FOOD MICROBIOME AND ITS IMPLICATIONS IN FOOD SAFETY AND FOOD QUALITY

by

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(Under the Direction of Hendrik C. den Bakker)

ABSTRACT

Understanding the food microbiome is crucial for identifying microorganisms that impact food quality and safety. My studies aimed to contribute further research in this field. Specifically, one of my research objectives was to investigate the microbial content of eight edible insect product types using traditional microbiological methods and whole genome sequencing. Results showed that insect product type significantly influenced total viable counts, bacterial spore counts, and lactic acid bacteria counts ($P = 0.00391$, $P = 0.0065$, and $P < 0.001$), with counts ranging from <1.70 to 6.01, <1.70 to 5.25, and <1.70 to 4.86 Log_{10} CFU/g, respectively. Whole genome sequencing revealed the presence of 12 different bacterial genera among the analyzed isolates, with a majority belonging to the *Bacillus* genus. Some isolates from the *Bacillus* cereus group were identified as biovar Emeticus. My second research initiative explored the microbiome of various retail food products, including fresh produce, deli meats, and cheese, using a 16S rRNA sequencing approach. We observed that alpha diversity (Shannon and Simpson indices) was significantly higher ($P < 0.001$ for both) in fresh produce (2.4 to 4.1, 0.82 to 0.95, respectively) compared to other products. Beta diversity analyses showed distinct microbial community compositions across product types. Cheese, hard salami, and turkey breast

were dominated by fermentation-associated genera such as *Lacticaseibacillus*, *Latilactobacillus*, and *Pediococcus*, while fresh produce harbored genera commonly associated with food spoilage or plant diseases, such as *Pseudomonas*, *Pantoea*, *Psychrobacter*, and *Serratia*. My last research objective was to investigate how the retail environment's microbiome interacts with foodborne pathogens within biofilms. This study examined biofilm formation, structural variability, and susceptibility to quaternary ammonium compounds (QUATs) in various biofilm structures involving retail-derived bacteria (*Serratia liquefaciens* and *Pseudomonas simiae*) and foodborne pathogens (*Salmonella* Typhimurium and *Listeria monocytogenes*). In mono-species biofilms, *S. liquefaciens* exhibited the highest biofilm-forming ability, while *L. monocytogenes* was the most susceptible to QUATs, with a mean log reduction of 3.83. In binary biofilms, *L. monocytogenes* demonstrated reduced sanitizer susceptibility. Overall, my research has provided important insights into microbial communities in food products and their implications for food safety.

INDEX WORDS: Microbiome, edible insects, biofilms, *Salmonella*, *Listeria*, retail

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DEDICATION

I would like to dedicate this dissertation to my family, whose unwavering support and encouragement have been invaluable throughout the years. I also dedicate this research to the pursuit of improving the quality of life and well-being of individuals everywhere.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The term microbiome is defined as a community of microbes, including fungi, viruses, and bacteria, that inhabit a specific environment (National Human Genome Research Institute, 2025). In food systems, the food-associated microbiome is a major factor influencing the quality and safety of food products. Beneficial microorganisms play a role in enhancing quality traits, such as the rheological and organoleptic properties of fermented products (De Filippis et al., 2018). However, unwanted microorganisms can result in food spoilage and raise food safety concerns (De Filippis et al., 2018). Research on food-related microbial ecology has historically relied heavily on culture-based techniques. However, traditional culture methods provide limited information about the identity of microorganisms based on their morphological and biological characteristics. These methods are also ineffective at detecting unculturable microbes (Sadurski et al., 2024). The term "unculturable" does not indicate that these microbes “can never be cultivated” but rather highlights the limitations of traditional methods due to factors such as insufficient knowledge of microbes’ nutritional requirements, temperature conditions, and growth time (Chaudhary et al., 2019; Stewart, 2012). Despite being considered the "gold standard," culture-based methods can capture only about 0.1 percent of complex microbial communities, such as the human gut microbiome (Cao et al., 2017). Over the years, significant advancements in culture-independent methods have allowed researchers to overcome the limitations of culture-based techniques (Cocolin et al., 2013). Specifically, next-generation

sequencing techniques, capable of high-throughput screening, have improved our ability to perform in-depth characterization of microbial communities in complex niches, such as food and environmental samples (Cao et al., 2017). The study of microbiomes in food and food environments typically utilizes two different sequencing approaches: targeted amplicon sequencing and shotgun sequencing. Targeted amplicon sequencing involves selecting and amplifying a specific genomic target of interest, which is then sequenced (Billington et al., 2022). In contrast, shotgun sequencing is an untargeted method that sequences the entire genomic content present in the sample (De Filippis et al., 2018).

Published research on the microbiomes of food commodities and food environments, either independently or combined with other microbiological methods analyses, provide important insights into various aspects such as food quality, safety, authenticity, processing (such as fermentation), and the factors that influence food microbial ecology. For instance, Liu et al. (2020) conducted microbiome analyses of fresh shellfish and revealed significant differences in the beta diversity of microbial communities of samples collected from Nova Scotia and Quebec. Their findings highlight the potential of targeted amplicon sequencing for determining the geographical origin of food products. In the context of food safety, a two-year longitudinal study focusing on the environmental microbiomes of tree fruit packing facilities identified several bacterial taxa, including *Pseudomonas*, *Stenotrophomonas*, and *Microbacterium*, as well as fungal taxa such as *Yarrowia*, *Kurtzmaniella*, *Cystobasidium*, *Paraphoma*, and *Cutaneotrichosporon*, which were found to co-occur with *Listeria monocytogenes*. The study concluded that these taxa could potentially act as indicator microorganisms for the presence of *Listeria monocytogenes*. Additionally, the research found significant differences in the microbial communities across various facilities, with these communities changing over time in each

inspected facility (Rolon et al., 2023). Another study revealed the contribution of the food environmental microbiome in increasing the tolerance of *Listeria monocytogenes* to sanitizers when present in multi-species biofilms along with selected bacterial species of food processing environmental microbiota (Rolon et al., 2024). Moreover, the microbiome analyses conducted on retail packaged broiler meat and broiler abattoirs provided insights into the presence and types of putative spoilage bacteria and the role of shelf-life storage in shifting the microbial communities on these retail products. Additionally, the same study traced the contamination pathway of *Janthinobacterium lividum*, a bacterium occasionally linked to meat spoilage. This research identified production environments, particularly the cooling condenser, as the source of *Janthinobacterium* (Lauritsen et al., 2019). A study conducted by Dugat-Bony et al. (2015) on surface-ripened cheese demonstrated how microbiome insights can aid in understanding food processing. Specifically, in the fermentation of surface-ripened cheese, when microbiome analyses were combined with metatranscriptomic and biochemical analyses, the authors identified key bacterial species and their associated functions during the fermentation process. For example, *Lactococcus lactis* and *Kluyveromyces lactis* were involved in the rapid consumption of lactose during the early ripening stages, while *Debaryomyces hansenii* and *Geotrichum candidum* were responsible for consuming lactate, which is produced from lactose. Overall, these examples demonstrate the important role of food-related microbiome analyses in advancing the field of food microbiology in various aspects. Despite significant advancements, there are still notable limitations in the field of microbiome analyses. These include biases linked to the extraction of nucleic acids from samples, the preferential selection of certain taxa during amplicon sequencing, and the absence of a standardized bioinformatics pipeline (De Filippis et al., 2018).

The objective of this literature review was to provide an overview of the advancement in sequencing technologies, the sequencing approaches used for microbiome analysis, potential applications of microbiome analysis in food systems, and the scope of microbiome analyses in my research projects.

Overview of Advancements in DNA Sequencing Technologies

DNA sequencing techniques have evolved significantly since the introduction of the first successful sequencing technique by Sanger et al. (1977). These techniques are generally categorized into three generations: first generation, second generation, and third generation (Eren et al., 2022). Each generation has its advantages and limitations. This section aims to briefly explain the principles, advancements, and limitations of these sequencing technologies across the three generations.

First generation of DNA sequencing technology

The first generation of DNA sequencing technology was successfully developed by Sanger et al. (1977) based on the "chain-termination" method. Briefly, in this technology, a single strand of DNA is used to determine the sequence of its nucleotides. DNA polymerase incorporates deoxyribonucleotides (dNTPs) into a primer, using single-stranded DNA template to guide complementary base paring. Separate reactions are conducted for each type of dNTP. Additionally, modified versions of each dNTP, known as dideoxyribonucleotides (ddNTPs), are used to terminate the DNA chain during the extension step. These ddNTPs lack a 3’ hydroxyl group, which prevents them from bonding with the 5’ phosphate of incoming dNTPs. The concentration of ddNTPs is lower than that of dNTPs, ensuring that chain termination occurs randomly at varying positions. Once the chain growth is halted, DNA fragments of varying lengths are produced, with the length of each fragment depending on where the ddNTP is

incorporated. The DNA fragments can then be separated via electrophoresis on polyacrylamide gel across four separate lanes. The resulting fragment patterns are used to deduce the original DNA sequence (Heather and Chain, 2016; Kchouk et al., 2017; Sanger et al., 1977). The first automated Sanger sequencing machine, ABI 370A, was developed by Applied Biosystems Inc. in 1987 (Hood et al., 1987). The major limitations of this technology include its lower efficiency in sequencing larger genomes, time consumption, and the associated costs (Chelliah et al., 2022).

Second generation of DNA sequencing technology

The second generation of sequencing technologies has overcome the limitations of first-generation methods by allowing for the simultaneous sequencing of multiple DNA fragments at a large scale. This advancement enables the rapid sequencing of larger genomes at a lower cost and in a shorter timeframe (Chelliah et al., 2022). Several platforms within this generation have emerged, with the most prominent being Roche/454, Ion Torrent, and Illumina, each employing its own distinct sequencing methodologies.

The principle of the Roche/454 sequencing method was described by Margulies et al. (2005). Briefly, double-stranded DNA is fragmented, and these fragments are ligated to oligonucleotide adapters, and then attached to synthetic beads for emulsion PCR, which amplifies each fragment on its respective bead. Following emulsion PCR, the double-stranded DNA fragments are denatured to convert them into single-stranded DNA. The DNA-carrying beads are then loaded into “picoliter-sized wells”. Next, the loaded DNA amplicons undergo pyrosequencing (Ronaghi et al., 1996). During this process, dNTPs (adenosine, thymidine, cytosine, and guanine) are added sequentially, one at a time, to the reaction. When the correct dNTP, complementary to the base of the DNA template strand, is incorporated by DNA polymerase, it releases pyrophosphate. This pyrophosphate triggers an enzymatic reaction that

produces light, with the intensity of the light being proportional to the number of dNTPs incorporated. The resulting light signals are detected and analyzed computationally to determine the DNA sequence. A major limitation of this technique is the difficulty in reading through homopolymeric regions (Cao et al., 2017). Currently, this sequencer is no longer available on the market.

Rothberg et al. (2011) explain the principles behind the Ion Torrent platform. In this platform, emulsion PCR is conducted on DNA fragments prior to sequencing. The resulting beads, each containing amplified DNA fragments, are then loaded into the ion chip, with each bead placed into its own well. Next, in the presence of a primer and DNA polymerase, the sequential addition of each of the four dNTPs leads to the incorporation of a specific nucleotide that is complementary to the DNA template strand. When the nucleotide is incorporated into the extending DNA strand, it releases a single proton which results in a pH change of the surrounding solution. This pH change is directly related to the number of specific bases incorporated into the extending strand. A sensor located at the bottom of each well detects this pH change which then gets converted into a voltage that can be digitized. However, this platform has a limitation when it comes to accurately interpreting the sequences of homopolymer regions (Loman et al., 2012).

Illumina sequencing platform relies on a clonal array formation and reversible terminator technology (Bentley et al., 2008). Briefly, genomic DNA is fragmented, and these fragments are ligated with adapters on both ends. Next, the DNA fragments are loaded into a flow cell, where each fragment undergoes bridge amplification, resulting in clonal clusters of each fragment. Illumina employs a sequencing-by-synthesis approach to sequence the generated clonal clusters. During the sequencing cycles, fluorescently labeled reversible terminator dNTPs are used. Each

type of dNTP is attached to a unique fluorophore, and the 3' end is modified to prevent the addition of multiple dNTPs simultaneously. In each cycle, only one modified dNTP is incorporated into the extending DNA strand. The identity of the incorporated dNTP is determined by its fluorescence, which is excited by a laser and captured through simultaneous imaging. Following the incorporation step, the attached fluorescent reporter and terminator are cleaved, allowing the next dNTP to be added. This method enables "base-by-base" sequencing, which helps minimize errors in repetitive regions or homopolymers.

Third generation of DNA sequencing technology

Despite the significant success of second-generation DNA sequencing technologies, there are some limitations with these methods. Specifically, they require a PCR amplification step and cannot sequence the long reads (Kchouk et al., 2017). Third-generation DNA sequencing technologies have addressed these challenges and can provide real-time sequencing results. The most common platforms in this generation include PacBio SMRT (Single Molecule, Real-Time) sequencing and Oxford Nanopore sequencing.

PacBio SMRT sequencing platform employs a specialized nanophotonic structure called a zero-mode waveguide (ZMW) to monitor the process of DNA polymerization in real-time (Levene et al., 2003). ZMWs are tiny holes in a metallic film that cover a chip, confining laser light to an extremely small area at the bottom of the wells. This setup minimizes background noise and enables the precise detection of a single dNTP (Levene et al., 2003). DNA polymerase is immobilized at the bottom of these ZMWs. In this technique, dNTPs are attached to unique fluorophores. When dNTPs and a DNA library are introduced to the ZMWs, the DNA polymerase incorporates a single nucleotide into the elongating DNA strand. This incorporation produces a real-time fluorescence signal based on the specific dNTP added. After a dNTP is

incorporated, the fluorescent label is cleaved from the nucleotide, stopping the signal at that position, and the process continues with the addition of subsequent nucleotides (Eid et al., 2009). The Nanopore sequencing platform uses a nanopore, which is a tiny protein or synthetic pore embedded in a membrane that separates two chambers filled with an ionic solution. An electric potential is applied across this membrane, creating an ionic current that flows through the nanopore. For DNA sequencing, a motor protein unwinds the double-stranded DNA and translocates it through the membrane. As a single strand of DNA passes through the nanopore, each nucleotide type causes a unique disruption in the ionic current. These changes in the ionic current are recorded in real-time and analyzed using algorithms to determine the DNA sequence (Eren et al., 2022; Feng et al., 2015). Third generation sequencing still has limitations, such as the high cost of PacBio SMRT technology and the high error rate, which can be 15% in the nanopore method (Satnam et al., 2023).

Common Sequencing Approaches for Microbiome Analysis

There are two common approaches currently used to study the microbiome of food or food-associated environments: amplicon and shotgun sequencing. Both approaches do not require the isolation and culturing of microorganisms, making them suitable for analyzing even unculturable microbes in the respective food or environment samples (Forbes et al., 2017).

Amplicon sequencing approach is based on targeting and PCR amplification of a specific region of nucleic acid which is then sequenced (Billington et al., 2022). The selection of the target region of genomic nucleic acid is based on the objective of a particular research, and these target regions are generally conserved across a particular group of microbes. Specifically, the 16S rRNA gene is targeted for the analysis of bacteria and archaea, the 18S rRNA gene for eukaryotes, and the internal transcribed spacer (ITS) for fungi (Jagadeesan et al., 2019). After

sequencing these target genomic regions, the next step involves bioinformatics analysis to draw conclusions from the raw data. Common bioinformatics pipelines used for bacterial microbiome assessment in sequences obtained from amplicon sequencing approach include QIIME2, MOTHUR, and DADA2 (Callahan et al., 2016; Caporaso et al., 2010; Schloss et al., 2009). Amplicon sequencing in the food system helps obtain the taxonomic classification of microorganisms present in a given sample and determine their relative abundance (De Filippis et al., 2018). Moreover, amplicon sequencing is being utilized to study microbial diversity such as alpha and beta diversity. Alpha diversity metrics provide information about the richness and evenness within the microbial community of a given sample, while beta diversity helps determine how different the samples or environments are from each other based on the abundance or presence-absence of microbial sequences (Kers and Saccenti, 2022). Richness refers to the number of taxonomic groups present, while evenness describes their relative distribution (Kers and Saccenti, 2022). The main limitations of amplicon sequencing include the need for prior knowledge of target genomic region for analyzing specific microbial groups, biases introduced during PCR amplification, and limited resolution in taxonomic classification, specifically at the species level (Forbes et al., 2017).

On the other hand, shotgun sequencing is an untargeted method where the entire genomic content of a sample is sequenced after being fragmented into small pieces (De Filippis et al., 2018). When used to study complex microbial communities, this approach is called metagenomic (Jagadeesan et al., 2019). The basic workflow of metagenomic analysis can be summarized as follows (Weinroth et al., 2019): First, DNA is extracted, fragmented, and prepared into a library before being sequenced using second or third-generation sequencers. This process generates FASTQ files, which store the information of DNA sequences (reads) along with quality scores

and unique identifiers. After sequencing, the next step involves the bioinformatic analysis of these reads. The first step of the bioinformatics analysis is quality control, which includes removing adaptors and low-quality reads. Following quality control, the reads can either be assembled or aligned. During assembly, short reads are merged into "contigs" using de Bruijn graph-based or overlap-layout-consensus algorithms (Miller et al., 2010). These contigs are then compared to reference databases using matching algorithms such as BLAST to infer taxonomic identity and similarity scores such as sequence identity (Altschul et al., 1990). In read alignment-based analyses, unassembled reads are directly matched to the reference database using algorithms like the Burrows-Wheeler Aligner (Li and Durbin, 2010). Matching sequences to a reference database enables the identification and quantification of microorganisms and functional genes of interest. Once this information is obtained, descriptive or statistical analyses can be performed based on the metadata related to experimental conditions or study design. The major advantage of shotgun sequencing over amplicon sequencing is its ability to characterize microorganisms at lower taxonomic levels, such as species or strain (Cao et al., 2017). It can also provide information about specific genes, functional pathways, and evolutionary relationships among microbial species, therefore, make this approach a more suitable choice to study microbial ecology (Cao et al., 2017). However, this method has significant limitations, such as the difficulty in identifying low-abundance taxa, high costs, and the need for advanced computational resources (Forbes et al., 2017).

Application of Microbiome Analysis in Food Systems

Food microbiome studies using 16S rRNA sequencing or shotgun sequencing, with or without other microbiological methods, have been conducted for various purposes.

One of the major focuses of microbiome analyses has been to understand microbial ecology in food environments and how foodborne pathogens interact with other microbial species in these settings. For instance, Rothrock Jr et al. (2019) analyzed the microbiome of diverse sample types collected from production houses and the processing environment of pasture-raised broilers. They observed significant shifts in the microbiome profiles across the life cycle, with brood, fecal, and cecal samples being distinct from pre-hatch and final product samples. However, a core microbiome consisting of 13 taxa, including *Salmonella* and *Campylobacter*, was consistently present across all collected samples. Notably, *Salmonella* and *Campylobacter* were most abundant in fecal samples.

