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The Invisible Meat Microcosmos - Investigations of Processed Meats' Specific Spoilage Organisms

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THE INVISIBLE MEAT MICROCOSMOS– INVESTIGATIONS OF PROCESSED MEATS' SPECIFIC SPOILAGE ORGANISMS

by

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THE INVISIBLE MEAT MICROCOSMOS– INVESTIGATIONS OF PROCESSED MEATS' SPECIFIC SPOILAGE ORGANISMS

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As humankind grows in population, so will our need to compete against bacterial populations which spoil foodstuffs before we are able to consume them. This dissertation aims to identify the bacterial communities responsible for meat spoilage, and the mechanisms that govern the behaviors of these organisms. The literature review summarizes the state of knowledge regarding factors that influence meat microbiome composition, and how analytical methods can influence research outcomes. Study 1 aimed to identify the impact of additional ingredients and processing steps on the microbial ecology of processed turkey products. The main specific spoilage organisms (SSOs) belonged to the orders Pseudomonadales, Enterobacteriales, and Lactobacillales. While thermal processing and subsequent contamination altered abundances of lactic acid bacteria, *Pseudomonas* spp. were observed in all samples, affirming their prevalence in products produced in the Loeffel Meat Laboratory. In an attempt to modulate raw turkey microbiomes away from rapid SSOs like pseudomonads, Study 2 utilized packaging systems with varying atmosphere compositions. While some packaging systems steered accumulation of greater abundances of lactic acid bacteria or *Brochothrix*, *Pseudomonas* spp. were still recovered from all samples and were a substantial portion of the microbial communities. This incessant microbe illustrates that starting material and processing

environment play an important role in setting a microbiome prior to additional hurdles. We hypothesize that the processing environment, as a proxy effect of its geographic location, plays a role in establishing the meat microbiome. The problematic pseudomonads from the previous studies were then submitted for whole genome sequencing to gain insight into the genetic mechanisms that may allow their survival in low oxygen conditions is discussed in Chapter 4.

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CHAPTER ONE: REVIEW OF LITERATURE

1. INTRODUCTION

As the global population increases, so does the demand for nutritious foods, and the resources to produce them. While enough calories are currently harvested to sustain the global demand, there is increased pressure to generate and utilize these calories in responsible ways as nearly 40% of all food produced is wasted (Gunders & Bloom, 2017). Around 55.9% percent of food waste in the United States is currently lost to landfills, so considering the journey of foods, from farm to fork to disposal is of great interest (US EPA, 2020). One reason foods are lost prior to consumption, is due to spoilage rendering products unsuitable for eating due to quality or safety concerns. This often happens at a consumer level, where meat and muscle foods comprise 41% of the value lost (Buzby & Hyman, 2012). Muscle foods, often having high moisture content and requiring refrigeration, are particularly vulnerable to bacterial spoilage. Thus, striving to understand bacterial communities to delay their outgrowth can result in shelf-life extension, ensuring product has time to be consumed rather than disposed.

Bacterial spoilage of meat products is typically characterized by the production of off-odors, off-flavors, discoloration, gas production, or slime formation. Typically, total growth above 7 log CFU/g is considered sufficient biomass to cause spoilage, and spoilage generally coincides with a loss of bacterial diversity (Chaillou et al., 2015). While the typical specific spoilage organisms of meat and poultry products are psychrophilic members of Pseudomonadaceae, Enterobacteriaceae, Listeriaceae, and Lactobacilliaceae, the dominant community members will change on a product-to-product basis, and exceptional species pervade the meat landscape under the appropriate

conditions (Odeyemi et al., 2020). Determining which organisms are likely to dominate the spoilage microbiome of a product requires the consideration of many intrinsic and extrinsic factors, such as pH, storage temperature, processing parameters, and initial inoculum. Due to the complex assembly of microbial spoilage communities, researchers and processors must consider a multi-tiered approach when determining which organisms are of concern in their systems and determining methodologies to delay their outgrowth. A general overview of factors determining the spoilage microbiome composition is supplied in Figure 1, and further described in this review.

Additionally, when investigating the meat spoilage microbiome, researchers must consider the impact on their methodology, both in "wet lab" procedures and in computational analyses to adequately assess the phenomena that influence community structure. Through proper use of available tools and thorough investigation of factors of interest, we may be better able to target problematic SSOs, not only reducing food waste but wisely picking the best antimicrobial scheme for the organism to avoid extraneous energy costs during product development and production.

2. EXTRINSIC FACTORS INFLUENCING MICROBIAL SPOILAGE

2.1. Processing Contamination Routes

Across the meat and poultry processing microbiome, meat scientists are looking to reduce routes of contamination and keep bacterial counts low once contaminants are introduced on the muscle surface. Knowing where these contamination routes arise, and which bacteria to expect can vary from product to product, but generally, the core microbiome of meat and poultry products is believed to be firstly governed by the cold processing environment selecting for psychrotrophs, mostly of environmental origins,

such as soil or water. After this major group, an additional contamination route is from the outer surface of the animal during harvest, with poultry items having bacteria related to poultry litter, and ruminant species having bacteria from rumen contents (Chaillou et al., 2015). Bacterial communities do not seem to be impacted by facility size, with small and large facilities exhibiting similar profiles (Stellato et al., 2016). This concept is sensible in nature when comparing processing environments with similar environmental inoculum, as a study observing the establishment of a small processing plant over time observed that psychrotrophic environmental bacteria, capable of forming biofilms persisted in stable communities, setting the core microbiome of the facility (Belk et al., 2022).

The amount of inoculum, and exposure to sanitizers, however, varies across a meat facility, and can further impact community development. In packaging areas that are heavily sanitized, sanitizer-resistant bacteria may dominate, leading to communities with a higher propensity to contaminate products. Additionally, in locations that may be difficult to sanitize, such as meat slicers, biofilms may aggregate on product blades and contaminate the product, so products produced in the same facility that interface with different implements, could have varied communities. Sliced hams produced in the same facility as unsliced hams were observed to have notably higher plate counts over time, exhibiting *Lactobacillus* and *Leuconostoc* spp. that likely arose from post-process contamination (Samelis et al., 1998).

Considering meat processing microbiomes are largely influenced by contaminants of the environment, the influence of the geographical location of the processing plant, and incoming materials is called into question. While differences in community

composition have been observed in both bulk tank milk from different farms and clams from two coastal locations, the geospatial variation in meat microbiomes in underexplored (Liu et al., 2020; Porcellato et al., 2021).

2.2. Production Parameters

While the meat processing environment and raw materials provide a community baseline, operators can modulate a variety of parameters to modulate bacterial communities. For cooked items, it is proposed that thermal processing parameters, temperature, and time, may play a role in determining spoilage microbiomes. When chub-packed luncheon meat was processed at different temperatures, different microbial profiles emerged, insinuating different bacterial species have differing thermal resistances (Bell, 1983). While cook cycles are developed to inactivate pathogens, some spoilage organisms have been observed to endure said temperatures. *Pseudomonas* isolates from turkey meat, when inoculated into a beef system appear to survive thermal process cycles which were adequate for pathogen reduction (Watson et al., 2021).

Some microbes are geared for more aerobic metabolism, and thus can be suppressed by the removal of oxygen from the packaging system, such as in vacuum packaging and modified atmosphere packaging (MAP) systems. A potential issue for these reduced oxygen systems is the proliferation of unwanted anaerobes, such as clostridia as observed in vacuum-packaged red meat species, or *Yersinia* in poultry in low oxygen MAP (Höll et al., 2016; P. Zhang et al., 2020). To supplement the advantages of oxygen removal, carbon dioxide is commonly used in MAP systems, as it exhibits an antimicrobial effect though decreasing substrate pH, and interfering with intracellular enzymes (Daniels et al., 1985). While carbon dioxide has notable effect on some bacteria,

such as some *Pseudomonas* spp. and *Yersinia*, others are still able to prevail and spoil the product, such as *Enterobacter* (Gill & Tan, 1980). *Brochothrix thermosphacta* TMW 2.2101 and *Carnobacterium maltaromaticum* TMW 2.1581 exhibited upregulation of fatty acid synthesis, modifying their cell membranes to be more resistant to the diffusion of carbon dioxide and oxygen upon exposure (Kolbeck, Kienberger, et al., 2021).

2.3. Formulation Ingredients

Many ingredients added to processed meats are incorporated not only for their beneficial impacts on sensorial aspects of product quality such as color and flavor but because they have additional efficacy as antimicrobial agents. One of the most utilized ingredients, in this case, are salts, sodium chloride, or potassium chloride. Salts generally reduce product water activity, limiting the outgrowth of many species (Taormina, 2010). Reduction of salt in pork sausage decreased bacterial diversity and shelf-life, as salt-sensitive Enterobacteriaceae and Leuconostocaceae dominated the product rendering it spoiled (Fougy et al., 2016).

In addition to salt, nitrite has historically been used in meat production, due to its ability to provide “cured” color and flavor, but also due to its observed bacteriostatic properties. In order to induce a bacteriostatic effect, nitrite related compounds employ various chemical interferences. One of the main methods of action is a direct interference with the Fe-S clusters present in the enzymes of glycolysis and the tricarboxylic acid cycle. Nitrite has been shown to inhibit aldolase (EC 4.1.2.13) in *E.coli*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis*, as well as inhibiting active transport of proline, and glucose uptake in *E.coli* (Yarbrough et al., 1980). Researchers concluded that nitrite had the capability of disrupting a wide range of enzymes,

uncoupling electron transport, and diminishing the impact of the proton gradient (Meijer et al., 1979).

In addition to using glucose to modulate cellular pH, many processors utilize organic acids (such as lactic, benzoic, and sorbic acid) and organic acid salts. These undissociated acids can cross cellular membranes to dissociate inside the cell, acidifying the cytoplasm and limiting cellular function. Sodium lactate and acetate have been found effective against many spoilage species, such as *Pseudomonas* spp., *Serratia* spp., *Carnobacterium* spp., and *Lactobacillus* spp. (Drosinos et al., 2006; Ouattara et al., 1997). This approach has a differential effect on different species. As many LAB, such as *Carnobacterium* spp. produce organic acids themselves, they are more resistant to these treatments (Zhang et al., 2019), and other bacteria can develop acid-tolerant responses to overexposure. Because of these attributes, researchers and producers should consider which antimicrobial agents are best tailored toward SSOs relevant to their systems.

Outside of these components that directly interfere with microbial processes and structures, sugars may also play a notable role in determining product shelf life. Not only can sugars reduce the water activity of the meat substrate like salts, but they can also support preferential metabolic activities of the microbial community, limiting the production of sensorial defects from proteolytic degradation. Glucose is a preferential medium for bacterial growth, producing lactic acid and reducing product pH. Aerobically stored ground beef supplemented with 2 – 10% glucose had three to five days extended shelf life as the product pH reduced, and bacteria used glucose, rather than protein or lipid as an energy source preventing the formation of rancid products (Shelef, 1977).

Additionally, vacuum-packaged lamb had favorable sensorial parameters with surface treated with glucose, invoking a 76% increase in shelf-life (Rood et al., 2022).

While widely used for their ability to improve the water binding of meats, phosphates modify product pH while providing an additional benefit of metal chelation, issuing some merit as antimicrobial agents. Magnesium is an integral component of bacterial cell walls and thus researchers have hypothesized its chelation by phosphate could provide antibacterial action (Post et al., 1963). Washing chicken carcasses with tripolyphosphate provided a three-day shelf-life extension, illustrating this could be possible (Vareltzis et al., 1997). *Pseudomonas* spp. isolated from chicken meat grown in presence of phosphate exhibited delayed growth despite increased pH, which could be attributed to phosphate outcompeting their natural chelating pyoverdine for iron acquisition (Elliott et al., 1964). Seeing as chelation is a valid mechanism for bacterial suppression, novel applications have considered the use of ethylenediaminetetraacetic acid (EDTA) in meat systems as well, delayed outgrowth (Leelaphiwat et al., 2022).

Seasonings and other flavor additives are a point of interest when it comes to bacterial communities. Some components of spices exhibit antibacterial activity when extracted, such as allicin from garlic disrupting *Pseudomonas* spp. quorum sensing and biofilm formation, or fractions of rosemary and licorice inhibiting microbial growth in fresh pork (Harjai et al., 2010; H. Zhang et al., 2009). One must consider these results in context, however, as whole spices themselves may carry spoilage bacteria which can inoculate the product, contributing to the spoilage microbiome (Säde et al., 2016).

3. INTER-COMMUNITY INFLUENCE ON MICROBIAL SPOILAGE

3.1. Metabiotic Factors

To fully understand the dynamics of meat spoilage microbes, one must consider the community as a whole. There is strength in flexibility and numbers, and in some cases, the presence of certain bacterial taxa can benefit another. Metabiosis, a condition where one organism favorably primes the environment for another, influences how the meat microbiome takes shape, and which organisms may ultimately contribute to product spoilage. One example of metabiosis in the processed meat environment is the protection conferred by multi-species biofilms. While some species, like *Listeria monocytogenes*, may be sensitive to sanitizers on their own, polysaccharide structures created by neighboring *Pseudomonas*, *Acinetobacter*, and *Janithobacterium* shield the community from disinfecting agents (Zwirzitz et al., 2021). Beyond sanitizer protection, aerobic organisms have been found to have metabiosis with microaerophiles or anaerobes, as they quickly utilize oxygen which stresses the later organisms. *Campylobacter* spp., known microaerophiles, grown in co-culture with chicken-isolated *Pseudomonas putida* were able to survive ambient oxygen conditions, as they were aggregated together with fiber-like structures in microenvironments with reduced oxygen due to pseudomonad metabolism (Hilbert et al., 2010). It is possible that oxygen utilization from aerobic organisms could also play a role in providing environments for anaerobes like *Clostridium estertheticum* to establish and incur gaseous, “blown-pack” spoilage. Beyond removing components, some bacteria transform substrates into metabolites that other bacteria can utilize to spoil products more rapidly. Amino acid degradation is a multi-step process, often converting arginine to ornithine, and further into malodorous putrescene. Many *Pseudomonas* and LAB spp. utilize arginine deaminase to generate ornithine,

which Enterobacteriaceae convert to putrescene at rates much quicker than if these organisms were grown in isolation (Jørgensen et al., 2000). Microbial community analysis should consider the enzymatic spoilage capabilities of the community, rather than as individual components when determining flux of spoilage metabolites.

3.2. Quorum Sensing Systems

Food spoilage, is at least in part, driven by quorum sensing (QS) behaviors of the microbial community of the product. Bacteria are metabolically geared to find environments and ways to utilize every component available for their survival. Often, this means working in "teams" to communicate information regarding stresses and cross-feeding metabolites. While there are many different signaling molecules, many gram-negative SSOs utilize N-acyl-homoserine lactones (AHLs) to signal community load, and regular cellular processes collectively.

As a population increases in numbers, nutrient availability decreases, and biofilms form, oxygen penetration becomes limited, so the community must work to shuttle components to the core as necessary, or altruistically slough-off to allow nutrient penetration further into the biofilm, and dispersal of cells to colonize other portions of the substrate (Coughlan et al., 2016). Cells will signal their stresses, and the behavior of the group changes to account for this, including changing their metabolic processes to utilize different components of the food matrix, furthering spoilage. *Pseudomonas fluorescens* for example, have proteolytic enzymes that are transcriptionally regulated by their QS system (Wang et al., 2022). Some organisms have a particular advantage of having multiple QS system receptors, allowing them to "eavesdrop" from AHL signals produced by other bacteria in the community, and adjust their behaviors to account for their

presence as well (Li et al., 2019). Generally, it is understood that strong QS strong infer favorable outcomes for bacterial survival, as they are better informed about the cellular landscape and can manage cellular processes to throttle energy usage and acquire necessary substrates.

QS systems are often employed in cases of anaerobic environments. In an aerobic system, there is less stress on the community, and bacteria are more allowed to act individually. There is evidence that bacterial signaling may be involved with a switch from aerobic respiration to denitrifying or fermentative metabolism through the Anr regulon in pseudomonads (Hammond et al., 2015). Under low oxygen conditions, this regulon operates on genes, steering central metabolism in a way to conserve energy, such as arginine fermentation (Tribelli et al., 2019). While anaerobic activity is not generally associated with *Pseudomonas* spp., this metabolic flexibility can help explain why this SSO predominates in a variety of stressful situations, such as vacuum-packaged meats (Kolbeck, Abele, et al., 2021).

Because of the broad changes QS systems and regulons can exact in cellular processes, there is particular interest to target these processes as a means for shelf-life extension. Just as humans are interested in suppressing bacteria, other bacterial members of the community target QS systems to interfere with competitor strains. *Lactobacillus crustorum* ZHG 2-1 has been identified for its ability to interfere with QS systems, as its extract can degrade the AHLs other bacteria utilize, suppressing protease, pyoverdine, and exopolysaccharide synthesis in *Pseudomonas aeruginosa* (Cui et al., 2020). Perhaps quorum interference by LAB allows for their predominance in some meat systems.

3.3. Resource Shunting (Indirect Attack)

Beyond modifying macronutrient utilization, some bacteria additionally have cellular processes that allow them to shunt or embargo critical substrate components so only a subset of the population and utilize them and proliferate with such an advantage. One such shunt is that of iron and extracellular siderophores. Iron is a key cofactor for many central metabolic enzymes, thus maintaining sufficient levels of soluble iron is pertinent for cellular function. Many bacteria excrete siderophores, small molecules that can bind and solubilize iron from the environment. Once bound, these structures exhibit unique confirmations which interact with surface receptors on cells, usually specific to the organisms which produced them. Many *Pseudomonas* spp. for example, produce pyoverdine and can intake this molecule, blockading iron from other members of the bacterial community. Interestingly though, some spoilage bacteria exhibit xenosiderphore piracy, containing surface receptors for siderophores they do not produce, freely benefiting from the energy costs other cells paid producing said siderophores. *Pseudomonas fragi*, for example, does not produce its own siderophores, yet can intake pyoverdines of other pseudomonads, and enterobactin from *Escherichia coli* (Champomier-Vergès et al., 1996).

Outside of iron shunting and piracy, some bacteria fast track the shunting of readily accessible sugars as energy sources. While most organisms rely on the glycolysis pathway for the conversion of glucose to pyruvate, many gram-negative bacteria possess an alternate route, the Entner-Doudoroff (ED) pathway. While the usual glycolytic pathway yields more energy from glucose in the form of ATP, it requires a greater “start-up” cost to produce the enzymes responsible for maintaining such flux. The ED pathway, in contrast, produces less ATP, but requires less protein to function (Flamholz et al.,

2013). In stress-induced, energy-limited conditions, organisms that have the ED pathway can readily use sugar without necessitating extraneous energy costs, giving them an energetic "head start" and endurance to remain viable in the substrate. This "long-term" metabolic strategy has been observed in *Pseudomonas* spp. isolates from anoxic packaged beef (Kolbeck, Abele, et al., 2021), and conferred favorable stationary phase survival outcomes for *Campylobacter* spp. isolates which had the ED pathway, compared to those without it (Vegge et al., 2016).

3.4. Community – Produced Antibacterial Agents (Direct Attack)

While the competition for resources selects for certain microbes, many bacteria much more directly attack other members of the meat microbiome to secure their place in the community. Across a variety of biomes, lactic acid bacteria (LAB) have utilized a wide variety of bacteriocins, bacterially produced antibacterial peptides, to suppress organisms that are in contest for their niches, often closely related bacterial taxa. Nisin, one of the most commercially utilized bacteriocins, for example has been observed to delay outgrowth of gram positive carnobacteria, LAB, and *Brochothrix* (Ercolini et al., 2010). Pediocins from *Pedicoccus* and additional molecules from *Weisella* spp. have a greater spectrum of microbes they can inhibit across both gram positive and negative organisms, however, resistance mechanisms to pediocin seems more frequent than nisin resistance mechanisms (Woraprayote et al., 2016). For these reasons, LAB with minimal sensory impact or their bacteriocins have been utilized as bioprotectants, dosing the community with bacteria of known favorable behavior. Regardless of artificial addition or natural occurrence on a product, bacteriocin producers play a role in selecting the spoilage community composition.

Not only do bacteria play a role in "selecting out" other bacteria, but growing interest has centered around the influence of processing plant a meat virome influence on bacterial community (Mahony & van Sinderen, 2022). While targeted use of bacteriophage against pathogens of interest is used commercially, phages against a variety of organisms are naturally available in the environment (Xu et al., 2022). Interestingly, researchers have found that soil viromes are more indicative of geospatial location than bacterial communities. If certain viromes are associated with a particular processing plant, this could impact the survival of bacteria brought in from external raw materials. While many studies have observed *Brochothrix* as the main spoiler aerobically stored raw meat (Stanborough et al., 2017), De Filippis et al. observed *Pseudomonas* instead. This discrepancy was supported by finding the presence of three *Brochothrix* lytic phages, which could have reduced *Brochothrix* in the environment, easing competition for the pseudomonads (De Filippis et al., 2018).

4. UNSEEN SSOs – ADVANTAGES OF SEQUENCING METHODS

4.1. Blind Spots in Traditional Microbiology

Traditional methods of microbial analysis typically differentiate between groups of bacteria rather than at an individual genus or species level. In meat products, standard methods agar (SMA) or brain heart infusion agar (BHI) can be incubated in aerobic, anaerobic, or refrigerated conditions to enumerate aerobic, anaerobic, and psychrotrophic plate counts, respectively. Selective agars are commonly used to identify groups of organisms such as coliforms, and lactic acid bacteria (LAB), and in some cases specific genera such as *Pseudomonas, Brochothrix,* and *Enterobacteriaceae*. These methods, however, impart practical and physical barriers for many researchers. Firstly, one must

anticipate the types of bacteria present in the foodstuff that will likely be present to select the media which best supports their growth. While this works for many substrates, it presents blind spots for assessing spoilage communities from novel foodstuffs, as well as products manufactured under new processes or locations or assuming certain bacterial strains will not be present in the matrix and incorrectly excluding its selective media. When organisms of interest are not known, both economic and time cost is put on the laboratory servicing these samples, as multiple plate media will be needed to assess the route bacterial group driving spoilage. Additionally, some organisms are not readily culturable, so these methodologies may miss relevant strains altogether.

4.2. Culture-Independent Methods

Recently, however, metagenomic assays, such as 16S rDNA sequencing, have allowed for culture-independent resolutions of the bacterial communities associated with meat spoilage. While traditional plating methods depend on sufficient amounts of readily culturable organisms from meat samples, sequencing methods simply require low amounts of bacterial DNA. Particularly advantageous, is the ability to map sequenced amplicons to 16S databases for taxonomic grouping. By having a fuller picture of the spoilage community, researchers have been better able to identify problematic organisms by longitudinally monitoring the relative abundance of bacteria in the community over time. While 16S sequencing does capture sequences from both alive and dead bacteria, sequences with increased abundance over time can be attributed to the actively growing proportion of that community.

