacillus, Leuconostoc, Carnobacterium, Meiothermus, Photobacterium, Yersinia, Brochothrix* and *Hyrdrogenophilus*.

Core microbiota are classified as microbiota that were present in at least 50% of all samples. Without removing any samples, bacterial sequencing results identified genera *Pseudomonas,* and species, *Lactobacillus algidus* and *Pseudomonas fragi* as core microbiota for bacterial community composition (**Figure 2.1)**. *Pseudomonas* organisms are psychrotrophic and are the predominant spoilage organisms found on meat during chilled aerobic storage (Wickramasinghe et al., 2019). *Pseudomonas fragi, Pseudomonas lundensis, Pseudomonas fluorescens,* and *Pseudomonas putida* are the predominant four *Pseudomonas* organisms that are found to be responsible for meat spoilage (Filippis et al., 2019). Dainty et al. (1988) reported that beef inoculated with *Pseudomonas fragi* had a sensory profile that was described as sweet, putrid, and fruity (Dainty et al., 1988). *Pseudomonas fragi* has also been reported to have a shorter lag period when compared to other *Pseudomonas* species, allowing for *Pseudomonas fragi* to become the dominant species on aerobically chilled beef (Wickramasinghe et al., 2019).

Lactic acid bacteria are commonly found on meat products and are often used as starter cultures for fermented meats. However, *Lactobacillus algidus* (8.5 log CFU/ml) has been shown to have a negative impact on sensory properties of vacuum packaged meats due to the production of intense, sour, and undesirable odors (Schirmer et al., 2009).

Fungal data reported *Mucor flavus* as the only fungal taxa present in approximately 60% of all samples and observed at all locations except location G (**Figure 2.2**). The *Mucor* genera is a well-known and common mold found in many environments worldwide. The presence of *Mucor* species has been observed on the surface of cave-aged cheese during the ripening process and the proteolytic capability of this genus may be desirable for the development of unique flavor compounds (Zhang and Zhao, 2010). Other investigators have

reported that *Mucor flavus* is beneficial to the dry-aging process as it produces gamma-aminobutyric acid (GABA), proline, and aspartic acid throughout its life cycle on dry-aging beef products resulting in savory odors that contribute to a unique sensory profile (Hanagasaki and Asato, 2018). Additionally, *Mucor flavus* is known to be active at low temperatures and maintains the ability to produce proteases. Use of *Mucor flavus* in the fermentation of soybean at low temperatures results in an increase in free amino acids that contribute to unique flavor profiles in Sufu, a strong Chinese liquor (Cheng et al., 2009).

The genus *Penicillium* was present at three locations (D, E, J) before and after the removal of low recounts. Unfortunately, not all sequencing read data were assigned a species level taxonomic assignment within the *Penicillium* genus. Many *Penicillium* species are utilized in food production systems, specifically during the ripening of cheeses and fermented meats, and contribute to product quality and safety (Laranjo et al., 2019; Button and Dutton, 2012). *Penicillium nagliovense* and *Penicillium chrysogenum* are commonly used as surface starter cultures on fermented sausages and dry-cured hams in order to inhibit the growth of molds that can produce mycotoxins (Laranjo et al., 2019). *Penicillium bialowiezense* was present at location C and D prior to low read removal. After removal of low reads *P. bailowiezense* was still present at location D (~25%). *P. bialowiezense* is a close relative to *P. brevicompactum* which has shown to produce desirable sensory characteristics when inoculated into coffee. The key microorganism used during the ripening of blue cheeses is *Penicillium roqueforti* and its presence has also been documented on dry-aged beef (Capouya et al., 2020). Additionally, *Penicillium carneum*, which belongs in the *Penicillium roqueforti* group, was identified at aging location J (<1%) in this study.

The fungal genera *Cladosporium* was present at two of the aging locations (A and J).

These organisms are slow growing and often responsible for grapevine rot in maturing grapes destined for wine production (Briceno and Latorre, 2008). Ryu et al. (2018) reports that *Cladosporium* species are potentially harmful during high levels of exposure, however during the dry-aging process these organisms disappeared after a 60-day aging period. For this study, sequencing results do not define if, or when, the organisms are viable on dry-aged beef. Nevertheless, *Cladosporium* organisms have been isolated from baijiu, an ancient Chinese liquor, and are associated with the metabolite acetoin, a compound responsible for a buttery odor and flavor.

Due to low sequencing read counts for fungal sequences, samples with less than 200 reads per sample were removed from the dataset before further analysis and should be viewed with caution. Bacterial sequencing data from all locations were retained (**Figure 2.3**) following low read removal. Locations D, F, G, and I reported the use of UV light in order to inhibit microbial growth. However, bacterial sequencing results suggest that relative abundances of bacterial organisms were not impacted by UV light in this study. After filtering for low read counts, fungal data were removed from locations: C, F, and wet-aged samples and not included in further analyses (**Figure 2.4**). Location F reported the use of UV light, which could be the reason these samples had very low fungal sequence read counts. However, locations: D, G, and I reported the use of UV light during the aging period and still included samples with over 200 fungal sequencing reads. This observation suggests that UV treatment was not effective at these locations and may be the cause of improper placement of UV lights. Location F did not have any fungal samples with more than 200 reads, which suggests this location may also not have a well-established mycobiota within the aging facility. Additional environment factors (wind speed, relative humidity, and temperature) may also suppress

optimal conditions for fungal growth. The wet-aged control group did not include any fungal samples with more than 200 reads, as most fungal organisms are aerobic and struggle to thrive in anaerobic conditions.

Diversity Measures

Principal Coordinate Analysis plots did not show clear clustering by aging location for bacterial and fungal data (**Figure 2.5 & 2.6**). This suggests that bacterial and fungal community compositions were not unique to each individual aging location in this study. Shannon diversity indices calculated at the species level were not different between aging locations for unrarefied or rarefied bacterial data (P = 0.29 and P = 0.31, respectively) (**Figure 2.7** & **2.8**). Unrarefied fungal data did not show significant differences (P = 0.08) in Shannon diversity measures between aging locations (**Figure 2.9**). After fungal data was rarefied, Shannon diversity measures were significant (P = 0.03), however Tukey comparison results suggests the groups are not significant (**Figure 2.10**).

Strengths of this study were more dry-aging facilities were sampled than any previously known studies (Capouya et al., 2020) while 6 experimental units were observed at each aging facility. A sufficient number of bacterial reads were obtained in the current study which allowed for conclusions to be drawn. Limitations of the study however, included low sequencing reads for fungal data. In this study samples that had a minimum of 200 reads remained in the fungal data and thus caution is warranted in interpreting the results broadly.

Conclusion

Dry-aging of beef is a process that has been classified as more of an art than a science. During this process microorganisms can colonize the surface of dry-aged products and can potentially contribute to unique flavors observed in the final product. This study demonstrated

the presence of bacterial and fungal communities that can be found on dry-aged beef throughout the US. Though the presence of some organisms were shared across aging locations, additional organisms were unique for individual locations and likely due to the environment surrounding each aging facility. Further studies should investigate the relationships between the microorganisms and flavor profiles found on dry-aged beef. Additionally, investigating the inoculation of organisms thought to be beneficial during the dry-aging process could develop a more thorough understanding of their contribution to end product quality attributes.