Additionally, investigations into the microbiome of a meat-cutting facility revealed bacterial genera positively associated with the presence of *Listeria* spp. across different sample sites. *Acinetobacter* and *Janthinobacterium* were correlated with *Listeria* in both meat and environmental samples, *Brachybacterium* and *Carnobacterium* were associated with *Listeria* in environmental samples, and *Pseudomonas* was linked to *Listeria* specifically in meat samples (Zwirzitz et al., 2021). Similarly, research exploring the microbiome of tree fruit packing house environments identified microbial taxa that could serve as potential indicators of *Listeria monocytogenes*. The microbial taxa included both bacterial genera such as *Pseudomonas*, *Stenotrophomonas*, and *Microbacterium*, and fungal genera like *Yarrowia*, *Kurtzmaniella*, *Cystobasidium*, *Paraphoma*, and *Cutaneotrichosporon* (Rolon et al., 2023). In line with these findings, microbiome analysis of distribution centers handling fresh produce also revealed that *Psychrobacter* and *Pseudomonas_E* were present at significantly higher abundance in *Listeria*-positive samples compared to *Listeria*-negative samples (Townsend et al., 2023).

Further studies have focused on understanding biofilms formed by resident microbiota of food facilities, particularly in relation to foodborne pathogens. For instance, it was found that resident microbiota co-occurring with *Listeria monocytogenes* in tree fruit packing facilities had the potential to enhance *Listeria's* tolerance to sanitizers (benzalkonium chloride and peracetic acid) when present in mixed-species biofilms. Additionally, *Listeria* exhibited increased growth in mixed-species biofilms formed along with resident microbiota compared to its mono-species biofilm (Rolon et al., 2024; Voloshchuk et al., 2025).

Moreover, microbiome studies have played a key role in identifying cross-contamination routes in food environments, such as poultry establishments and food service facilities, while also highlighting factors that shape microbiome profiles, such as the prevalence of psychrotrophic bacteria in low-temperature rooms of a small meat processing facility (Belk et al., 2022; Lim et al., 2021; Telli et al., 2024).

Another focus of food microbiome-related research has been exploring whether metagenomics can serve as an alternative method for detecting and characterizing foodborne pathogens in food or food-related environmental samples. These studies often involve using food or environmental samples spiked with pathogens for testing. For instance, Maguire et al. (2021) employed qPCR and a long-read metagenomics approach to detect and assemble Shiga toxin-producing *Escherichia coli* (STEC) in irrigation water that was artificially spiked with STEC and enriched for 24 hours. They found that the qPCR method had a detection limit of 30 CFU per reaction, which is equivalent to 5 Log CFU/mL in the enrichment. Using metagenomics, performed on a nanopore sequencing platform, they were able to detect STEC at a concentration as low as 3 Log CFU/mL. The study also successfully obtained a fragmented or completely closed *Escherichia coli* O157:H7 metagenome-assembled genome at concentrations of 5–8 Log

CFU/mL and 7–8 Log CFU/mL, respectively. Similarly, Leonard et al. (2015) confirmed the use of metagenomic sequencing for detecting STEC in spiked spinach samples. They reported that the method could detect Shiga toxin-producing *Escherichia coli* at a concentration as low as 10 CFU per 100 g of the sample, with an enrichment time as short as 8 hours. In a subsequent study, the same group demonstrated that shotgun metagenomics could accurately differentiate between contaminating STEC strains in spinach spiked with a range of genomically diverse STEC strains, further illustrating the method's resolution and effectiveness (Leonard et al., 2016).

Additionally, amplicon and shotgun sequencing have been successful in identifying food safety risks directly in natural samples. For example, amplicon sequencing has helped identify pathogenic genera in the fecal samples of livestock animals. Specifically, *Staphylococcus* and *Clostridium* were detected in the feces of cattle, pigs, and chickens, while *Bacillus*, *Listeria*, and *Salmonella* were found in chicken feces, and *Campylobacter* and *Vibrio* were detected in cattle feces (Kim et al., 2021). Moreover, metagenomic studies performed using nanopore sequencing platform have identified pathogens such as *Clostridium botulinum, Acinetobacter baumannii*, and *Vibrio parahaemolyticus* directly in food samples, raw meat and sashimi (Lee et al., 2024).

Research has also explored whether metagenomics can be used for foodborne outbreak investigations. For example, Huang et al. (2017) employed shotgun metagenomics to investigate the "pathogen-specific signature on the microbiome" in human stool samples collected from foodborne outbreaks in Alabama and Colorado, where culture-dependent methods had confirmed the presence of two distinct strains of *Salmonella* Heidelberg. Their shotgun metagenomics results were consistent with the culture-dependent findings and also highlighted that metagenomics was faster than traditional methods. In another study, shotgun metagenomics was used to detect the food source linked to a *Salmonella* outbreak in Belgium, where freshly

prepared tartar sauce was identified as the source through current outbreak investigation methods, including isolation, serotyping, multilocus variable-number tandem repeat analysis, and whole-genome sequencing. The researchers applied shotgun metagenomics to two suspect food samples associated with this outbreak. Without prior isolation of *Salmonella*, they successfully detected the genome of *Salmonella* Enteritidis and phylogenetically linked it to human isolates (Buytaers et al., 2021). Additionally, Ahlinder et al. (2022) highlighted the use of combining traditional methods with amplicon and shotgun sequencing for source tracking in food or waterborne outbreaks. Their study demonstrated the effectiveness of this approach in tracing *Cryptosporidium* back to its source, which was romaine lettuce.

Metagenomics has also been instrumental in identifying genes of interest, particularly antibiotic resistance genes and virulence factors. For example, Lee et al. (2024) performed nanopore metagenomic of 260 food samples, including raw meat, sashimi, and ready-to-eat vegetables. Their study detected foodborne pathogens and revealed that ready-to-eat vegetable products had a high abundance (49.9%) of multidrug resistance genes. Carbapenem resistance genes were most prevalent in poultry products, while cephalosporin resistance genes were notably abundant in ready-to-eat vegetables. Similarly, Rubiola et al. (2022) used shotgun metagenomics to analyze bulk tank milk filters and found that the majority of antibiotic resistance genes were harbored by Gram-negative genera such as *Enterobacter*, *Acinetobacter*, *Escherichia*, and *Pseudomonas*. They identified a total of 160 antibiotic resistance genes, conferring resistance to 12 different antibiotic classes, with aminoglycosides, β-lactams, tetracyclines, multidrug resistance, and macrolide-lincosamide-streptogramin being the predominant classes. In another study, Li et al. (2020) applied shotgun metagenomics to retail chicken breast and observed a low abundance of antimicrobial resistance genes, with no

significant difference in the resistome between antibiotic-free and conventional poultry samples. Additionally, a study using shotgun sequencing to characterize virulence factors in cattle fecal samples identified 1,383 virulence-associated genes across 28 samples, corresponding to 63 distinct virulence factors. The predominant virulence factor superfamilies included adhesion and invasion, secretion systems, toxin production, and iron acquisition (Yang et al., 2016).

Microbiome investigations have been promising in better understanding food spoilage. For instance, Bassi et al. (2015) analyzed the bacterial communities in 83 hard cheese samples exhibiting blowing defects to study the clostridia involved in spoilage and their ecological interactions with other bacterial species present within the cheese microbiota. They found that *Lactobacillus*, *Streptococcus*, and *Clostridium* were the dominant genera in the spoiled samples, with *Clostridium tyrobutyricum* and *Clostridium butyricum* identified as the primary spoilage-causing clostridia. Notably, cheese samples with high abundance of *Streptococcus thermophilus* and *Lactobacillus rhamnosus* were predominantly spoiled by *Clostridium tyrobutyricum*, while samples with high abundance of *Lactobacillus delbrueckii* showed *Clostridium butyricum* as the main spoilage species.

Also, microbiome analyses of food products were also conducted to investigate the role of food preservatives in preventing spoilage. In a previous study, the microbiota of pork sausage samples with and without preservatives, such as lactate and acetate, was analyzed after 12 days of storage under modified atmosphere packaging (70% oxygen, 30% carbon dioxide) at chilled temperatures. The authors found a high abundance of *Brochothrix* spp., a key food spoilage bacterium, in the samples without preservatives, highlighting the critical role of preservatives in controlling microbial growth (Bouju-Albert et al., 2018).

The impact of specific spoilage bacteria was further explored by Pothakos et al. (2015), who studied the indigenous microbiome in a ready-to-eat meal facility. Although *Leuconostoc gelidum* was present at lower abundance initially, it became the dominant bacterium by the end of the shelf life of ready-to-eat meals, outgrowing other spoilage-related bacteria such as *Pseudomonas*, *Brochothrix*, and *Lactobacillus*. This shift highlighted the strong growth potential of *Leuconostoc gelidum* and its ability to spoil food under cold storage conditions. The study also found that the environment of the processing facility and raw ingredients were the sources of contamination of finished products with *Leuconostoc gelidum*. Similarly, Säde et al. (2017) investigated the microbiome of commercial beef products sourced from different lots within the same production plant. They found that although bacterial communities varied in the early stages of shelf life in these products, after 8 to 12 days of storage at 6 °C, spoilage-associated bacterial genera such as *Carnobacterium* spp., *Brochothrix* spp., *Leuconostoc* spp., and *Lactococcus* spp. became dominant, regardless of the production lots.

Lastly, microbiome community analyses were combined with assessments of genes involved in stress responses in high-oxygen modified-atmosphere-packaged beef products. The products were categorized into three groups, such as acceptable product, early spoilage, and late spoilage based on sensory odor scores. The study found that *Bacillales* were predominant in acceptable products, while lactic acid bacteria dominated early spoilage. In late spoilage, both lactic acid bacteria and yeast were abundant. Additionally, the expression of genes related to cold-shock stress decreased as shelf life progressed, while genes involved in respiration and oxygen stress increased (Hultman et al., 2020), providing further insights into the genetic mechanisms driving food spoilage.

Another application of microbiome-related studies is in food authentication. For instance, microbiome analyses of fresh soft-shell clams obtained from two different locations on the east coast of Canada revealed distinct microbial profiles among these products. Clams from Nova Scotia had a higher abundance of *Proteobacteria* and *Acidobacteria* and a lower abundance of *Actinobacteria* compared to samples collected from Quebec in both 2015 and 2018. Additionally, the alpha diversity of samples from Nova Scotia was significantly higher. Beta-diversity analysis also showed that the microbial profiles from both locations were significantly different, supporting the use of microbiome analyses for food authenticity determination (Liu et al., 2020). Similarly, Peruzza et al. (2024) used 16S rRNA sequencing along with machine learning tools to trace the origin of Manila clams harvested from geographically close areas. They collected samples from five locations across different seasons (winter and summer) between 2018 and 2020, including four farming areas and one banned area due to potential area or site contamination. By analyzing the microbiome of clams' gills and digestive glands, they successfully predicted the origin of clams from the banned area with 95% accuracy.

Furthermore, while not directly related to microbiome analysis, sequencing approaches have also been used for food ingredient detection. Previously, Haiminen et al. (2019) developed a bioinformatics pipeline called Food Authentication from Sequencing Reads (FASER) to aid in food authenticity. This tool analyzes the relative composition of eukaryotic species based on RNA or DNA sequencing and includes a reference genome collection of 6,160 unique plants and vertebrate organisms. When tested on 31 raw high-protein powder samples (poultry meal), FASER correctly identified the expected chicken in most samples. However, three samples unexpectedly contained pork and beef.

Overall, this section provides a comprehensive overview of the diverse applications of food microbiome analysis based on amplicon or shotgun sequencing methods, highlighting its significant contributions to advancing food microbiology.

Scope of Microbiome Assessment in my Research Projects

Two of my research initiatives focused on understanding the microbial communities present in retail derived ready-to-eat food products, including edible insects, deli meats, cheese, and fresh produce such as peaches, pears, red onions, mini cucumbers, and nectarines.

Previous studies on edible insects have reported the presence of various gut-associated microbes in processed edible insects, some of which have the potential to act as opportunistic pathogens in humans (Garofalo et al., 2017). Specifically, this study identified *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp., and *Bacillus* spp. within these products (Garofalo et al., 2017). Despite the growing availability of edible insect products in the U.S., research on their microbial risks remains limited. Therefore, assessing the microbial communities of these less studied products is essential to understanding the food safety risks associated with them. Initial attempts to analyze the microbiome of these products using 16S rRNA sequencing were hindered by low DNA yield. Consequently, we employed traditional culture methods and whole genome sequencing to assess their microbial composition.

Similarly, the other retail-derived food products mentioned above have been linked to foodborne illness outbreaks in the United States (CDC 2025; FDA 2025). Therefore, understanding the microbiome of these retail-derived products is crucial for identifying potential food safety hazards and spoilage risks. This knowledge is especially important in the final stages of the food supply chain, as it helps ensure the safety and quality of products before they reach consumers. Additionally, studying the microbiome of retail products provides valuable insights

into bacterial populations that could potentially transfer or cross-contaminate the surrounding retail environments.

The final chapter of my dissertation focused on investigating the interactions between retail-derived bacteria, specifically *Serratia liquefaciens* and *Pseudomonas simiae*, and foodborne pathogens, namely *Salmonella* Typhimurium and *Listeria monocytogenes*, in various biofilm combinations. Additionally, the study aimed to evaluate the effectiveness of quaternary ammonium compounds, which are commonly used antimicrobials in the food industry, against these biofilm configurations. The selected retail-derived bacteria were based on previous research that identified these genera as dominant in retail microbiomes (Britton et al., 2023). This study is of particular importance because prior literature suggests that resident microbiota can form robust biofilms and may interact with pathogens in mixed biofilm communities, aiding their establishment. For instance, Habimana et al. (2010) studied the microbiota of feed environments and their relationship with *Salmonella* and revealed that the resident isolates exhibited higher resistance to desiccation, disinfection, and had greater biofilm-forming capabilities. Furthermore, comparisons of monomicrobial biofilms of *Salmonella* with mixed-species biofilms formed alongside resident isolates such as *Staphylococcus* and *Pseudomonas* showed an increase in biovolume by 2.8-fold and 3.2-fold, respectively (Habimana et al., 2010). Therefore, it is essential to understand how the microbiomes in retail environments contribute to the formation of biofilms by foodborne pathogens, as well as the protective effects that these mixed-species biofilms may have against antimicrobial agents. This understanding is crucial for enhancing food safety strategies and improving the effectiveness of disinfectants in controlling pathogenic biofilms in the food industry.

Conclusion

Advancements in nucleic acid sequencing technologies and ongoing improvements in bioinformatics tools have transformed food microbiome research. These technological innovations provide deeper insights into the complex microbial communities that influence various ecosystems, including food systems. They have addressed many limitations of traditional microbiological methods, which often fail to capture the full microbial diversity and complexity among food or food environment samples. Research on the food microbiome has significant implications for food safety and quality. By studying the microbiome present in food products, researchers can identify factors that contribute to spoilage or contamination. Insights gained from microbiome research can offer the food industry opportunities to mitigate cross-contamination risks, enhance process controls for pathogen elimination or mitigation, and develop more effective packaging and storage conditions to reduce food spoilage. However, challenges remain in food microbiome analysis. Issues such as biases in nucleic acid extraction, the preferential amplification of certain taxa during amplicon sequencing, and the absence of standardized bioinformatics pipelines can hinder the consistency and reliability of microbiome data (De Filippis et al., 2018). Nonetheless, continued improvements in these areas could lead to more standardized, reproducible, and widely applicable food microbiome analyses, further unlocking the potential of microbiome research to enhance food safety and quality practices.

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CHAPTER 2

ANALYSIS OF MICROBIAL COMPOSITION OF EDIBLE INSECT PRODUCTS AVAILABLE FOR HUMAN CONSUMPTION WITHIN THE UNITED STATES USING TRADITIONAL MICROBIOLOGICAL METHODS AND WHOLE GENOME SEQUENCING[1]

[1] Pal, A., Amy, M., & den Bakker, H. C. (2024). *Journal of Food Protection, 87(6), 100277*. Reprinted here with permission of the publisher.

Abstract

Edible insects offer a promising protein source for humans, but their food safety risks have not been previously investigated within the United States. Therefore, the aim of this study was to investigate the microbial content of processed edible insect products. A total of eight different types of edible insect products, including diving beetles, silkworms, grasshoppers, Jamaican crickets, mealworms, mole crickets, whole roasted crickets, and 100 % pure cricket powder, were purchased from a large online retailer for the analysis. All the products were purchased in August 2022 and examined between August 2022 to November 2022. Traditional microbiological methods were employed to determine microbial counts for each product type using three replicates (total number of samples = 24). This included assessing aerobic bacterial spore, lactic acid bacteria, *Enterobacteriaceae*, total viable counts and the presence of *Salmonella*. Additionally, whole genome sequencing was employed to further characterize selected colonies (n = 96). Microbial counts data were statistically analyzed using one-way ANOVA, while sequence data were taxonomically classified using Sepia. *Bacillus cereus* group isolates underwent additional characterization with Btyper3. Product type significantly influenced total viable counts, bacterial spore counts, and lactic acid bacteria counts (P = 0.00391, P = 0.0065, and $P < 0.001$, respectively), with counts ranging from < 1.70 to 6.01 Log_{10} CFU/g, < 1.70 to 5.25 Log_{10} CFU/g, and < 1.70 to 4.86 Log_{10} CFU/g, respectively. *Enterobacteriaceae* were only detected in mole crickets (< 2.30 Log_{10} CFU/g) and house cricket powder (< 2.15 Log_{10} CFU/g). All samples were negative for *Salmonella*. Whole genome sequencing revealed the presence of 12 different bacterial genera among the analyzed isolates, with a majority belonging to the *Bacillus* genus. Some of the isolates of *Bacillus cereus* group were identified as biovar Emeticus. Overall, although edible insects offer a promising food

alternative, the presence of *Bacillus cereus* group in some products could raise concerns regarding food safety.

Keywords: Aerobic bacterial spore, *Bacillus cereus*, Edible insect, Emetic, *Enterobacteriaceae*, Whole genome sequencing

Introduction

The rise in global population and the subsequent surge in food requirements have stimulated unsustainable agricultural methods worldwide, resulting in habitat degradation, deforestation, excessive exploitation of animals, and a surge in greenhouse gas emission. To address this issue, suggested strategies encompass decreasing meat consumption, enhancing agricultural productivity, and exploring alternative food options that demand fewer land and natural resources for production (Ordoñez-Araque and Egas-Montenegro, 2021). The cultivation of edible insects as a viable option for human consumption presents a potential solution to these challenges for two key reasons. Firstly, when compared to conventional livestock, insects may offer a more environmentally friendly approach by leaving a smaller ecological impact. Their production necessitates lesser quantities of food and water, occupies less space, and boasts superior biomass conversion rates (Fernandez-Cassi et al., 2020). Secondly, edible insects possess a diverse range of nutritional benefits. They typically exhibit a protein content ranging from 30 % to 65 % of the total dry matter, and they are abundant in micronutrients (Dobermann et al., 2017). There are essentially three potential methods for insect consumption. The first entails consuming whole insects in their recognizable form. The second involves processing whole insects into powder or paste, while the third method involves utilizing insect extracts, for example, protein isolates (Klunder et al., 2012). Insects are a significant component of traditional diets for a minimum of 2 billion individuals, with over 1,900 species reported to be consumed as food. Entomophagy, the practice of eating insects, is deeply intertwined with cultural and religious customs, and insects serve as a common food source in numerous regions worldwide. Nevertheless, in many Western nations, entomophagy is often met with aversion, with people associating insect consumption with primitive behavior (van Huis et al., 2013). Furthermore,

ensuring the safety, traceability, and quality of edible insects is a matter of utmost importance for both producers and consumers. These factors significantly influence the level of acceptance of edible insects as a part of the human diet (Frigerio et al., 2020; House, 2016).