4.2. The genus *Photobacterium*

The advantages of this methodology have been observed in a multitude of meat matrixes, allowing researchers to observe strains not typically associated with these substrates, and improve methodologies for their further isolation. One such example is the genus *Photobacterium*, of the family Vibrionaceae. While this group of organisms has been traditionally associated with marine environments, culture-independent methods have identified their prevalence in red meat systems, particularly when hurdles such as sanitation agents, salts or antimicrobial agents shift the microbial community away from more fastidious, yet chemically sensitive organisms (Bouju-Albert et al., 2018; Pennacchia et al., 2011; Stoops et al., 2015). This organism was likely previously missed, as it had been associated only with the marine environment and foodstuffs, and media commonly used for meat assessment more readily promoted the growth of other bacteria from the sample. By enriching samples with marine broth, researchers have found readily harvestable *Photobacterium* spp. from a variety of meat substrates (Hilgarth et al., 2018). Interestingly, a novel species, *Photobacterium carnosum*, isolated from poultry meat, was found to be a terrestrial strain separate from its marine counterparts, with enhanced flexibility in sugar metabolism, potentially conferred from horizontal gene transfer (Fuertes-Perez et al., 2021).

4.3 The genus *Pseudomonas*

Instances of preconceptions about a genus and its limited proliferation do not stop there; *Pseudomonas* spp. have been widely misunderstood by researchers and have maintained a position as a dominant SSO in a multitude of unexpected situations. Traditionally, *Pseudomonas* were thought to be obligate aerobes, so modified

atmospheric packaging (MAP) or vacuum packaging was viewed as means of suppression (Rossaint et al., 2015; Sun & Holley, 2010). More recently, this obligate aerobic notion been disrupted. It is now thought that some *Pseudomonas spp*. are obligate respirators, allowing for anaerobic growth due to terminal electron acceptors besides oxygen (Su & Hassett, 2012). Anaerobic growth of *Pseudomonas* has also been observed in MAP packaged meats, giving further incentive to find alternative strategies to prevent *Pseudomonas* outgrowth (Hilgarth et al., 2019; G. Wang et al., 2017).

5. IMPORTANCE OF COMPUTATIONAL METHODS

While sequencing methods have allowed for over a decade of intensive microbiome research, the computational workflows used to evaluate obtained reads are just as important as wet-laboratory methodologies. In general, 16S workflows follow a general framework wherein a subregion of the 16S region is sequenced following wet-lab procedures designed to minimize contaminant reads and ensure the quality of desired reads. Once sequence reads are obtained, researchers must decide on relevant filter criteria (quality score cutoffs, singletons, host contaminates), how to infer meaningful gene reads, and if the taxonomy is desired, which databases to compare to (Bharti & Grimm, 2021).

A vast majority of work conducted in the meat microbiome space, utilizes operational taxonomic units (OTUs) to bin samples into identifiers that are within a certain dissimilarity threshold, with 3% dissimilarity roughly estimating a species, obscuring the impact of nonsensical reads. While this approach minimizes the impact of

error, it also leads to less precise assignment of reads. Amplicon sequence variants (ASVs), on the other hand, consider the estimated error rates of the sequencing run, and instances of an exact sequence occurring, to estimate whether or not that sequence itself is sensible. In this system, single nucleotide differences are conserved, meaning samples have inherent biological meaning and can be compared across studies (Callahan et al., 2017).

Once OTUs or ASVs are assigned, scientists often seek to assign taxonomy, which comes with its own variety of issues. Depending on the region sequenced and your sample matrix, different databases may have bacterial species more representative of those in your sample and can affect community assignment and subsequent analyses (Ramakodi, 2022; Soriano-Lerma et al., 2020). Choices to rarefy, normalize, cutoff spurious sequences, and impute zero-values all additionally impact the final analysis matrix, and thus can influence study results (Baruzzo et al., 2022; McMurdie & Holmes, 2014; Reitmeier et al., 2021). Due to the many discrepancies that arise from computational methods, properly documented methods, and public accessibility of raw sequence reads with thorough metadata detailing sample origin and sequence run parameters are highly encouraged. By reporting reproducible analyses, researchers set examples of best practices or leave important metadata for researchers aggregating information in the future. For example, by reporting the sequence subregion and database used, future researchers may notice that a new database update could further categorize previously unknown organisms, or how to proceed with a metanalysis given these constraints.

6. PROPOSED WORKFLOW FOR SPOILAGE RESEARCH

As researchers and producers alike attempt to navigate the invisible microscopic worlds of the meat and processing environment microbiome, there are many pertinent questions they seek to answer, and tools that can be used. When it comes to reducing food losses due to microbial spoilage, processors must know which organisms are present in their food matrix, to then either identify contamination sources or if the organisms are unavoidable, reduce their outgrowth with hurdles that consider the physiological components of said organism. We propose, that, when possible, both culture-dependent and culture-independent methods are used to identify organisms, and sequence reads and isolates are stored in a public repository. In this system, novel SSOs may be identified via sequencing methods and collected strains can be subjected to challenge studies using various hurdle technologies to determine which combinations are effective for the SSO, or to develop novel antimicrobial solutions, giving producers processing schemes tailored for their product.

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Figures

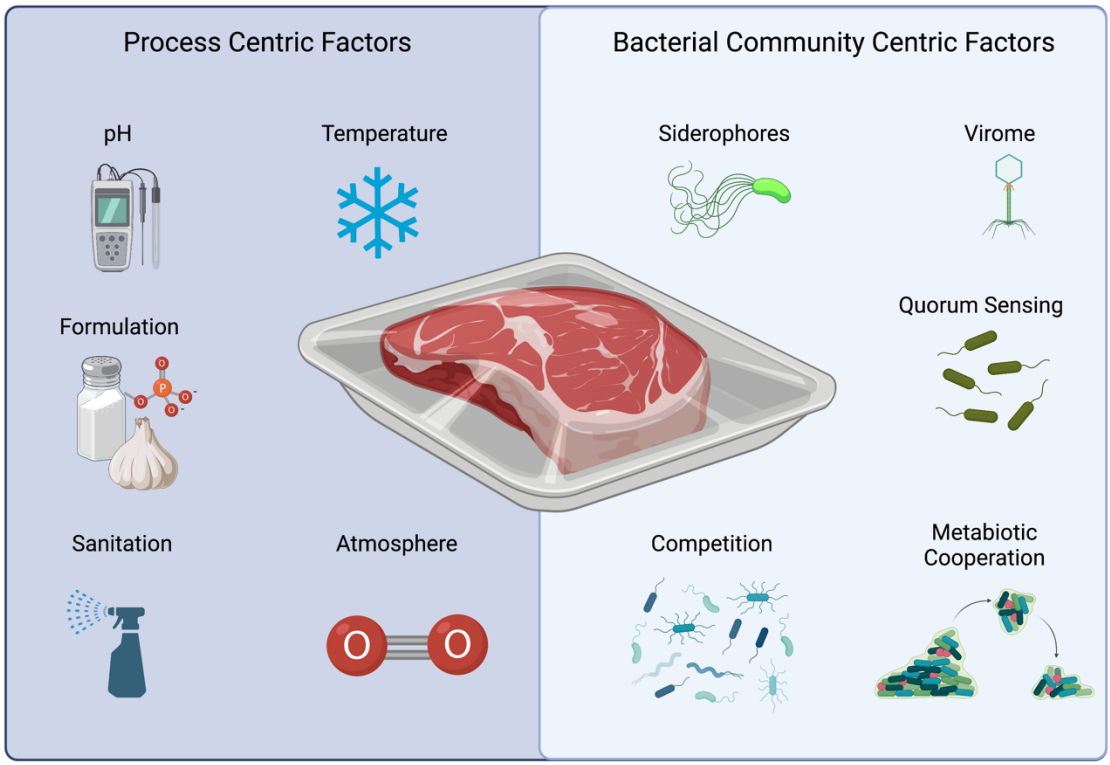

Figure 1. Overview of factors modulating bacterial communities in meat products (Created in BioRender)

CHAPTER TWO: EFFECT OF PROCESSING PARAMETERS AND STORAGE TIME ON THE SPOILAGE MICROBIOME AND QUALITY CHARACTERISTICS OF TURKEY PRODUCTS

ABSTRACT

Microbial contamination of muscle foods leads to unnecessary food waste; thus processors and consumers alike aim to reduce losses attributed to microbial outgrowth. To identify areas of process control to limit specific spoilage organisms (SSOs), this "stepwise" study has been devised to investigate the impact of additive ingredients (salt, spices, nitrite) and processing procedures (stuffing, cooking, slicing) on microbial communities and product quality. Three separate lots of boneless skinless turkey breast were used to manufacture products of varying ingredient inclusion and processing steps on three separate replicates of production day. Manufactured treatments are as follows: T1) Ground Turkey, T2) Ground Turkey and Salt, T3), Ground Turkey, Salt and Seasonings, T4) Cooked Links, T5) Cooked Cured Links, T6) Sliced Deli Meat, T7) Sliced Cured Deli Meat. Treatments were sampled throughout shelf life for physicochemical, microbial, and metagenomic parameters. Both treatment and storage time exhibited significant effects ($P < 0.01$) on beta diversity metrics. Differential amplicon sequence variants (ASVs) (Linear Discriminant Analysis score > 3.5) mapping to *Lactococcus* and *Carnobacterium* were identified between raw and cooked samples, portraying the influence of processing steps and environment. Microbial communities are modulated by degree of processing, ingoing ingredients, and storage time. SSOs identified is this study belonged to the orders Pseudomonadales, Enterobacteriales, and Lactobacillales. By using processes or ingredients to target these organisms, processors may see favorable shelf-life outcomes, however, it is possible that residential microbial

communities vary across facilities, and further microbial profiling work may need to be done to identify SSOs of interest in additional environments.

1. INTRODUCTION

Muscle foods are an important source of nutrition worldwide, however, the attributes that make foods nutritious for human consumption, also make them a substrate amiable to bacterial growth. These microorganisms readily metabolize meat, poultry, and fish products rendering them spoiled, and contributing to food losses. The U.S. Environmental Protection Agency estimates 63,132,123 tons of food is wasted per year in the U.S., and of that 55.9% is lost to landfills. Despite efforts to divert products to energy production or animal feeds, the tons directed toward landfills has consistently increased since the 1970s, partially because much of the waste happens at a residential level where consumers trusted option for spoiled foods, is disposal (US EPA, 2020). Understanding the factors which govern the outgrowth and assembly of spoilage communities in consumer goods is pertinent for extending product shelf life and mitigating food waste. While healthy muscle tissue is considered nearly sterile, its microbiotic landscape begins to take shape as soon as the tissue is exposed to the outside environment (Huffman, 2002). After this point, meat products can undergo a broad series of manipulations comprising ingredients addition, physical manipulation with processing equipment, and further changes through thermal processing, all potentially influencing product quality and microbial composition.

Currently, many investigations have centered around connecting regions of the processing environment to specific contaminates in exceptional cases of spoilage, where

shelf-life is reduced compared to normal production basis. Examples of such work include identifying poor hygiene harborage sites of specific spoilage organisms (SSOs) in a processing environment contaminating sausage (Hultman et al., 2015), and identification of gas-producing *Clostridium* spp. isolates, rupturing vacuum packages intended for the export market. These studies, however, often focus on identifying SSOs when a known, exceptional case was ongoing, rather than identifying the microbiota that products would normally encounter, and the sources that these organisms originated. By studying the changes in microbiome throughout modes of processing, processors and microbiologists can work to design more robust solutions against the expected organisms, as well as identify which substrates or surfaces are likely contributors of new organisms to capture for further novel challenge studies. The aim of this study is to identify how commonly utilized practices in poultry processing, modulate the bacterial community of the final product.

2. MATERIALS AND METHODS

2.1. Treatment Formulations of Turkey Products

Treatments were selected to represent a breadth of commonly available turkey products in North America, with each subsequent treatment adding additional ingredients (salt, seasonings, water, sodium nitrite, and sodium ascorbate) or processing parameters (thermal processing, slicing) to assess their effects on the finished product attributes throughout shelf life. Treatment descriptions are as follows:

T1 (Raw Ground Turkey): Boneless skinless turkey breast was ground through a 3mm plate with a Hobart Meat Grinder (Model #4734, Hobart Mfg. Co., Troy, OH) and stuffed

into white opaque plastic chubs with a vacuum stuffer (Vegmag Robot 1000 DC; Reiser, Canton MA) and sealed with plastic tape.

T2 (Raw Ground Turkey and Salt): Boneless skinless turkey breast was ground through a 3mm plate as in T1, and mixed with 2% added salt in a double-action mixer (Leland Southwest, Fort Worth, TX). Meat batter stuffed and taped as in T1.

T3 (Raw Ground Turkey, Salt, and Seasoning): Follows as T2, with seasoning blend (0.5% dextrose, 0.12% garlic, 0.3% black pepper) added during the mixing step.

T4 (Cooked Link): Boneless skinless turkey breast was ground through a 12.70mm plate, mixed with previously described salt, seasonings, and 10% added water. Meat batter was then fine ground through a 3.715mm plate. Links were then stuffed into cellulose casings with a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton MA) and subjected to thermal processing.

T5 (Cooked Cured Link): Follows as T4 ingredients and processing with the addition of curing ingredients (sodium nitrite at 156ppm and sodium erythorbate at 547ppm).

T6 (Sliced Deli): Boneless skinless turkey breast was coarse ground through a 25.40mm plate and mixed with a 25% brine extension including relevant non-meat ingredients (2% salt, 0.5% dextrose, 0.12% garlic, 0.3% black pepper, 0.3% sodium phosphate) in a vacuum tumbler (Model DVTS R2-250; Daniels Food Equipment, Parkers Prairie, MN). Tumbling proceeded under vacuum (66.7 kPa) at 4°C for 45 minutes. Meat batter was then stuffed into plastic casings, clipped using a Tipper Clipper (Model PR465L; Tipper Tie, Inc., Apex, NC), and subjected to thermal processing.

T7 (Sliced Cured Deli): Follows as T6, with additional curing ingredients (sodium nitrite at 156ppm, sodium erythorbate at 547ppm).

2.2 Manufacturing and Storage of Turkey Products

Vacuum packaged turkey breast meat (pectoralis) obtained from a commercial abattoir and placed in frozen storage at -20°C and tempered 48 hours at 4°C prior to usage. Three independent replications of the treatments described were manufactured at the Loeffel Meat Laboratory (University of Nebraska, Lincoln, NE), utilizing raw turkey of three separate lots of production.

Thermal processing (T4, T5, T6, T7) was conducted in a smokehouse (Alkar-Rapid Pak, Lodi, WI) to an internal temperature of 71°C in accordance with USDA FSIS Appendix A (FSIS-GD-2017-0008) and chilled overnight to 4°C per USDA FSIS Appendix B (FSIS-GD-2017-0007). Casings were removed from all products, and deli meats were sliced into 13mm slices for physiochemical analyses, and 2 mm slices for microbial analyses (SE 12D manual slicer; Bizerba, Piscataway, NJ). Approximately 100 grams worth of slices or two links were designated for each analysis, packaged into 3 mil standard vacuum pouch (Bunzl Koch, Riverside MO), and vacuum sealed to approximately at 1.4 kPa (Multivac Model C500; Multivac Inc., Kansas City, MO). Treatments were grouped into individual, covered white plastic totes in dark storage at 4°C for the duration of the experiment (3 weeks for raw treatments, 8 weeks for cooked treatments).

2.3. Physicochemical Methods

Initial measures

Samples were homogenized with a food processor prior to water activity and sodium concentration measurements on the day after grinding for raw samples, or the day after slicing for cooked samples. Water activity measurements were conducted using an AquaLab 4TE water activity meter (Decagon Devices, Inc., Pullman, WA). Sodium chloride concentration was measured as described in Sebranek, Lonergan, King-Brink, Larson, & Beermann (2001) by measuring chloride ion concentration and converting to sodium chloride. Quantab high range chloride titration strips (Hach Company, Loveland, CO) were placed in a Whatman filter, which was lowered into a stirred mixture of 10g sample and 90mL boiling double distilled deionized water in a 150mL plastic beaker. Samples were allowed to cool to room temperature prior to measuring chloride ions. Two measurements were taken per treatment, in duplicate. Proximate composition (moisture, fat, , and ash) of pulverized samples were determined. Samples were manually diced, submerged in liquid nitrogen until completely frozen, and pulverized using a Hobart commercial blender (Model 51BL32; Waring Commercial, Torrington, CT). In duplicate, 2 g of pulverized tissue were used to quantify moisture and ash content using a LECO thermogravimetric analyzer (Model TGA701, LECO Corporation, St. Joseph, MI). In triplicate, total fat was determined as outlined by AOAC using the Soxhlet extraction procedure.

pH

For each treatment, 10g of meat and 90mL DDD water was added to a 150mL beaker and mixed with a magnetic stir bar on a stir plate (Thermolyne Cimarec-top stirring hotplate; Barnstead Themolyne, Dubuque, IA). A pH meter calibrated with standards of pH 4.01, 7.00, and 10,01 (Orion 410A+ with 910104, 910107, and 910110 standards ThermoScientific, Waltham, MA) was used to measure sample pH in duplicate. Raw ground samples were measured on days 0, 7, 14, and 21 and cooked samples on days 0, 28, 56 and 84.

Objective Color

Objective color, stated as CIE L*, a*, b*, as measured with a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ), calibrated with a white calibration plate through polyvinyl cling wrap to avoid contamination of the lens during measurements. Six readings were taken on two samples per treatment using a 2° standard observer with an 8mm aperture and the D65 illuminant setting. Raw ground samples were measured on days 0, 7, 14, and 21 and cooked samples on days 0, 28, 56 and 84.

Residual Nitrite

For cured treatments (T5, T7) on days 28, 56, and 84 residual nitrite was measured using methods adapted from AOAC 973.31 methods with modifications (Redfield & Sullivan, 2015). Flasks containing 5 g of ground sample and approximately 350 ml of hot deionized, distilled water were heated in 82°C water baths for 2 h and

uncorked, swirled, and recorked every 30 min, and then cooled to room temperature. Deionized, distilled water was added to fill 500 ml volumetric flask and the solutions were filtered through Whatman No. 1 filters (GE Healthcare UK Ltd., Buckinghamshire, UK). Four ml of filtrate was combined with 0.22 ml of sulfanilamide solution (0.5 g sulfanilamide in 150 ml 15% glacial acetic acid, w/v). After a five min incubation period, 0.22 ml N-(1-naphthyl) ethylenediamine dihydrochloride (NED) solution (1.5 g NED in 150 ml 15% glacial acetic acid, w/v) was added, and incubated 15 min. Samples were read at 540 nm with a spectrophotometer blanked with a representative water/sulfanilamide solution (DU 800 Spectrophotometer; Beckman Coulter, Fullerton, CA). All samples were measured absorbance readings converted to ppm of sodium nitrite using a standard curve of known nitrite concentrations.

2.4. Microbiological Plate Methods

Approximately 100g of meat was aseptically transferred from packaging to filtered stomacher bags (Interscience USA, Woburn, MA) with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ). Bags were placed in a stomacher (bioMerieux Inc., Durham, NC) for 2 minutes to homogenize the sample. Two, 1.75 ml samples of fluid were pipetted from the bag and stored at -20°C until DNA Extraction. Portion of remaining sample was subjected to microbial plating methods in duplicate. Fifty µl of sample was administered to 100mm agar plates utilizing an Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain). Brain, Heart, Infusion agar (Thermo Fisher Scientific, Waltham, MA) was used to conduct aerobic plate counts (APC), anaerobic plate counts (AnPC), and psychrotrophic (PSY) plate count. DeMan Rogosa Sharpe agar

(Thermo Fisher Scientific, Waltham, MA) was used to enumerate lactic acid bacteria (LAB). Cephaloridine Fucidin cetrimide agar (CFC) and Pseudomonas supplement (Thermo Fisher Scientific, Waltham, MA) was used to enumerate *Pseudomonas* spp. Plates for APC, AnPC, CFC, and LAB were incubated at 37°C and counted at 48 hours. AnPC were held in anaerobic chamber (BD GasPak EZ Large Insulation Container; Becton, Dickinson, and Company, Sparks, MD) with three oxygen absorbent packs (BD GasPak EZ sachet; Becton, Dickinson, and Company, Sparks, MD). Psychrotrophs were incubated at 4°C and counted at 10 days. Raw treatments were sampled on days 0, 7, 14, and 21 and cooked treatments on days 0, 28, 56 and 84.

2.5. Microbial Sequencing Methods

Bacterial communities were investigated for each sample using the MiSeq Illumina Sequencing Platform, targeting the bacterial-specific 16s rRNA gene (Kozich et al., 2013). DNA was extracted from samples using DNA QuickExtract Solution 1.0 (Epicentre, Madison, WI). Obtained DNA was amplified via the polymerase chain reaction (PCR) with a solution that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc.), approximately 1-5 ng of extracted DNA, and 0.5 μM barcoded universal primers. The PCR reaction was performed alongside negative controls in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), with the following PCR cycle: initial denaturation at 98°C for 3 min, followed by 30 cycles of 98°C for 30s, 58°C for 30s, and 68°C for 45s, and a final extension of 68°C for 4 min.

PCR products were then analyzed on a 1.5% agarose gel to ensure amplification occurred

successfully, without contamination of negative controls. Samples were then normalized using the Norgen NGS Normalization 96-Well Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to manufacturer protocol. Pooled sample was then placed in 50°C water bath to remove excess ethanol from the normalization kit and ran through a spin column. DNA was found to be insufficient in concentration, so samples were subjected to additional PCR using a 5-cycle rendition of the previously described protocol. Products were then analyzed on a 2% agarose gel, which yielded two bands. The band corresponding to the bp size of the 16s rRNA V4 subregion was removed with a scalpel and DNA was recovered using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Concentration and bp size of the 16S rRNA libraries were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library concentration was confirmed with a DeNovix QFX Fluorometer and the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc., Wilmington, DE). The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) using the V2 500 cycle kit.

2.6. Statistical Analyses

Physicochemical and Plate Counts

Physicochemical and microbial growth data were analyzed using R (R Core Team, 2013). For salt and water activity (measured day 0 only), data were analyzed using R (lm and anova functions), and means were separated using the emmeans package (lsmeans and cld functions;(Lenth, 2020). For longitudinal physicochemical measures and plate counts, cooked and raw samples were analyzed separately. For pH, color, and plate counts, data were analyzed as a factorial arrangement with 3 treatments by 4

sampling times for raw samples, and 4 treatments by 4 sampling times for cooked samples, with storage time as a repeated measure with an independent covariance structure using the nlme package, lme function (Pinheiro J, Bates D, DebRoy S, Sarkar D, 2020). Means were separated using the emmeans package, lsmeans and cld functions (Lenth, 2020). Significance was declared at $\alpha = 0.05$ throughout the study.