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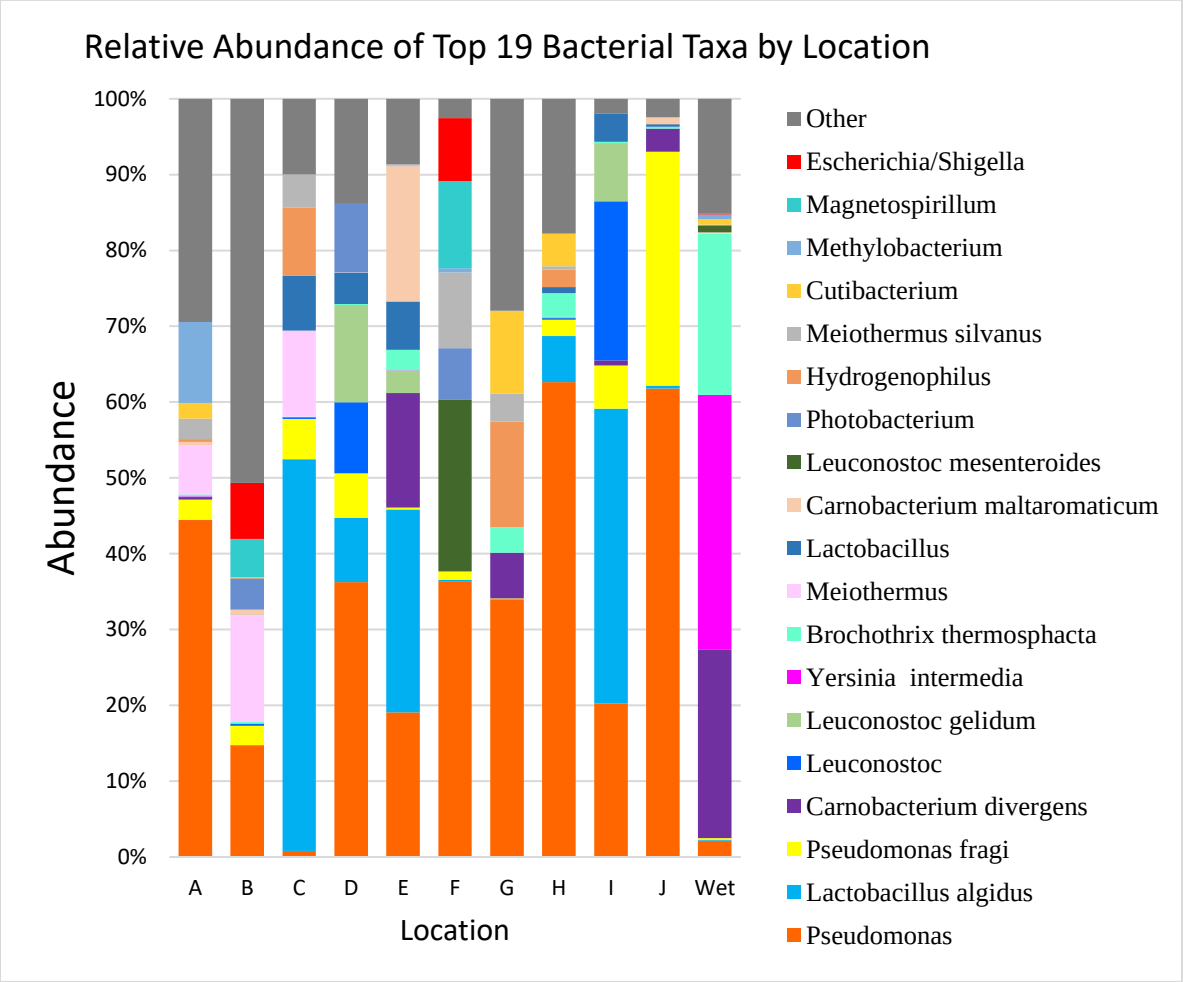


Figure 2.1:
Relative abundance chart of top 19 bacterial taxa by location of all samples. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were assigned using the SILVA 16S rRNA version 138 database. *Psuedomonas, Lactobacillus algidus,* and *Pseudomonas fragi* are core taxa present in at least 50% of all samples.

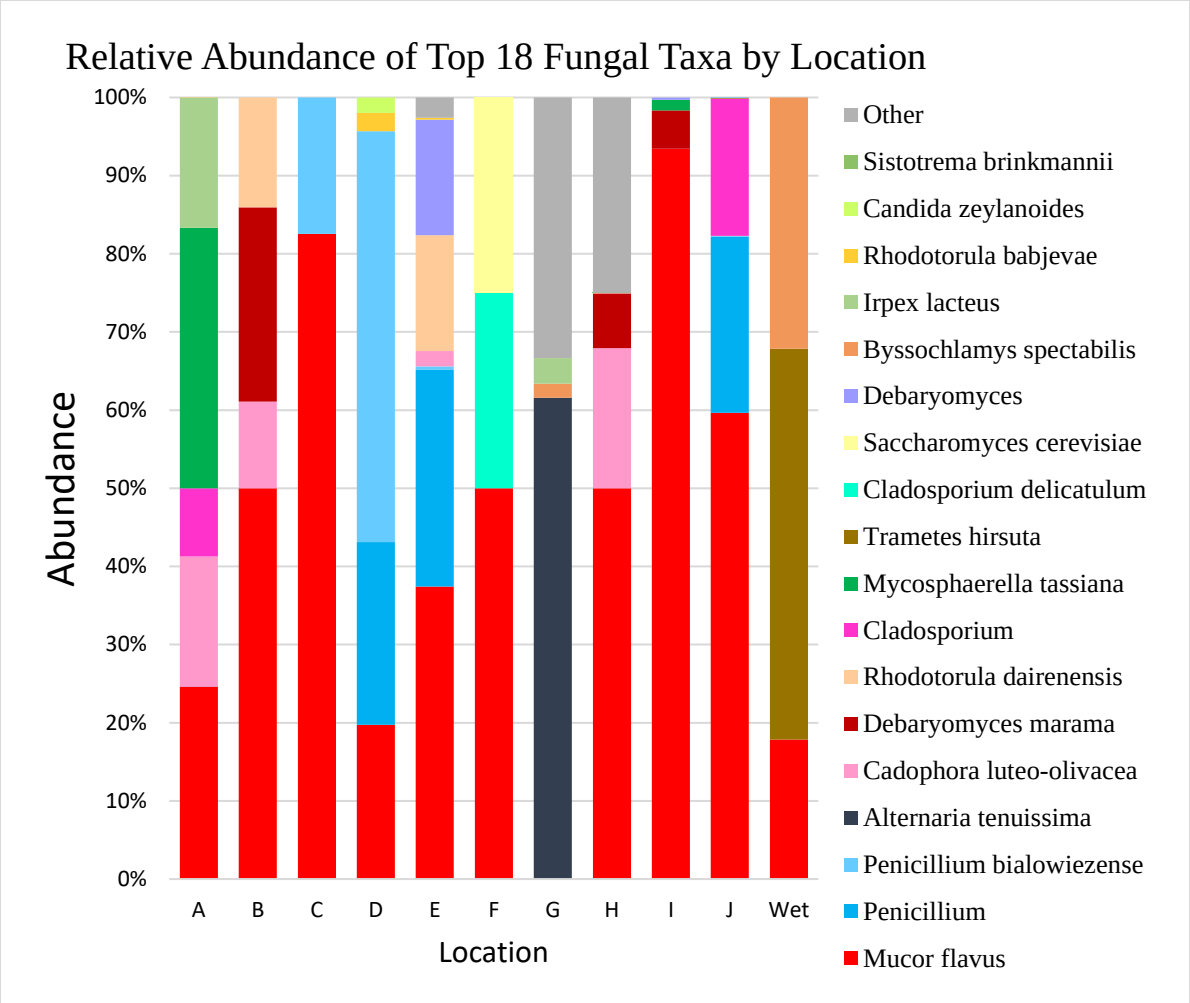


Figure 2.2:
Relative abundance chart of top 18 fungal taxa by location prior to removing samples with less than 200 reads. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the ITS1 domain. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were completed using the UNITE version 8.2 database. *Mucor flavus* was the dominant taxa present in approximately 60% of all samples and identified at all locations except location G.