Despite being consumed in various regions globally, there is lack of comprehensive scientific data regarding the safety of consuming edible insects, especially when compared to the extensive knowledge available on conventional protein sources such as dairy products, meat, and eggs (Osimani et al., 2018c). Numerous researchers have identified instances of microbial contamination in these products. A study utilizing pyrosequencing has discovered the presence of various insect gut-associated microbes in processed edible insects, some of which have the potential to act as opportunistic pathogens in humans (Garofalo et al., 2017). Specifically, this study revealed the presence of *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp., and *Bacillus* spp., while viable pathogens like *Salmonella* spp. and *Listeria monocytogenes* were not detected (Garofalo et al., 2017). Osimani et al. (2018c) showed spore-forming bacteria were prevalent in the microbiota of edible insects and were primarily represented by *Bacillus* and *Clostridium*. In addition, Ulrich et al. (1981) reported the presence of human opportunistic or pathogenic bacterial genera such as *Yersinia* spp., *Klebsiella* spp., *Citrobacter* spp., and *Fusobacterium* spp. in the gut of common house crickets (*Acheta domesticus*).

Studies investigating the microbial profile of edible insects intended for human consumption uncovered that the microbial data exhibit intricate ecosystems, displaying notable variations in microbial load and genera or species across different edible insect species. For example, certain popular edible insect species, such as mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria*), were found to possess stable and species-specific microbiota (Garofalo et al., 2019). Likewise, the microbial loads in edible insect products may differ based

on their raw microbial content, the reduction or increase of bacteria during processing, and the potential for secondary contamination after processing (Grabowski & Klein, 2017). Additionally, Frigerio et al. (2020) discovered microbial variability between raw and processed food derived from the same insect species or similar products manufactured by different companies. Consequently, it becomes crucial to identify the microbiota of edible insects accurately and reliably to assess the potential presence of pathogens, spoilage, and beneficial bacteria within this context (Garofalo et al., 2017).

Remarkably, there is a noticeable lack of research regarding the microbial risks associated with edible insects within the United States, despite the availability of various edible insect products on e-commerce platforms. Furthermore, there is a notable absence of stringent food regulation governing the manufacturing and marketing of edible insects in the United States. Consequently, it becomes imperative to examine the food safety hazards posed by edible insect products within the United States. Therefore, the objective of our research was to investigate the microbial content of edible insect products currently available for human consumption via e-commerce platforms in the United States. In addition to employing conventional microbiological methods, we incorporated whole genome sequencing technique to explore the microbial communities inhabiting edible insects. This high-throughput sequencing technology enables a comprehensive and precise analysis of the microbial composition within these products, improving our ability to identify potential microbial risks with heightened accuracy (Garofalo et al., 2017).

Materials and Methods

Sampling plan

Diving beetles, silkworms, grasshoppers, Jamaican crickets, mealworms, mole crickets, whole roasted crickets, and 100 % pure cricket powder were purchased from a large online retailer to perform this study (Table 2.1). The desired products were purchased in August 2022 and studied between August 2022 to November 2022. The edible insects used in this study were selected to represent the major groups of edible insects consumed worldwide (van Huis et al., 2013). Briefly, we conducted microbiological sampling on each of the eight types of edible insects, employing three biological replicates for every insect product. This means that each edible insect variant was tested three times using distinct samples (Number of replications = 3; total number of samples = 24). Each replicate sample per product type was weighing 25 g. To ensure uniform sample sizes for microbiological analyses across all edible insect types, we combined the requisite number of packages of the same product whenever the individual package weight was less than 25 g (Table 2.1). Enumeration of total viable counts, *Enterobacteriaceae*, lactic acid bacteria, and aerobic bacterial spores within these products was performed using plate count method on appropriate media. *Salmonella* prevalence in these products was analyzed using enrichment (Pal et al., 2021). Additionally, whole genome sequencing was used to further characterize selected colonies of the enumerated bacteria. The criteria of colonies selection for whole genome sequencing are described in subsequent paragraph.

Sample processing for enumeration and detection of bacteria using traditional microbiological methods

Replicate samples of each product type were used for enumerating total viable counts, *Enterobacteriaceae*, lactic acid bacteria, and aerobic bacterial spores, and detecting *Salmonella*. Briefly, 25 g of each sample was aseptically transferred into a sterile filter bag (Nasco whirl-pak® sample bag, Madison, WI) and then crushed with a mortar. Next, the crushed 25 g sample was diluted in 225 mL of sterile buffer peptone water (BBL™, Becton Dickinson and Company, Sparks, MD, USA), and the mixture was homogenized for one minute in a stomacher (Laboratory Blender Stomacher 400, Seward, UK). This direct homogenate, mixture of a 25 g sample added to 225 ml of buffered peptone water, was then subjected to a 10-fold dilution with physiological saline solution [0.85 % (w/v) of NaCl; autoclaved for 15 min at 121 ℃]. Later, a 0.1 ml aliquot from either the direct homogenate or its further dilution was spread plated on appropriate media in duplicate for enumerating the above-mentioned bacteria. Specifically, the sample was spread plated onto Plate Count Agar (BBL™, Becton Dickinson and Company, Sparks, MD, USA) and then incubated at 30 ℃ for 48-72 h before enumerating the total viable counts. For *Enterobacteriaceae* counts, the sample was plated on Voilet Red Bile Glucose agar (Neogen® Culture Media, Lansing, MI, USA) and incubated for 24 h at 37 ℃. Lactic acid bacteria numbers were obtained by culturing sample onto de Man, Rogosa and Sharpe agar (Neogen® Culture Media, Lansing, MI, USA) and then incubating at 37 ℃ for 72 h under an anaerobic environment obtained by the AnaeroGen™ 2.5L atmosphere generation system (Oxoid Ltd, Basingstoke, UK). For aerobic bacterial spore counts, the diluted sample (10 mL) was subjected to heat shock at 80 ℃ for 10 minutes in a water bath before plating onto Plate Count Agar. Aerobic bacterial spore counts were noted after incubating media plates for 24 h at

37 °C. For *Salmonella* detection, direct homogenate (around 200 mL) of sample was enriched at 37 °C for 24 h. Enriched samples were duplicate streaked onto Xylose Lysine Tergitol-4 agar plates (Neogen® Culture Media, Lansing, MI, USA). After that, streaked plates were incubated at 37 °C for 24 h before confirming presumptive isolated *Salmonella* colonies.

Whole genome sequencing of selected bacterial isolates

A subset of the bacteria that were enumerated were used for sequencing. A total of 48, 24, and 24 bacterial isolates from 1st, 2nd, and 3rd replication were characterized using whole genome sequencing. This selective sequencing approach was adopted to optimize resources while ensuring representation of microbial variety within the edible insect products under investigation. The colonies were selected to capture as much variation in colony morphology found on a plate as possible. This methodological strategy aimed to capture a diverse range of phenotypic characteristics, potentially reflecting the underlying genetic variability of microbial populations. By deliberately selecting colonies with varied morphologies, the study sought to enhance the comprehensiveness of the genomic analysis and provide deeper insights into microbial species and strains present in the samples. Each selected colony was transferred to a tube containing 10 mL of nutrient broth (BBL^{TM}, Becton Dickinson and Company, Sparks, MD, USA). Next, the tubes were incubated for further growth of bacteria at the same time and temperature as used for enumeration of respective bacterial types. After that, the broth containing growing bacteria was used for DNA extraction using the Dneasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of purified DNA was determined using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). The extracted DNA was then sequenced on an Illumina MiSeq benchtop

sequencer using the Nextera XT Library Prep Kit (Illumina, San Diego, CA) to obtain paired-end 300 bp reads.

Statistics and bioinformatics analyses

Microbial counts data were analyzed using one-way ANOVA ($\alpha = 0.05$), and means were separated employing Tukey's HSD test from the "agricolae" (de Mendiburu, 2023). These analyses were performed in R version 4.2.3. The limit of detection for the spread plate method was determined to be 1.70 Log_{10} CFU/g. Sequence data were assembled using Shovill (Seemann et al, 2020) and the resulting assemblies were taxonomically classified into organism by utilizing Sepia v1.1.0 (den Bakker and Katz, 2021), which employed a reference database containing type and representative genomes of the Genome Taxonomy Database version r207 (Parks et al., 2022). Assemblies with an estimated k-mer similarity of < 0.5 (which correlates to an average nucleotide identity of < 0.95), as inferred by the Sepia software, were submitted to the Type (Strain) Genome Server (https://tygs.dsmz.de) to confirm that whether or not the sequenced strain belonged to a known bacterial species. *Bacillus cereus* group isolates were further characterized using Btyper3 v3.3.4 (Carroll et al., 2020a). Btyper3 v3.3.4 was enabled to search for the presence of the following genes: *cesABCD, hblABCD, nheABC, and cytK-2* among the *Bacillus cereus* group isolates. In our study, we adopted the nomenclature for the *Bacillus cereus* group as established by Carroll et al. (2020b).

Results and Discussion

Total viable counts, aerobic bacterial spores, and lactic acid bacteria vary significantly among edible insect products

The microbial quality of the edible insect products was assessed by analyzing total viable counts, aerobic bacterial spores, lactic acid bacteria, and *Enterobacteriaceae*. These microbial

groups have been widely used by other researchers to evaluate the microbial quality and safety of edible insects. Table 2.2 presents the microbial counts and their variation among various types of tested processed edible insect products. Our results showed significant variation in total viable counts, aerobic bacterial spore counts, and lactic acid bacteria counts among different product types ($P = 0.00391$, $P = 0.0065$, and $P < 0.001$, respectively). The total viable counts ranged from < 1.70 to 6.01 Log_{10} CFU/g, while the aerobic bacterial spore counts ranged from < 1.70 to 5.25 Log_{10} CFU/g. Jamaican crickets had total viable counts below the limit of detection. Among the different product types, mole crickets had the numerically highest total viable counts, which were significantly higher than the counts of grasshoppers, Jamaican crickets, and mealworms. Aerobic bacterial spore levels were significantly higher in mealworms, mole crickets, and 100% pure cricket powder compared to the rest of the products.

Regarding lactic acid bacteria, the counts were ranging from < 1.70 to 4.86 Log_{10} CFU/g. Diving beetles, grasshoppers, Jamaican crickets, and mealworms all exhibited lactic acid bacteria counts below the limit of detection. *Enterobacteriaceae* were only found in mole crickets (< 2.30 Log_{10} CFU/g) and 100 % pure cricket powder (< 2.15 Log_{10} CFU/g). Notably, all tested edible insect products were negative for *Salmonella*, indicating a satisfactory level of food safety in terms of this particular pathogen.

While our findings may not directly correlate with previous studies due to variations in the types of products analyzed and the lack of available information on the rearing and precise processing parameters of the products used in this research, we can still derive valuable insights through comparisons. For instance, Fasolato et al. (2018) discovered variations in the total viable counts, ranging from less than 2.0 Log_{10} CFU/g to over 7.0 Log_{10} CFU/g, and total aerobic spore counts, ranging from 1.6 Log_{10} CFU/g to 8.1 Log_{10} CFU/g, when comparing processed edible

insect products from four different insect species such as crickets, silkworm, mole crickets, and mealworms. The study found that processed silkworm had the highest median values for both total viable counts and total aerobic spore counts. Lactic acid bacteria and *Enterobacteriaceae* were mostly below the limit of detection in this study. Similarly, Osimani et al. (2017) observed variations in total mesophilic aerobes, ranging from 2.6 Log_{10} CFU/g to 5.0 Log_{10} CFU/g, and lactic acid bacteria, ranging from less than 1 Log_{10} CFU/g to 5.5 Log_{10} CFU/g, across cricket powder (*Acheta domesticus*), whole dried small crickets (*Acheta domesticus*), whole dried locusts (*Locusta migratoria*), and whole dried mealworms (*Tenebrio molitor*). The same study found that the cricket powder had the highest counts of *Enterobacteriaceae*, with 3.1 Log_{10} CFU/g, while all the remaining products had the same and lowest counts (less than 1 Log_{10} CFU/g). Likewise, Garofalo et al. (2017) observed very low counts of *Enterobacteriaceae* in all the analyzed processed edible insects obtained from the market. Consequently, the microbial variation in processed edible insect products is common and can be attributed to various factors, including but not limited to insect species, rearing conditions, processing methods, batch variability during processing, and storage. In our investigation, no measurable levels of total viable counts were found in Jamaican crickets, and viable lactic acid bacteria and *Enterobacteriaceae* were absent in many of the products we examined. Based on these findings, it appears that the primary microbial load of these products in our study was attributed to bacterial spores, or the processing method used by manufacturing companies successfully destroyed these bacteria within the products. Previous research has shown that a brief heating step in processing can reduce total viable counts and *Enterobacteriaceae* in edible insects. However, some spore-forming bacteria that likely enter these products through soil were able to survive the boiling treatment (Klunder et al., 2012). Given our findings and previous research

indicating high levels of total aerobic spore-forming bacteria in processed edible insect products, it is imperative to establish appropriate processing parameters for edible insects in order to effectively manage these bacterial populations. Moreover, it is worth mentioning that we observed the presence of *Enterobacteriaceae* in a few products. This can pose a serious food safety concern regarding the consumption of edible insects, as the *Enterobacteriaceae* family includes some bacterial species known to be pathogenic or opportunistic. Like our findings, several other researchers observed the absence of viable *Salmonella* in processed edible products (Garofalo et al., 2017; Grabowski & Klein, 2017). Interestingly, in some of the investigated products, aerobic bacterial spore counts were found to be higher than their respective total viable counts. The observed phenomenon could not be plausibly interpreted due to the inadequate information of the processing treatment (temperature and time) mentioned on the labels of the edible insects. It can be suspected that the provided processing treatments were insufficient to eliminate bacterial spores from the products. Consequently, this inadequacy might have contributed to elevated counts of aerobic bacterial spore formers compared to the total viable counts. Alternatively, the different incubation conditions used for total viable counts and aerobic spore-forming bacteria in this study could result in these observations.

Bacillus cereus group and non-cereus _Bacillus_ group were the most prevalent species among the analyzed isolates, and some toxin-producing _Bacillus_ were identified

Figure 2.1 presents the results of whole genome sequencing conducted on a total of 96 bacterial isolates. The sequencing analysis revealed the presence of 12 distinct bacterial genera among the isolates. Detailed information of sequencing results and Btyper3 analysis is available in the supplementary material (Table S1 and Table S2). These identified genera include

Acinetobacter, *Bacillus*, *Brevibacillus*, *Curtobacterium*, *Enterobacter*, *Enterococcus*, *Exiguobacterium*, *Klebsiella*, *Luteibacter*, *Mixta*, *Priestia*, and *Pseudobacillus*.

Among the sequenced isolates, the majority of them belonged to *Bacillus cereus* group (total number of isolates = 34) and the non-cereus *Bacillus* group (total number of isolates = 25). The presence of the *Bacillus* genus was detected in bacterial isolates obtained from all the investigated products, except diving beetles. The *Bacillus cereus* group encompasses a set of bacteria that are responsible for two distinct gastrointestinal syndromes in humans. Firstly, there is an intoxication (known as emetic disease) caused by a heat-stable toxin called cereulide, which is produced directly within the food itself. Secondly, there is an infection (known as diarrheal disease) resulting from the production of *Bacillus cereus* enterotoxins within the small intestine of the affected individual (Osimani et al., 2018a). The diarrheal syndrome is caused by two enterotoxin complexes, namely hemolysin BL and nonhemolytic enterotoxin, together with two proteins known as cytotoxin K and enterotoxin T (Osimani and Aquilanti, 2021). By conducting Btyper3 analysis on the *Bacillus cereus* group, we found a small number of isolates (a total of 3) from mealworms and mole crickets to be biovar Emeticus. These particular isolates possessed genes encoding cereulide synthetase (*cesABCD*), which is associated with the production of the toxin cereulide. Additionally, our investigation revealed that among the *Bacillus cereus* group isolates, nine isolates contained all genes (*hblA*, *hblB*, *hblC*, and *hblD*) encoding for hemolysin BL, while 24 isolates possessed all the genes (*nheA*, *nheB*, and *nheC*) encoding for nonhemolytic enterotoxin. Only six of the isolates had all genes encoding for both hemolysin BL and nonhemolytic enterotoxin. The gene (*cytK*-2) encoding for cytotoxin K was observed among 21 isolates. In our study, we also observed *Bacillus cereus sensu stricto* biovar Thuringiensis from silkworm. It is worth noting that the insecticidal properties of *Bacillus* Thuringiensis can pose a

potential risk in insect-rearing scenarios (Bravo et al., 2007). Furthermore, the non-cereus *Bacillus* isolates we observed in our study represented several species, including *Bacillus velezensis*, *Bacillus tequilensis*, *Bacillus sonorensis*, *Bacillus siamensis*, *Bacillus safensis*, *Bacillus subtilis*, *Bacillus paralicheniformis*, *Bacillus licheniformis*, *Bacillus glycinifermentans*, *Bacillus amyloliquefaciens*, and *Bacillus altitudinis*.

Previous research has also documented the presence of *Bacillus* genus in edible insects. In one study, for example, a total of 20 bacterial isolates derived from non-processed yellow mealworms and 79 bacterial isolates obtained from non-processed house crickets were identified as members of the *Bacillus cereus* group. Additionally, a total of 16 bacterial isolates were classified as non-cereus *Bacillus* spp. These non-cereus *Bacillus* spp. in the same study included *Bacillus pumilus*, *Bacillus altitudinis*, *Bacillus siamensis*, *Bacillus licheniformis*, *Bacillus vallismortis*, and *Bacillus subtilis* (Vandeweyer et al., 2020). Moreover, in a study conducted by Fasolato et al. (2018), the researchers examined processed edible insects and observed the presence of viable presumptive *Bacillus cereus* counts. Among the samples, mole crickets exhibited the highest level, reaching 6.6 Log_{10} CFU/g. Additionally, within the *Bacillus cereus* group strains analyzed in the same study, around 26 % were found to possess all the genes responsible for encoding the three components of the hemolysin BL. Moreover, approximately 30 % of the strains carried genes encoding for the nonhemolytic enterotoxin, suggesting the potential pathogenic activity of those particular strains.