Bacterial Community Analysis

Reads were paired, trimmed, and filtered in DADA2 with *truncLen=c(200,130)* to ensure proper region and quality (Callahan et al., 2016). Screened reads were assigned to amplicon sequence variants (ASVs) and taxonomy was assigned according to SILVA rRNA SSU 138. Reads pertaining to non-bacterial sequences and spurious ASVs were removed, leaving an average 10,488 reads per sample. Good's coverage is not considered as an index for DADA2 denoised datasets, as the algorithm removes singletons by default and Good's coverage explicitly relies on singletons for coverage estimation (Kleine Bardenhorst et al., 2022). Thus, ASV table was rarefied to 1,500 reads for alpha diversity estimation to allow retention of all treatment and day combinations, while curves were reaching their asymptotes. This number of reads was determined sufficient to capture sufficient alpha diversity and identification of SSOs per the aim of the study. The ASV table was additionally proportionally normalized for beta diversity and composition analyses. Additionally, a "core" microbiome set was created by selecting only ASVs which were present in all 3 replications to remove noise in differential abundance and overlap analyses. A phylogenetic tree was generated in Mothur version 1.46 for use in beta diversity estimates (Schloss et al., 2009). Alpha diversity estimates (Chao1 and

observed ASVs) were calculated for the entire community with the phyloseq package (McMurdie & Holmes, 2013). The effects of treatment and storage time on alpha diversity metrics were evaluated with Kruskal-Wallis tests. Bray-Curtis, Weighted and unweighted UniFrac distances describing beta diversity were ordinated in phyloseq. Bacterial community composition differences across treatments and storage time were evaluated using permutational multivariate analysis of variance with Bray-Curtis, weighted and unweighted UniFrac distances with the vegan package in R (Oksanen, 2019) and pairwise.Adonis (Martinez Arbizu, 2020). As UpSetR plot (Conway et al., 2017) was generated to visualize ASVs shared between treatments, and linear discriminant analysis of effect size (LefSE) (Segata et al., 2011) was used to identify differentially abundant bacterial community members between raw and thermally processed products with the MicrobiomeMarker package (Cao, 2022).

2.7. Data Availability

The 16S rRNA gene sequences are available at the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under BioProject PRJNA877355. The scripts and data objects for the reproduction of figures in this study are found in a Zenodo repository at the following address: https://doi.org/10.5281/zenodo.7178685.

3. RESULTS AND DISCUSSION

3.1. Initial Measures

Salt percentage (measured as chloride ion), water activity, and proximate composition were measured on Day 0 to ensure treatments exhibited the expected behaviors per their formulation. Table 1 summarizes these parameters. Ash and salt percentage were significantly different ($P = 0.0001$) across treatment as expected, as treatments with added salt or nitrite contributed to these parameters. Water activity was significantly different ($P = 0.0074$) across treatments in a similar manner, as formulations containing salt had reduced water activity compared to treatment 1, and observed values are comparable to those measured for commercial poultry meat, sausage, and smoked deli turkey (Segata et al., 2011). Proximate moisture was significantly different ($P = 0.0001$) across treatments as expected. Previous work has determined boneless, skinless, denuded turkey breast to contain around 75% moisture, thus the values for the raw variables are sensible in nature (Yalçın & Şeker, 2016). The decreased moisture in the cooked links can be attributed to cook losses. Deli meats contained a 25% brine extension, so while some cook loss occurred, the moisture values were similar to those of the raw formulations. Raw materials and processed products were indeed representative of industrially produced formulations.

3.2. Longitudinal Physicochemical Measures

Objective color and pH were measured throughout shelf-life for raw and cooked treatments separately. Results are compiled for treatment effects in Table 1, and storage time effects in Table 2. In raw products, there was no significant effect of treatment on pH ($P = 0.9022$), but there was a significant effect of storage time ($P = 0.0002$). Over

storage, the pH of raw treatments increased, which may be attributed to the production of biogenic amines by genera such as *Pseudomonas* (Zhang et al., 2016). Additionally, in cooked products there was no significant effect observed for treatment ($P = 0.0830$), but there was a significant effect observed for storage time ($P = 0.0029$). Contrastingly, the pH of cooked products actually fluctuated over time, which could be attributed to different bacterial metabolites, such as aforementioned biogenic amines in combination with acids from lactic acid bacteria in these items (Hanna et al., 1983).

A significant treatment effect ($P < 0.05$) was observed in raw treatments for objective color measures L* and a*, but not b* ($P = 0.4757$), and a significant day ($P < 0.05$) effect was observed for all color measures in raw treatments as detailed in Tables 1 and 2. All objective color values decreased over time, describing a general dull color as myoglobin oxidized, and the beginning development of greenish hues. Other authors have observed a greening effect in raw poultry, which could be attributed to hydrogen sulfide production, or green siderophore pigments from pseudomonads (Katiyo et al., 2020).

In cooked treatments, significant treatment ($P < 0.05$) and storage time ($P < 0.05$) effects were observed for L*, and a significant treatment by storage time observed for a* ($P = 0.0011$) and b* ($P = 0.0384$). The L* results, as displayed in Table 1 and 2, are explained by the links having darker color than their brine diluted deli counterparts, and all L* values dulling over time. The a* results are visualized in Figure 1, and b* in Figure 2. Generally, these parameters were governed by the cured treatments maintaining stable cured color throughout shelf-life while the non-cured treatments started to discolor. Residual nitrite was monitored in cured treatments. While not significant day effect was

found (P =0.2467), a decreasing trend over time was observed in Figure 3. Reduction in residual nitrite over shelf-life is typical for cured meats, as it is utilized in the nitrite oxidation-reduction cycle to continually stabilize myoglobin, or it is exhausted as an antimicrobial agent (Xi et al., 2012).

3.3. Microbial Parameters

A significant treatment effect ($P < 0.05$) was observed for APC and PSY counts for raw treatments, but not for AnPC ($P = 0.1381$), CFC ($P = 0.4260$), and LAB ($P = 0.9227$) as displayed in Table 1. This is due to the salt-tolerant nature of lactic acid bacteria, anaerobes, and some pseudomonads (Vermeiren et al., 2004). While salt in T2 and T3 suppressed salt sensitive organisms on other plate media, these treatments did not have as much effect on LAB as seen in Figure 4. A significant effect of storage time ($P < 0.05$) was observed for all plate media counts of raw treatments, simply describing growth over time as displayed in Table 2.

There were significant treatment ($P = 0.0015$) and storage time ($P = 0.007$) effects for CFC counts of cooked treatments as displayed in Tables 1 and 2. Treatment 6 has the most pseudomonad outgrowth, and growth increased over time. There was a significant treatment by storage time interaction for APC ($P = 0.0029$), AnPC ($P = 0.0393$), LAB ($P = 0.0012$), and PSY ($P < 0.0001$) counts, thus they are represented in Figure 5. While cured deli meat T7 overperformed its noncured counterpart, T6 at the beginning of shelf life, it eventually intersects T6 as residual nitrite is depleted and microorganisms have more favorable growth conditions.

Observed ASVs, Chao1 estimates, and Shannon's index were used as indices of alpha diversity of samples. There was an observed treatment by storage time effect ($P < 0.001$) on all indices as visualized in Figure 6. Generally, alpha diversity decreased over time, which is typical of meat microbiomes as they reach spoilage (Fougy et al., 2016; Johansson et al., 2020). Raw treatments generally had higher alpha diversity than cooked treatments. Previous work has identified that while some bacteria can survive certain cook processes, others are less adept, which could explain this alpha diversity reduction in cooked products (Bell, 1983)

These trends in alpha diversity are further aided by beta diversity estimates. Bray-Curtis, Weighted UniFrac, and UniFrac distances were calculated and ordinated in Figure 7. Significant effects of treatment ($P < 0.001$) and day ($P < 0.001$) were observed for Bray-Curtis, Weighted UniFrac, and Unweighted UniFrac distances. As shown in Figure 7, Bray Curtis and Unweighted distances explained less variation than the cumulative 75.6% of the Weighted UniFrace, meaning the additional phylogentic information and abundances supplied in the Weighted estimates play an important role in explaining microbiome composition. In Figure 7c, treatments are observed to have started in the lower left quadrant, where they have the most diverse microbiomes. Overtime, many raw treatments gather in a new cluster in the lower right, while linked treatments do not migrate as far. Treatment 7 is of note, as it drifts on its own accord to the top of the plot, with Treatment 3 and 6 endpoints in closest proximity. These migrations can be better explained with the aid of Figure 8. At the beginning timepoints, samples all contain diverse microbiomes which populate the lower left quandrant. Then, many samples are dominated by Pseudomonadales, noted as this rightward motion along Axis 1 in Figure

7c. Other samples, such as T3, T6, and T7 begin to have more Lactobacillales or Enterobacteriales, which reflects the upwards motion along Axis 2 in 7c.

To further explain these drifts in community composition, further consideration must be given to the root sources of these changes. Treatment 3 movement along Axis 2 could be attributed to lactic acid bacteria contamination of spices added to this formulation modulating community composition (Säde et al., 2016). While treatments 6 and 7 also follow this movement pattern, the source of their increased LAB abundance is likely from a separate source. As the same spice blend was used in treatments T3-T7, and T4 and T5 did not display a similar abundance of LAB, it would be sensible that these strains did not survive cooking, and additional LAB strains contaminated T6 and T7 post-processing. To further investigate this, LefSE was used to compare raw and cooked items. Resulting LDA scores for differentially abundant organisms are displayed in Figure 9. As suspected, *Lactococcus* spp. are the more predominant LAB in raw items, while *Carnobacterium* show increased abundance in cooked items. In previous work, *Carnobacterium* isolates were the only LAB found at all sampled sites in a beef abattoir, as well and the only LAB isolates identified prior to starting work on the sampling day (Wang et al., 2018). This cold-persistent organism is able to establish as a residential organism in the meat-processing environment, and may have contaminated T6 and T7 during slicing, shifting their microbiomes accordingly.

While LDA scores illustrated differences among LAB strains, *Pseudomonas* isolates were not differentially abundant, and were a major contributor to the microbial community composition of all treatments. To further investigate source attribution of the *Pseudomonas* spp. and other major contributing bacterial taxa, Figure 10, a dendrogram

of the top 30 most abundant ASVs in the study was generated, with tips annotated with samples they are present in on a raw (Y, triangle) and cooked (N, circle) basis. Interestingly, only one pseudomonad was unique to raw samples, with all others present in both raw and cooked items. While the presence of a sequence read does not mean a cell is viable, increased CFC counts in cooked treatments corroborate the sentiment that *Pseudomonas* spp. are active in these products. This outcome asserts multiple possibilities regarding the source of *Pseudomonas* spp. in these products. The first proposed route of contamination is from the raw material itself, or equipment used to grind, tumble, and stuff all treatments. Under this scenario, products are inoculated during this early processing and persist through the cooking cycle. Continuing work utilizing *Pseudomonas* strains harvested from turkey products in thermal process challenge studies asserts this may be a possibility. Another explanation is that these *Pseudomonas* spp. are ubiquitous organisms in the cold environment of the Loeffel Meat Laboratory, and contaminate products regardless of equipment used in processing, as they are present at every step of the process. Studies of the meat processing environment have supported this notion, in one instance finding a few oligotypes tend to dominate the meat and dairy processing environment (Stellato et al., 2017), and another noting that the fabrication and processing area of a small meat facility was dominated by *Pseudomonas* spp., and mainly driven by three ASVs (Belk et al., 2022). In our study, 73% of ASVs were found in both raw and cooked samples, and furthermore, the intersection containing all 7 treatments had the most ASVs as displayed in Figure 11.

The observed ubiquity of *Pseudomonas* isolates from products processed in the Loeffel Meat Laboratory has remained constant in our studies (Furbeck et al., 2022), but

the variability in LAB, and the assertion that *Pseudomonas* spp. are the main residential bacterial taxa of meat processing facilities come with various assertions. While these strains are residential to Nebraska, it is possible that processing plants in other locations have different communities, leading to similar products with differing compositions, as observed in retail hams from different commercial facilities. Researchers and processors should consider the results of this study in the context of their facility of interest.

4. CONCLUSION

This study suggests that ingoing ingredient contamination, ingredient antimicrobial activity, cook cycle selection, and processing equipment contamination play a role in modulating the meat microbiome, and thus sampling of these substrates or modulation of processes could provide meaningful isolation of SSOs for use in source attribution investigation or microbial challenge studies. In the context of turkey products produced at the Loeffel Meat Laboratory, SSOs were Pseudomonadales, Lactobacillales, or Enterobacterales. Looking forward, the aggregation of sequence information from various facilities and substrates may shed light on which organisms are most problematic at global scale, and the factors that influence their outgrowth.

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Tables

Table 1. Least squared means for main effect of treatment on physicochemical and microbiological analysis of turkey products.

	Raw Treatments					Cooked Treatments					
	T1	**T2**	**T3**	**SEM**	***P* value**	**T4**	**T5**	**T6**	**T7**	**SEM[1]**	***P* value**
Moisture†	75.2^{b}	74.1^{b}	73.3^{b}	0.902	0.001	67.4^{y}	67.8^{y}	76.7^{z}	76.4^{z}	0.902	0.001
Fat†	4.24	3.11	3.54	0.463	0.484	3.16	3.95	2.54	3.02	0.463	0.483
Ash†	1.26^{a}	2.74^{bc}	2.91^{bc}	0.114	0.001	3.10^{yz}	3.20^{z}	2.60^{y}	2.74^{yz}	0.114	0.001
Salt %†	0.00^{c}	1.61^{b}	1.80b	0.102	0.001	1.95^{yz}	2.33^{y}	1.70^{z}	1.75^{z}	0.102	0.001
a_w†	0.990^{b}	0.975^{ab}	0.974^{a}	0.003	0.007	0.976^{yz}	0.975^{y}	0.986^{yz}	0.985^{yz}	0.003	0.007
pH	6.08	6.06	6.05	0.014	0.902	6.14	6.13	6.23	6.26	0.037	0.083
L*	58.73^{b}	57.36^{ab}	55.32^{a}	0.740	0.001	71.56^{y}	70.26^{y}	76.93^{z}	75.64^{z}	0.534	0.001
a*	6.43^{b}	6.6^{b}	5.34^{a}	0.165	0.001	4.74	6.3	3.33	5.65	0.247	0.001‡
b*	6.94	7.22	8.05	0.635	0.476	8.75	8.12	10.12	7.69	0.600	0.109‡
APC[1]	4.91^{b}	4.54^{ab}	4.43^{a}	0.100	0.005	2.91	1.90	5.15	4.09	0.230	0.001‡
AnPC[1]	3.96	3.89	3.96	1.000	0.138	2.68	1.77	4.64	3.71	0.352	0.002‡
PSY[1]	5.36	4.94	4.86	0.150	0.004	3.50	2.42	4.81	4.38	0.397	0.001‡
LAB[1]	3.56	3.13	3.48	0.454	0.923	1.30	1.35	2.35	2.45	0.391	0.001‡
CFC[1]	4.46	3.98	3.77	0.356	0.426	1.72^{y}	1.21^{y}	4.47^{z}	3.16^{y}	0.274	0.002

†Salt, water activity and proximate composition were analyzed across all treatments for day 0 measurements. For pH, objective color measures, and plate counts, analysis was split between raw (T1-T3) and cooked (T4-T7) products, as these measures were collected at separate sampling points, relevant to the shelf-life expectancy of these products.

a,b,c,d y,z Means in the same row lacking a common superscript are significantly different ($P < 0.05$). Raw $^{c \text{ being highest}}$ Cooked$^{z \text{ being highest}}$

‡Indicates a significant ($P < 0.05$) treatment by storage time interaction, therefore main effects cannot be analyzed. T1) Ground Turkey, T2) Ground Turkey and Salt, T3), Ground Turkey, Salt and Spices, T4) Cooked Link, T5) Cooked Cured Link, T6) Sliced Deli Meat, T7) Sliced Cured Deli Meat.

1 SEM: Standard error of the overall mean, APC: aerobic plate count, AnPC: anaerobic plate count, PSY: psychrotrophic plate count, LAB: Lactic acid bacteria plate count, CFC: Pseudomonad plate count.

Table 2. Least squared means for main effect of storage time(days) on physicochemical and microbiological analysis of turkey products.

	Raw Treatments						Cooked Treatments					
	0	**7**	**14**	**21**	**SEM**	***P* value**	**0**	**28**	**56**	**84**	**SEM**	***P* value**
pH	6.07^{b}	6.04^{ab}	6.04^{a}	6.10^{ab}	0.0221	0.0002	6.20^{yz}	6.28^{y}	6.12^{z}	6.17^{yz}	0.023	0.0029
L*	59.1^{b}	56.5^{a}	56.5^{a}	56.4^{a}	0.5780	0.0001	74.3^{y}	73.7^{yz}	73.2^{z}	73.1^{yz}	0.376	0.0160
a*	7.81^{b}	5.75^{a}	5.34^{a}	5.60^{a}	0.2520	0.0001	4.46	4.99	5.25	5.32	0.158	0.0001*
b*	10.12^{b}	7.12^{a}	6.26^{a}	6.10^{a}	0.4500	0.0001	8.46	8.63	8.75	8.84	0.313	0.2229*
APC	3.41^{b}	3.58^{b}	5.34^{a}	6.18^{a}	0.2340	0.0001	1.92	2.87	3.92	5.34	0.211	0.0001*
AnPC	2.93^{b}	3.31^{b}	4.88^{a}	4.62^{ab}	0.7270	0.0002	1.86	2.38	3.94	4.62	0.352	0.0001*
PSY	3.12^{c}	4.15^{c}	5.85^{b}	7.09^{a}	0.184	0.0001	1.68	3.40	4.73	5.29	0.263	0.0001*
LAB	2.98^{b}	2.99^{b}	2.76^{b}	4.85^{a}	0.332	0.0005	1.53	1.81	2.23	1.87	0.422	0.2073*
CFC	1.97^{c}	2.67^{c}	5.25^{b}	6.39^{a}	0.284	0.0001	1.49^{y}	1.68^{y}	2.46^{yz}	3.18^{z}	0.274	0.0007

Analyses were split between raw (0, 7, 14, 21 days of storage) and cooked (0, 28, 56, 84 days) products, as these measures were collected at separate sampling points, relevant to the shelf-life expectancy of these products.

a,b,c (Raw, c being highest) y,z (Cooked, z being highest) Means in the same row lacking a common superscript are significantly different ($P < 0.05$).

* Indicates a significant ($P < 0.05$) treatment by storage time interaction, therefore main effects cannot be analyzed.

[1] SEM: Standard error of the overall mean, APC: aerobic plate count, AnPC: anaerobic plate count, PSY: psychrotrophic plate count, LAB: Lactic acid bacteria plate count, CFC: Pseudomonad plate count.

Figures

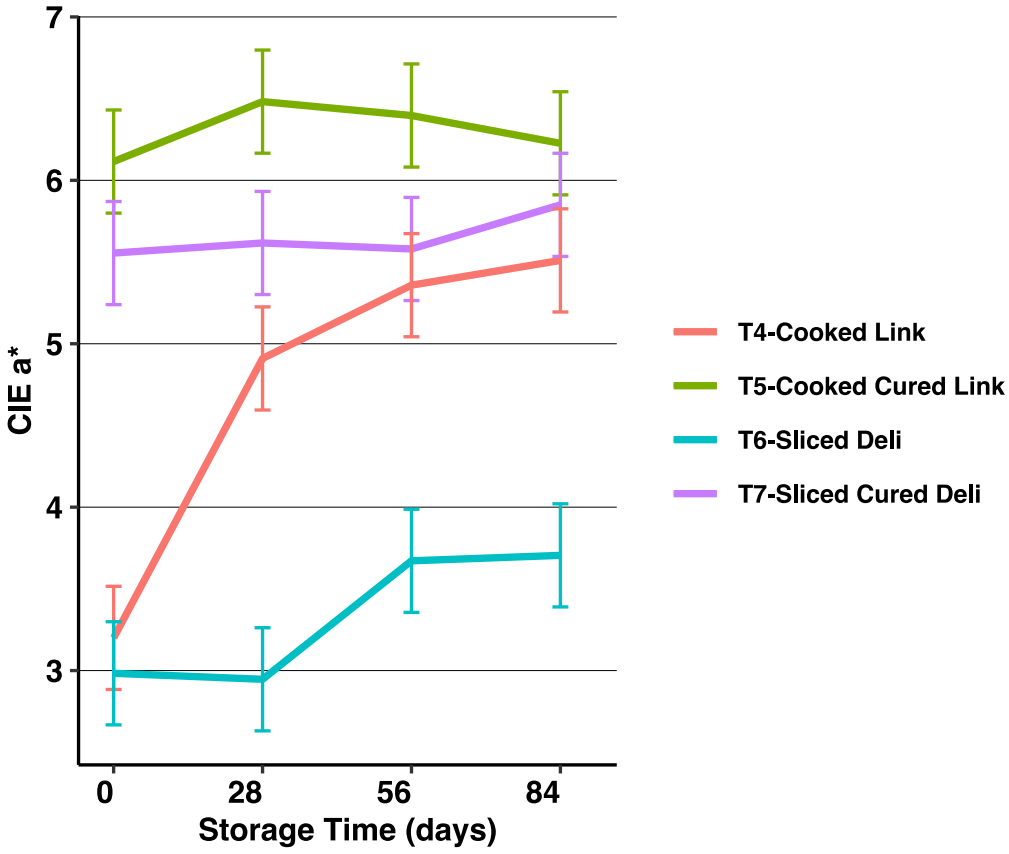

Figure 1. Objective color a* values for cooked turkey products over storage time.

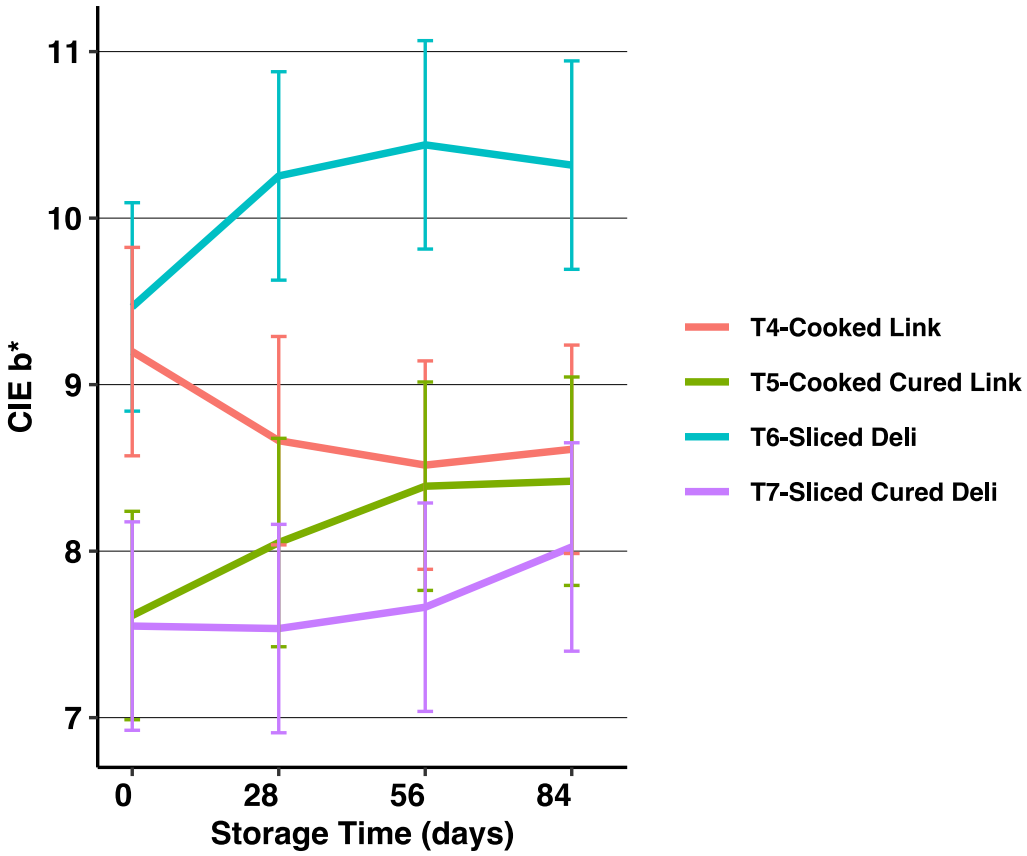

Figure 2. Objective color b* values for cooked turkey products over storage time.