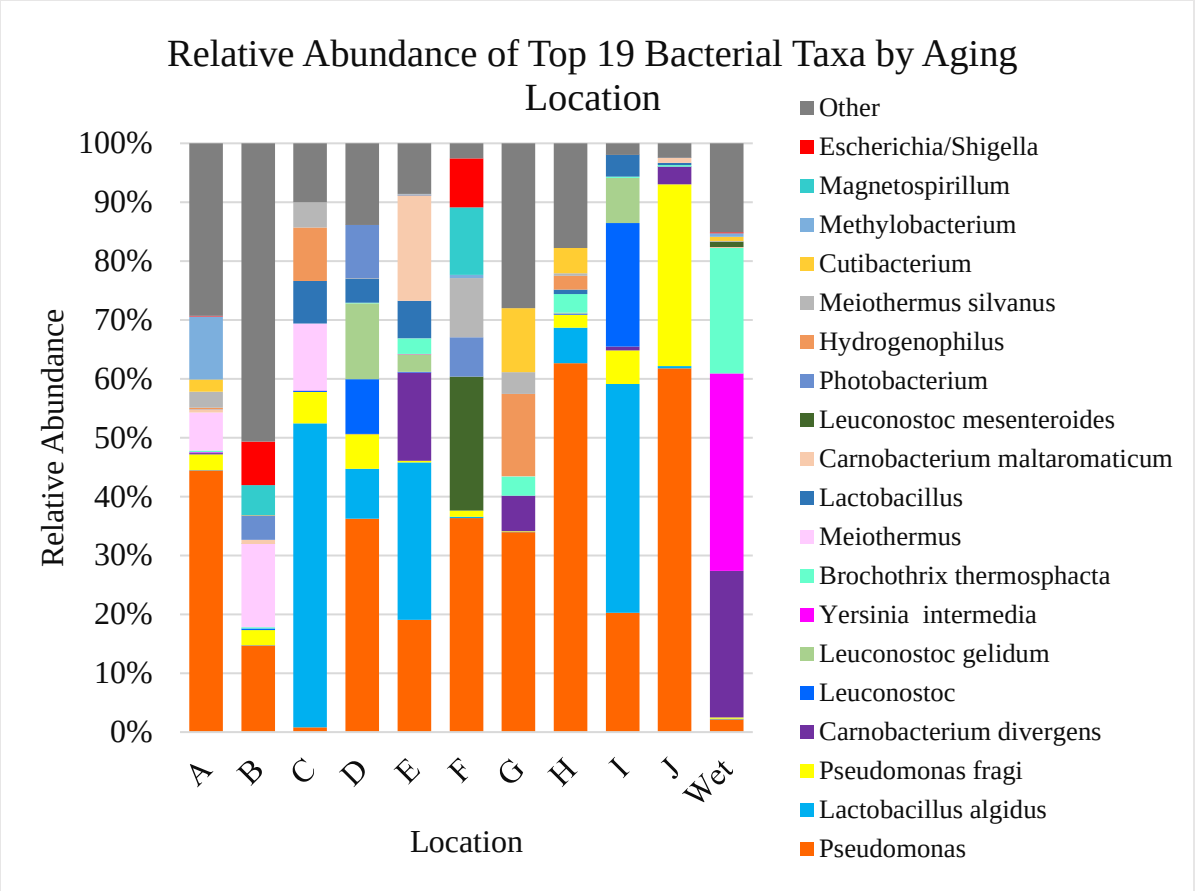


Figure 2.3:
Relative abundance of top 19 bacterial taxa after removing samples with less than 200 reads. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were assigned using the SILVA 16S rRNA version 138 database. Bacterial DNA was found at all locations, suggesting UV light was not effective in inhibiting bacterial growth.