Potential opportunistic or pathogenic bacteria such as *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* were found in certain insect products

Our study also identified a few bacterial isolates from the *Enterobacteriaceae* and lactic acid bacteria families. Specifically, we found *Enterobacter hormaechei* (total number of isolates

= 1) in 100 % pure cricket powder, *Enterococcus lactis* (total number of isolates = 6) in roasted whole crickets and 100 % pure cricket powder, and *Enterococcus faecium* (total number of isolates = 3) in mole crickets and 100 % pure cricket powder. Previous findings of Garofalo et al. (2017) have already established the presence of *Enterobacter* and *Enterococcus* genera in processed edible insects like mealworms and powdered crickets, suggesting that these genera naturally exist in the microbiota of edible insects' gut. Furthermore, the *Enterococcus* genus encompasses species that are highly adaptable to various environments, including the gut of humans, animals, insects, plants, soil, and water. Among this genus, *Enterococcus faecalis* and *Enterococcus faecium* are well-known for their potential to cause human infection (Garofalo et al., 2017).

One of the isolates in our experiment, obtained from 100 % pure cricket powder, was identified as *Klebsiella pneumoniae*. The presence of the *Klebsiella* genus has been previously reported in various edible insects such as laboratory-reared mealworms, ready-to-eat mealworms, and crickets (Fernandez-Cassi et al., 2020; Osimani et al., 2017; Osimani et al., 2018b). In humans, *Klebsiella* can pose a significant health risk as an opportunistic pathogen, particularly affecting infants, the elderly, and individuals with weakened immune systems (Osimani et al., 2018b). In this study, two bacterial isolates belonged to the *Acinetobacter* genus. Specifically, *Acinetobacter baumannii* was observed in 100 % pure cricket powder, while *Acinetobacter radioresistens* was found in whole roasted crickets. *Acinetobacter* spp. have been documented in diverse environments and are recognized as important opportunistic pathogens in healthcare settings, known for their multi-drug resistant nature (Al Atrouni et al., 2016). Previous research has also detected *Acinetobacter* spp. in crickets (*Acheta domesticus*) reared under controlled

conditions (Fernandez-Cassi et al., 2020). Notably, *Acinetobacter baumannii* was detected in lesser mealworms (*Alphitobius diaperinus*) (Wynants et al., 2018).

Bacterial genera harboring potential new species were identified within a few insect products

We encountered four bacterial types that could only be classified at the genus level, namely *Curtobacterium, Brevibacillus, Luteibacter*, and *Exiguobacterium*. An average nucleotide identity (ANI) of less than 95% with type strains in Genome Taxonomy Database version r207 (Parks et al., 2022) and further analysis with the Type (Strain) Genome Server (https://tygs.dsmz.de) suggests that these particular isolates may represent new species within their respective genera. Interestingly, one of the presumptive bacterial isolates obtained from diving beetles could not be taxonomically classified, and further examination by nucleotide blast (https://blast.ncbi.nlm.nih.gov/, accessed September 2023) identified this isolate as a fungus, the yeast *Rhodotorula mucilaginosa*. Previously, the presence of *Exiguobacterium* was observed in protein bars made from *Acheta domesticus* and other non-insect ingredients (Frigerio et al., 2020). Additionally, Vandeweyer et al. (2017) observed *Brevibacillus* spp. in living mealworms obtained from an industrial rearing company. *Brevibacillus* spp. can act as spoilage organisms and affect the quality of insect-based food products (Lücking et al., 2013). Similarly, in our study, we observed *Exiguobacterium* (total number of isolates = 1) in whole roasted crickets and *Brevibacillus* (total number of isolates = 10) in mealworms. Furthermore, we identified a few other bacterial species in this study including *Pseudobacillus badius, Priestia flexa, Priestia megaterium, Priestia aryabhattai,* and *Mixta calida*. Previous findings have identified the presence of *Priestia* genus in the soil, suggesting that its occurrence in the investigated edible insect products could potentially be attributed to soil contamination (Esikova et al., 2021). The

presence of *Mixta tenebrionis* sp. nov has been observed within the gut of mealworms (Xia et al., 2020). Consequently, the presence of this genus within edible insect products can be attributed to its transmission from the gut of insects to final processed edible insects, which in our case were mole crickets.

Limitation of the current study

It is important to note that the microbial analysis conducted in this study focused on specific edible insect types. Consequently, the results might not be broadly applicable to all edible insect species, as microbial communities can exhibit species-specific associations. The composition of microbial communities may vary significantly among different insect species, highlighting the need for caution when extrapolating these findings to a broader range of edible insects.

Conclusion

Overall, edible insects have the potential to serve as a valuable food source for humans. However, the ongoing concern lies in their inadequate microbial quality. The high presence of total viable counts and aerobic bacterial spores in this study indicates the necessity for proper rearing and processing of edible insects. Additionally, edible insect products harbor microorganisms from various genera/species, including those from soil or the insect's gut. The existence of emetic strains of *Bacillus cereus* group, as well as other opportunistic pathogens like *Klebsiella pneumoniae* and *Acinetobacter baumannii*, raises a critical food safety issue regarding their consumption.

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Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work the authors used ChatGPT, version 3.5 tool in order to enhance readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Table 2.1. List of edible insect products and their specifications.

Sr. No.	Name of the product	Ingredients	Weight per package	Processing method	Prescribed use
1	Diving beetles	Asian diving beetle (*Dytiscidae*), Salt	15 g	Boiled and dehydrated	Ready-To-Eat
2	Silkworms	Silkworm pupae (*Bombyx mori* Sp)	50 g	Microwave dried	N/A[1]
3	Grasshoppers	Grasshoppers (*Oxya yezoensis*), Salt	15 g	Microwave dried	N/A
4	Jamaican crickets	Jamaican field crickets (*Gryllus assimilis*), Salt	15 g	Boiled and dehydrated	Ready-To-Eat
5	Mealworms	Mealworms (*Tenebrio molitor*), Salt	15 g	Microwave dried	Ready-To-Eat
6	Mole crickets	Mole crickets (*Gryllotalpidae*), Salt	15 g	Boiled and dehydrated	Ready-To-Eat
7	Whole roasted crickets	Crickets (*Acheta domesticus*)	15 g	-	N/A
8	100 % pure cricket powder	Cricket flour (*Acheta domesticus*)	45 g	-	N/A

[1]N/A = Information was not provided on product package.

Table 2.2. Microbial counts of edible insect products (number of sample tested/product type = 3).

Product types	Microbial counts (Log_{10} CFU/g ± Standard error) and number of positive samples (n[1])		
	Total viable counts	Bacterial spores	Lactic acid bacteria
Diving beetles	$< 2.59 \pm 0.89^{abc}$ (n=2)	$< 2.51 \pm 0.81^{b}$ (n=1)	$< 1.70^{b}$ (n=0)
Silkworms	$< 3.41 \pm 1.27^{abc}$ (n=2)	$< 1.80 \pm 0.10^{b}$ (n=2)	$< 2.06 \pm 0.36^{b}$ (n=2)
Grasshoppers	2.41 ± 0.23^{bc} (n=3)	2.06 ± 0.18^{b} (n=3)	$< 1.70^{b}$ (n=0)
Jamaican crickets	$< 1.70^{c}$ (n=0)	$< 1.70^{b}$ (n=1)	$< 1.70^{b}$ (n=0)
Mealworms	2.10 ± 0.20^{bc} (n=3)	3.17 ± 0.20^{ab} (n=3)	$< 1.70^{b}$ (n=0)
Mole crickets	6.01 ± 1.08^{a} (n=3)	5.25 ± 1.26^{a} (n=3)	4.75 ± 0.90^{a} (n=3)
Whole roasted crickets	4.01 ± 0.15^{abc} (n=3)	2.52 ± 0.34^{b} (n=3)	3.91 ± 0.15^{a} (n=3)
100 % pure cricket powder	5.34 ± 0.53^{ab} (n=3)	3.44 ± 0.09^{ab} (n=3)	4.86 ± 0.06^{a} (n=3)
P value	0.00391	0.0065	< 0.001

[1]n = Number of positive samples. It indicates the number of samples that yielded bacterial counts greater or equal to the limit of detection (1.70 Log_{10} CFU/g).

[a-c]Values within a column with different superscripts differ significantly ($P \leq 0.05$). Data values for samples that fell below the level of detection were included in mean calculation, one-way ANOVA, and mean separation. Statistical analyses utilized their value equivalent to the limit of detection, which was determined to be 1.70 Log_{10} CFU/g.

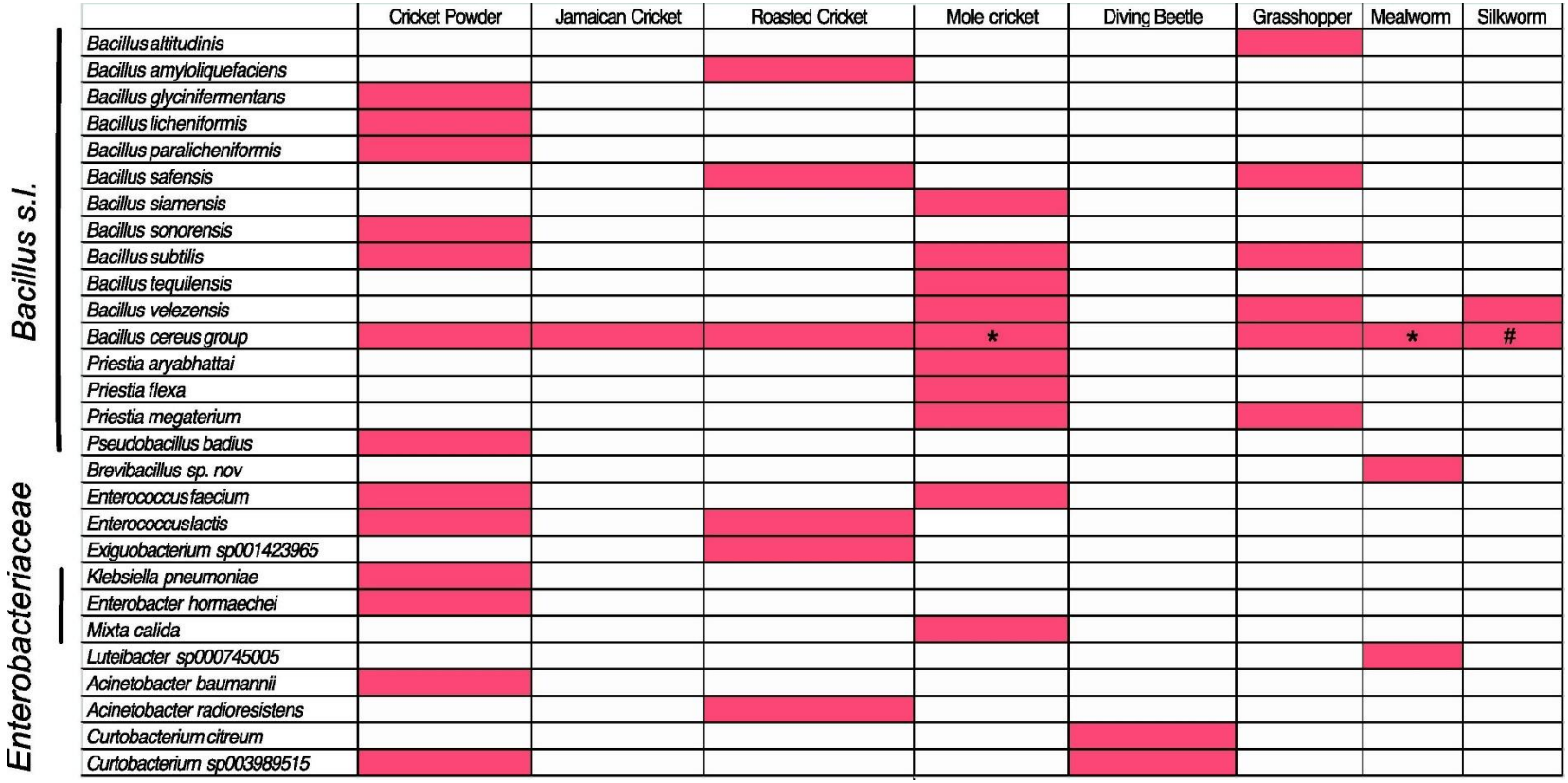

Figure 2.1. Overview of bacterial taxa recovered from insect products and identified using whole genome sequencing. Legend; red = present, white = absent, ∗ = *B. cereus* biovar Emeticus, # = *B. cereus* biovar Thuringiensis.

CHAPTER 3

MICROBIOME ANALYSIS OF SELECTED RETAIL-DERIVED PRODUCTS USING 16S AMPLICON SEQUENCING

Introduction

The microbiome, defined as the community of microorganisms, including bacteria, fungi, and viruses, inhabiting a specific environment (National Human Genome Research Institute, 2024), plays a crucial role in various ecosystems, including those involved in food supply chain. Microbiome analysis of food products can be performed using targeted amplicon sequencing, a high-throughput sequencing method. In this approach, nucleic acids are extracted directly from the food sample, and the polymerase chain reaction (PCR) is used to amplify a genomic target, most commonly the entire or part of the 16S rRNA gene, which is widely regarded as a universal marker for analyzing bacterial populations (De Filippis et al., 2018). In general, 16S amplicon sequencing protocols focus on amplifying one of hypervariable regions (V5-V6, V3-V4, or V4) of 16S rRNA gene for performing microbiome analyses (Bharti and Grimm, 2021). High-throughput sequencing methods are considered more sensitive than traditional culture-independent techniques, enabling the detection of non-dominant and difficult to culture microorganisms in a sample. These methods also allow for the quantification (abundance) of microbial communities based on the number of sequencing reads (De Filippis et al., 2018).

Previous research into food microbiomes has offered valuable insights into the factors influencing microbial dynamics within the food continuum and has been instrumental in identifying sources of microbial contamination, both of which are crucial for improving food

safety and quality. For instance, processing conditions and packaging types have been shown to make a significant difference in the microbiome profiles of retail chicken breast products (Li et al., 2020). Similarly, research on microbiome of retail packaged broiler meat and broiler abattoirs revealed the presence and types of putative spoilage bacteria, along with changes in microbial population composition during shelf-life periods. It also traced the contamination pathway of *Janthinobacterium lividum*, a bacterium occasionally linked to meat spoilage. This research identified production environments, particularly the cooling condenser, as the source of *Janthinobacterium*, leading to the contamination of final products (Lauritsen et al., 2019). In addition, a study in a small meat processing establishment identified a relationship between the facility environment's microbiome and the presence of *Listeria* spp. (Belk et al., 2022), highlighting the importance of understanding food-related microbiomes to develop targeted interventions and improve precision in managing food safety risks. Furthermore, in addition to safety or quality concerns, food microbiomes can also serve as geographic markers, distinguishing food products based on microbial profiles. For example, beta diversity analysis has identified distinct microbial signatures between unprocessed shellfish from Nova Scotia and Quebec (Liu et al., 2020).

In light of these findings, our study specifically focuses on analyzing the microbiome of retail food products. Food in retail environments is often exposed to various environmental factors that can influence its microbial composition, introduce non-native microorganisms as contaminants, and promote the growth of pathogens. These factors include refrigeration temperature, cleanliness of food contact surfaces, cross-contamination, and overall hygiene conditions (Higgins et al., 2018). Moreover, the bacterial ecology of similar retail food products can vary depending on the size of the retail store, as demonstrated by high-throughput

pyrosequencing of the microbiota in food samples (Higgins et al., 2018). Analyzing the microbiome of retail food products is vital for identifying potential microbial risks and ensuring food safety and quality at the final stages of the supply chain.

Therefore, the primary objective of this study was to analyze the microbiomes likely present on the surfaces of peaches, pears, red onions, mini cucumbers, nectarines, queso fresco cheese, pre-sliced hard salami, and pre-sliced oven-roasted turkey breast obtained from retail stores to understand the potential quality and food safety risks within these products. The selection of these product categories was based on their previous history in foodborne illness outbreaks (CDC, 2024; FDA, 2024).

Materials and Methods

Description of sample types

This study investigated the microbiomes of various food products, including peaches (Product of USA), pears (Product of Argentina), red onions (Product of USA), mini cucumbers (Product of Canada), nectarines (Product of USA), queso fresco cheese (Product of Mexico), pre-sliced hard salami ((Product of USA), and pre-sliced oven-roasted turkey breast (Product of USA). All food items were purchased from a local retail store in Griffin, GA.

For fresh produce (peaches, pears, red onions, mini cucumbers, and nectarines), each sample comprised five individual units (such as five pieces of peaches for one sample of peach). Mini cucumbers were packaged together in a plastic bag. For queso fresco cheese, each sample consisted of two packages, with each package weighing 284 g. Hard salami and turkey breast were analyzed as single packages per sample, with weights of 153 g and 453 g, respectively.

A total of 12 samples were collected and analyzed for each product type, resulting in 96 samples overall. Sample collection and processing were conducted over 13 separate days

between April 2024 to May 2024. On each processing day, a control sample was included, yielding 13 control samples for the entire experiment. To maintain consistency, food items from the same company brand were purchased for each product type across all sampling days.

Description of sample processing

For each food product type, the designated sample was sonicated with 250 mL of wash solution comprising 1X Tris-EDTA buffer (G-Biosciences, Saint Louis, MO, USA) supplemented with 2% Tween 80 (Research Products International, Mount Prospect, IL, USA). To dislodge microorganisms from the surface of the samples, the stomacher bag with the product sample was sonicated for 5 minutes using a sonicator (VWR International, LLC, Radnor, PA, USA). Fresh produce items, including peaches, pears, red onions, mini cucumbers, and nectarines, were individually sonicated for 1 minute per unit as part of the 5-minute dislodging procedure. For queso fresco cheese, each package was sonicated for 2 minutes and 30 seconds. Hard salami and oven-roasted turkey breast packages were divided into five parts, with each part sonicated for 1 minute. During sonication, samples were securely contained within sterilized sampling bags to maintain aseptic conditions. The resulting wash solution, likely containing dislodged microorganisms, was divided into seven aliquots of 30–35 mL in sterilized conical centrifuge tubes. These aliquots were centrifuged at 3900 rpm for 15 minutes at 4 °C. Following centrifugation, the clear supernatant was carefully removed, leaving a dense pellet in each aliquot. The pellets from all aliquots were combined into a single tube and centrifuged again at 3900 rpm for 15 minutes at 4 °C. After discarding the supernatant, the consolidated pellet was transferred to a microcentrifuge tube and subjected to a final centrifugation at 14,000 × g for 10 minutes. The remaining concentrated pellet served as the sample for DNA extraction.

DNA was extracted from the pellet using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The concentration of the purified DNA was measured using the Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). The extracted DNA was subsequently used for 16S rRNA sequencing. During 16S rRNA sequencing, library preparation followed the 16S metagenomic sequencing library preparation protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) from Illumina (San Diego, CA). Amplicon libraries targeting the V3 and V4 regions of the 16S rRNA gene were generated using Polymerase Chain Reaction (PCR) with specific forward and reverse primers. After PCR amplification, the dsDNA concentration of the libraries was re-evaluated using the Qubit system to ensure sufficient yield. The amplified libraries were purified using AMPure XP beads to remove unwanted fragments and contaminants. Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA), producing 2 × 300 bp paired-end reads for downstream microbiome analysis.

For control samples, 250 mL of wash solution, consisting of 1X Tris-EDTA buffer supplemented with 2% Tween 80, was used. Control samples, which did not contain food products, underwent the same processing steps as the food product samples, including sonication, centrifugation, DNA extraction, and sequencing.