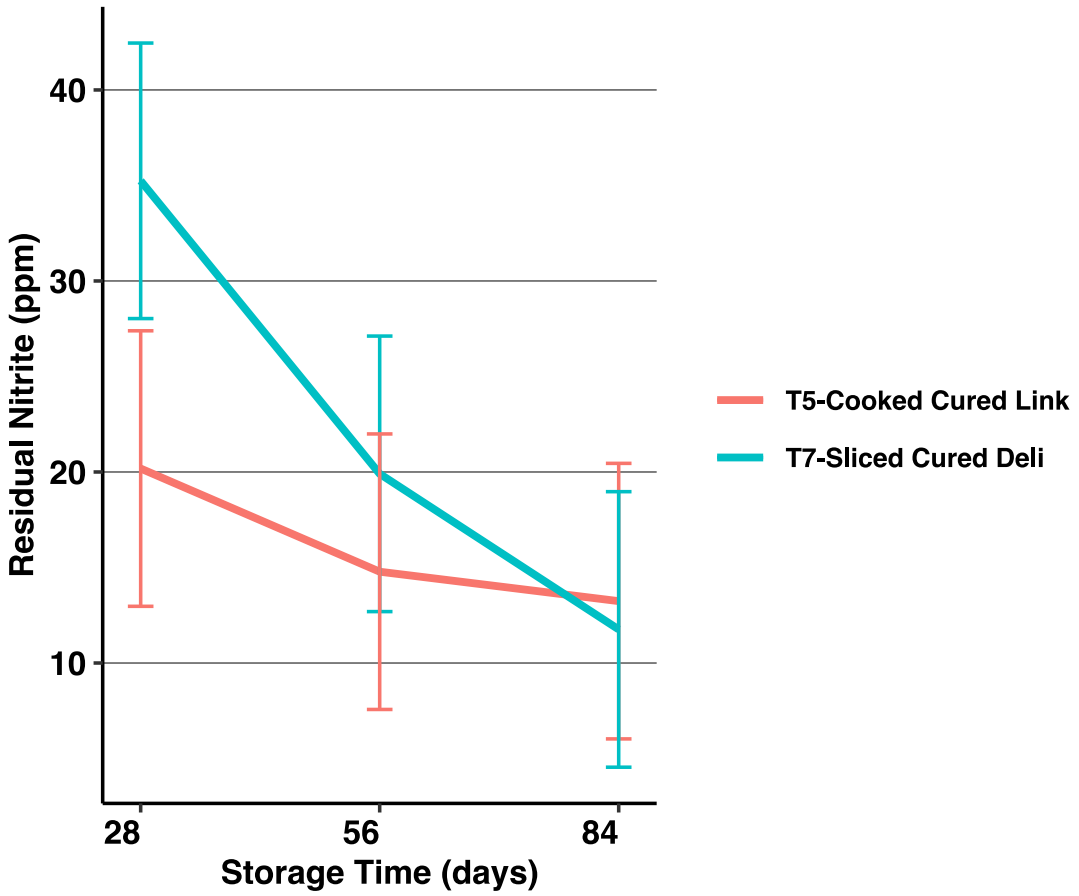

Figure 3. Residual nitrite levels (ppm) of cured turkey products over storage time (days).

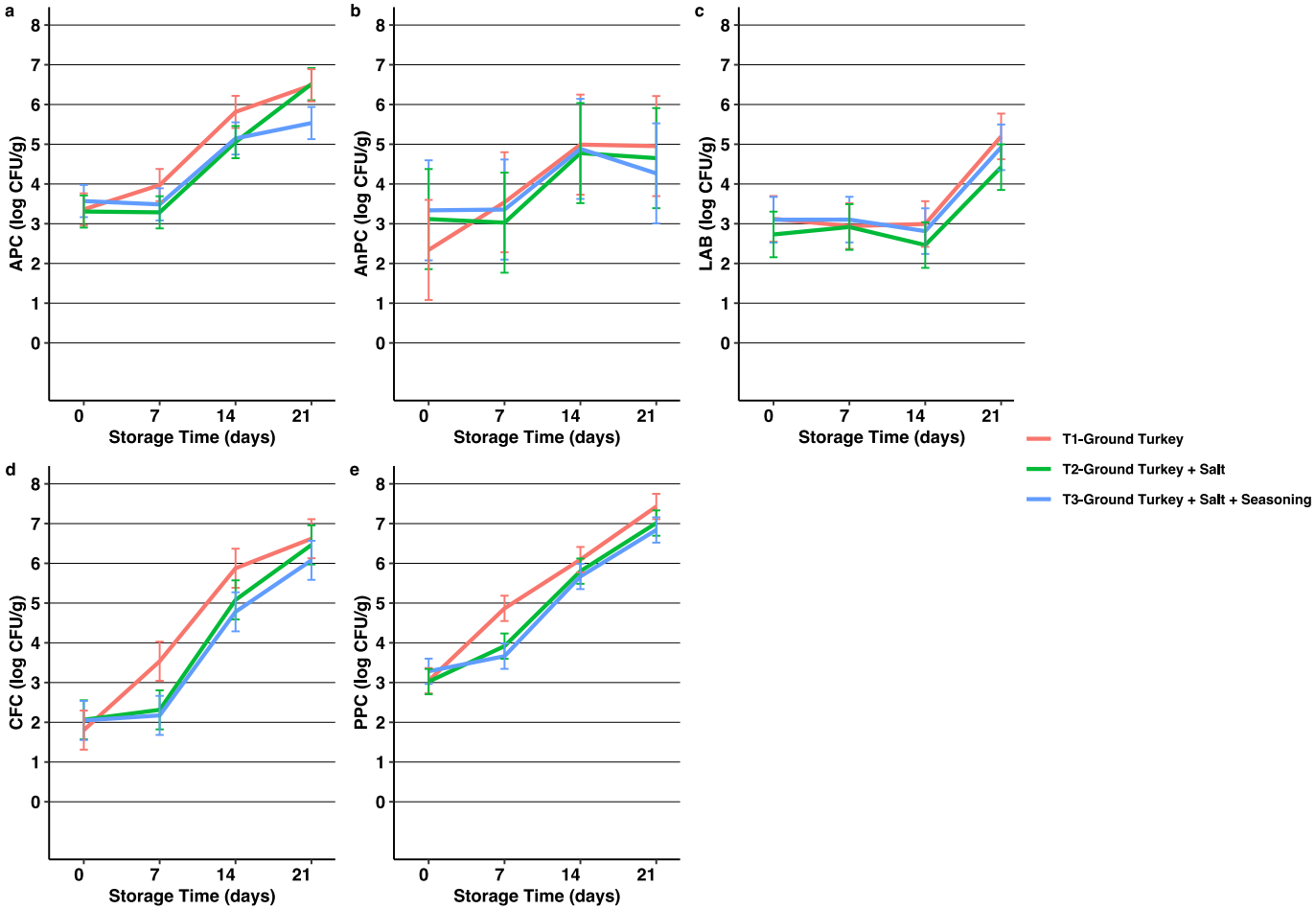

Figure 4. Microbial enumeration of raw turkey products over storage time. APC: aerobic plate count, AnPC: anaerobic plate count, PSY: psychrotrophic plate count, LAB: Lactic acid bacteria plate count, CFC: Pseudomonad plate count.

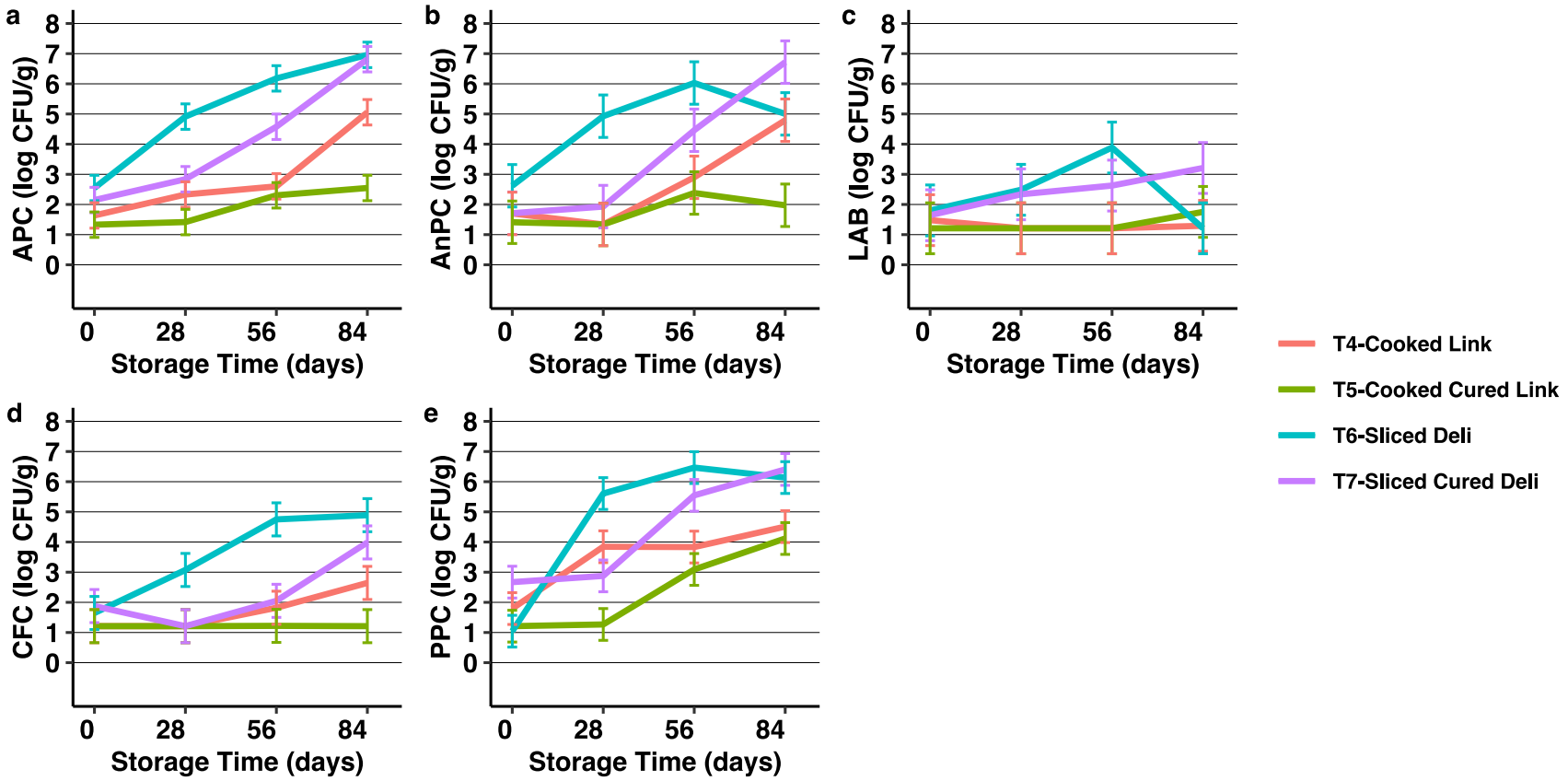

Figure 5. Microbial enumeration of cooked turkey products over storage time. APC: aerobic plate count, AnPC: anaerobic plate count, PSY: psychrotrophic plate count, LAB: Lactic acid bacteria plate count, CFC: Pseudomonad plate count.

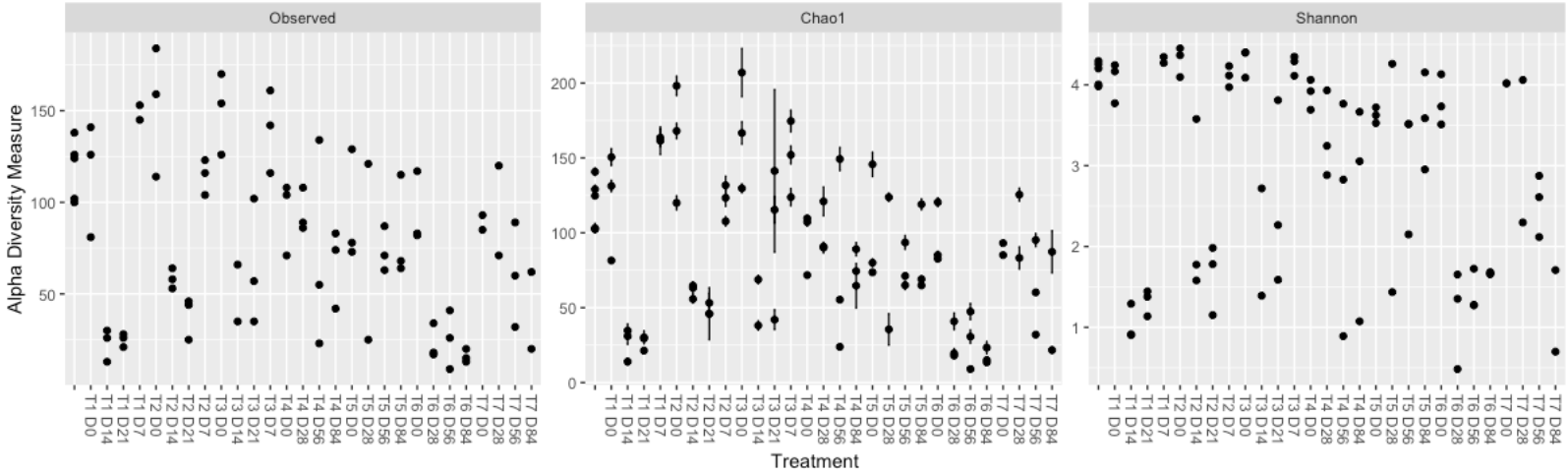

Figure 6. Alpha diversity indices for processed turkey products over storage time. T1) Ground Turkey, T2) Ground Turkey and Salt, T3), Ground Turkey, Salt and Seasonings, T4) Cooked Link, T5) Cooked Cured Link, T6) Sliced Deli Meat, T7) Sliced Cured Deli Meat

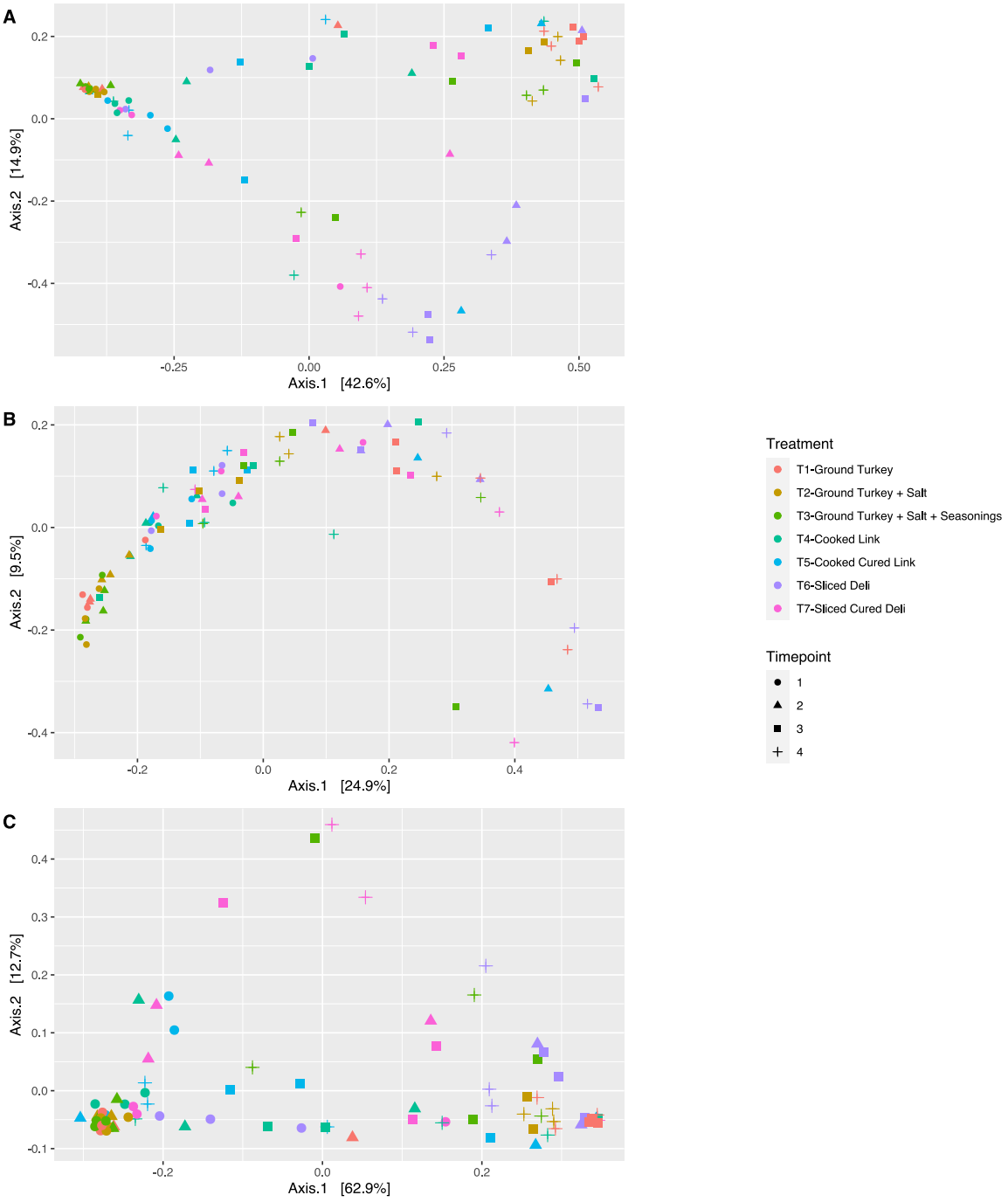

Figure 7. Beta Diversity Indices for Processed Turkey Products, A: Bray Curtis, B. Unweighted UniFrac, C: Weighed Unifrac.

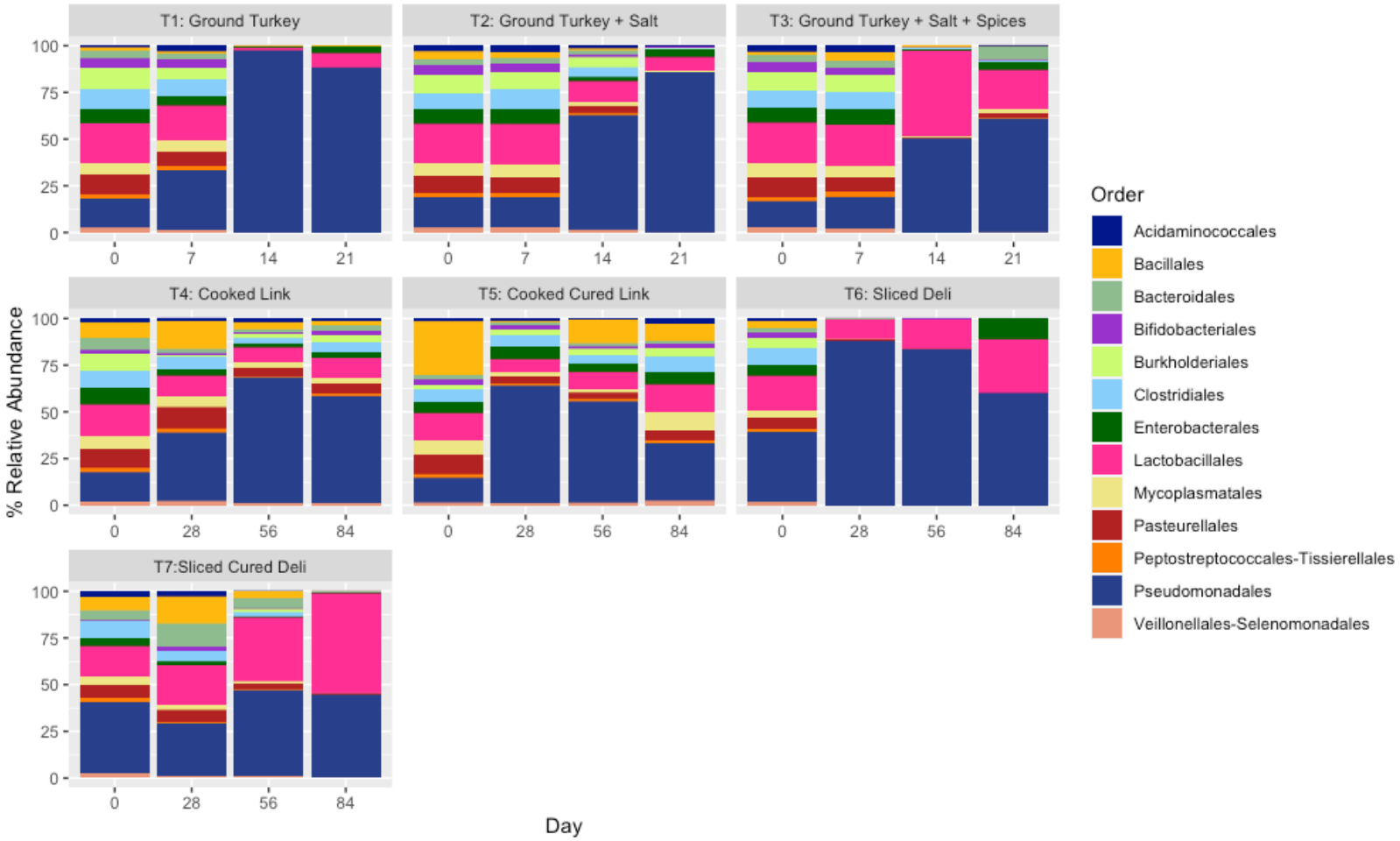

Figure 8. Top 300 Most Abundant ASVs Relative Abundance Plot for Processed Turkey Products