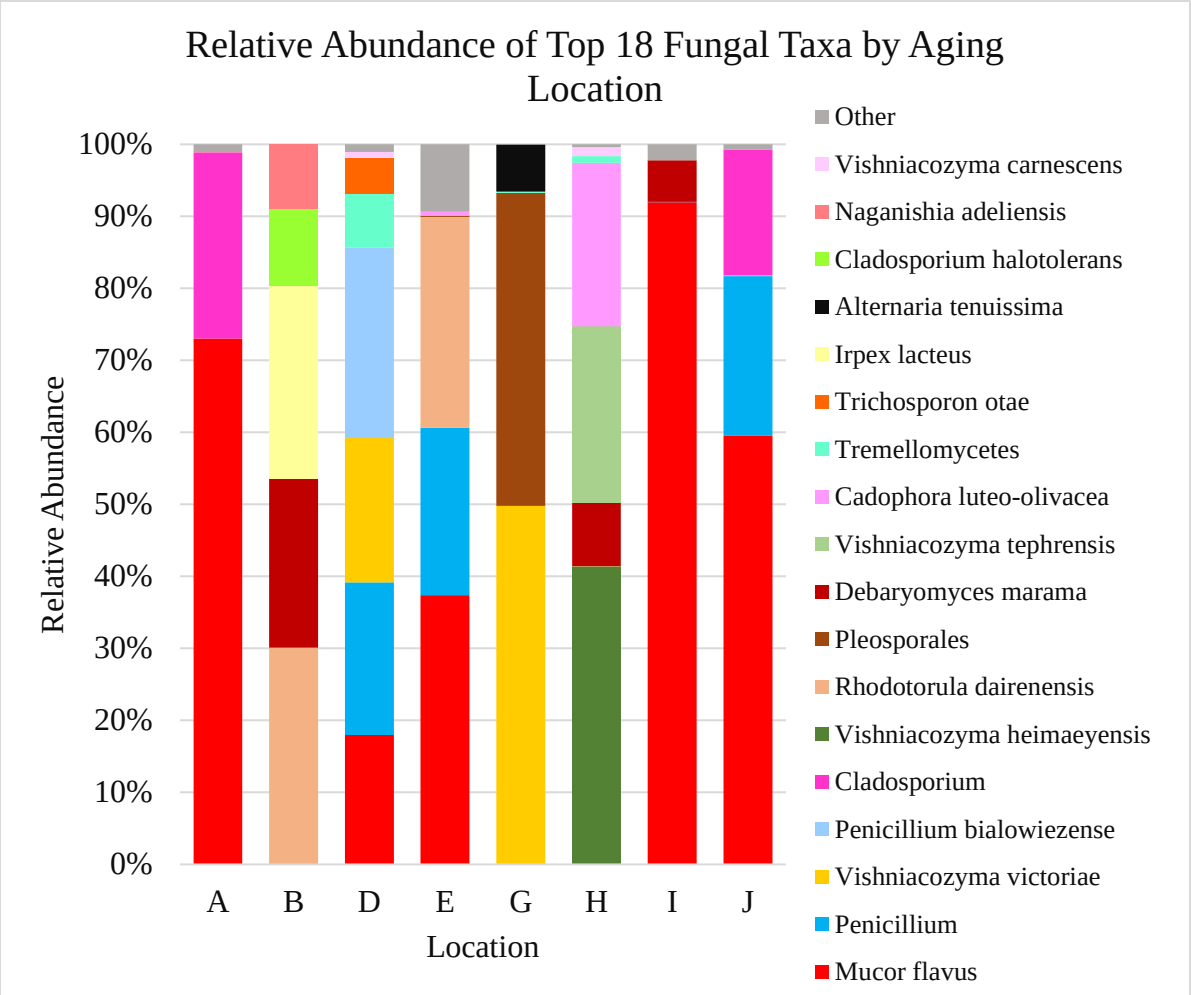


Figure 2.4:
Relative abundance of top 18 fungal taxa after removing samples with less than 200 reads. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the ITS1 domain. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were completed using the UNITE version 8.2 database.

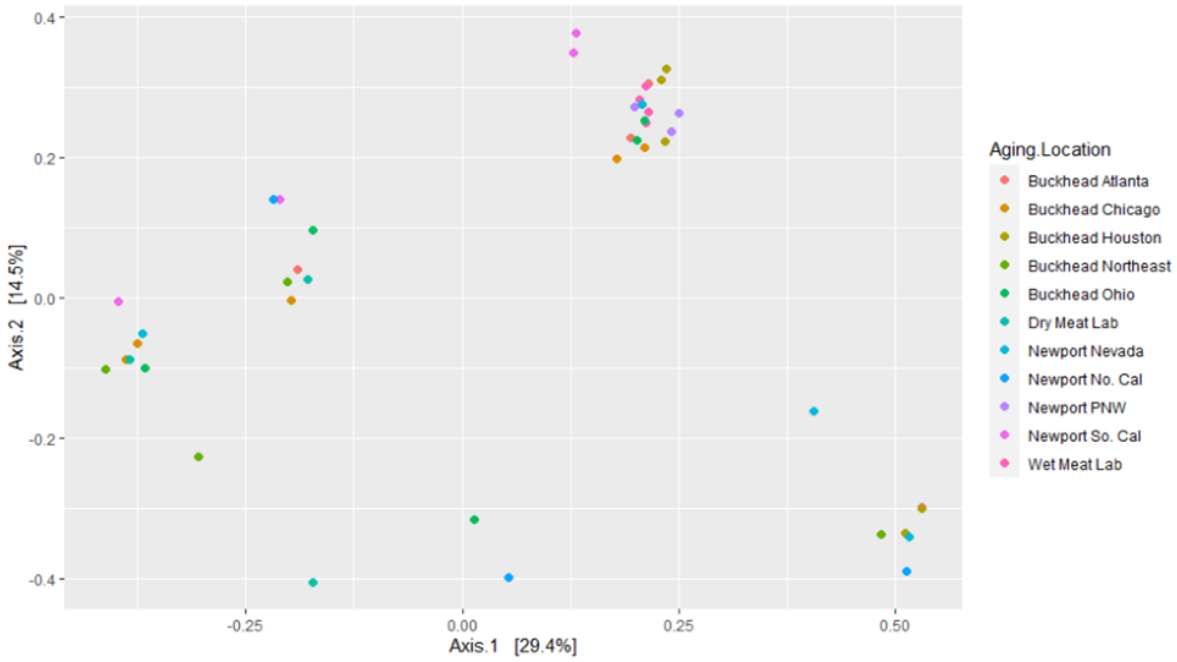


Figure 2.5:
Principal Coordinate Analysis plot (PCoA) for rarefied bacterial data at species-level. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were assigned using the SILVA 16S rRNA version 138 database.

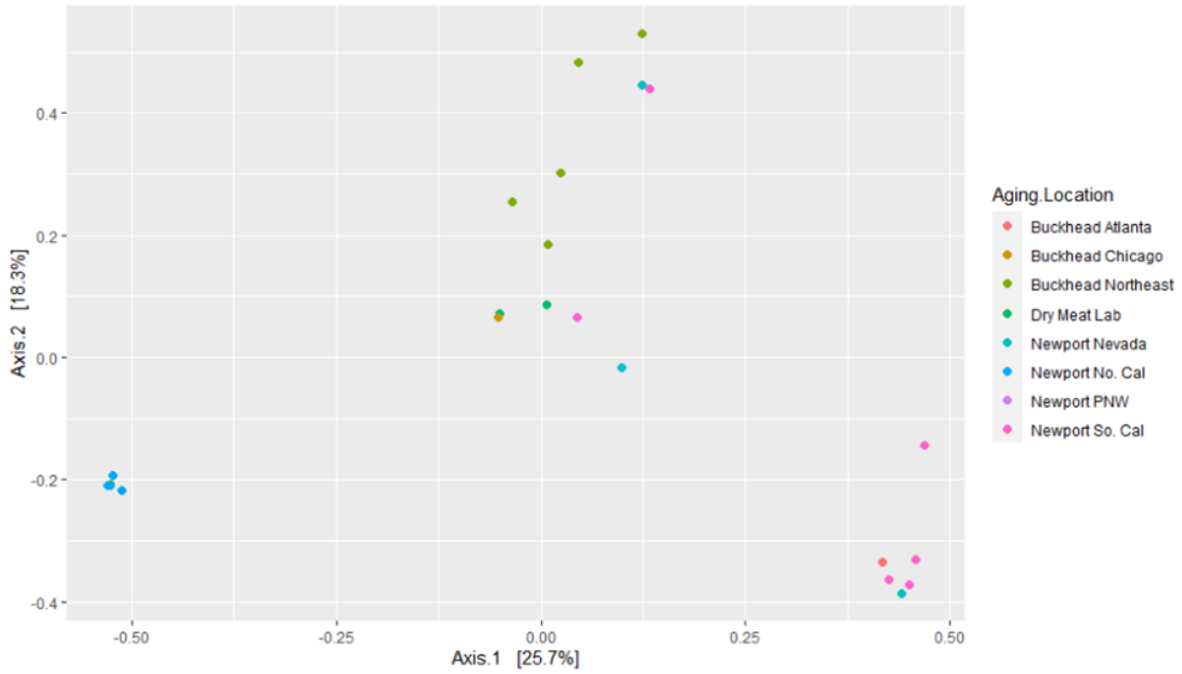


Figure 2.6:
Principal Coordinate Analysis plot (PCoA) for rarefied fungal data at species-level. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the ITS1 domain. Sequencing was performed on an Illumina Miseq 300 bp for 600 cycles. Taxonomic assignments were completed using the UNITE version 8.2 database. Samples with less than 200 reads were removed.