Data analyses

Demultiplexed 16S rRNA amplicon sequences were processed using the DADA2 package (v1.30) (Callahan et al., 2016) in the R version 4.3.3 (R Core Team, 2024). The DADA2 workflow (https://benjjneb.github.io/dada2/tutorial_1_8.html) involved the removal of read primers, quality filtering, and trimming to eliminate low-quality bases and sequences. Following

this, reads were dereplicated, paired-end reads were merged, and chimeric sequences were identified and removed to ensure the accuracy of downstream analyses. After these steps, Amplicon sequence variants (ASVs) were inferred and subsequently used for taxonomic classification. Negative control samples were used as a quality check to exclude product samples where the read counts fell below the maximum read counts observed in negative control samples after chimeric sequence removal.

Taxonomic classification of ASVs was performed using the SILVA database (v138.1) as reference database. Alpha and Beta diversity metrics were calculated and visualized using the Phyloseq package (McMurdie & Holmes, 2013) in R version 4.3.3 (R Core Team, 2024). The top 20 bacterial families and genera were identified and visualized using the same package. Beta diversity effects were evaluated using permutational multivariate analysis of variance (PERMANOVA) implemented with the adonis function from the Vegan package (v2.6.8). Alpha diversity metrics, including Shannon and Simpson indices, were compared across product types using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) test. The codes used for alpha and beta diversity and top taxa analyses in Phyloseq package were run based on the information given in the following Github repository: https://github.com/hcdenbakker/RetailBiofilmStudy and https://github.com/joey711/phyloseq/issues/1701.

Results and Discussion

This study analyzed the alpha and beta diversity of microbiota, as well as major bacterial families and genera associated with peaches, pears, red onions, mini cucumbers, nectarines, queso fresco cheese, pre-sliced hard salami, and pre-sliced oven-roasted turkey breast.

Figure 3.1 illustrates the alpha diversity (Shannon and Simpson indices) across the investigated food products. The mean values of both indices differed significantly across product types ($P < 0.001$, $P < 0.001$, respectively). For the Shannon index, higher mean values were observed for pears (4.12), red onions (3.83), and peaches (3.50), whereas nectarines (3.13) and cucumbers (2.42) had intermediate values. The lowest mean values were recorded for pre-sliced oven-roasted turkey breast (1.68), pre-sliced hard salami (1.36), and queso fresco cheese (0.70). Similarly, the Simpson index was highest for pears (0.95), red onions (0.93), peaches (0.91), and nectarines (0.84). In contrast, pre-sliced oven-roasted turkey breast (0.69), pre-sliced hard salami (0.67), and queso fresco cheese (0.38) exhibited the lowest mean values. The findings show that fresh produce, particularly pears, red onions, and peaches, supports greater microbial diversity and evenness, indicating a diverse microbial ecosystem in these products that is not dominated by only a few bacterial species. In contrast, processed food products such as deli meats and queso fresco cheese displayed lower microbial diversity and Simpson index, indicating that their microbial communities were dominated by few specific microbial species. This reduction in microbial diversity aligns with previous research, which reported that food processing can decrease the diversity found in microbial communities, often leading to the dominance of specific species in fermented products compared to their raw ingredients (Jeong et al., 2013; Mitchell, 2024). Notably, the fermented products observed in this study contained dominant bacterial taxa associated with fermentation (Figure 3.3 and 3.4), which generally are intentionally added to enhance product safety due to their ability to produce antimicrobial compounds like bacteriocins, as well as to accelerate the ripening process and improve product quality attributes (Laranjo et al., 2019).

Beta diversity analysis using Bray-Curtis dissimilarity (Figure 3.2) revealed distinct microbiome compositions across different food product types. This finding was further supported by a significant PERMANOVA test ($P = 0.001$) and pairwise comparisons among food product types. Notably, tighter clustering was observed among samples of queso fresco cheese, pre-sliced hard salami, and nectarines, suggesting that the microbiome compositions of their samples were more similar to each other. The observed separation of microbiome profiles among the food products may be attributed to various factors such as differences in product composition, processing methods, harvesting practices, and cultivation conditions. Consistent with our findings, previous studies using Bray-Curtis dissimilarity have also demonstrated distinct separations among the microbiomes of minimally processed food products, including fennel, leafy greens, tomatoes, and pears (Sequino et al., 2022).

Figure 3.3 shows the average relative abundance of the top 20 bacterial families within different food product types that were analyzed within this study. In queso fresco cheese, the predominant families were *Lactobacillaceae* (67%) and *Aerococcaceae* (29%). Cucumbers were mainly dominated by *Bacillaceae* (23%), *Erwiniaceae* (18%), *Microbacteriaceae* (15%), and *Pseudomonadaceae* (14%). In nectarines, *Moraxellaceae* accounted for 40%, followed by *Erwiniaceae* (27%). Red onions were primarily dominated by *Yersiniaceae* (29%) and *Erwiniaceae* (15%). For peaches, *Erwiniaceae* made up 26%, with *Lactobacillaceae* at 18%. In pears, *Pseudomonadaceae* (38%) was the most abundant family. Hard salami showed a dominance of *Lactobacillaceae* (94%), while turkey breast had *Lactobacillaceae* (48%), *Streptococcaceae* (25%), and *Carnobacteriaceae* (19%).

The dominance of *Lactobacillaceae* and *Aerococcaceae* in queso fresco cheese and deli meats aligns with their role in fermentation processes, as these families include lactic acid

bacteria widely used for this purpose (Lawson, 2014; Walter & O'Toole, 2023). Similarly, the presence of *Streptococcaceae*, which includes *Pediococcus* spp., is consistent with its common use as a starter culture in salami production (Feiner, 2006). In fresh produce, the presence of *Bacillaceae* in cucumbers likely stems from soil contamination, as this family is commonly found in soil environments (Mandic-Mulec et al., 2016). *Erwiniaceae*, prevalent in multiple fresh produce samples such as nectarines, red onions, peaches, and cucumbers, has also been identified in other fresh produce like lettuce and bean sprouts in previous studies (Leonard et al., 2021; Solcova et al., 2021). These studies also reported the presence of *Pseudomonadaceae* and *Moraxellaceae* in these products. The *Pseudomonadaceae* family has been associated with spoilage and shown to enhance the tolerance of *Listeria monocytogenes* to sanitizers, when both were present in mixed biofilms, raising potential food safety concerns (Voloshchuk et al., 2024; Zhang et al., 2018).

Figure 3.4 presents the average relative abundance of the top 20 bacterial genera identified across the different food product types. In queso fresco cheese, the dominant genera were *Lacticaseibacillus* (66%) and *Aerococcus* (29%). Cucumbers were primarily populated by *Bacillus* (24%), *Erwinia* (17%), *Pseudomonas* (14%), and *Microbacterium* (12%). In nectarines, *Psychrobacter* was the most abundant genus (36%), followed by *Pantoea* (22%) and *Pseudomonas* (10%). Red onions were mainly dominated by *Rahnella1* (20%), *Pantoea* (13%), and *Serratia* (7%). For peaches, the most abundant genus was *Pantoea* (19%), with *Pseudomonas* (14%) and *Latilactobacillus* (8%) also present. In pears, *Pseudomonas* (40%) was the dominant genus, followed by *Acinetobacter* (6%) and *Pantoea* (6%). Hard salami was largely dominated by *Latilactobacillus* (49%) and *Pediococcus* (46%). Lastly, in turkey breast, the most abundant genera were *Lactococcus* (25%), *Leuconostoc* (25%), *Carnobacterium* (18%), and

Dellaglioa (16%). Detailed information on the top genera within each food product type, with average relative abundances exceeding 1%, is provided in Table 3.1.

These findings align with previous research on food-associated microbial communities. For example, *Lacticaseibacillus casei*, widely used as an adjunct starter culture in cheese manufacturing, is known to enhance proteolysis and gas and aroma production during ripening, thereby improving cheese flavor and texture (Sviridenko et al., 2024; Zheng et al., 2020). Similarly, the presence of *Aerococcus* in Mexican-style cheeses has been reported, with potential bioprotective effects against *Salmonella* in dairy products (Besnard et al., 2021; Murugesan et al., 2018). Species from the genera *Latilactobacillus*, *Pediococcus*, and *Lactococcus* are commonly employed as bacterial starter cultures in meat fermentation (Cocconcelli and Fontana, 2010; Zheng et al., 2020). Their detection in products such as hard salami and turkey breast is likely attributable to their intentional addition during the fermentation process. These genera have also been previously identified in the microbiome of deli meats (Wang et al., 2018). The genus *Carnobacterium* includes species known for their spoilage potential in meat products; however, certain strains, such as *Carnobacterium* maltaromaticum exhibit promising meat preservation capabilities by producing organic acids and bacteriocins, which exert antibacterial effects on spoilage bacteria. (Casaburi et al., 2011; de Andrade Cavalari et al., 2024; Zhang et al., 2019a). Recent findings have also highlighted the presence of *Dellaglioa* species in high-oxygen modified-atmosphere packaged meats (Werum and Ehrmann, 2024). Among this genus, *Dellaglioa algida*, a psychrotolerant species, has shown antibacterial activity against psychrotrophic *Pseudomonas* (Sun et al., 2022). The *Dellaglioa* genus is one of the newly defined genera resulting from the reclassification of *Lactobacillus* genus, divided its species into 25 genera, including 23 novel genera (Zheng et al., 2020).

In fresh produce, the predominant genera we detected included *Bacillus*, *Pseudomonas*, *Pantoea*, *Erwinia*, *Acinetobacter*, and *Psychrobacter*. Previously, strains of the *Bacillus cereus* group carrying toxin genes have been isolated from fresh vegetables, presenting a potential health risk when present in high concentrations (Fiedler et al., 2019). Additionally, some species of the *Bacillus* genus are commonly used as biopesticide in agriculture settings (Mnif and Ghribi, 2015). In our study, after blasting some ASVs (50/204) assigned to the *Bacillus* genus against the NCBI 16S rRNA database, we found a few ASVs (2/50) with a high percentage identity (≥ 99.7%) to the *Bacillus cereus* group, and these ASVs were observed in cucumbers, peaches, pears, nectarines, and red onions. The genus *Pseudomonas*, identified as one of the most abundant taxa in the majority of fresh produce types in our study, is well-documented for its significant role in the spoilage of fruits and vegetables (Raposo et al., 2016). The *Pantoea* genus is commonly isolated from soil, water, and plants and exhibits diverse traits, such as nitrogen fixation, plant growth promotion, biocontrol activity for disease management, and, in some cases, pathogenicity in plants (Walterson and Stavrinides, 2015). This genus has also been reported in various fruits and vegetables, including cabbage, carrots, capsicum, lettuce, radishes, and watermelon (Al-Kharousi et al., 2016). *Erwinia*, another significant genus detected, is well-known for its role in soft rot disease in plants (Wasendorf et al., 2022). Additionally, *Acinetobacter baumannii* has been reported in fruits and vegetables, with some isolates exhibiting drug resistance, raising concerns about its potential impact on food safety (Ababneh et al., 2022). Also, *Acinetobacter* species have been previously reported in the skin microbiome of healthy humans (Wang et al., 2021), which may suggest the occurrence of this genus in our analyzed food products due to contamination by human-associated microbiota along the food supply chain. In nectarines, we observed a high abundance of *Psychrobacter*, *Pantoea*, and

Pseudomonas. These cold-resistant bacteria are known for their ability to thrive under low-temperature storage conditions and contribute to spoilage in food (Zhang et al., 2019b). It is important to note that although spoilage-related genera were observed in our sample products, none of the products were actually spoiled. This could be attributed to several factors, such as low microbial loads of these spoilage bacterial genera, the inability of 16S rRNA sequencing to differentiate between viable and non-viable cells, and its limited resolution in identifying the exact strain type within the respective genera. Therefore, these results should be interpreted cautiously when relating the presence of spoilage-related genera to the actual risk of food spoilage.

Conclusion

Our study revealed that fresh produce supports greater microbial diversity compared to queso fresco cheese and deli meats. This indicates a more diverse microbial ecosystem in minimally processed foods, which may contribute to the introduction of a broader range of bacterial species in a retail setting. Beta diversity analyses demonstrated distinct microbial community compositions across food product types. Queso fresco cheese, pre-sliced hard salami, and oven-roasted turkey breast were dominated by fermentation-associated genera such as *Lacticaseibacillus*, *Latilactobacillus*, *Pediococcus,* and *Lactococcus*. In contrast, fresh produce harbored spoilage-associated genera like *Pseudomonas*, *Pantoea*, and *Psychrobacter*.

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Table 3.1. Bacterial genera with a relative abundance greater than 1% in each food product type.

Product Type	Bacterial Genera
Queso fresco cheese	*Lacticaseibacillus, Aerococcus, Staphylococcus*
Cucumbers	*Bacillus, Erwinia, Pseudomonas Microbacterium, Stenotrophomonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Staphylococcus, Methylobacterium-Methylorubrum, Acinetobacter, Glutamicibacter, Rosenbergiella, Enterococcus, Sphingomonas Exiguobacterium, Sanguibacter-Flavimobilis Rhodococcus, Paenarthrobacter*
Nectarines	*Psychrobacter, Pantoea, Pseudomonas, Rahnella1, Erwinia, Acinetobacter, Serratia, Leuconostoc, Janthinobacterium, Aerococcus*
Red onions	*Rahnella1, Pantoea, Serratia, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Pseudomonas, Leuconostoc, Bacillus, Paenibacillus, Yersinia, Sphingobacterium, Microbacterium, Stenotrophomonas, Flavobacterium, Enterobacter*
Peaches	*Pantoea, Pseudomonas, Latilactobacillus, Erwinia, Serratia, Pediococcus, Rahnella1, Actinomyces, Leuconostoc, Staphylococcus, Janthinobacterium, Bacillus, Microbacterium*
Pears	*Pseudomonas, Acinetobacter, Pantoea, Carnobacterium, Kocuria, Enhydrobacter, Staphylococcus, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Aerococcus, Sphingomonas, Frigoribacterium, Curtobacterium, Skermanella, Rahnella1, Psychrobacter, Pediococcus, Turicibacter, Glutamicibacter, Bacillus, Paracoccus*
Pre-sliced hard salami	*Latilactobacillus, Pediococcus, Staphylococcus*
Oven-roasted turkey breast	*Lactococcus, Leuconostoc, Carnobacterium, Dellaglioa, Latilactobacillus, Brochothrix, Pediococcus, Pseudomonas*

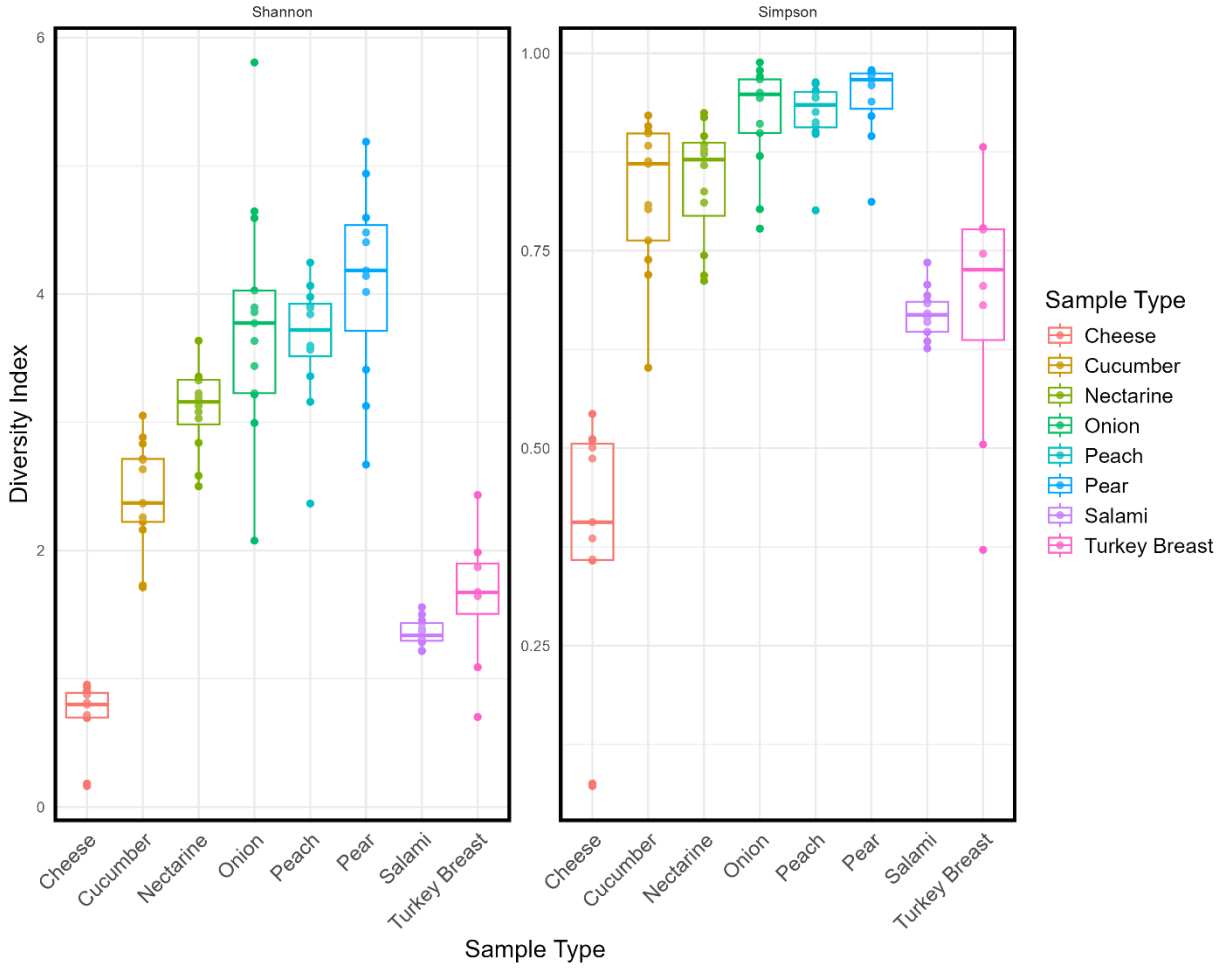

Figure 3.1. Shannon and Simpson alpha diversity indices across food product types, displayed as boxplots showing the median, first and third quartiles, with whiskers representing the minimum and maximum values.