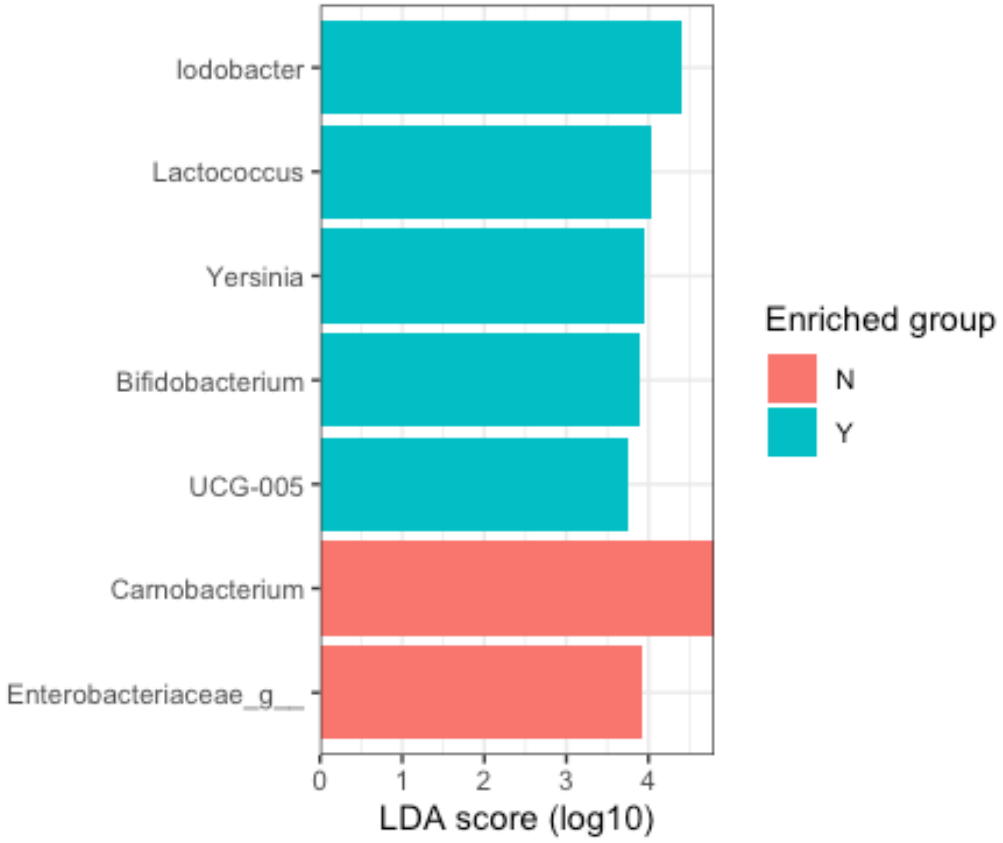

Figure 9. Differentially abundant bacterial taxa in Raw (Y) and Cooked (N) turkey products

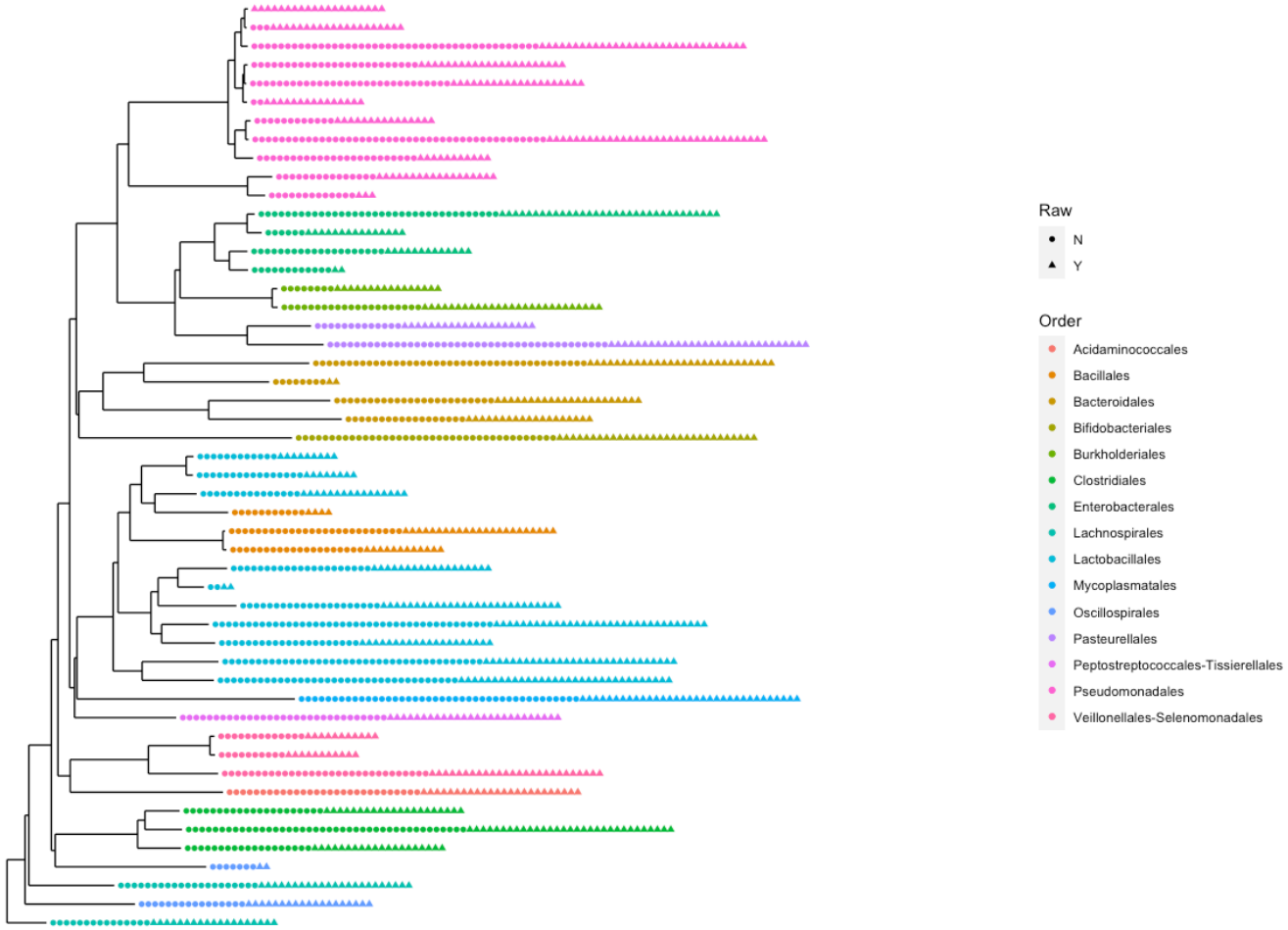

Figure 10. Dendrogram of the Top 30 most abundant ASVs in processed turkey items, and if they occur in raw (Y) or cooked (N) samples

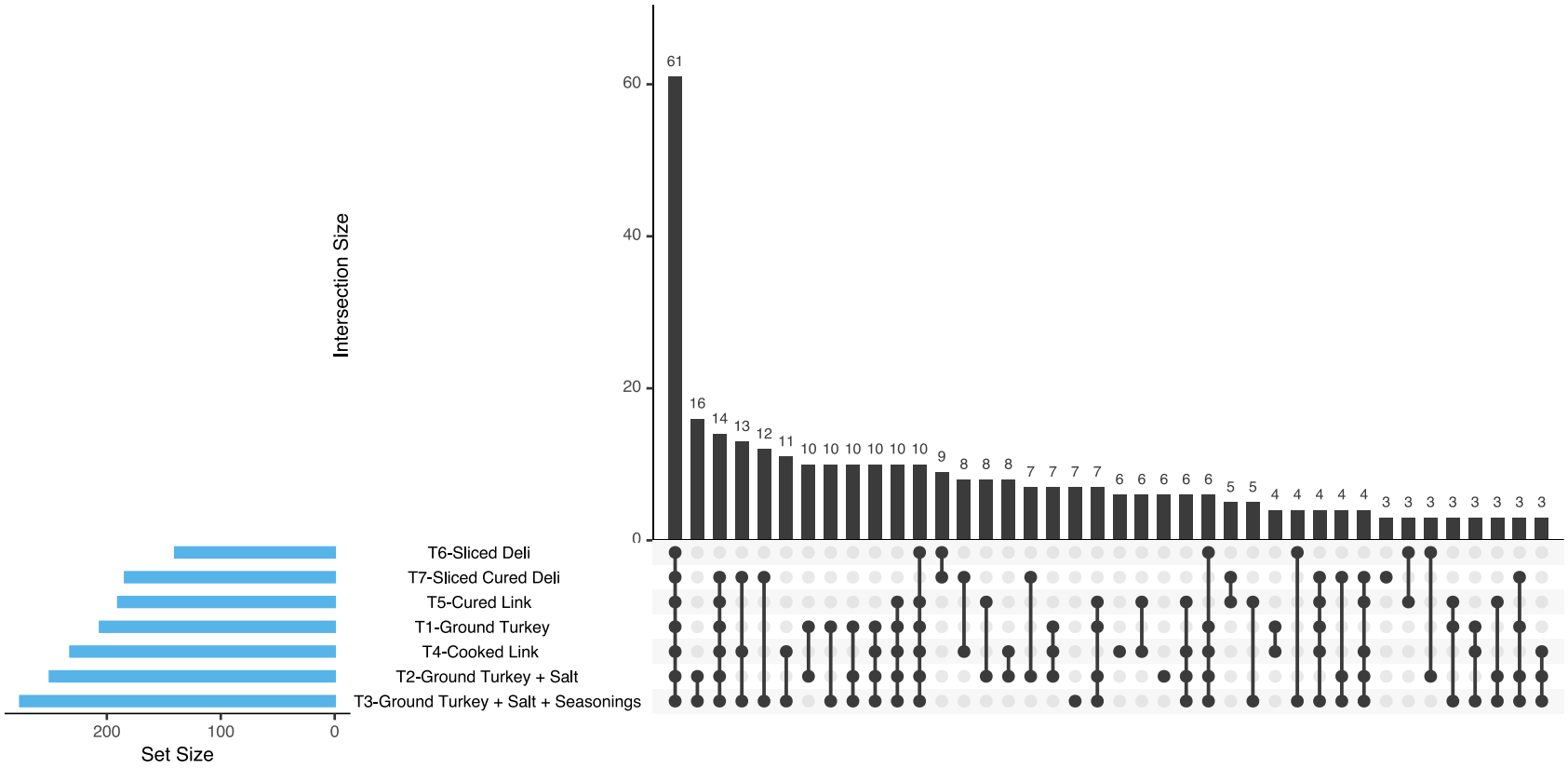

Figure 11. UpsetR Intersection Plot for ASVs in processed turkey products.

CHAPTER THREE: IMPACT OF PACKAGING SYSTEM ON THE MICROBIAL ECOLOGY OF RAW GROUND TURKEY

ABSTRACT

Ground poultry products are an economical source of high-quality protein worldwide. Unfortunately, the high nutrient content, mildly acidic pH, and incorporated oxygen in these products make them particularly susceptible to microbial spoilage from rapidly growing organisms such as *Pseudomonas* spp. Deterring the outgrowth of specific spoilage organisms (SSOs) and shifting bacterial taxa to slow-growing organisms may increase product shelf life allowing more time to distribute and consume these products. This may be accomplished with modified atmosphere packaging (MAP), which is known to limit certain microbial respiration. The objective of this study is to determine the impact of packaging system on the bacterial community composition of raw ground turkey over time. Three separate lots of boneless skinless turkey breast were ground on three separate replicates of production day. Product was placed in packaging treatments: T1) High oxygen MAP (80% oxygen / 20% carbon dioxide), T2) Low oxygen MAP (80% nitrogen / 20% carbon dioxide), T3) Mother Bag (Trays covered with an oxygen permeable film on Styrofoam trays and packaged in a modified atmosphere bag of 80% nitrogen / 20% carbon dioxide, T4) Plastic chub, T5) Vacuum packaging. Treatments were sampled throughout storage time for physicochemical, microbial, and metagenomic parameters. A significant effect of storage time by treatment interaction was observed for beta diversity metric Weighted UniFrac distances ($P = 0.030$). Packaging systems did modulate bacterial communities to include more relative abundance of *Brochothrix* spp. or lactic acid bacteria over time, however, *Pseudomonas* spp. occurred regardless of

treatment, illustrating the influence of the initial processing environment, and starting materials.

1. INTRODUCTION

Food losses occurring at retail or consumer levels comprise a bulk of wasted foods and impart direct economic losses on the consumer. Recent surveys suggest that the average American spends $1,300 on wasted foods each year (Conrad, 2020). Meat, poultry, and fish represent 41% of the total value, of which a vast majority of the waste occurs at the consumer level (Karwowska et al., 2021). The shelf life of fresh meat products is particularly susceptible to specific spoilage organisms present in the microbial communities of the high water activity substrate. These bacteria can more readily spoil goods by producing malodorous compounds and slimes that render products unfit for consumption.

By increasing understanding of the spoilage organisms in meats throughout shelf life, manufacturers may be able to employ specific strategies to extend shelf life. Recent work has revealed *Pseudomonas* spp. as a problematic organism. Due to its complex metabolism and tolerance of a wide variety of stresses, *Pseudomonas* spp. are ubiquitous in the environment and can readily contaminate and spoil products if not controlled (Peix et al., 2018). Particularly detrimental, pseudomonads have been shown to grow in anaerobic conditions, contrasting the traditional notion that they are obligate aerobes (Kolbeck, Abele, et al., 2021). Anaerobic growth capablity undermines the basis of vacuum packaging for shelf life extension, which would be particularly detrimental to meat systems and export. Alternative packaging strategies may provide an opportunity to shift the microbial community of meat products away from one dominated by

Pseudomonas spp., toward preferable slow-growing bacterial taxa producing less putrid metabolites.

Modified atmosphere packaging including carbon dioxide as an inhibitory compound, or "mother bag" packing systems with oxygen scavengers have been widely used in the red meat industry, as they provide shelf-life extension and additional color stability enhancements (McMillin, 2017); however, they could additionally be applied in the poultry industry specifically to extend shelf life in the context of microbial suppression. If the composition of the microbial community can be identified before time of spoilage, then organisms that are known SSOs, or are associated with certain packaging types can be better targeted by packaging with atmospheres or additional antimicrobial hurdles to suppress their outgrowth. These microbiome "snapshots" provide a group picture of which organisms are adequately suppressed, giving producers targeted packaging solutions against these organisms, and which continue to flourish, signaling additional hurdles may be necessary, or additional solutions developed to delay their outgrowth. The objective of this study is to evaluate the effects of commercially available packaging systems on the microbial communities and shelf-life outcomes of raw ground turkey.

2. MATERIALS AND METHODS

2.1. Packaging Treatments of Ground Turkey

Identified packaging treatments were chosen to represent a diversity of packaging atmospheres that could be readily serviceable for meat processors and capture a range of anoxic pressures on bacterial communities. Packaging treatments consisted of 5 case-

ready packaging systems currently used or emerging in fresh meat and poultry products, as follows:

- **T1 (High Oxygen MAP**): Packaged in a high oxygen modified atmosphere tray (80% nitrogen / 20% carbon dioxide). This system is case ready as is.
- **T2 (Low Oxygen MAP):** Packaged in a low oxygen modified atmosphere tray (80% nitrogen and 20% carbon dioxide). This system is case ready as is.
- **T3 (Mother Bag):** Packaged in trays covered with an oxygen permeable film and placed in modified atmosphere "mother bag" containing 80% nitrogen and 20% carbon dioxide. In this system, mother bags are distributed to point of sale, bags are opened, and trays are placed on retail display in the case.
- **T4 (Plastic Chub):** Packaged in plastic chub. This system is case ready as is.
- **T5 (Vacuum Bag):** Packaged in vacuum packaging. This system is case ready as is.

2.2 Manufacturing and Storage of Ground Turkey

Three independent replications of ground turkey were manufactured at the Loeffel Meat Laboratory (University of Nebraska, Lincoln, NE); each replication utilized a separate production lot of raw turkey. Vacuum packaged turkey breast meat (pectoralis) was obtained from a commercial abattoir, placed in frozen storage at -20°C, and tempered 48 hours at 4°C prior to usage. Pectoralis muscles were coarse ground through a 12.5 mm plate and then fine ground through a 3.2 mm plate using a Hobart Meat Grinder (Model 4734, Hobart Mfg. Co. Troy, OH). Meat was then divided into one-pound portions for packaging.

Treatments 1 and 2, the MAP treatments, were packaged on a tray sealer with a gas flush capability (Koch Equipment LLC, Kansas City, MO) with 2.4 mil high barrier transparent lidding film with an oxygen transmission rate (OTR) of 3 cc/m^2/24 hr at standard temperature and pressure (STP; 0°C and 105 Pa; Ultra Source, Kansas City, MO) was used to cover 22.0 x 16.8 x 5.5 cm white opaque plastic food tray (Coextruded Plastic Technologies, Janesville, WI).

Treatment 3, mother bags, first had meat placed onto Styrofoam trays (21.6 x 15.9 x 2.1 cm, Stryo-Tech, Denver, CO) and overwrapped with transparent oxygen permeable film (Prime Source PSM 18 750003815, Bunzl Processors Division, North Kansas City, MO). Then, two of these trays were placed into a 3 mil high barrier transparent vacuum pouch (CLARITY pouch, Bunzl Koch, Riverside, MO) with an oxygen scavenger sachet (BD GasPak EZ sachet Becton Dickinson and Company, Sparks, MD). Atmospheric gases were removed from packages with a vacuum sealer, and then flushed on the same unit with 80% nitrogen / 20% carbon dioxide.

Treatment 4 was stuffed into opaque one-pound 2 mil polymer meat bags (UltraSource, Kansas City, MO) using a vacuum stuffer (Vemag Robot 1000 DC; Reiser, Canton, MA), and sealed using plastic tape.

Treatment 5 was placed in 3 mil high barrier transparent packages (CLARITY pouch, Bunzl Koch, Riverside, MO) and vacuum sealed at approximately 1.4 kPa (Multivac Model C500; Multivac Inc., Kansas City, MO).

2.3 Storage of Packaged Treatments

Packages were kept in dark storage for a specified number of days and then placed in a simulated retail display case (RD) for 4 days. Trays were removed from mother bag prior to placement in RD. Packaged were placed under simulated retail display (RD) conditions for 4 d (3°C under white fluorescence lighting at 1000 to 1800 lux) and randomly rotated daily. Day 0 is considered the date of grinding and packaging. All treatments were sampled on days 0, 1, 14, 18, 21, and 25 with additional sampling for treatments T1 and T5 (days 7, 11, 28, 32) to ensure all treatments were sampled pre- and post-spoilage. Detailed storage and sampling schematic is displayed in (Table 1).

2.4. Physicochemical Methods

Objective Color (L, a*, b*)*

Samples were assessed for objective color on days 1, 14, 18, 21 and 25 with a calibrated colorimeter (Chroma Meter CR0400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ). Packages were opened and allowed to rest for 30 minutes prior to color reading to allow for equivalent blooming time, as Treatment 4 had opaque packaging that could not be read through, and the package had to be opened for assessment. Six readings were taken on two samples per treatment using a 2° standard observer with an 8 mm aperture and the D65 illuminant setting.

pH

Samples were assessed for pH on days 1, 14, 18, 21 and 25. For each treatment, 10g of meat and 90mL DDD water were added to a 150mL beaker and mixed with a magnetic stir bar on a stir plate (Thermolyne Cimarec-top stirring hotplate; Barnstead

Themolyne, Dubuque, IA). A pH meter calibrated with standards of pH 4.01, 7.00, and 10,01 (Orion 910104, 910107, and 910110, ThermoScientific, Waltham, MA) was used to measure sample pH in duplicate.

2.5. Microbiological Plate Methods

Approximately 100 g of meat was aseptically transferred from packaging to filtered stomacher bags (Interscience USA, Woburn, MA). Bags were placed in a stomacher (bioMerieux Inc., Durham, NC) with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ) for 2 minutes to homogenize the sample. Two, 1.75 ml samples of fluid were pipetted from the bag, and stored at -20°C until DNA Extraction. A portion of remaining sample was used for microbial plating methods in duplicate. Fifty µl of sample was administered to 100mm agar plates utilizing an Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain). Brain, Heart, Infusion agar (Thermo Fisher Scientific, Waltham, MA) was used to conduct aerobic plate counts (APC), anaerobic plate counts (AnPC), and psychrotrophic (PSY) plate count. DeMan Rogosa Sharpe agar (Thermo Fisher Scientific, Waltham, MA) was used to enumerate lactic acid bacteria (LAB). Cephaloridine Fucidin cetrimide agar (CFC) and Pseudomonas supplement (Thermo Fisher Scientific, Waltham, MA) was used to enumerate *Pseudomonas* spp. APC, AnPC, CFC and LAB were incubated at 37°C and counted at 48 hours. AnPC were held in anaerobic chamber (BD GasPak EZ Large Insulation Container; Becton, Dickinson, and Company, Sparks, MD) with three oxygen

absorbent packs (BD GasPak EZ sachet; Becton, Dickinson, and Company, Sparks, MD). Psychrotrophs were incubated at 4°C and counted at 10 days.

2.5. Microbial Sequencing Methods

Bacterial communities were investigated for each sample using the MiSeq Illumina Sequencing Platform, targeting the bacterial-specific 16s rRNA gene (Kozich et al., 2013). DNA was extracted from samples using DNA QuickExtract Solution 1.0 (Epicentre, Madison, WI). Obtained DNA was amplified via the polymerase chain reaction (PCR) with a solution that contained 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA), 0.75 U Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc.), approximately 1-5 ng of extracted DNA, and 0.5 μM barcoded universal primers. The PCR reaction was performed alongside negative controls in a Veriti 96 well thermocycler (Thermo Fisher Scientific, Walther, MA), with the following PCR cycle: initial denaturation at 98°C for 3 min, followed by 30 cycles of 98°C for 30s, 58°C for 30s, and 68°C for 45s, and a final extension of 68°C for 4 min.

PCR products were then analyzed on a 1.5% agarose gel to ensure amplification occurred successfully, without contamination of negative controls. Samples were then normalized using the Norgen NGS Normalization 96-Well Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to manufacturer protocol. Pooled sample was then placed in 50°C water bath to remove excess ethanol from the normalization kit and ran through a spin column. DNA was found to be insufficient in concentration, so samples were subjected to additional PCR using a 5-cycle rendition of the previously described protocol. Products were then analyzed on a 2% agarose gel, which yielded two bands. The band corresponding to the bp size of the 16s rRNA V4 subregion was removed with

a scalpel and DNA was recovered using the MinElute PCR Purification kit (Qiagen Inc., Germantown, MD). Concentration and bp size of the 16S rRNA libraries were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library concentration was confirmed with a DeNovix QFX Fluorometer and the Denovix dsDNA High Sensitivity reagent kit (Denovix Inc., Wilmington, DE). The 16S libraries were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) using the V2 500 cycle kit.