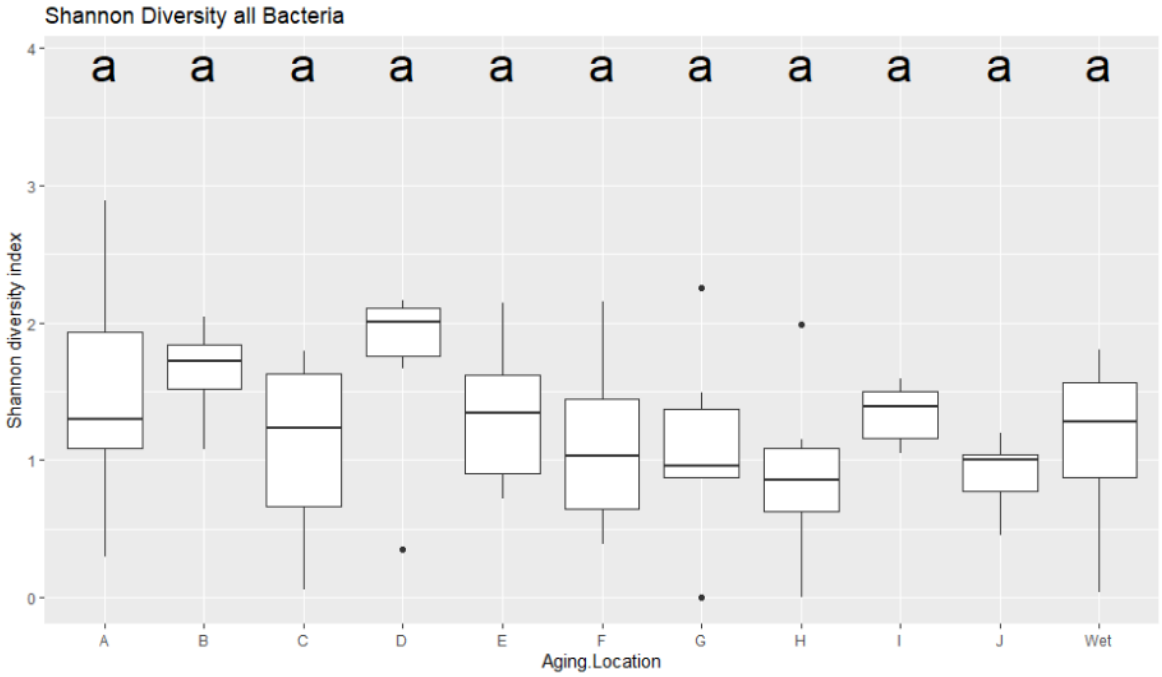


Figure 2.7:
Boxplots depicting Shannon diversity indices by aging location calculated from unrarefied bacterial count data (P = 0.29). Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Taxonomic assignments were assigned using the SILVA 16S rRNA version 138 database.

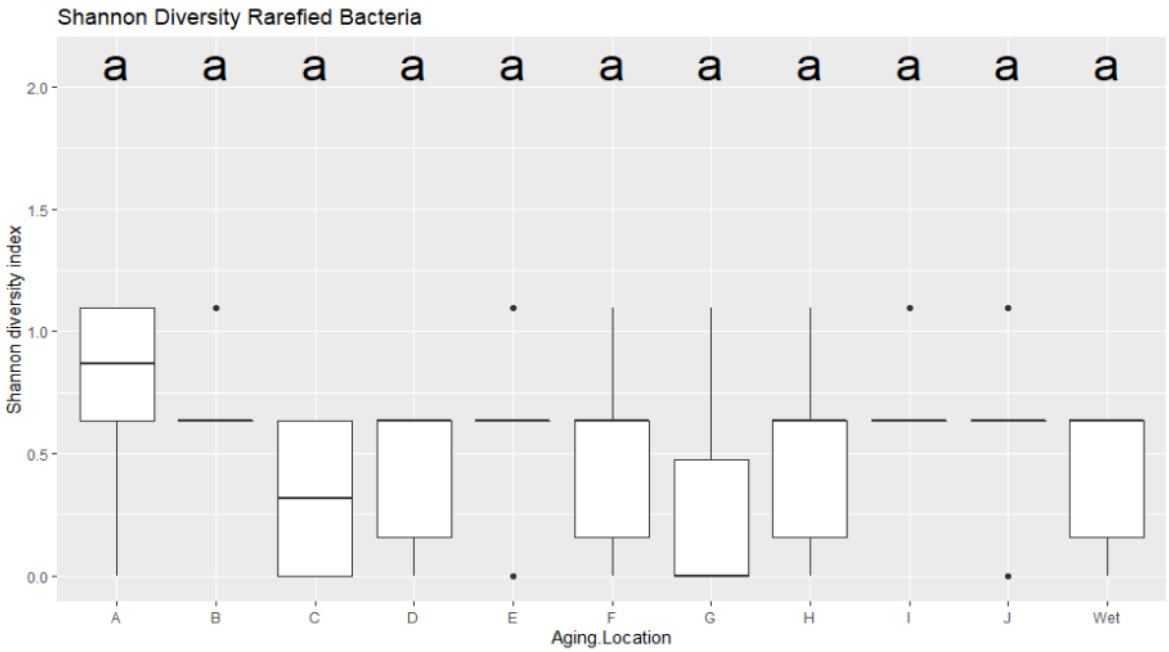


Figure 2.8:

Boxplots of Shannon diversity indices by aging location for bacterial samples rarefied at 1000 reads (P = 0.31). Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the V1-V3 hypervariable region of the 16S rRNA gene. Taxonomic assignments were assigned using the SILVA 16S rRNA version 138 database.