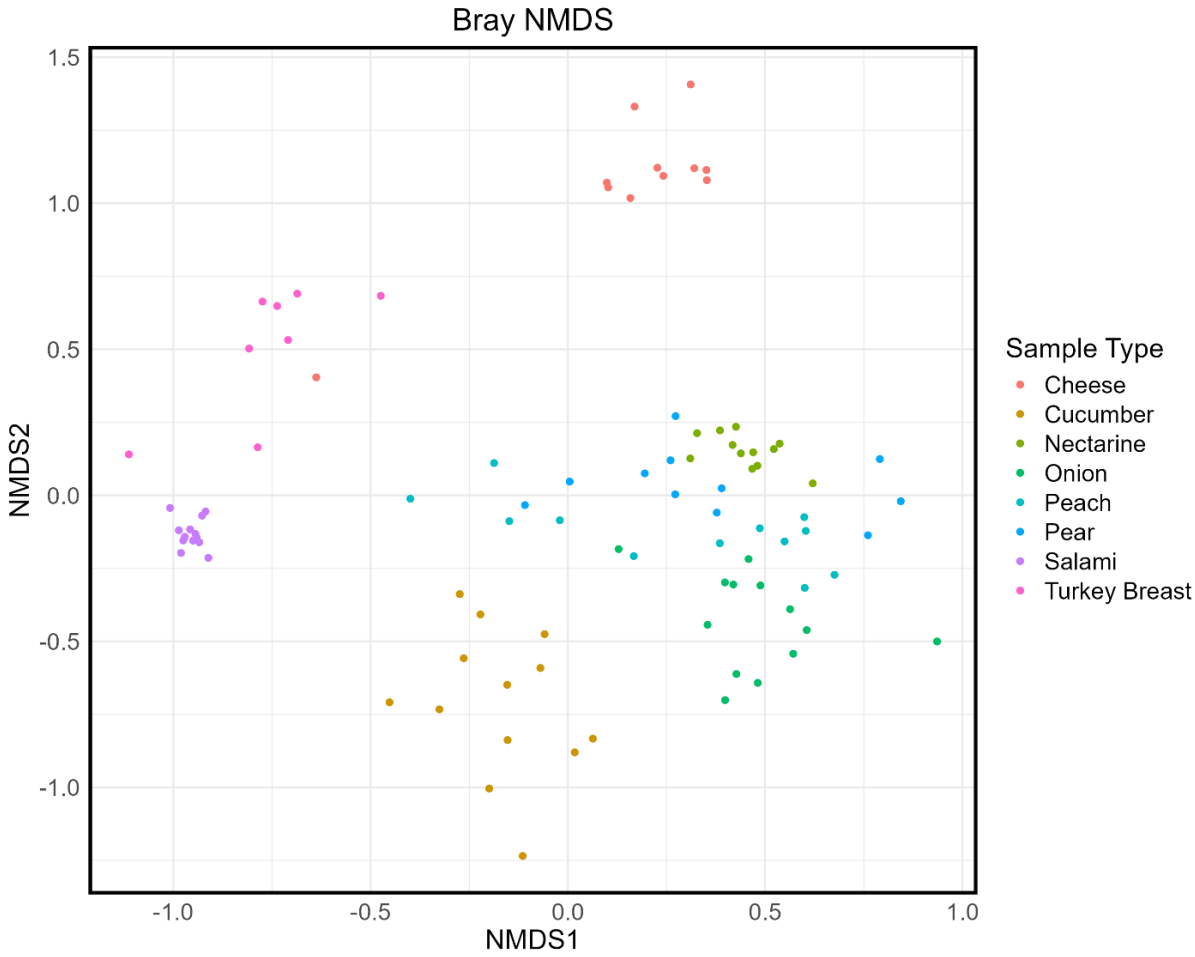

Figure 3.2. Non-metric multidimensional scaling (NMDS) ordination of beta diversity analysis based on Bray-Curtis dissimilarity.

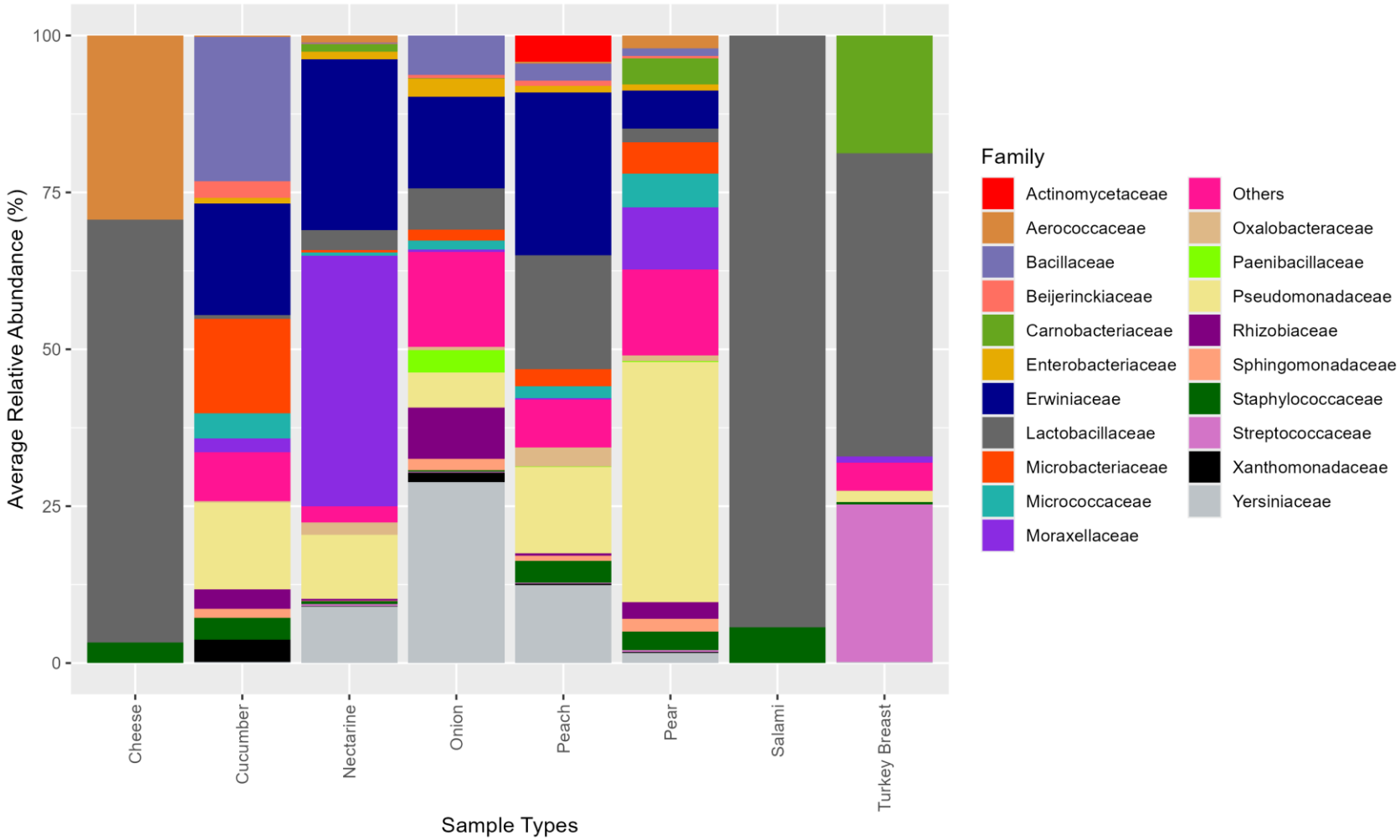

Figure 3.3. Average relative abundance of the top 20 bacterial families within each food product type, with "others" representing the remaining identified families.

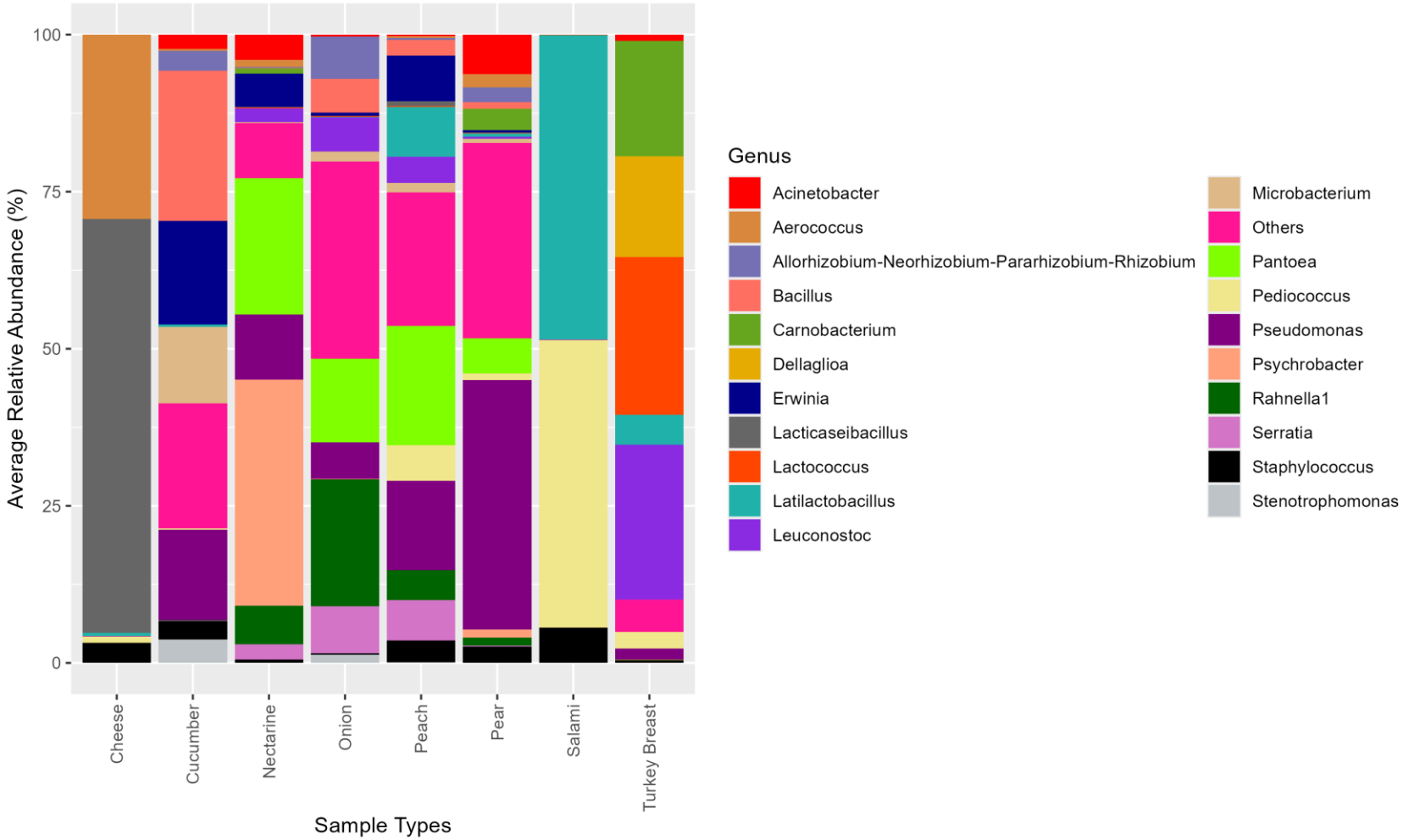

Figure 3.4. Average relative abundance of the top 20 bacterial genera within each food product type, with "others" representing the remaining identified genera.

CHAPTER 4

INTERACTIONS AND INACTIVATION OF *SALMONELLA* TYPHIMURIUM AND *LISTERIA MONOCYTOGENES* IN BIOFILMS WITH DOMINANT GENERA OF RETAIL ENVIRONMENTS[2]

[2] Pal, A., Amy, M., Parra, A., Olszewska, M. A., & den Bakker, H. C. To be submitted to a peer-reviewed journal (*Food Control*).

Abstract

The retail food environment may present food safety risks by enabling foodborne pathogens to coexist with native microbiota on surfaces. Therefore, this study investigated the biofilm formation, structural variability, and susceptibility to quaternary ammonium compounds (QUATS) in various biofilms involving retail-derived bacteria (*Serratia liquefaciens* HDI-166 and *Pseudomonas simiae* HDI-178) and foodborne pathogens (*Salmonella* Typhimurium strain 96037-1 and *Listeria monocytogenes* strain 2011L-2626). The study tested monomicrobial, binary (one retail bacterium + one foodborne pathogen), and ternary (both retail bacteria + one foodborne pathogen) biofilms. After biofilm formation, one set was left untreated, while others were exposed to 50 ppm or 200 ppm QUATS for 2 minutes. Untreated biofilms were also observed using confocal laser scanning microscopy after cultivation in 8-well chamber slides. In monomicrobial biofilms, *S. liquefaciens* exhibited the highest biofilm-forming ability, while *L. monocytogenes* was most susceptible to QUATS, with a mean log reduction of 3.83. In binary biofilms, *L. monocytogenes* demonstrated reduced QUATS susceptibility, with log reductions ranging between 1.84 to 2.01. This decreased susceptibility may be attributed to *Listeria's* localization within the biofilm and the protective role of dead cells in lowering disinfection efficacy. *S.* Typhimurium had decreased biofilm formation in mixed-species biofilms, likely due to competitive interactions with *S. liquefaciens* and *P. simiae*. Under confocal microscope, *P. simiae* and *S.* Typhimurium were observed to form distinct microcolonies in monomicrobial biofilms, whereas *L. monocytogenes* monomicrobial biofilms were thin. Overall, this study underscores the varied biofilm-forming capabilities and interspecies interactions in mixed biofilms, which significantly affect their growth, QUATS susceptibility, and spatial organization.

Keywords: Biofilm, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Serratia liquefaciens*, *Pseudomonas simiae*, Retail, Quaternary ammonium compounds

Introduction

Biofilms are matrix-encased microbial communities, composed of one or more species of bacteria, which represent the dominant form of microbial life. Biofilms or biofilm-like structures are essential for the growth and survival of microorganisms in adverse environments, as they facilitate access to nutrients and help the removal of metabolites while limiting the penetration of biocides into deeper layers of the biofilm (Álvarez-Ordóñez and Briandet, 2016). In the food industry, biofilms are of particular concern because they act as reservoirs for pathogenic microorganisms. Insufficient hygiene or ineffective disinfection in the food processing or retail facility can lead to the colonization of working surfaces by pathogens, facilitating their transmission to food (Coughlan et al., 2016). The inability to effectively eliminate biofilms, especially with traditional treatments like antimicrobials, poses a challenge to ensuring food safety. Studies have consistently shown that biofilms exhibit significantly higher tolerance to antimicrobial agents compared to their planktonic counterparts. For example, *Salmonella* isolates in biofilm form demonstrated greater tolerance to peroxyacetic acid and acidified hypochlorite than their planktonic forms (Chylkova et al., 2017). Similarly, *Pseudomonas* isolates display enhanced resistance to peracetic acid-based disinfectants in biofilms. Moreover, when *Pseudomonas* and *Listeria* co-exist in mixed biofilms, *Pseudomonas* provides protection to *Listeria* from peracetic acid-based disinfectants (Thomassen et al., 2023). The nature of this protection, however, is still unclear and requires further investigation.

Two major foodborne pathogens, *Salmonella* spp. and *L. monocytogenes*, are recognized for their ability to attach to surfaces and form biofilms, frequently leading to recurrent contamination and foodborne illness outbreaks (Bai et al., 2021; da Silva and De Martinis, 2013; Steenackers et al., 2012). According to the Centers for Disease Control and Prevention (CDC),

Salmonella causes approximately 1.35 million infections and 420 deaths per year, while *Listeria* leads to around 1,600 infections and 260 deaths (CDC 2024a, 2024b). Their biofilms are often composed of bacteria from diverse genetic backgrounds, forming complex consortia where both cooperative and competitive relationships shape the population's structure and function (Giaouris et al., 2015). There is increasing interest in studying mixed-species biofilms, as they provide a more accurate representation of the complexity of naturally occurring biofilms, particularly in food processing and retail environments. More specifically, in recent years, polymicrobial biofilms composed of pathogens and resident microbiota of food environments have received much attention (Rolon et al., 2024). Studies have shown that certain species of resident microbiota can increase the tolerance of *Listeria monocytogenes* to sanitizers in multi-species biofilms (Voloshchuk et al., 2025). Additionally, the presence or absence of pathogen can influence resident microbial communities in food environments. For example, microbiome analysis of distribution centers handling fresh produce revealed that *Carnobacterium_A*, *Psychrobacter*, and *Pseudomonas_E* were significantly more abundant in environmental samples that tested positive for *Listeria* compared to *Listeria*-negative samples (Townsend et al., 2023). Similarly, in a study exploring the microbiota of feed environments and their relationship with *Salmonella*, it was found that resident isolates exhibited higher resistance to desiccation and disinfection, as well as greater biofilm-forming ability. Also, when they compared *Salmonella* monomicrobial biofilms to dual-species biofilms formed with resident isolates such as *Staphylococcus* and *Pseudomonas*, the biovolume of the *Salmonella* cells increased by 2.8-fold and 3.2-fold, respectively (Habimana et al., 2010).

Despite these findings, there is still limited knowledge regarding the interactions of *Salmonella* or *Listeria* with bacteria prevalent in retail settings. Biofilms in retail environments

present significant challenges, particularly in ensuring food safety at the final stage of the supply chain. At retail, the persistence of foodborne pathogens has been previously linked to their ability to form biofilms. For instance, a study examining isolates recovered from both food and non-food contact surfaces in 30 retail delicatessens found that persistent strains of *L. monocytogenes* exhibited significantly stronger adhesion compared to non-persistent strains (Wang et al., 2015).

Given these concerns, the objective of this study was to investigate the interactions between retail-derived bacteria (*Serratia liquefaciens* HDI-166 and *Pseudomonas simiae* HDI-178) and foodborne pathogens (*Salmonella* Typhimurium strain 96037-1 and *Listeria monocytogenes* strain 2011L-2626) in various biofilms. Additionally, the study sought to evaluate the efficacy of quaternary ammonium compounds (QUATS), commonly used sanitizers in the food industry, against these biofilms. The retail-derived bacteria were selected based on previous research, which identified these genera as dominant in retail microbiomes (Britton et al., 2023). To achieve this objective, we employed a microtiter plate assay, a commonly used method to grow and study biofilm due to its high-throughput screening capacities (Azeredo et al., 2017). Considering that the complexity and thickness of biofilm layers significantly impact their formation and eradication, we employed confocal laser scanning microscopy (CLSM) to accurately quantify biofilm growth. CLSM is a widely used technique for studying fragile structures by enabling direct, in situ, and non-destructive analysis of biofilms with specific fluorescent markers (Bridier et al., 2010). Also, we used image analysis software to extract detailed quantitative structural parameters from confocal image stacks (Heydorn et al., 2000; Vorregaard, 2008). This offers deeper insights into the biofilm architecture and allows examination of how specific biofilm characteristics relate to pathogen susceptibility to QUATS.

Materials and Methods

Bacterial species

This study focused on different biofilm assemblages using a selection of foodborne pathogens and retail environment isolates. Specifically, two well-known pathogens, *Salmonella enterica* serovar Typhimurium strain 96037-1 and *Listeria monocytogenes* strain 2011L-2626, were studied alongside two environmental isolates, *Serratia liquefaciens* HDI-166 and *Pseudomonas simiae* HDI-178. *S.* Typhimurium and *L. monocytogenes* were sourced from the culture collection at the Center for Food Safety, University of Georgia. *S. liquefaciens* and *P. simiae* were isolated from mushrooms and Italian parsley, respectively, purchased from a retail store in Griffin, Georgia, representing typical retail environment bacteria. Bacterial species identification was carried out via Whole Genome Sequencing on the Illumina MiSeq platform using the Nextera XT Library Prep Kit (Illumina, San Diego, CA, USA). Next, the resulting reads were classified using Sepia 1.1.0 (Den Bakker & Katz, 2021) with GTDB r220 (PMID: 34520557) as a reference database.