2.6. Statistical Analyses

Physicochemical and Plate Count Analyses

Microbial plate counts, color parameters and pH were analyzed with independent covariance structure using the nlme package using R using timepoints when samples from each treatment were collected (Pinheiro J, Bates D, DebRoy S, Sarkar D, 2020). Means were separated using the emmeans package. Significance was declared at $\alpha = 0.05$ throughout the study.

Bacterial Community Analysis

Reads were paired, trimmed, and filtered in DADA2 (Callahan et al., 2016) with *truncLen=c(200,130)* to ensure proper region and quality. Screened reads were assigned to amplicon sequence variants (ASVs) and taxonomy was assigned according to SILVA rRNA SSU 138. Reads pertaining to non-bacterial sequences and spurious ASVs were removed, leaving an average of 26,504 reads per sample. The ASV table was rarefied to 3000 reads for alpha diversity analysis, and samples below this threshold were removed. A phylogenetic tree was generated in Mothur version 1.46 for use in beta diversity

estimates (Schloss et al., 2009). Alpha diversity estimates (Chao1 and observed ASVs) were calculated for the entire community with the phyloseq package (McMurdie & Holmes, 2013). The effects of treatment and storage time on alpha diversity metrics were evaluated with Kruskal-Wallis tests. Weighted UniFrac distances describing beta diversity were ordinated in phyoseq. Bacterial community composition differences across treatments and storage time were evaluated using permutational multivariate analysis of variance with weighted UniFrac distances using PERMANOVA in the vegan package (Oksanen et al., 2019) and pairwise analyses via the Adonis function from pairwise.Adonis (Martinez Arbizu, 2020). Differential abundance analysis was performed using DESeq2 to determine the influence of retail display (Love et al., 2014).

2.7. Data Availability

The 16S rRNA gene sequences are available at the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under BioProject PRJNA877347. The scripts and data objects for the reproduction of figures in this study are found in a Zenodo repository at the following address: https://doi.org/10.5281/zenodo.7110592.

3. RESULTS AND DISCUSSION

3.1. Physicochemical and Plate Counts

There was a significant effect ($P < 0.05$) of storage time on pH as it declined over time in all treatments, likely attributable to the generation of acidic components from microbial fermentation. There were no observed effects for treatment ($P = 0.6288$) or treatment by day interaction ($P = 0.9589$) on pH. All three color measures (L*, a*, b*) exhibited significant treatment-by-day interactions ($P = 0.0145$, 0.001, 0.0306

respectively), as displayed in Figure 1. This phenomenon can be explained by the high oxygen T1 and T4 displaying bright red/pink color of oxygenation initially, which were then more quickly oxidized than other treatments displaying a brown color at the end of the testing period. Storage time had a significant effect on all plate counts, as they increased over time ($P < 0.05$) as displayed in Figure 2. Spoilage of meat products is correlated to around 7 log CFU/g, as this is generally when slime production and off-odors are shown to emerge, and thus is used as a proxy for spoilage in this study to benchmark performance (Nychas et al., 2008). A treatment by storage time interaction ($P = 0.0061$), was observed in AnPC because T1 (High Oxy MAP) took longer to reach 7 log CFU/g as fastidious aerobic organisms outcompeted anaerobes during high oxygen storage illustrating how packaging plays a role in selecting organisms with differential metabolic flexibility and preferences. Additionally, Treatment 4 (plastic chub) was observed to reach spoilage level prior to its counterparts on CFC agar, demonstrating the treatment's propensity for pseudomonad growth.

3.2. Microbial Communities

Alpha Diversity

Alpha diversity metrics across treatments have been visualized in Figure 3. There was no observed treatment effect on Observed, Shannon, or Chao 1 diversity indices as calculated using the Kruskal-Wallis analysis of variance ($P = 0.1627$, 0.0501, 0.1527 respectively). However, there was an observed treatment effect of day on all three diversity metrics ($P < 0.0001$), and moreover, there was a significant treatment-by-day interaction effect for all attributes, thus previous P values must be considered in the context of interactions ($P = 0.0271$, 0.0022, and 0.0321 respectively). As shown in Figure

4, alpha diversity tends to decrease with time in all samples, however the plastic chub is dominated by *Pseudomonas* throughout the entirety of storage, as shown in Figure 5, eliciting this interaction effect. Others have observed this phenomenon and associated this loss of diversity with spoilage (Fougy et al., 2016). As meat systems approach the end of shelf-life, many of the readily consumable metabolites like sugars, and precious metabolic compounds like metals or oxygen have been already utilized by the broad range of microbes. The more nutrient sparse environment niches are filled by bacterial taxa with high metabolic flexibility for proteolysis, or a means of outcompeting the other taxa that have mechanisms to remain viable in the changed environment, such as self-serving metal chelator siderophore reserves. This concept is further illustrated for all treatments in Figure 5, where the wide array of colors representing different bacterial taxa are truncated into a few dominating bands of specific spoilage organisms.

Beta Diversity

Weighed UniFrac distances were significantly impacted by retail display ($P = 0.006$), Day ($P < 0.001$), Treatment ($P < 0.001$) and Day by Treatment interaction ($P = 0.030$). Retail display has been observed to impact the relative abundance of *Pseudomonas* spp. in raw beef samples, and light conditions of retail display could promote the growth of some phototrophic bacteria (Hanlon et al., 2021). To further evaluate this, significant differential abundances of bacterial taxa from packages with and without retail display are displayed in Table 2. While no *Pseudomonas* spp. were found to change significantly, some organisms that better endure anaerobic conditions, like the *Lactobacillus* genus, were less frequent in retail display, which could be partially explained by the opening on the mother bag in Treatment 3. Another possibility is that

these organisms were more dependent on light-sensitive components of the meat for growth, such as riboflavin or other vitamins. These compounds have been shown to degrade in model systems exposed to daylight, suppressing growth of *Lactobacillus casei* (Anderson & Cowan, 1968), and degradation has been observed in dairy products under retail display (Deger & Ashoor, 1987; Wang et al., 2020). Outside of these few bacterial taxa, the treatment-by-day interaction generally steered the microbial profile throughout shelf life. Figure 6 gives a spatial visualization in changes in treatments, with some treatments close to a central starting point, while others deviate away as they are shifted by their packaging conditions over time. The treatments generally start clustered in the bottom left, from which two separate groups emerge to the top and to the right. Treatment 4, the plastic chub tended to stay close to the central hub, which signifies a predominance of *Pseudomonas* throughout storage. More anaerobic leaning treatments (T2, T3, T5) strayed away to the right with communities dominated by lactic acid fermentative genera like *Lactobacillus*, *Lactococcus*, or *Carnobacterium.* Treatment 1 drifted to the top on its own accord, as it had a higher relative abundance of *Brochothrix*, an aerobic bacterium that can withstand carbon dioxide exposure. Overall, packaging system did alter the microbial composition and shelf life of processed turkey with different degrees of efficacy over time.

3.3 Implications for Packaging Utilization

Treatment 4, the plastic chub was the poorest performing treatment, as it was the first to reach the defined spoilage associated plate counts and provided an oxygen-rich environment where the initial *Pseudomonas* spp. continued to dominate. Interestingly, Treatment 1 outperformed Treatment 4 in plate count benchmarks. Despite having high

oxygen in the package, the supplementary carbon dioxide provided additional pseudomonad suppression (CFC plots in Figure 2). Because of this outcome, high oxygen MAP with carbon dioxide for other antimicrobial hurdles may be considered as an option for shelf life extension, which enhances a* values in early shelf life. Low oxygen MAP tray (T2) also appeared to have an inhibitory effect on *Pseudomonas spp.* due to carbon dioxide in the starting gas mixture making this a potentially viable option for ground poultry products. This low oxygen MAP shift to lactic acid bacteria has also been observed in low oxygen packaged raw chicken meat, where *Latilactobacillus sakei* was determined as a SSO (Tsafrakidou et al., 2021). The mother bag system also functioned as a method to shift to lactic acid bacteria at the time of spoilage with the added benefit of minimized *Yersinia*. While no pathogens were identified in this study, the genus Yersinia does contain the notable pathogen, *Yersinia enterocolitis*, thus processors should be aware of how processing choices, including packaging, influence the meat microenvironment. Regardless of these shifts, certain ASVs mapping to *Pseudomonas* spp. endured across a multitude of the packaging treatments compared to other pseudomonads, as seen in bright lateral bands in Figure 7. Further investigation into these exact strains could help elucidate the mechanisms by which certain pseudomonads an persist in anoxic environments.

As a result of choices made throughout processing, the exact composition of the meat microbiome varies across facilities, products, and production lots, making a universally effective packaging system impractical to identify. Products in this study and prior studies performed at the Loeffel Meat Laboratory often have a high relative abundance of *Pseudomonas* spp. at day 0, and thus, MAP systems with increased carbon

dioxide and decreased oxygen showed the greatest shifts in biome composition and suppression of outgrowth. Additional studies have observed such influence of initial composition and processing facility on packaging effect. When four separate facilities' fresh minced pork was packaged in oxygen permeable overwrap and high oxygen MAP system (30% carbon dioxide, 70% oxygen), two facilities were dominated by *Pseudomonas* spp. initially had shifted to *Brochothrix*, *Leuconostoc*, or *Lactobacillus* at end of storage in congruence with results from this manuscript, while the two facilities that initially had more *Photobacterium* spp. retained a high abundance of *Photobacterium* at end of storage (Cauchie et al., 2020). As *Pseudomonas* and *Brochothrix* can utilize oxygen to grow, other researchers had hypothesized that sensors detecting the decrease in headspace oxygen could be used as spoilage indicators. While the sensor was able to identify a decrease in oxygen level associated with spoilage with *Brochothrix* and *Leuconostoc* strains, the approach was ineffective with *Carnobacterium* spp as the oxygen levels remained steady for these fermentative organisms (Kolbeck, Hilgarth, et al., 2021) illustrating that novel packaging systems must consider which SSOs are of relevance for particular products.

4. CONCLUSION

In the context of this study, packaging atmosphere composition containing carbon dioxide or reduced oxygen modulated bacterial communities to include a greater relative abundance of *Brochothrix* or lactic acid bacteria over time, however, *Pseudomonas* spp. were still present regardless of treatment. This pseudomonad persistence and varying results from other locations illustrate the predominant influence of the initial processing environment and starting materials on the meat microbiome and spoilage outcomes.

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Tables

Table 3. Sampling schematic of differentially packaged ground turkey in dark storage and retail display over 32 days

DAYS:	0	1	7	11*	14	18*	21	25*	28	32*
T1	X	X	X	X	X	X	X	X		
T2	X	X			X	X	X	X		
T3	X	X			X	X	X	X		
T4	X	X			X	X	X	X		
T5	X	X			X	X	X	X	X	X

X = Microbial sampling took place for row treatment on specified day. Columns denoted with * are representative of samplings from products after 4 days of retail display. T1) High Oxy MAP, T2) LowOxy Map, T3) Mother Bag, T4) Plastic chub, T5) Vacuum Packaging.

Table 4. Differentially abundant bacterial taxa from retail display

Family pertaining to ASV	**Genus pertaining to ASV**	**Log2Fold Change**	**Adjusted *P*-value**
Veillonellaceae	Megasphaera	-5.27	0.0043
Oscillospiraceae	UCG-002	-5.57	0.0136
Peptostreptococcaceae	Terrisporobacter	-6.18	0.0136
Prevotellaceae	Prevotella	-5.74	0.0063
Streptococcaceae	Streptococcus	-6.74	0.0035
Lactobacillaceae	Lactobacillus	-5.63	0.0017

Figures

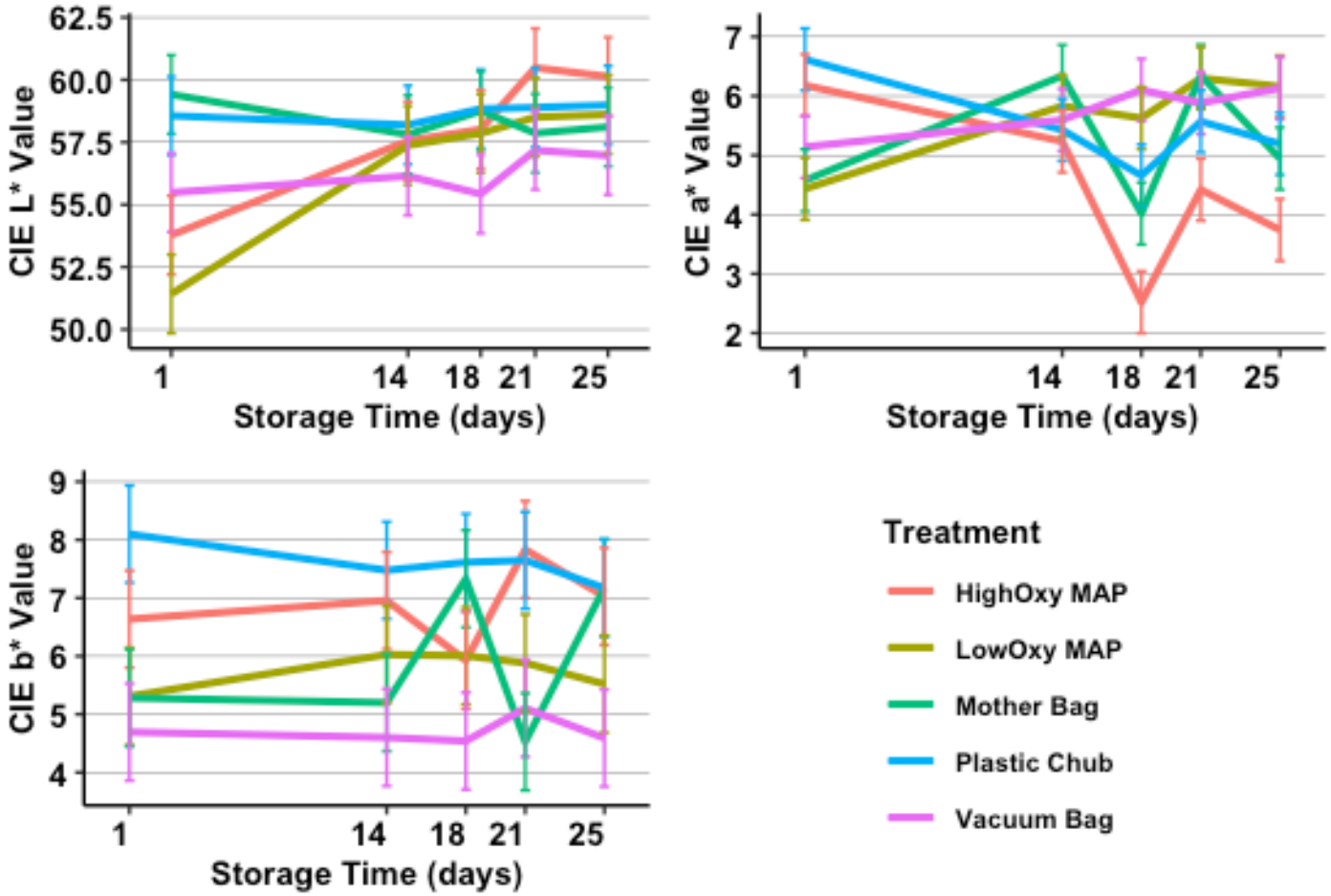

Figure 1. Average objective color measurements of differentially packaged raw ground turkey over time. Days 18 and 25 are following 4 days of retail display.

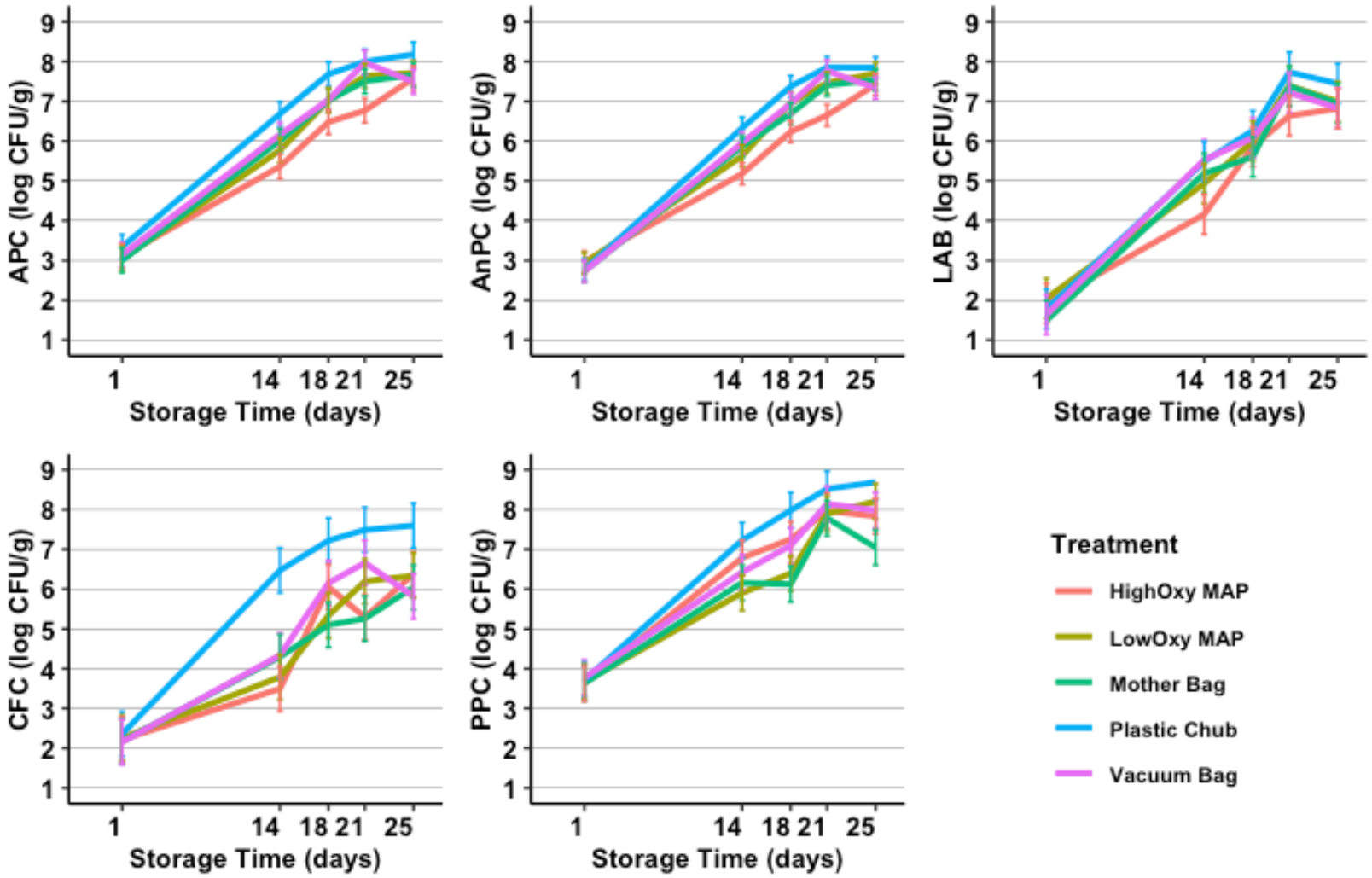

Figure 2. Average microbial plate counts (log CFU/g) of differentially packaged raw ground turkey over time

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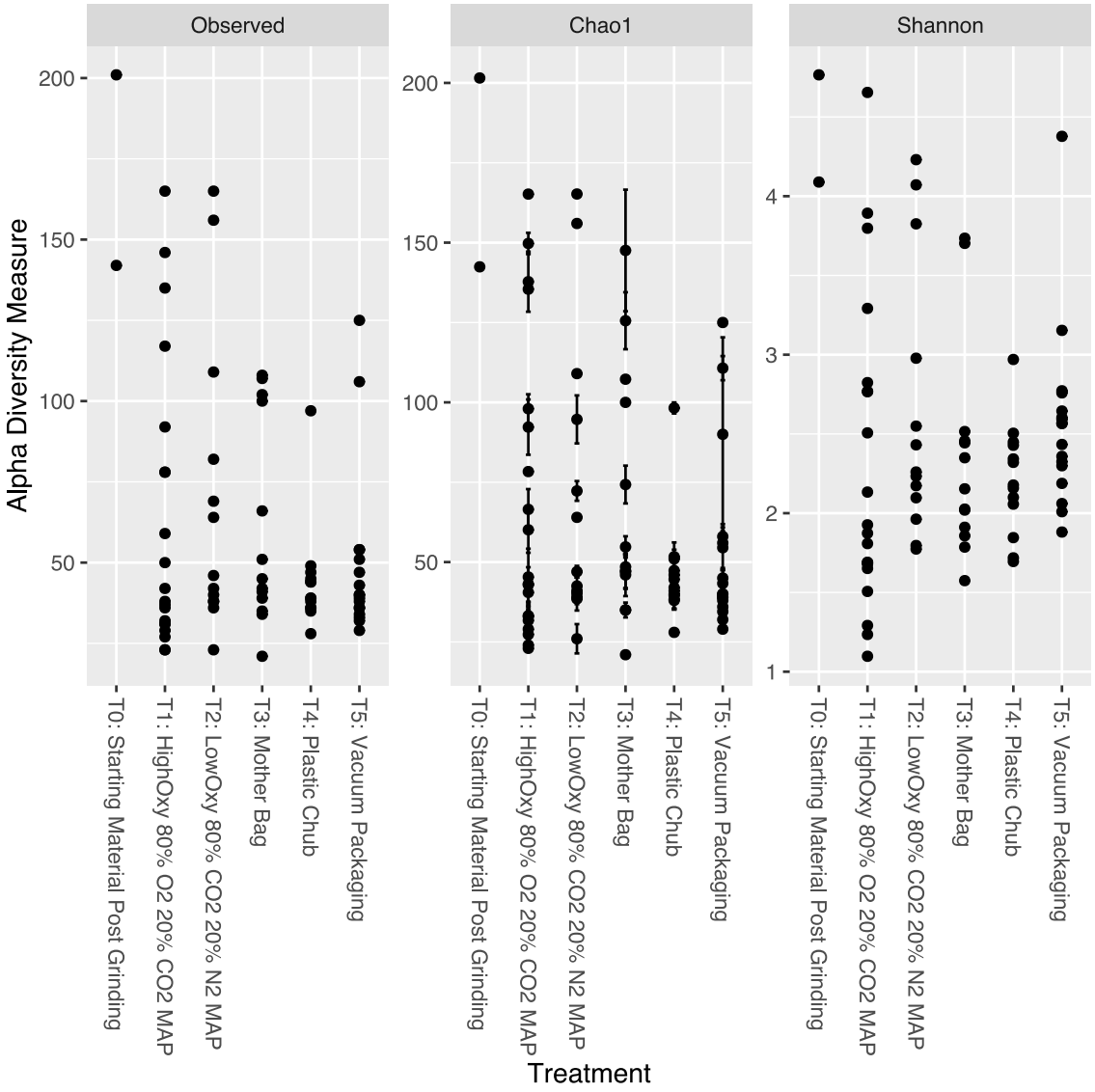

Figure 3. Alpha diversity metrics of raw ground turkey across various packaging treatments.