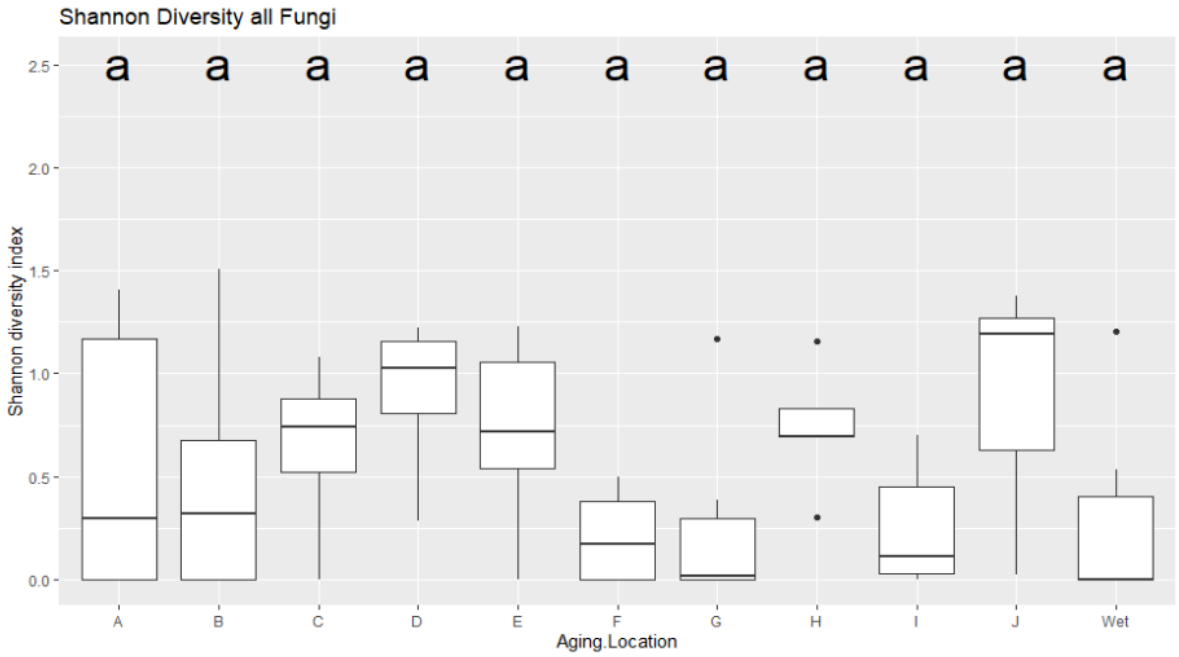


Figure 2.9:
Boxplots of Shannon diversity indices by aging location calculated from unrarefied fungal count data at the species-level (P = 0.08). Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the ITS1 domain. Taxonomic assignments were completed using the UNITE version 8.2 database. All aging locations retained all six samples expect: A (n = 5), C (n = 5), F (n = 4) and H (n = 5).

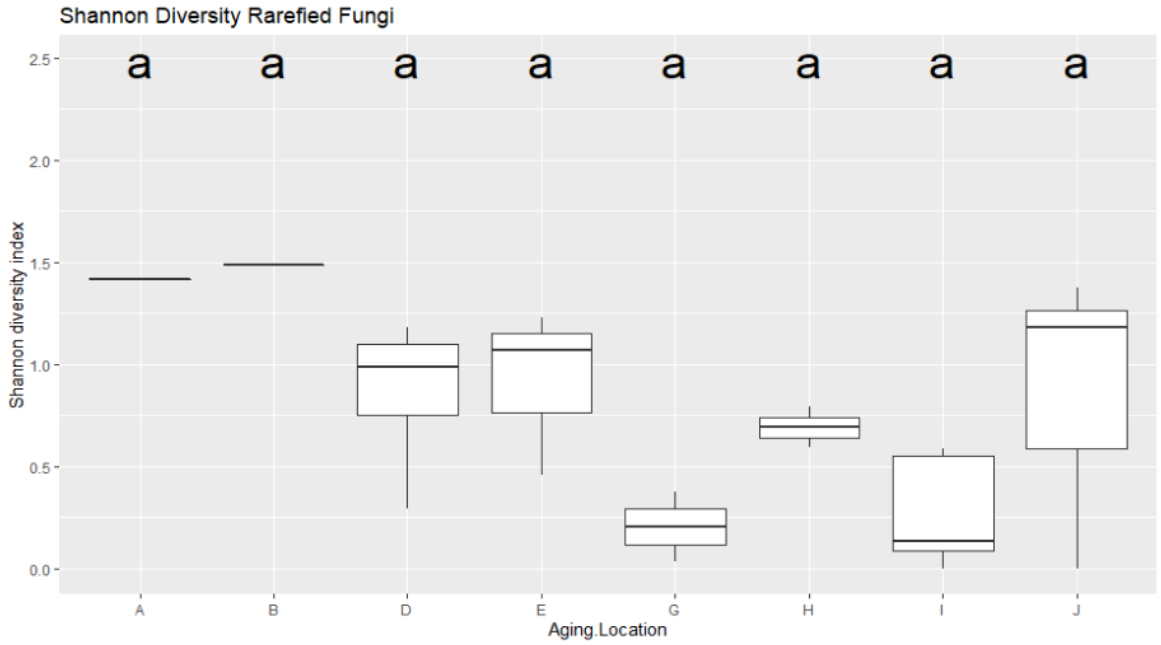


Figure 2.10:
Boxplot of Shannon diversity indices by aging location for fungal data rarefied at 200 reads. Each aging location consisted of 6 *Certified Angus Beef*® bone-in strip loins (NAMI #175). Strip loins were dry-aged for 45 days at each facility and returned to Vandal Brand Meats, Moscow, ID. Surface crust (200mg) was removed from the anterior face of each strip loin. DNA was extracted and targeted PCR was performed to amplify the ITS1 domain. Taxonomic assignments were completed using the UNITE version 8.2 database. Samples with less than 200 reads were removed. A (n = 1), B (n = 1), D (n = 6), E (n = 3), G (n = 2), H (n = 3), I (n = 5), J (n = 6).

Appendix A: Sampling for microbiome analysis on Dry-Aged Beef

Microbiome Sampling Protocol

Note: Sterile technique is very important when collecting samples for microbiome analysis in order to minimize contamination (Sterile gloves, scalpels, collection area and other instruments used for sampling should be first priority).

1) Place short loin on collection area– with the vertebrae flat on the table surface.
    a. Grab the vertebrae and tail of short loin when moving to collection area.
        i. Avoid contact with the face(s) (exposed muscle) of short loin in order to prevent contamination.
2) Swab surface of the anterior face of short loin with Copan Floqswab, after dipping the swab in nuclease-free water, and place swab on ice after collection.
    a. Make sure Floqswab is sealed prior to use.
    b. Once you have broken the seal of the Floqswab, dip end of swab in nuclease-free water and proceed to swab ventral corner of the anterior face of the short loin.
        i. Be sure **not** to touch anything (table, outside of water vial, gloves, etc.) with end of swab, other than area of sampling.

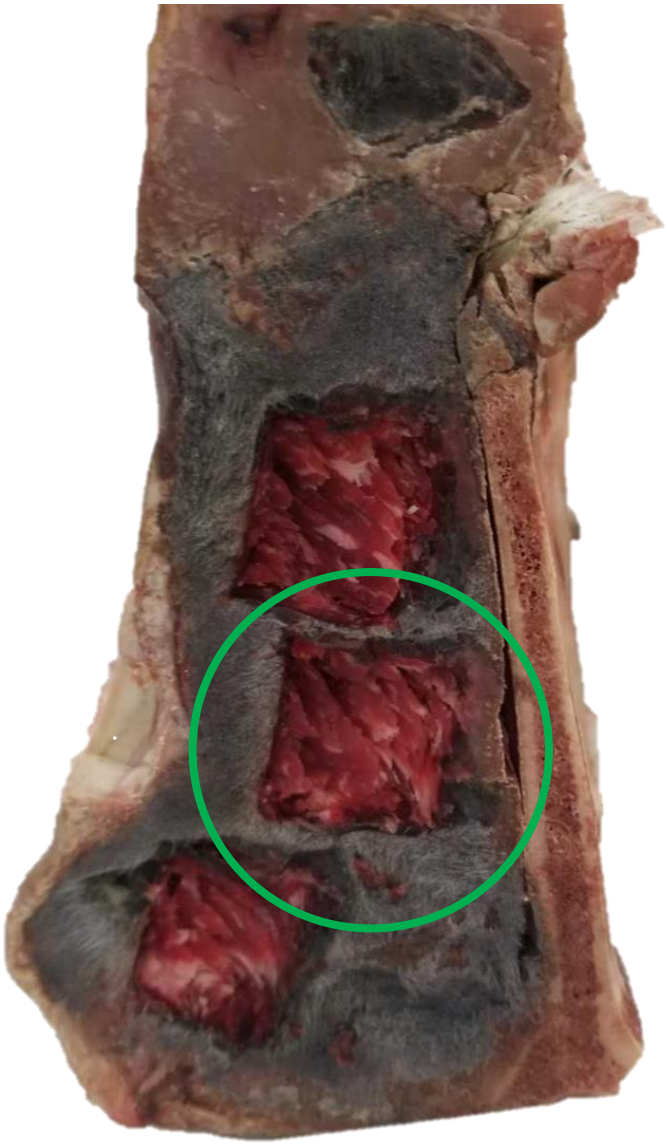
Note: Avoid swabbing area (step #2) when collecting meat/fat samples (step 3-6). See diagram for further explanation on locations for steps 2-6.