Biofilm assemblages

Ten different biofilms were investigated, including monomicrobial biofilms of *S. liquefaciens* (1), *P. simiae* (2), *S.* Typhimurium (3), and *L. monocytogenes* (4); binary biofilms of *S. liquefaciens* × *S.* Typhimurium (5), *S. liquefaciens* × *L. monocytogenes* (6), *P. simiae* × *S.* Typhimurium (7), and *P. simiae* × *L. monocytogenes* (8); and ternary biofilms of *S. liquefaciens* × *P. simiae* × *S.* Typhimurium (9) and *S. liquefaciens* × *P. simiae* × *L. monocytogenes* (10). Monomicrobial biofilms were initiated with an inoculum concentration of approximately 6 log CFU/mL. For mixed biofilms, including both binary and ternary biofilms, the respective

bacterial inoculum were combined in equal volumes to create a mixed inoculum, which was then used to initiate the formation of polymicrobial biofilms.

Bacterial inoculum preparation

Bacterial inocula were prepared by transferring a loopful of each bacterial species from their respective stock cultures into 10 mL of tryptic soy broth (TSB) (BBL™, Becton, Dickinson and Company, Sparks, MD, USA) in individual tubes. The cultures were incubated at 37 °C for 24 hours, except for *P. simiae*, which was incubated at 25 °C for 24 hours. Following initial incubation, the cultures were transferred into fresh TSB and incubated under the same conditions to obtain the final working cultures.

Post-incubation, the bacterial inocula were diluted to the desired concentrations and spread-plated on selective media to verify growth and ensure the target inoculum concentration for biofilm formation. The selective media and incubation conditions were as follows: *S.* Typhimurium was plated on Xylose Lysine Tergitol-4 agar (Neogen® Culture Media, Lansing, MI, USA) and incubated at 37 °C for 24 hours; *L. monocytogenes* on Modified Oxford Agar (Neogen® Culture Media, Lansing, MI, USA) and incubated at 37 °C for 24 to 48 hours; *S. liquefaciens* on Chromagar Serratia (CHROMagar, Paris, France) and incubated at 37 °C for 24 hours; and *P. simiae* on *Pseudomonas* Isolation Agar (Neogen® Culture Media, Lansing, MI, USA) and incubated at 25 °C for 24 to 48 hours.

Biofilm formation and treatment using quaternary ammonium compounds in polystyrene microtiter plates

Biofilms were grown in 96-well polystyrene microtiter plates (Corning Inc., Kennebunk, ME, USA) to study the growth of individual bacterial species and to conduct sanitizer testing with QUATS against these species in their respective biofilm assemblages. The experimental

protocol for biofilm formation and sanitizer treatment was adapted with modifications from Olszewska and Dies-Gonzalez (2021). Each biofilm type was prepared in triplicate wells, with one set serving as control (untreated) and the other two sets treated with 50 and 200 ppm of the QUATS (Professional Lysol® No Rinse Sanitizer, Lysol, Parsippany, NJ, USA). The QUATS used in this study contained Alkyl (C_{14} 50%, C_{12} 40%, C_{16} 10%) dimethyl benzyl ammonium chloride as the active ingredient. The QUATS was diluted to 50 or 200 ppm following the manufacturer's instructions provided on the product label. The QUATS sanitizer concentration was further confirmed using QAC dual range test strips (LaMotte Company Inc., Newark, NJ, USA).

Biofilm formation was initiated by transferring 200 µL of each bacterial inoculum into the individual wells, followed by static incubation at 25 °C for 3 hours to facilitate initial attachment. The wells were then washed with 200 µL of phosphate-buffered saline (PBS) (Fisher Scientific, Fair Lawn, NJ, USA) and replenished with 200 µL of TSB, followed by further incubation at 25 °C for 24 hours to allow biofilm development. Post-incubation, the biofilms were washed with PBS and subjected to either control or QUATS treatment. For the control group, 200 µL of PBS was added to each well, incubated at room temperature for 2 minutes, and then replaced with 200 µL of Dey/Engley broth (General Laboratory Products, Yorkville, IL, USA) for 5 minutes. In the treatment groups, 200 µL of 50 or 200 ppm QUATS was added to each well, and incubated for 2 minutes at room temperature, followed by the addition of Dey/Engley broth as described for the control. After treatment, each well was emptied and washed twice with 200 µL of PBS, and then biofilms were scraped with pipette tip, serially diluted in PBS, and plated on the respective agar media as specified above in section 2.3.

Microbial counts were recorded for each bacterial species within their respective biofilms, and the experiment was repeated 3 times.

Confocal laser scanning microscopy analysis of untreated biofilm

Untreated biofilms were observed using confocal laser scanning microscopy, following a modified version of the method described by Olszewska and Dies-Gonzalez (2021). Biofilms were cultivated in 8-well chamber slides (LAB-TEK® Brand Products, Rochester, NY, USA). Each well was inoculated with 400 µL of a bacterial inoculum and incubated at 25 °C for 3 hours to facilitate initial attachment. After incubation, the inoculum was removed, and each well was washed with 400 µL of 0.85 % NaCl solution to remove the unattached cells. Subsequently, 400 µL of TSB was added to each well, and the chamber slides were incubated at 25 °C for 24 hours. At the end of the incubation period, each well was emptied and washed again with 400 µL of 0.85 % NaCl solution.

Following biofilm cultivation, the biofilms were stained with Syto 9 and propidium iodide dyes (LIVE/DEAD™ BacLight™ Bacterial viability kit, Life Technologies Corporation, Eugene, OR, USA) to differentiate live and dead cells. Specifically, biofilms were stained with 6 µM Syto 9, which emits green fluorescence for live cells, and 30 µM propidium iodide, which emits red fluorescence for dead cells, in 400 µL of deionized water. The chamber slides were incubated in the dark at room temperature for 30 minutes, followed by three washes of each well with 200 µL of 0.85 % NaCl solution. After washing, the chambers were carefully removed from the slides. Sterile saline was applied to the biofilms, which were separated by a gasket, and coverslips were placed on top and sealed with mounting oil and nail polish. The prepared slides were then stored overnight at 4 °C before imaging.

Imaging of the biofilms was performed using a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Fluorescence was detected within the ranges of 500–600 nm for the green channel and 610–710 nm for the red channel. Z-stack images were captured to visualize the biofilms, and the resulting images were analyzed using Comstat 2.1 software (www.comstat.dk), which provided quantitative measurements of structural parameters including biomass, maximum thickness, roughness coefficient, and surface-to-volume ratio) (Heydorn et al., 2000; Vorregaard, 2008). The entire experiment was repeated three times.

Statistical analysis

Microbial counts (log CFU/well) and image-derived structural parameters (biomass, maximum thickness, roughness coefficient, and surface-to-volume ratio) were analyzed using one-way ANOVA with a significance level of $\alpha = 0.05$, followed by Duncan's post hoc test or Dunnett's multiple comparisons test for mean separation. The minimum detection limit for microbial counts was 0.69 log CFU/well. All statistical analyses were performed using R software version 4.2.3 (R Core Team, 2024). The efficacy of QUATS treatment was evaluated by calculating log reductions, comparing microbial counts between treated and untreated biofilms.

Results

Analysis of biofilm formation ability and QUATS susceptibility among bacterial species in monomicrobial and mixed biofilm

Figure 4.1 illustrates the biofilm-forming capacities (viable counts, log CFU/well) of *S. liquefaciens*, *P. simiae*, *S.* Typhimurium, and *L. monocytogenes* in monomicrobial biofilms, along with the observed log reductions following treatment with 50–200 ppm QUATS. A

significant variation in biofilm formation among the species was detected. *S. liquefaciens* exhibited the highest biofilm-forming ability, with a mean value of 7.15 log CFU/well, while *L. monocytogenes* and *P. simiae* showed the lowest capacities, with values of 5.68 and 5.87 log CFU/well, respectively. *S.* Typhimurium displayed an intermediate capacity, averaging 6.27 log CFU/well.

Furthermore, significant differences in bacterial susceptibility to QUATS were observed across monomicrobial biofilms after treatment. *L. monocytogenes* exhibited the highest susceptibility, with a mean reduction of 3.83 log, indicating QUATS' pronounced efficacy against *L. monocytogenes* monomicrobial biofilms. In contrast, *S.* Typhimurium showed the lowest susceptibility, with a reduction of only 1.04 log, suggesting relative tolerance to QUATS treatment. *S. liquefaciens* and *P. simiae* demonstrated intermediate reductions of 2.27 and 2.53 log, respectively.

Figure 4.2 illustrates the bacterial loads and reductions after treatment with 50–200 ppm QUATS in biofilms containing *L. monocytogenes* as a companion microorganism. The biofilm-forming ability of *S. liquefaciens* remained consistent when paired with *L. monocytogenes* in binary (*S. liquefaciens* × *L. monocytogenes*) and ternary (*S. liquefaciens* × *P. simiae* × *L. monocytogenes*) biofilms, showing stable average counts of 6.33 log CFU/well. Similarly, the biofilm-forming capacity of *P. simiae* was largely unaffected by the presence of *L. monocytogenes* in the *P. simiae* × *L. monocytogenes* (5.61 log CFU/well) and *S. liquefaciens* × *P. simiae* × *L. monocytogenes* (5.50 log CFU/well) biofilms. In direct comparisons, *S. liquefaciens* exhibited significantly higher counts than *L. monocytogenes* in the *S. liquefaciens* × *L. monocytogenes* biofilms, suggesting that *S. liquefaciens* outcompeted *L. monocytogenes* in these binary biofilms. However, this competitive advantage was not observed in the *P. simiae* ×

L. monocytogenes or *S. liquefaciens* × *P. simiae* × *L. monocytogenes* biofilms. Lastly, the biofilm-forming ability of *L. monocytogenes* did not significantly differ when present in binary or ternary biofilms compared to monomicrobial biofilms, with mean viable counts ranging from 4.52 to 5.68 log CFU/well.

Data on biofilm reduction involving *L. monocytogenes* as a companion (Figure 4.2) showed that the presence of *L. monocytogenes* had no significant impact on the reduction of *S. liquefaciens*; although the calculated log reductions were slightly lower. The mean log reductions were 1.70 for the *S. liquefaciens* × *L. monocytogenes* biofilms and 1.76 for the *S. liquefaciens* × *P. simiae* × *L. monocytogenes* biofilms, while a reduction of 2.27 log was observed for *S. liquefaciens* in monomicrobial biofilms. A similar pattern was observed for *P. simiae*, which showed reductions ranging from 2.47 to 2.53 log in both monomicrobial and *Listeria*-containing biofilms. Notably, *L. monocytogenes* was less susceptible to QUATS when in binary biofilms with *S. liquefaciens* (2.01 log reduction) and *P. simiae* (1.84 log reduction), compared to a 3.83 log reduction observed in its monomicrobial biofilms.

Figure 4.3 illustrates the bacterial loads and reductions after biofilm treatment with 50–200 ppm QUATS in biofilms containing *S.* Typhimurium as a companion microorganism. The biofilm-forming ability of *S. liquefaciens* was significantly reduced in the *S. liquefaciens* × *S.* Typhimurium biofilm, averaging 5.42 log CFU/well. However, *S. liquefaciens* counts did not differ significantly from monomicrobial levels when in the ternary (*S. liquefaciens* × *P. simiae* × *S.* Typhimurium) biofilms. The biofilm-forming capacity of *P. simiae* seemed less affected by the presence of *S.* Typhimurium, with counts of 4.62 log CFU/well in the *P. simiae* × *S.* Typhimurium biofilms and 5.25 log CFU/well in the *S. liquefaciens* × *P. simiae* × *S.* Typhimurium biofilms. Lastly, we observed that the biofilm-forming ability of *S.* Typhimurium

was significantly reduced when mixed with other bacteria compared to its monomicrobial biofilm growth, with mean viable counts ranging from 4.94 to 6.27 log CFU/well.

Data on biofilm reduction involving *S.* Typhimurium as a companion indicated that its presence did not significantly affect the reduction of *S. liquefaciens*. However, the mean log reductions were 1.07 for the *S. liquefaciens* × *S.* Typhimurium biofilms and 1.98 for the *S. liquefaciens* × *P. simiae* × *S.* Typhimurium biofilms, compared to a reduction of 2.27 log observed for *Serratia* in monomicrobial biofilms. A similar trend was noted for *P. simiae,* indicating a somewhat neutral relationship of *S.* Typhimurium with both retail-derived microorganisms. Additionally, *S.* Typhimurium reduction did not show significant differences between its monomicrobial biofilms and binary or ternary biofilms. Although non-significant, a slight increase in the reduction of the pathogen was observed, likely due to the negative impact of resident microorganisms on the pathogen.

Characterization of biofilm architecture based on quantitative and qualitative insights from confocal microscopy images

Figures 4.4 and 4.5 provide a quantitative analysis of four key biofilm structural parameters—biomass, maximum thickness, roughness coefficient, and surface-to-volume ratio—evaluated separately for live-cell populations (Figure 4.4) and dead-cell populations (Figure 4.5).

Among live-cell populations (Figure 4.4, Column A), *S. liquefaciens* (3.8 $\mu m^3/\mu m^2$) and *L. monocytogenes* (3.4 $\mu m^3/\mu m^2$) monomicrobial biofilms exhibited significantly higher biomass compared to *P. simiae* and *S.* Typhimurium. Maximum thickness was highest for *S. liquefaciens* (15.3 µm). Roughness coefficients were notably higher for *P. simiae* (1.3) and *S.* Typhimurium (1.0), and the surface-to-volume ratio was highest for *S.* Typhimurium (3.5 $\mu m^2/\mu m^3$) and lowest for *S. liquefaciens* and *L. monocytogenes*.

L. monocytogenes biofilms exhibited distinct structural variations depending on the biofilm type (Figure 4.4, Column B). Biomass was lowest in binary biofilms with *P. simiae* (1.6 $\mu m^3/\mu m^2$) compared to other biofilms. Maximum thickness was significantly greater in binary biofilms with *S. liquefaciens* (12.9 µm). The roughness coefficient remained consistent across all *L. monocytogenes* biofilms and surface-to-volume ratio increased in binary biofilms of *L. monocytogenes* compared to its monomicrobial or ternary biofilms. Distinct differences were also observed when comparing *S.* Typhimurium monomicrobial biofilms with binary and ternary biofilms (Figure 4.4, Column C). The highest biomass was observed in binary biofilms of *P. simiae* × *S.* Typhimurium (4.6 $\mu m^3/\mu m^2$) and ternary biofilms of *S. liquefaciens* × *P. simiae* × *S.* Typhimurium (3.6 $\mu m^3/\mu m^2$). Roughness coefficient decreased significantly when *Salmonella* Typhimurium was present in mixed biofilms with *P. simiae* (0.4) or *S. liquefaciens* × *P. simiae* (0.3). Lastly, *S.* Typhimurium monomicrobial biofilms exhibited the highest surface-to-volume ratios compared to others.

For dead-cell populations, significant variations in the four parameters were observed among the monomicrobial biofilms of the tested bacterial species (Figure 4.5, Column A). Biomass was highest for *S. liquefaciens* (4.5 $\mu m^3/\mu m^2$). Maximum thickness was lowest for *L. monocytogenes* (5.4 µm) monomicrobial biofilms. Both the roughness coefficient and surface-to-volume ratio were significantly higher in *S.* Typhimurium and *L. monocytogenes* monomicrobial biofilms compared to the other bacterial species.

The presence of *L. monocytogenes* in mixed biofilms led to an increase in biomass, ranging from 3.2 $\mu m^3/\mu m^2$ to 4.9 $\mu m^3/\mu m^2$, in both binary and ternary biofilms compared to its monomicrobial biofilms (Figure 4.5, Column B). Maximum thickness was highest in the *S. liquefaciens* × *L. monocytogenes* biofilms (12.9 µm) compared to other *L. monocytogenes*-

containing biofilms. In contrast, both the roughness coefficient and surface-to-volume ratio decreased when *L. monocytogenes* were incorporated into mixed biofilms compared to its monomicrobial biofilms, being the lowest for the ternary biofilms. In *S.* Typhimurium biofilms (Figure 4.5, Column C), biomass increased in mixed biofilms, except for the combination with *P. simiae*, compared to its monomicrobial biofilms. Maximum thickness remained unaffected by the presence of other bacterial species. The roughness coefficient of mixed biofilms containing *S.* Typhimurium was significantly lower (ranging from 0.3 µm to 1.5 µm) than its monomicrobial biofilms, being the lowest in *P. simiae* × *S.* Typhimurium and *S. liquefaciens* × *P. simiae* × *S.* Typhimurium. Similarly, the surface-to-volume ratio was lowest when *S.* Typhimurium was in binary biofilms with *P. simiae* (2.1 $\mu m^2/\mu m^3$) and in ternary biofilms (1.4 $\mu m^2/\mu m^3$).

Furthermore, qualitative insights into biofilm structure were provided by the confocal microscopy images (Figure 4.6), offering a visual representation of the three-dimensional architecture of representative biofilms. Monomicrobial biofilms of *S. liquefaciens* appeared dense and thick, featuring evenly distributed live and dead cells within the biofilm matrix, along with larger gatherings of live cells. This is consistent with having the highest biomass and maximum thickness compared to others. In contrast, *P. simiae* and *S.* Typhimurium monomicrobial biofilms exhibited distinct microcolonies, correlating with the higher roughness coefficient values observed for these biofilms in live cell measurements. *L. monocytogenes* formed biofilms that were more uniform, thinner, and predominantly composed of live cells seldom decorated with dead cells, as confirmed by the thickness parameters. The binary biofilms of *S. liquefaciens* and *L. monocytogenes* were both thick and dense. While live cells comprised the majority of the structure, an increased proportion of dead cells was noted, which corresponds with the observed increase in maximum thickness. Finally, the binary biofilms of *P. simiae* and

S. Typhimurium were densely populated with live cells along with an even distribution of dead cells.

Discussion

In this study, we investigated the interactions between retail-derived bacteria (*S. liquefaciens* and *P. simiae*) and foodborne pathogens (*S.* Typhimurium and *L. monocytogenes*) in different biofilms and examined biofilm structures using CLSM to gain insights into their architecture. We also evaluated the efficacy of QUATS against various biofilms, which allowed us to speculate on how specific characteristics of biofilms relate to the susceptibility of pathogens to sanitizer.

Biofilm formation in monomicrobial conditions revealed significant variability among species, with *S. liquefaciens* demonstrating the highest biofilm-forming ability and *L.* monocytogenes the lowest. Previous studies have reported significant differences in biofilm-forming abilities among bacterial species. For instance, bacteria isolated from a meat processing environment, representing eleven different species, demonstrated varying levels of biofilm formation. A *Psychrobacter* spp. isolate was the weakest biofilm producer, with counts of 5.4 log CFU/cm², while a *Microbacterium* spp. isolate demonstrated the strongest biofilm-forming capacity, with counts of 8.7 log CFU/cm², after 10 days of growth at 7 °C on stainless steel (Wagner et al., 2021). In another study, *Listeria monocytogenes*, *Serratia liquefaciens*, *Shigella boydii*, *Staphylococcus aureus*, *Salmonella* Enteritidis, and *Bacillus cereus* were compared for biofilm formation using crystal violet method. It was found that *L. monocytogenes* and *S. liquefaciens* were strong biofilm formers (Xu et al., 2011). Biofilm formation can also vary significantly within a single species. For example, strains of *L. monocytogenes* show

considerable differences in their biofilm-forming abilities based on the crystal violet assay (Yang et al., 2024).