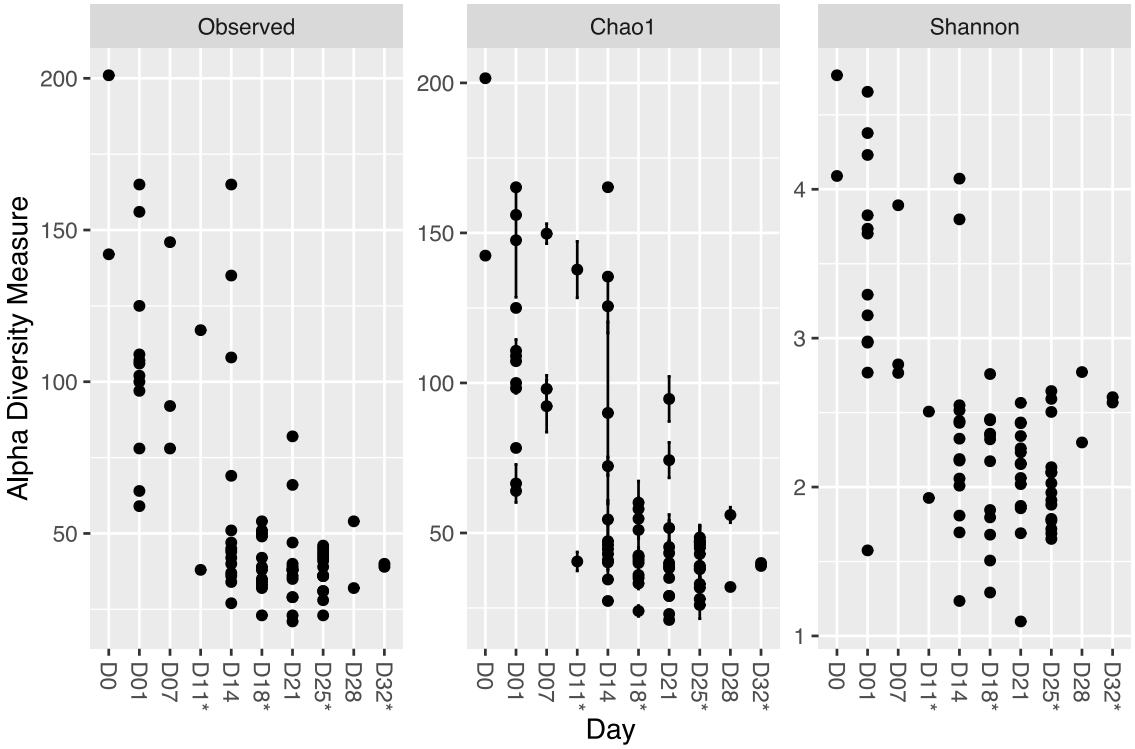

Figure 4. Alpha diversity metrics of raw ground turkey over shelf life. *Indicates samples that underwent four days of retail display.

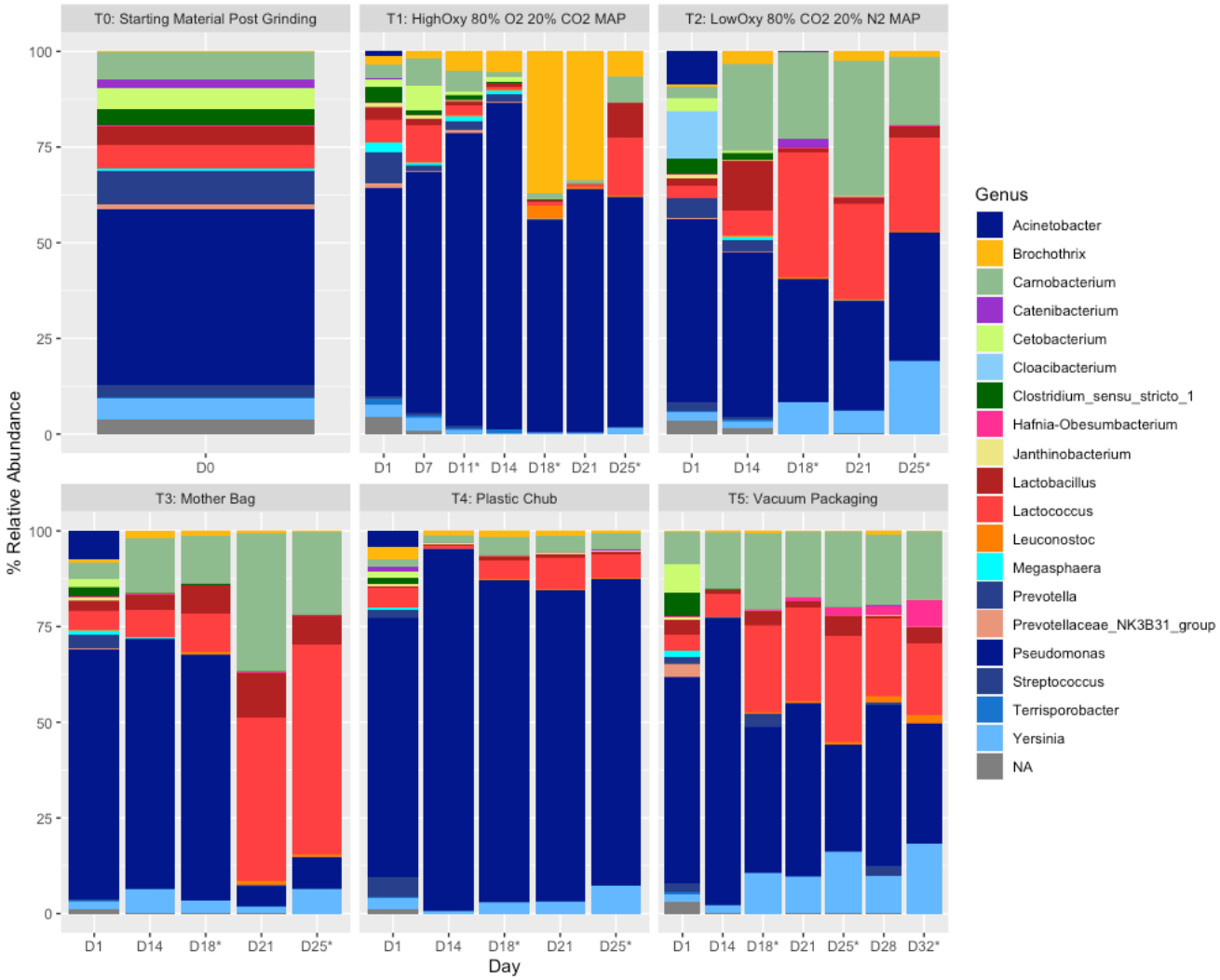

Figure 5. Bar plot of the top 50 most abundant ASVs truncated by Genus (others listed as NA) in ground turkey samples across various times and packaging methods. **Indicates sample underwent four days of retail display*

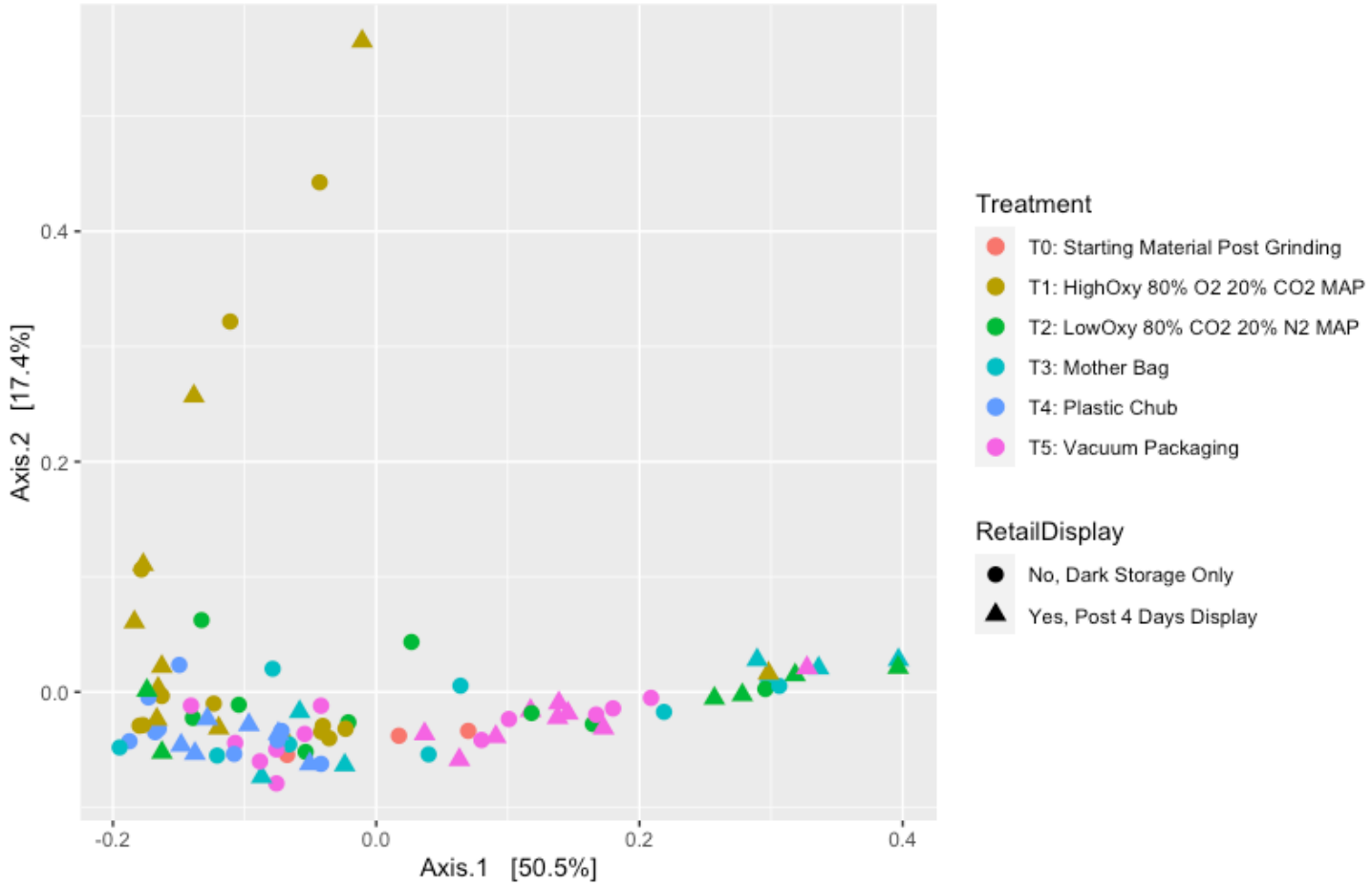

Figure 6.Principal coordinate analysis using the weighted UniFrac distance ordination. Relative distance between samples in the PCA plots indicates dissimilarity between bacterial community structure.

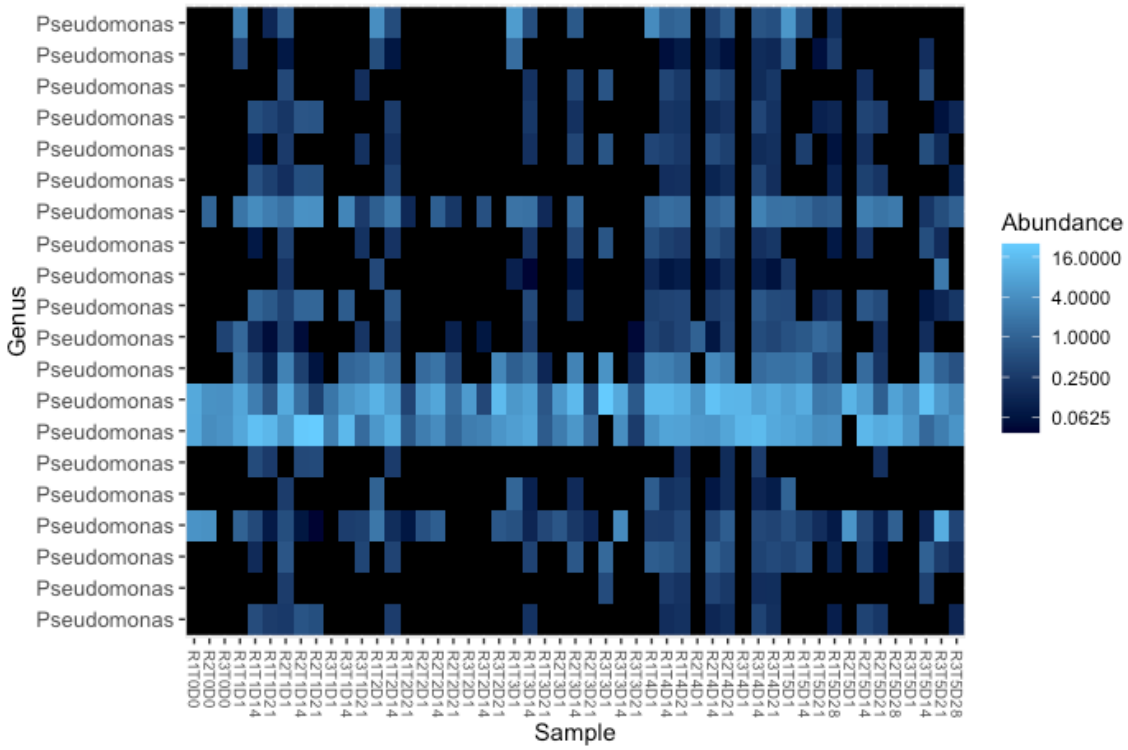

Figure 7. Relative abundance heatmap of Pseudomonas spp. across replication (R), Treatment (T), and day (D). T1) HighOxy MAP, T2) LowOxy MAP, T3) Mother Bag, T4) Plastic chub, T5) Vacuum Packaging.

CHAPTER FOUR: CHARACTERIZATION COMPLETE GENOME SEQUENCES OF *PSEUDOMONAS* SPP. STRAINS ISOLATED FROM GROUND TURKEY MEAT

ABSTRACT

Here, we report the genome sequences of *Pseudomonas* spp. strains UNL-A, UNL-C, UNL-E, and UNL-G, isolated from ground turkey meat processed at the Loeffel Meat Laboratory in Lincoln, NE. These genomes comprise complete chromosomes, with additional plasmid sequences. Featured genomes are from packages from a variety of atmospheres, and contribute to investigations of reduced oxygen tolerance among *Pseudomonas* spp.

1. INTRODUCTION

Pseudomonas spp. are well-known as meat spoilage organisms, which readily colonize the cold meat-processing environment. While past work asserts pseudomonads generally exhibit aerobic behavior, many researchers are obtaining isolates from modified atmosphere packages with significantly reduced oxygen (Kolbeck et al., 2021), and recent literature now describes pseudomonads as facultative aerobes.

This change in understanding is not without consequences. The ways we look to inhibit organisms, center around what we know of their physiology, thus vacuum packaging was thought to be a sufficient suppression technique under the past, “obligate aerobe” scheme. Given this new understanding, and observations of pseudomonads permeating in vacuum packaged systems (Furbeck et al., 2022), elucidating the mechanisms these strains utilize to predominate in the reduced oxygen environment

could provide insight into more efficacious ways to limit their outgrowth. Some pseudomonads can utilize terminal electron acceptors other than oxygen, such as nitrate or nitrite, however, Kolbeck et al. observed more substantial evidence for arginine fermentation in *Pseudomonas* spp. isolates from muscle foods. Further characterization of the genes associated with meat-associated anoxic phenotypes from various facilities and packaging atmospheres could shed light on new mechanisms to exploit. Because of this, we issue forward a genomic investigation of *Pseudomonas* spp. isolates from raw ground turkey in varying packaging systems, processed at the Loeffel Meat Laboratory in Lincoln, NE.

2. MATERIALS AND METHODS

2.1. Pure Culture Isolation

To further investigate *Pseudomonas* spp. ecology of modified atmosphere packaged poultry products, vacuum packaged boneless skinless turkey breast was purchased from a commercial abattoir, ground with a Hobart Meat Grinder (Model 4734, Hobart Mfg. Co. Troy, OH), and divided into portions for packaging treatments of varying packaging atmospheres. Packages were stored until time of spoilage. Approximately 100g of meat was aseptically transferred from packaging to filtered stomacher bags (Interscience USA, Woburn, MA) with 150 ml of sterile BBL Peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ). Bags were placed in a stomacher (bioMerieux Inc., Durham, NC) for 2 minutes to homogenize the sample. Fifty µl of sample was administered to 100mm agar plates utilizing an Eddy Jet spiral plater (IUL, S.A., Barcelona, Spain). Cephaloridine Fucidin cetrimide agar (CFC) and *Pseudomonas* supplement (Thermo Fisher Scientific, Waltham, MA) was used to harvest

Pseudomonas spp. Plates were incubated at 25°C and counted at 48 hours, then single colonies were harvested and streaked for isolation. Pure isolates then had DNA extracted and prepared for hybrid sequencing.

2.2. Sequencing and Assembly

Sample libraries were prepared using the Illumina DNA Prep kit and IDT 10bp UDI indices, and sequenced on an Illumina NextSeq 2000, producing 2x151bp reads. Demultiplexing, quality control and adapter trimming were performed with bcl-convert(v3.9.3). Long reads were also sequenced via an Oxford Nanopore Technology (ONT) sequencer, and trimming was performed with porechop (v0.2.3, Wick, 2017). Hybrid assembly with Illumina and ONT reads was performed with Unicycler (v0.4.8, Wick et al., 2017). Assembly statistics were recorded with QUAST (v5.0.2, Gurevich et al., 2013). Assembly annotation was performed with Prokka (v1.14.5, Seemann, 2014). Assemblies were identified using the Type (Strain) Genome Server (TYGS (Meier-Kolthoff & Göker, 2019), as displayed in Figure 1. Assembly metrics are provided in Table 1. Proposed taxonomies were validated by calculating average nucleotide identity with OrthoANI, with results displayed in Table 2 (Yoon et al., 2017).

2.3. Comparative Genome Analysis

To further compare these genomes and identify elements of interest, various comparative genomics and discovery tools were employed. Prokka annotated genomes were subjected to the MicrobeAnnotator workflow to group annotated genes by metabolism modules (Ruiz-Perez et al., 2021). These modules were evaluated for completeness (% of enzymes in pathway present) and displayed in Figure 2. Prokka annotated genomes, alongside genomes of type strain organisms, and other meat-

implicated strains of the same species were utilized for pangenome analysis in Roary v3.11.2 (Page et al., 2015), as visualized in Figure 3. Upon seeing variation in small fragments among species, additional consideration was given to the potential impact of gene transfer on composition, and signatures of genomic islands in all four genomes with IslandCompare v1.0 (Bertelli et al., 2022). Active prophages were predicted in novel strains with Prophage Hunter (Song et al., 2019).

RESULTS AND DISCUSSION

The first strain, *Pseudomonas* spp. UNL-A matches *Pseudomonas shahriarae.* This species was first described from a wheat source (Girard et al., 2021). To our knowledge, this is the first reported instance of the species from a meat substrate. This isolate was obtained from ground turkey stored in high oxygen-modified atmosphere packaging for 21 days. It exhibits strong production of florescent yellow pigment on cephaloridine Fucidin cetrimide (CFC) agar. This strain contained a predicted active prophage, which is most closely aligned to the known cold-active lytic phage, *Pseudomonas* phage VW-6S (Xiang et al., 2018).

Strain *Pseudomonas* spp. UNL-C matches *Pseudomonas haemolytica*, which has additionally been found in chicken products (Heir et al., 2021). This isolate was obtained from ground turkey stored in a mother bag system containing 80% N and 20% CO2 and oxygen scavengers for 21 days. It exhibits strong production of florescent yellow pigment on. This strain had notably more complete metabolic modules for cysteine and methionine metabolism than its counterparts (Figure 2), which aligns with a malodorous sulfur odor that was present in the package it was isolated from. While use of other amino acid metabolism pathways, such as the arginine deaminase pathway have been identified

as mechanisms of survival for pseudomonads in MAP packaged meats, perhaps this strain has increased propensity for the utilization of additional amino acids (Kolbeck et al., 2021). Using the KEGG Orthology (KO) numbers predicted from MicrobeAnnotator, unique KO pertaining only to strain UNL-C were identified and investigated with KEGG Mapper (Kanehisa et al., 2022). One feature of note, is enzyme 4.4.1.1 was only observed in Strain UNL-C. This multifunctional enzyme can facilitate a multitude of reactions pertaining to cysteine methionine metabolism, including conversions to pyruvate and thiocysteine. An overview of all observed enzymes in the cysteine methionine KEGG module in UNL-C is shown in Figure 4. By facilitating a multitude of reactions with one enzyme, this organism may be able to spend less energy to metabolize more reactions than the other strains.

Strain *Pseudomonas* spp. UNL-E and UNL-G match *Pseudomonas lundensis*, a species that is highly associated with the spoilage of meats, but also observed as an opportunistic pathogen in individuals with cystic fibrosis (Ravi et al., 2022). UNL-E was isolated from turkey meat stored for 21 days in an oxygen-permeable plastic chub package. UNL-G was isolated from ground turkey stored for 25 days in a vacuum package. These isolates exhibited weak production of florescent yellow pigment. Isolate UNL-G was able to survive limited oxygen-conditions. When streaked onto fresh plates and incubated in anaerobic chamber, this isolate was able to establish colonies that could be forward propagated again after 5 days of storage. It is of note, that more circular contigs were observed in UNL-G than UNL-E. Based on this, and evidence of genomic islands among all pseudomonads in the study, gene transfer may play a role in conferring fitness advantages in the meat microenvironment.

DATA AVAILABILITY

Raw reads can be found at the following repository: https://doi.org/10.5281/zenodo.7179539. Due to the anaerobic and florescent propensities of these isolates, and the implications these behaviors have on food spoilage and virulence, we are reporting these genomes to allow researchers to dig deeper into the genetic makeup of these organisms.