3) With clean gloves, sterile disposable scalpel w/No. 20 surgical blade and sterile disposable forceps remove approximately 1-1.5 inches of the thickest portion of subcutaneous fat on the anterior face of loin. Avoid scalpel/forceps contact with meat prior to fat collection. Store fat sample in labeled whirlpak bag on ice.
    a. Treat all tools and samples in the same sterile manner as the Floqswab in order to prevent cross contamination.
4) With the same scalpel/forceps used in step 3, remove approximately 1-inch X 1.5 inches X 1 centimeter (length X width X depth) of the medial (center) section of the anterior face and place in labeled whirlpak bag. Place sample on ice once collected.

5) Collect the dorsal (top) section of the anterior face of the short loin by using the same method as described in step 4.

6) Collect the ventral (bottom) section of the anterior face of the short loin by using the same method as described in step 4.

7) Once all areas have been collected, discard disposable scalpel into sharps container and disposable forceps into garbage. Repeat protocol with each loin. Each loin should have individual scalpel/forceps. Do not reuse scalpels/forceps on another loin.



Appendix B: ZymoBIOMICS DNA Miniprep Kit for Dry-aged Beef Microbiome

DNA Extraction Protocol

This portion of the protocol will be completed in the flow hood.

1) Thaw sample(s) on ice for approximately 1 hour prior to processing.
    a. Record samples and date of extraction.
2) Ethanol the interior (sides, base, glass) of flow hood; including scale.
3) Ethanol scalpel, forceps, sharpie, and tube rack prior to placing in hood. Place petri dish, and pre-labeled 1.7ml tubes (one per sample) into flow hood.
    a. Petri dishes, and pre-labeled 1.7ml tubes do not need to be cleaned with ethanol prior to entering the flow hood.
    b. Do not remove scalpel and forceps from package until ready to use.
4) After supplies are placed in flow hood, turn on UV light for approximately 15 minutes.
5) Once the flow-hood has been sterilized (ethanol and UV light) and the sample(s) have thawed, carefully place sample for processing into clean petri dish.
    a. Use bottom half of petri dish to hold sample.
    b. Use top half of petri dish as the "weigh boat".
6) Weigh approximately 200mg (±10 mg) of the surface crust and place into pre-labeled ZR BashingBead Lysis Tube.
    a. Weigh another 200mg (±10 mg) of the same sample and place into pre-labeled 1.7ml tube.
        i. Store in -80 freezer.
    b. Record weights of the amount placed in each tube.
7) Carefully put remaining portion of sample back into whirl-pack bag and place into -80 freezer.

Once 200mg of sample(s) are in the ZR BashingBead Lysis Tube, the remaining steps may be completed on the lab bench.

8) Add 750 µL of ZymoBIOMICS **Lysis Solution** to each BashingBead Lysis Tube containing sample and a negative control tube (BashingBead Lysis Tube).
    a. Treat negative control as if it were a sample and include in all remaining steps.
        i. A negative control should be included in each DNA extraction round performed.
        ii. A positive control should be included in each DNA extraction round performed.
            1. Add 37.5 µL of Zymo Microbial community standard to positive control tube.
9) Place each ZR BashingBead Lysis Tube into bead basher and run cycle:
    a. Speed: 6
    b. Time: 30 seconds (2x) = 60 seconds

- **c.** Rest on ice: 5 minutes
- **d.** Repeat: 5 cycles
    - **i.** Check for leaky caps between cycles. If leaking occurs, seal tubes with parafilm.

10) Following Bead-Bashing cycle, centrifuge samples for 60 seconds at ≥ 10,000 x g.

11) Add 400 µL of supernatant, from centrifuged BashingBead Lysis Tube, to Zymo-Spin III-F filter and place into new pre-labeled collection tube (label cap of filter and side of collection tube).
- **a.** Avoid bashing beads when removing supernatant.
- **b.** Centrifuge at 8,000 x g for 60 seconds.
    - **i.** Discard Zymo-Spin III-F filter.

12) Transfer flow-through, from step 11, to a new pre-prelabeled **2 mL** microcentrifuge tube.

13) Add 400 µL of **100% ethanol** to the 2 mL microcentrifuge tube from step 12.
- **a.** Add 100% ethanol to all samples before continuing.

14) Add 800 µL of ZymoBIOMICS **DNA Binding Buffer** and mix solution by aspirating with pipette. Once mixed, transfer 800 µL of solution to pre-labeled Zymo-Spin IICR Column in a new collection tube.
- **a.** Centrifuge at 10,000 x g for 60 seconds.
- **b.** Discard flow through and add the remaining solution (800 µL) to the Zymo-Spin IICR column.
    - **i.** Centrifuge at 10,000 x g for 60 seconds.
    - **ii.** Discard flow-through.
    - **iii.** Keep collection tube for step 15.

15) Add 400 µL ZymoBIOMICS **DNA Wash Buffer 1** to Zymo-Spin IICR Column.
- **a.** Centrifuge at 10,000 x g for 60 seconds.
- **b.** Discard flow through.
- **c.** Keep collection tube for step 16.

16) Add 700 µL ZymoBIOMICS **DNA Wash Buffer 2** to the Zymo-Spin IICR Column.
- **a.** Centrifuge at 10,000 x g for 60 seconds.
- **b.** Discard flow through.
- **c.** Keep collection tube for step 17.

17) Add 200 µL ZymoBIOMICS **DNA Wash Buffer 2** to the Zymo-Spin IICR Column.
- **a.** Centrifuge at 10,000 x g for 60 seconds.
- **b.** Transfer Zymo-Spin IICR Column to a new collection tube for step 18.
- **c.** Discard flow-through.
- **d.** Discard collection tube used in steps 14-17.

18) Add 50 µL of ZymoBIOMICS **DNase/RNase Free water** directly to the column matrix of the Zymo-Spin IICR Column that was placed in a new collection tube (step 17b).
- **a.** Incubate for 60 seconds at room temperature.
- **b.** Centrifuge at 10,000 x g.
- **c.** Leave Zymo-Spin IICR Column in collection tube and keep for step 20.

19) Place a Zymo-Spin III-HRC Filter in a new collection tube and add 600 µL ZymoBIOMICS **HRC Prep Solution** to filter.
   - **a.** Centrifuge at 8,000 x g for 3 minutes.
   - **b.** Discard flow-through and collection tube.

20) Place Zymo-Spin III-HRC filter (step 19) into a new pre-labeled microcentrifuge tube and transfer eluted DNA (step 18 (50 µL)) to the center of the filter.
   - **a.** Centrifuge at 16,000 x g for 3 minutes.

21) Supernatant in microcentrifuge tube (step 20) is now suitable for PCR.
   - **a.** Store in -80 freezer.

Appendix C: Accuclear High Sensitivity dsDNA Quantitation Protocol

ACCUCLEAR HIGH SENSITIVITY dsDNA QUANTITATION PROTOCOL

MATERIALS:

- 15 mL or 50 mL tubes

- Micropipettes: 1-10ul, 10-100ul

- Serological disposable pipettes (25mL)

- Multichannel micropipettes: 1-10uL, 300uL

- 50 or 100mL reservoir (2) *smaller internal channel than 25mL reservoir

- tips: 100uL, 20uL, 10uL

- black 96 well plates (Greiner Bio One 96 black)

- Microplate reader

- Foil

REAGENTS:

-AccuClear High Sensitivity dsDNA quantitation Kit with standards (Quantitation solution, dye, standards). Catalog Number _31028_ Biotium.

-Internal standard (pre-prepared)

Note: the kit does not come with a 0ng/ul standard. Use 10ul of the quantitation solution (without dye added) as the 0ng/ul standard. Also remember when calculating out reagent volumes, standards and blanks are done in duplicate.

-Buffer TE

PROCEDURE:

1. Perform calculations to determine quantity of reagents required (Attached calculation sheet below).
2. Warm kit at room temperature. Invert quantitation solution several times. Vortex the enhancer when thawed.
3. Prepare the Working Solution (WS): micropipette 10-100uL, pipette aid+ 25mL disposable pipette according to calculations.
4. Thaw the plate of PCR amplicons. Maintain it in ice.
5. Prepare the plate (samples, blank and standards) maintaining aseptic conditions:
   5.1. Pipette 200uL of WS per well (samples -unknowns-, blank and standards). Multichannel 300uL pipette
   5.2. Add 8uL of Buffer TE in each sample -unknowns- wells containing WS. Multichannel 1-10uL pipette

5.3. Add 10uL of Buffer TE in the blank well containing WS. Micropipette 1-10uL
5.4. Add 10uL of each dsDNA standard in duplicate (0, 0.03, 0.1, 0.3, 1, 3, 10, and 25ng/uL) into its own separate well containing WS. Vortex each standard before adding to appropriate well. Mix well by pipetting.
5.5. Pulse vortex plate of PCR amplicons and then do a brief spin down using plate spinner.
5.6. Add 2uL of each sample -unknown- amplicon into its own separate well containing WS and TE Buffer. Mix well by pipetting (↑10uL). Multichannel 1-10uL pipette.

6. Incubate during 10 min at room temperature in the dark: transport the plate covered with aluminum foil to the microplate reader, put it inside the reader.
7. Measure fluorescence.

USING THE MICROPLATE READER AND THE SOFTMAX 6.5.1 SOFTWARE.

1. Open the microplate reader case with the display of the microplate reader: put the plate, close the case.
2. Use the PC with your UI User and Password. Go to the SoftMax 6.5.1. program.
3. Set up the equipment:
   SETTINGS:

-Optical Configuration: Read mode: FL

Read type: Endpoint
Wavelength: Known
Number of wavelength pairs: 1
Bandwidth: Excitation-9/Emission-15nm
Excitation Lm 485nm/Emission 530nm

4. Design the plate: Plate 1 → Settings
   -Plate type: 96 well greiner blk/clr btm
   -Read area: select the area that you are going to use.
   -Define your samples (plate 1) template editor
   a. select unknowns → define it (select block) → assign
   b. select standards → define it (select block) → assign
5. Read (check that the equipment is online). After reading remember to take out the plate.
6. "Save as" the results (at the top left) .sda as a SoftMaxPro 6.5.1 file
7. Export as Excel File.

8. Manage the standard curve (intercept, R2, y) in excel..and the samples (x5 dilution).

AccuClear Record Sheet and Calculations

Samples and Plate Set Up:

= Samples

= Blanks

= Internal control

= Kit DNA Standards

	1	2	3	4	5	6	7	8	9	10	11	12
A											0	0
B											0.03	0.03
C											0.1	0.1
D											0.3	0.3
E											1	1
F											3	3
G											10	10
H											25	25

Calculations:

Total TE Buffer:

8 uL x [ ] Number of samples/internal control = [ ]

10 uL x [2] Number of blanks = [20]

= [ ]

Total

Working Solution:

Dye	Quantitation Solution	Number of wells/plates wells
200uL	20mL	96 wells (1 plate)
100uL	10mL	48 wells
75uL	7.5mL	36 wells
50uL	5mL	24 wells
25uL	2.5mL	12 wells

Appendix D: Primer Sequence Table

Target and Region	F Name	F Sequence	R Name	R Sequence
16S, V1-V3	CS1-27F_YM1-for	ACACTGACGACATGGTTCTACAGTAGAGTTTGATCCTGGCTCAG	CS2-534R_1-rev	TACGGTAGCAGAGACTTGGTCTCCATTACCGCGGCTGCTGG
	CS1-27F_YM2-for	ACACTGACGACATGGTTCTACACGTAGAGTTTGATCATGGCTCAG	CS2-534R_2-rev	TACGGTAGCAGAGACTTGGTCTGCCATTACCGCGGCTGCTGG
	CS1-27F_YM3-for	ACACTGACGACATGGTTCTACAACGTAGAGTTTGATTCTGGCTCAG	CS2-534R_3-rev	TACGGTAGCAGAGACTTGGTCTTGCCATTACCGCGGCTGCTGG
	CS1-27F_YM4-for	ACACTGACGACATGGTTCTACATACGTAGAGTTTGATTATGGCTCAG	CS2-534R_4-rev	TACGGTAGCAGAGACTTGGTCTATGCCATTACCGCGGCTGCTGG
	CS1-27F_Bif-for	ACACTGACGACATGGTTCTACAGTACGTAGGGTTCGATTCTGGCTCAG	CS2-534R_5-rev	TACGGTAGCAGAGACTTGGTCTCATGCCCATTACCGCGGCTGCTGG
	CS1-27F_Bor-for	ACACTGACGACATGGTTCTACACGTACGTAGAGTTTGATCCTGGCTTAG	CS2-534R_6-rev	TACGGTAGCAGAGACTTGGTCTTCATGCCATTACCGCGGCTGCTGG
	CS1-27F_Chl-for	ACACTGACGACATGGTTCTACAACGTACGTAGAAATTTGATCTTGGTTCAG	CS2-534R_7-rev	TACGGTAGCAGAGACTTGGTCTATCATGCCATTACCGCGGCTGCTGG
Target and Region	**F Name**	**F Sequence**	**R Name**	**R Sequence**
ITS1	CS2-ITS1_1	TACGGTAGCAGAGACTTGGTCTGGCTTGGTCATTTAGAGGAAGTAA	CS1-ITS2_1	ACACTGACGACATGGTTCTACACGGCTGCGTTCTTCATCGATGC
	CS2-ITS1_2	TACGGTAGCAGAGACTTGGTCTCGGCTTGGTCATTTAGAGGAAGTAA	CS1-ITS2_2	ACACTGACGACATGGTTCTACATCGGCTGCGTTCTTCATCGATGC

Black = Tag sequence

Red = phasing nucleotides

Green = linker

Purple = target-specific sequence

Appendix E: PCR Cycling Parameters