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Tables

Table 1. Genome Assembly Statistics of *Pseudomonas* spp. Isolates

Strain	Year of Isolation	No. Illumina Bases	No. Nanopore Reads	Genome size (bp)	No. of Contigs	N_{50} bp	G + C Content
UNL-A	2019	1227671033	504088585	6241539	3	6162084	60.37%.
UNL-C	2019	1100339745	388721574	6136762	3	6084387	59.90%
UNL-E	2019	1280748665	476944846	5135622	5	5080452	58.37%
UNL-G	2019	1185123504	337565008	5084666	11	4832490	58.61%

Table 2. Average Nucleotide Identity (ANI) of novel poultry associated genomes and corresponding type strain

Novel Strain	Proposed species	Type Strain	ANI
UNL-A	*Pseudomonas shahriarae*	ASM1426845v2	99.05%
UNL-C	*Pseudomonas haemolytica*	ASM164056v1	98.79%
UNL-E	*Pseudomonas lundensis*	2T-2-5-2v4	99.21%
UNL-G	*Pseudomonas lundensis*	2T-2-5-2v4	99.14%

Figures

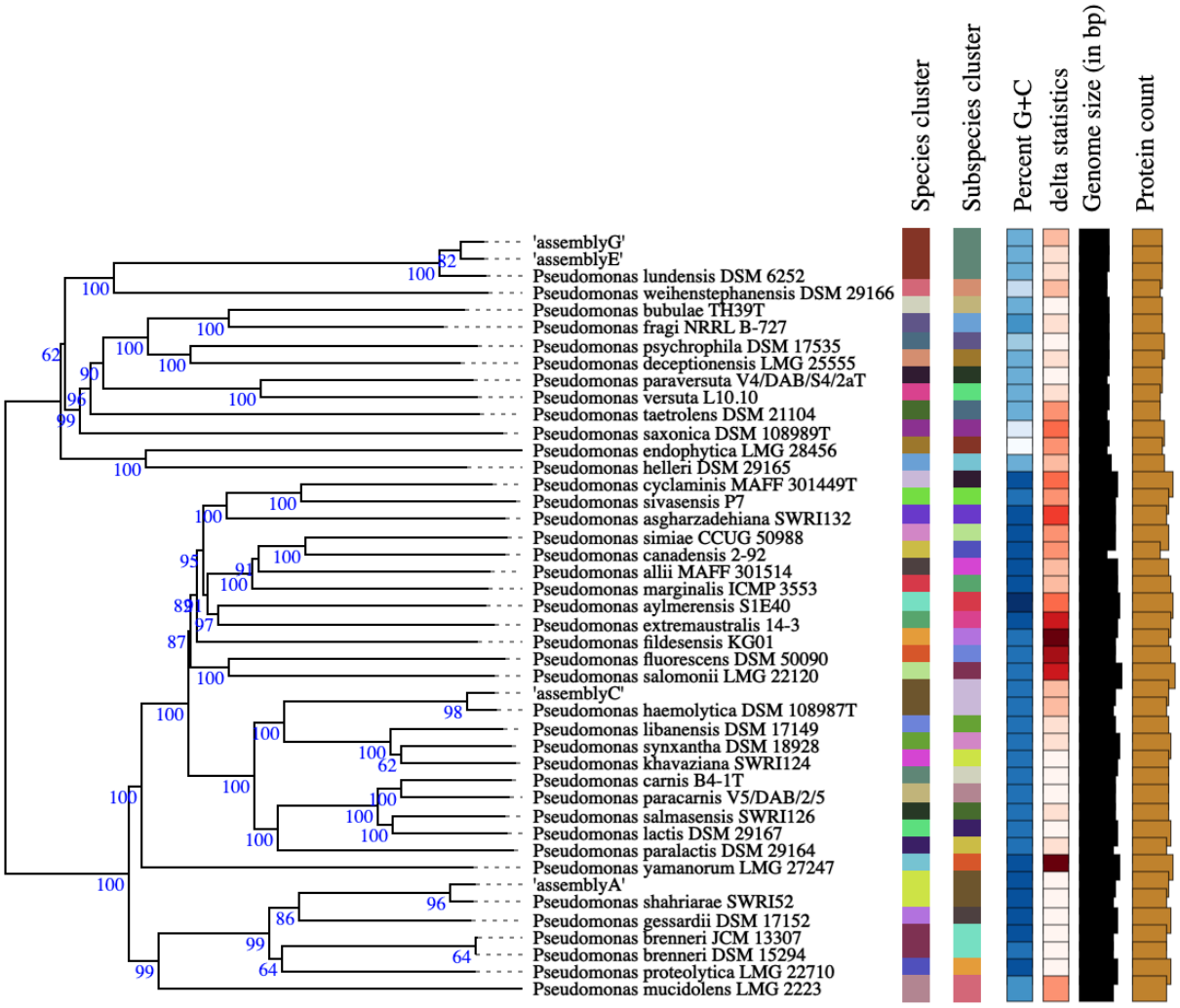

Figure 1. Phylogenetic relationships of sequenced novel strains and known type strains

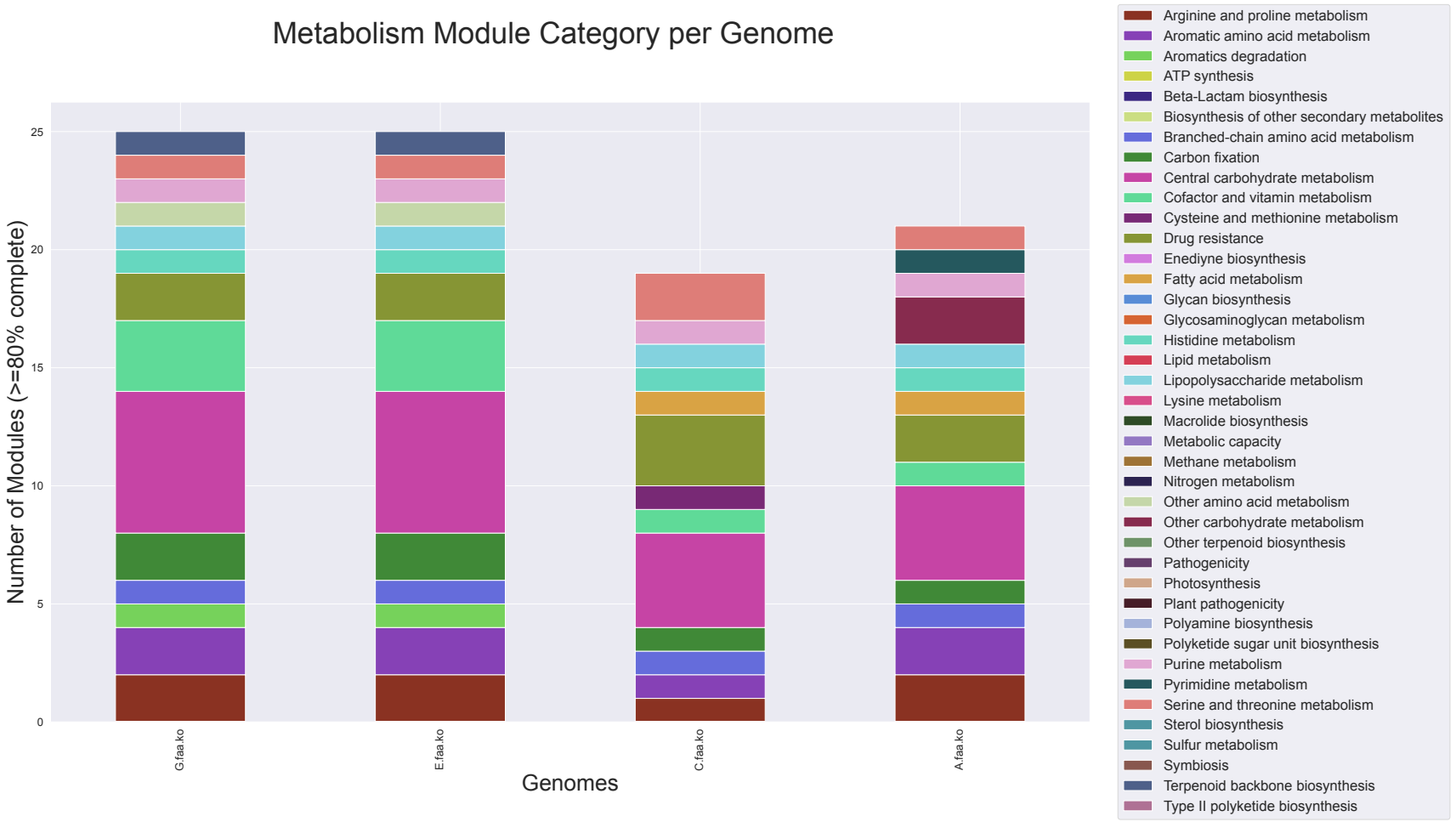

Figure 2. Metabolism Modules for *Pseudomonas* spp. UNL G,E,C, and A

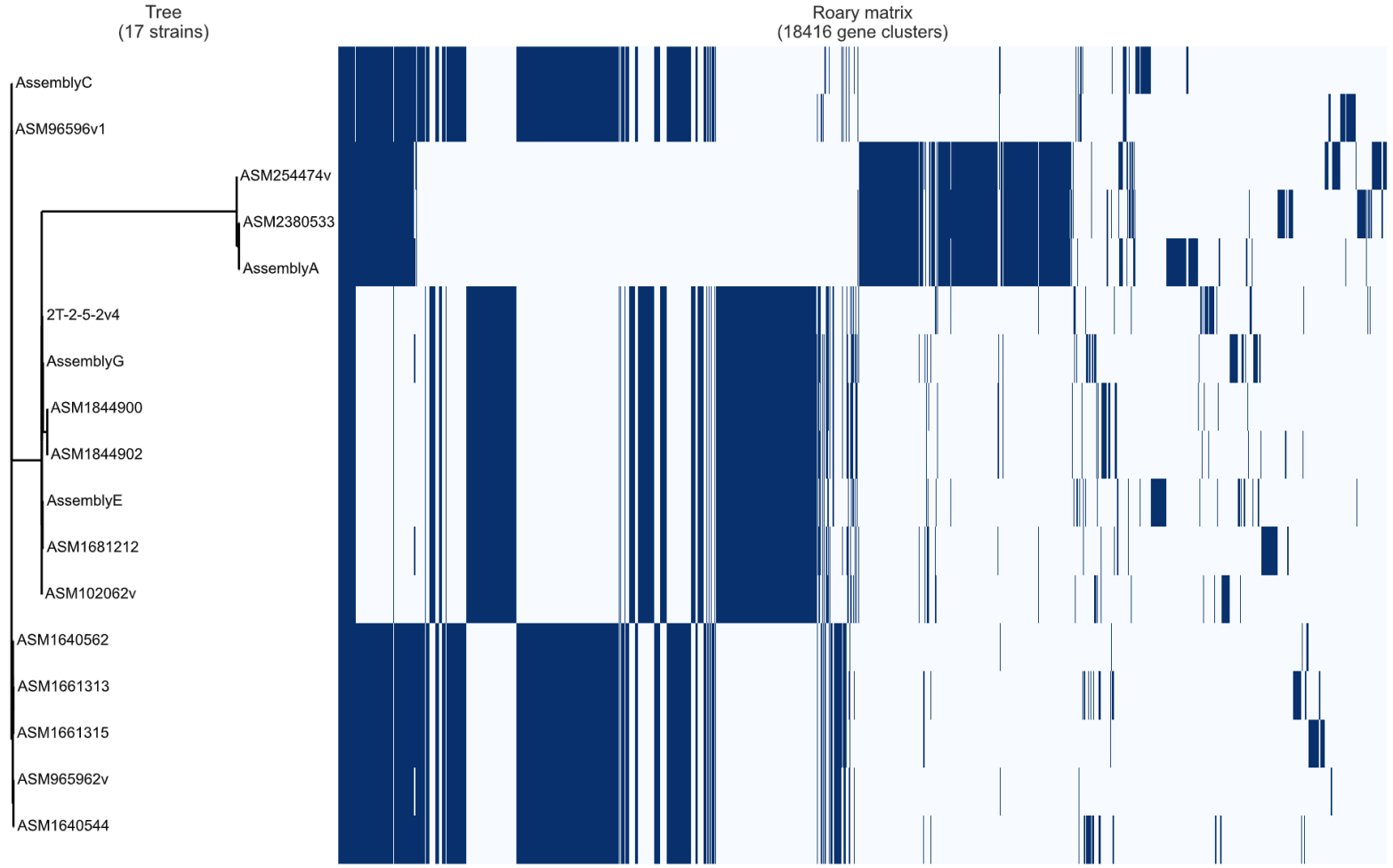

Figure 3. Visual summary of core and accessory genes of *Pseudomonas* spp. from Roary

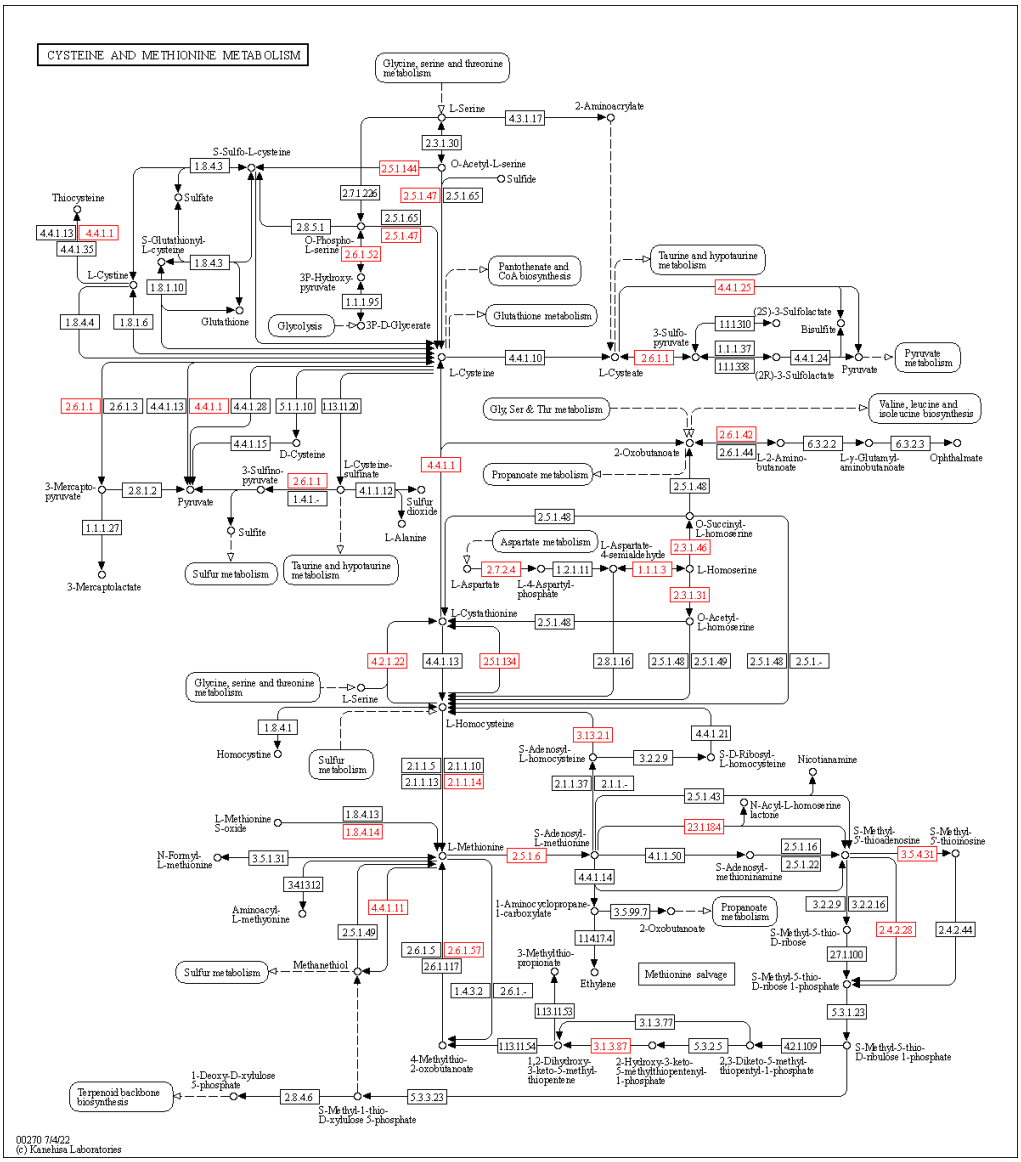

Figure 4. Predicted Cysteine and Methionine Metabolism of *Pseudomonas haemolytica* UNL-C KEGG Pathways, generated with KEGG Mapper

RECOMMENDATIONS FOR FUTURE RESEARCH

While a multitude of factors, such as processing parameters and packaging atmosphere, determine the spoilage microbiome of meat products, there are more causal agents yet underexplored. While 16S sequencing provides a list of potential SSOs, it discerns limited information as to how these organisms are acting as a collective, paying no attention to mobile elements or viral components of the system. Further investigation of the meat processing virome is suggested, as it could impact the bacterial community, and relevant phages could be isolated to target organisms of interest. Both new studies and computational evaluation of currently available shotgun sequenced libraries could identify new viruses of interest, similar to discoveries currently underway on the Serratus platform (Edgar et al., 2022).

While phages would suppress specific microbes, it is also important to consider more broad, metabolic components to target. Rather than thinking of specific bacteria as the determinants of shelf life, antimicrobial development should center around targeting spoilage-enabling cellular processes of the community. Now that researchers understand some of the components bacteria utilize to predominate the microbiome, such as siderophores, quorum signaling molecules, biofilms, and anaerobic regulons, schemes that interfere with these mechanisms should be developed in targeted manners. If a sequence or structure is known, computational prediction could aid the development of antimicrobial agents (Gupta et al., 2021). Bacteria themselves, are often good points of inspiration for such agents, as they themselves must compete in the environment. Co-culture experiments could provide insight into preserving diverse communities and limiting spoilage.

While sequencing methods do provide a holistic picture of the spoilage microbiome, they also necessitate the acquisition of costly sequencing platforms or reliance on service laboratories which add additional waiting time between sampling and results. Additionally, low biomass on processing surfaces can be a hurdle in sample processing, and proper methodology may require trained personnel to ensure adequate results. If processors are looking to treat their products with "precision shelf-life extension" methods comparable to "precision medicine" techniques, rapid diagnostics with minimal cost, quick results, and ease of use are necessitated. One proposed solution, Loop-mediated isothermal amplification (LAMP), has been shown as a viable method to detect photobacteria from meat in as few as two hours (Fuertes-Perez et al., 2020). By developing more LAMP assays against relevant SSOs, processors could potentially have a new quality control tool to monitor the presence of SSOs on facility surfaces, as well as designate relevant hurdles for products depending on their bacterial community composition.

References

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Gupta, R., Srivastava, D., Sahu, M., Tiwari, S., Ambasta, R. K., & Kumar, P. (2021). Artificial intelligence to deep learning: Machine intelligence approach for drug discovery. *Molecular Diversity*, *25*(3), 1315–1360. https://doi.org/10.1007/s11030-021-10217-3

APPENDIX A: FORMULATIONS FOR STUDY ONE

Product Name:	Treatment 1- Ground Turkey			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**100.00%**
Turkey Breast	25	11339.8	100.00%	100.00%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0**	**0**	**0.00%**	**0.00%**
Salt	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0	0.00 PPM	
Sodium Erythorbate	0	0	0.00 PPM	
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25**	**11339.8**		

Product Name:	Treatment 2 - Salt			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**98.04%**
Turkey Breast	25	11339.8	100.00%	98.04%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.5**	**226.796**	**2.00%**	**1.96%**
Salt	0.5	226.796	2.00%	1.96%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0	0.00 PPM	
Sodium Erythorbate	0	0	0.00 PPM	
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.5**	**11566.6**		

Product Name:	Treatment 3 : Salt and Spices			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**97.16%**
Turkey Breast	25	11339.8	100.00%	97.16%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**0.73**	**331.1222**	**2.92%**	**2.84%**
Salt	0.5	226.796	2.00%	1.94%
Dextrose	0.125	56.699	0.50%	0.49%
garlic	0.03	13.60776	0.12%	0.12%
black pepper	0.075	34.0194	0.30%	0.29%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0	0.00 PPM	
Sodium Erythorbate	0	0	0.00 PPM	
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**25.73**	**11670.92**		

Product Name:	Treatment 4: Cooked Link, Salt, Spices			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**88.56%**
Turkey	25	11339.8	100.00%	88.56%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**3.23**	**1465.102**	**12.92%**	**11.44%**
Salt	0.5	226.796	2.00%	1.77%
Dextrose	0.125	56.699	0.50%	0.44%
garlic	0.03	13.60776	0.12%	0.11%
black pepper	0.075	34.0194	0.30%	0.27%
water	2.5	1133.98	10.00%	8.86%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0	0	0.00%	0.00%
Sodium Nitrite (6.25% curing salt)	0	0		PPM
Sodium Erythorbate	0	0		PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**28.23**	**12804.9**		

Product Name:	Treatment 5: Cooked Link w Salt, Spices, Cure			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**88.32%**
Turkey	25	11339.8	100.00%	88.32%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**3.23**	**1465.102**	**12.92%**	**11.41%**
Salt	0.5	226.796	2.00%	1.77%
Dextrose	0.125	56.699	0.50%	0.44%
garlic	0.03	13.60776	0.12%	0.11%
black pepper	0.075	34.0194	0.30%	0.26%
water	2.5	1133.98	10.00%	8.83%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.07615	34.54103	0.30%	0.27%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0	0	0.00%	0.00%
Totals	**28.30615**	**12839.44**		

Product Name:	Treatment 6: Cooked Deli w Salt, Spices			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**80.00%**
Turkey	25	11339.8	100.00%	80.00%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**6.175**	**2800.931**	**24.70%**	**19.76%**
Salt	0.5	226.796	2.00%	1.60%
Dextrose	0.125	56.699	0.50%	0.40%
garlic	0.03	13.60776	0.12%	0.10%
black pepper	0.075	34.0194	0.30%	0.24%
water	5.445	2469.808	21.78%	17.42%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.075	34.0194	0.30%	0.24%
Sodium Nitrite (6.25% curing salt)	0	0		PPM
Sodium Erythorbate	0	0		PPM
Sodium Phosphate	0.075	34.0194	0.30%	0.24%
Totals	**31.25**	**14174.75**		

Product Name:	Treatment 7: Cooked Deli w Salt, Spices, Cure			
Meat Block:	25			
	lbs	g	% of meat block	%total formulation
Meat Ingredients:	**25**	**11339.8**	**100.00%**	**80.00%**
Turkey	25	11339.8	100.00%	80.00%
	0	0	0.00%	0.00%
	0	0		0.00%
	0	0		0.00%
Non-Meat Ingredients	**6.1**	**2766.911**	**24.40%**	**19.52%**
Salt	0.5	226.796	2.00%	1.60%
Dextrose	0.125	56.699	0.50%	0.40%
garlic	0.03	13.60776	0.12%	0.10%
black pepper	0.075	34.0194	0.30%	0.24%
water	5.37	2435.789	21.48%	17.18%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
	0	0		0.00%
Restricted Ingredients:	0.15115	68.56043	0.60%	0.48%
Sodium Nitrite (6.25% curing salt)	0.0624	28.30414	156.00	PPM
Sodium Erythorbate	0.01375	6.23689	550.00	PPM
Sodium Phosphate	0.075	34.0194	0.30%	0.24%
Totals	**31.25115**	**14175.27**		