Remarkably, the *L. monocytogenes* 2011L-2626 strain, one of the cantaloupe outbreak strains, was identified as a poor biofilm producer using crystal violet assay, similar to the findings in our study (Olszewska and Diez-Gonzalez, 2021). However, strains of *L. monocytogenes* from the 2011 cantaloupe outbreak also displayed strong adhesion, survival, and growth on cantaloupe surfaces, mainly on rind (Martinez et al., 2011), suggesting that specific environmental or surface conditions can promote more robust biofilm formation. In general, the differences in biofilm formation among bacterial species or strains can be attributed to several factors, including surface adhesion molecules associated with the bacteria, the characteristics of the attachment surface, gene regulation, quorum sensing, and environmental conditions (Zhao et al., 2017). Furthermore, the variation in biofilm formation observed across studies may also be attributed to differences in experimental conditions, including the assay methods used for quantifying biofilm formation and the specific environmental factors applied, even when the same bacterial species or strains are investigated.

In mixed-species biofilms, distinctive interactions between the pathogenic and retail-derived bacterial species were observed. Generally, synergistic interactions contribute to enhanced biofilm formation and increased cell density. In contrast, indifferent or neutral interactions do not affect biofilm production or cell counts, while antagonistic interactions may result in the inhibition of growth for one or more species (Teixeira-Santos et al., 2024). *P. simiae* and especially *S. liquefaciens* exhibited reduced biofilm cell counts when co-cultured with *S.* Typhimurium. Importantly, *S.* Typhimurium demonstrated significantly reduced biofilm cell counts in the presence of both these retail-derived microorganisms, whether introduced

individually or together. Also, we observed *S. liquefaciens* exhibited dominance in binary biofilms with *L. monocytogenes*, as indicated by significantly higher cell counts, a trend not observed in other mixed biofilms involving *L. monocytogenes*. These findings suggest that interspecies interactions within these biofilms negatively impact the pathogen's viability and biofilm formation abilities. Consistent with our results, Visvalingam et al. (2019) reported that *Serratia* spp. isolated from a beef processing plant environment exhibited antagonistic activity against *S.* Typhimurium. *Serratia* isolates were able to decrease *S.* Typhimurium biofilm development in dual-species biofilms and even reduce its population in planktonic co-cultures. Moreover, another study demonstrated that antagonistic interactions between *Salmonella* spp. and *Pseudomonas aeruginosa* led to lower planktonic population levels of *Salmonella* spp. in co-culture compared to mono-species cultures, as well as reduced *Salmonella* spp. density in dual-species biofilms compared to its mono-species biofilms (Pang et al., 2017). Common competitive interactions typically include competition for limited resources such as nutrients, oxygen, and available space for colonization (Giaouris et al., 2015). Additionally, species may compete by producing compounds (e.g., bacteriocins, organic acids, biosurfactants, enzymes) that inhibit the growth of other species, disrupt their attachment, or even promote the detachment of cells from biofilm structures (Rendueles and Ghigo, 2012).

The sanitizer treatments revealed significant differences in QUATS susceptibility among the tested bacterial species, with *L. monocytogenes* exhibiting the highest susceptibility in monomicrobial biofilms, while *S.* Typhimurium showed the lowest. This is consistent with previous studies demonstrating that QUATS exhibit stronger antimicrobial activity against Gram-positive bacteria, such as *L. monocytogenes*, compared to Gram-negative bacteria (Parish et al., 2003). Furthermore, a study by Kocot and Olszewska (2020) reported that *Listeria*

monomicrobial biofilms exhibit greater susceptibility to QUATS than to tertiary alkyl amines or chlorine. A different study also indicates that repeated exposure to QUATS can enhance disinfectant tolerance of *L. monocytogenes* biofilms, highlighting the need for a better understanding of the interactions between *L. monocytogenes* and QUATS (Olszewska et al., 2016). In polymicrobial biofilms, the presence of companion species (*S. liquefaciens* or *P. simiae*) resulted in reduced susceptibility of *L*. monocytogenes to QUATS compared to its monomicrobial biofilms, suggesting potential protective effects. Consistent with our findings, Thomassen et al. (2023) reported that *L. monocytogenes* received enhanced protection from disinfection in mixed biofilms with *Pseudomonas* spp. It was explained that the high disinfectant tolerance of *Pseudomonas* spp. contributed to the increased survival of *L. monocytogenes*. Similarly, Rolon et al. (2024) observed that *L. monocytogenes* in mixed biofilms with environmental microbiota of fruit packing facilities exhibited reduced susceptibility to benzalkonium chloride. They suggested that *Listeria's* localization within multi-species biofilms may aid survival under sanitizer exposure. Additionally, the increased production of extracellular matrix in mixed biofilms may limit the diffusion of chemicals, creating microenvironments with varying sanitizer concentrations that enhance tolerance. The protective effect of the extracellular matrix was observed in *L. monocytogenes* when co-cultured with *Lactobacillus* spp. in binary biofilms, enhancing its resistance to chlorine treatment (Olszewska and Diez-Gonzalez, 2021).

Quantitative analysis of biofilm structural parameters, coupled with visual observation of representative confocal images, provided valuable insights into the complexity and spatial organization of biofilms. Among the tested species, monomicrobial biofilms of *S. liquefaciens* exhibited the highest biomass and maximum thickness values. Principal Component Analysis (PCA) further supported these findings, with the loading scatterplot showing a strong association

between the biomass parameter of live cells and *S. liquefaciens* monomicrobial biofilms (Figure 4.7). This relation is also consistent with the highest *Serratia's* monomicrobial biofilms cell counts observed in microtiter plate assays, reinforcing the association between biofilm structure and bacterial density. These results highlight the *Serratia* 's distinctive ability to produce biofilms and thrive on abiotic surfaces. Previous studies have highlighted that food facility environments serve as a major reservoir for *Serratia* species. For instance, Xu et al. (2024) identified *Serratia proteamaculans* in microbiome samples collected from both food-contact and non-food-contact surfaces within the packaging area of a meat processing facility. Their study further demonstrated the biofilm-forming potential of *Serratia* species when co-cultured with other microbiome species isolated from the same surfaces. The co-culture experiments revealed the formation of robust mixed-species biofilms, highlighting synergistic interactions between *Serratia* and other microbiome species. This finding underscores the critical role of *Serratia* in facilitating microbial interactions, enhancing biofilm complexity, and contributing to the persistence of pathogens on environmental surfaces within the food continuum. This aligns with our findings when *S. liquefaciens* was co-cultured with *L. monocytogenes*. Specifically, *L. monocytogenes* alone exhibited the lowest maximum thickness and appeared thinner compared to monomicrobial biofilms of other tested bacterial species under the confocal microscope, which is consistent with its lowest biofilm cell counts. However, while with *S. liquefaciens* an increase in maximum thickness was observed for both live and dead cells. It was further revealed by PCA (Figure 4.7) that the maximum thickness for dead cells was highly associated with binary biofilms of *L. monocytogenes* with *S. liquefaciens*. These findings suggest the protective effect from dead cells towards the *L. monocytogenes* when co-existing with *S. liquefaciens*, and that the maximum thickness may be a parameter for predicting protection level to QUATS. The

increased thickness most likely impedes sanitizer penetration. Interestingly, a previous study by Barros et al. (2024) highlighted the role of dead cells in biofilm formation, showing that inocula containing dead cells promote the development of biofilms with higher cell culturability, cellular energy, and metabolic activity compared to biofilms formed with live cells alone. Additionally, biofilms with higher proportions of dead cells (up to 99.99%) tend to be more cohesive and compact, exhibiting higher total extracellular polymeric substance content (Barros et al., 2024). The reduced antimicrobial penetration through the extracellular polymeric substance is one of the key mechanisms by which biofilms resist antimicrobial agents (Yasir et al., 2018).

The interaction between *P. simiae* and *L. monocytogenes* warrants further investigation, as it resulted in enhanced protection of *L. monocytogenes* against QUATS. However, no increase in maximum thickness or biomass was observed, and therefore, *P. simiae* × *L. monocytogenes* did not associate with either of these parameters (Figure 4.7). Despite this, *P. simiae* and *P. simiae* × *L. monocytogenes* biofilms were associated with roughness and surface-to-volume ratio from live cells. A potential possible explanation for the protective effect in these biofilms (*P. simiae* × *L. monocytogenes*) could be the spatial arrangement of the species, with *L. monocytogenes* occupying the bottom layers and *P. simiae*, being an aerobic species, residing at the top. The favorable arrangement may contribute to the pathogen's protection. Previous studies have shown that multispecies biofilms are often spatially structured, with obligate aerobic species located at the surface and facultative aerobic or anaerobic species inhabiting the deeper layers of the biofilms (Elias and Banin, 2012; Nadell et al., 2016).

Monomicrobial biofilms of *P. simiae* and *S.* Typhimurium demonstrated higher roughness coefficients and surface-to-volume ratios compared to other monomicrobial biofilms, with confocal images revealing distinct microcolonies. The higher surface-to-volume ratio

suggests a larger exposed surface area for nutrient exchange, promoting more efficient nutrient and waste flow (Heydorn et al., 2000). This may encourage more robust bacterial growth within the biofilm. However, it is uncertain whether this translates into an increased area of interaction with potential sanitizers.

Despite observing an increase in biomass and a decrease in roughness and surface-to-volume ratio in *P. simiae* × *S.* Typhimurium biofilms compared to *S.* Typhimurium monomicrobial biofilms, no improvement in QUATS tolerance was seen for *S.* Typhimurium. Achieving this may be challenging, considering that *S.* Typhimurium monomicrobial biofilms already exhibit substantial tolerance to this sanitizer. Nonetheless, the structural outcome for *P. simiae* × *S.* Typhimurium indicates that these *Salmonella*-containing biofilms have a more compact structure, potentially due to interspecies interactions that facilitate denser packing or modify the composition of the extracellular matrix. In contrast, *P. simiae* or *S. liquefaciens* may benefit from their association with *S.* Typhimurium, as a decrease in QUATS susceptibility was observed compared to their respective monomicrobial biofilms, although this effect was not statistically significant.

Overall, based on our findings, it is recommended that cleaning and disinfection protocols in retail environments should not be exclusively targeted at foodborne pathogens. Consideration must also be given to resident microorganisms capable of biofilm formation, as these biofilms can confer protection to pathogens against disinfection.

Conclusion

This study investigated biofilm formation, structural variability, and QUATS susceptibility within monomicrobial and mixed-species biofilms involving retail-derived bacteria (*Serratia liquefaciens* HDI-166 and *Pseudomonas simiae* HDI-178) and foodborne pathogens

(*Salmonella* Typhimurium strain 96037-1 and *Listeria monocytogenes* strain 2011L-2626). Significant variations were observed in biofilm-forming capacities among bacterial species in monomicrobial biofilms, with *S. liquefaciens* exhibiting robust biofilm production (Figure 4.1). *L. monocytogenes* monomicrobial biofilms were most susceptible to QUATS treatment compared to monomicrobial biofilms of other bacterial species (Figure 4.1). In mixed biofilms, interspecies interactions significantly influenced biofilm formation, with competitive effects observed for *S.* Typhimurium when co-cultured with *S. liquefaciens* and *P. simiae* whether individually or together (Figure 4.3). Notably, *L. monocytogenes* had enhanced protection against QUATS when present in binary biofilms along with *S. liquefaciens* or *P. simiae* (Figure 4.2), emphasizing the impact of microbial interactions that translate into spatial organization (localization) within biofilm and the protective role of dead cells on disinfection efficacy. Structural analysis using confocal microscopy revealed distinct biofilm architectures of bacteria species (Figure 4.4 and 4.5). Monomicrobial *S. liquefaciens* biofilms demonstrated substantial biomass and thickness, while *L. monocytogenes* monomicrobial biofilms were comparatively thinner. Overall, these findings contribute to a deeper understanding of microbial interactions and biofilm tolerance mechanisms to QUATS, which are critical for designing more effective sanitation protocols. Addressing both foodborne pathogens and resident microbiota in retail environments is essential to mitigating biofilm-associated food safety risks.

Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work the authors used ChatGPT, version GPT-4o, in order to enhance readability and language. After using this technology, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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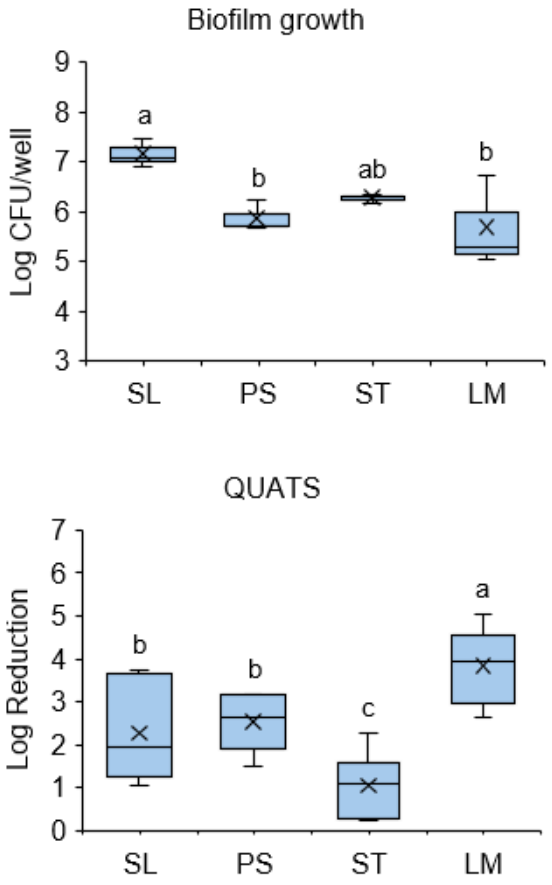

Figure 4.1. Bacterial loads (upper panel) and reductions (lower panel) in monomicrobial biofilms. Bacterial loads in monomicrobial biofilms were quantified prior to treatment with QUATS in polystyrene plates (n = 3). Reductions were quantified following QUATS treatment at concentration of 50–200 ppm (n = 6). Statistical significance was assessed using one-way ANOVA followed by Duncan's post hoc test, with different letters indicating significant differences in their mean values ($P < 0.05$). SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; ST: *Salmonella* Typhimurium; LM: *Listeria monocytogenes*.

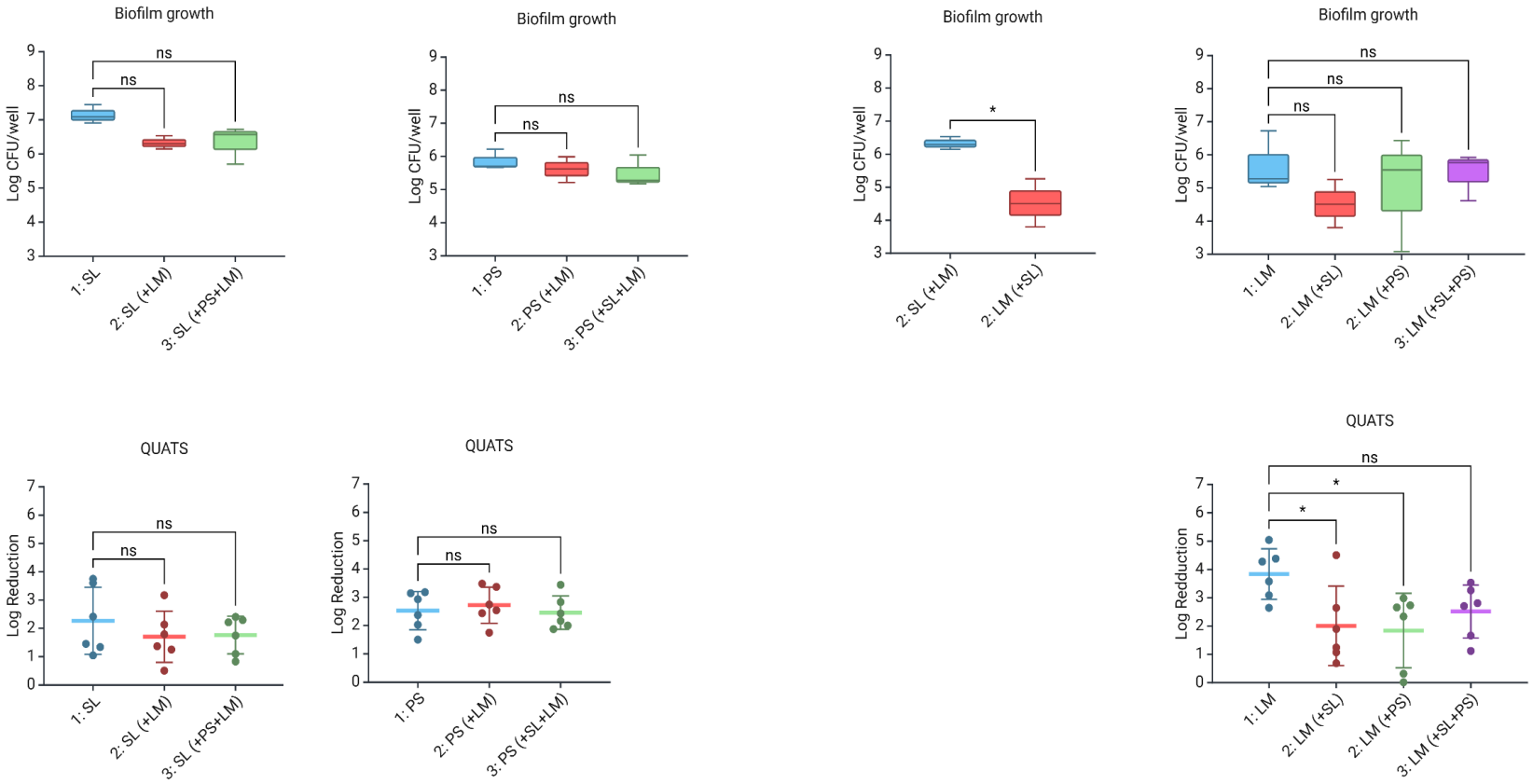

Figure 4.2. Bacterial loads (upper panel) and reductions (lower panel) in biofilms with *Listeria monocytogenes* as a companion microorganism. Bacterial loads in monomicrobial (1), binary (2), and ternary (3) biofilms were quantified prior to treatment with QUATS in polystyrene plates and plotted as min to max values (n = 3). Reductions were quantified following QUATS treatment at concentration of 50–200 ppm, and results are presented as mean ± SD (n = 6). Statistical significance was assessed using one-way ANOVA with Dunnett's multiple comparisons. *: $P < 0.05$ and ns: no significance. SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; LM: *Listeria monocytogenes*. Graphs were created using www.biorender.com.

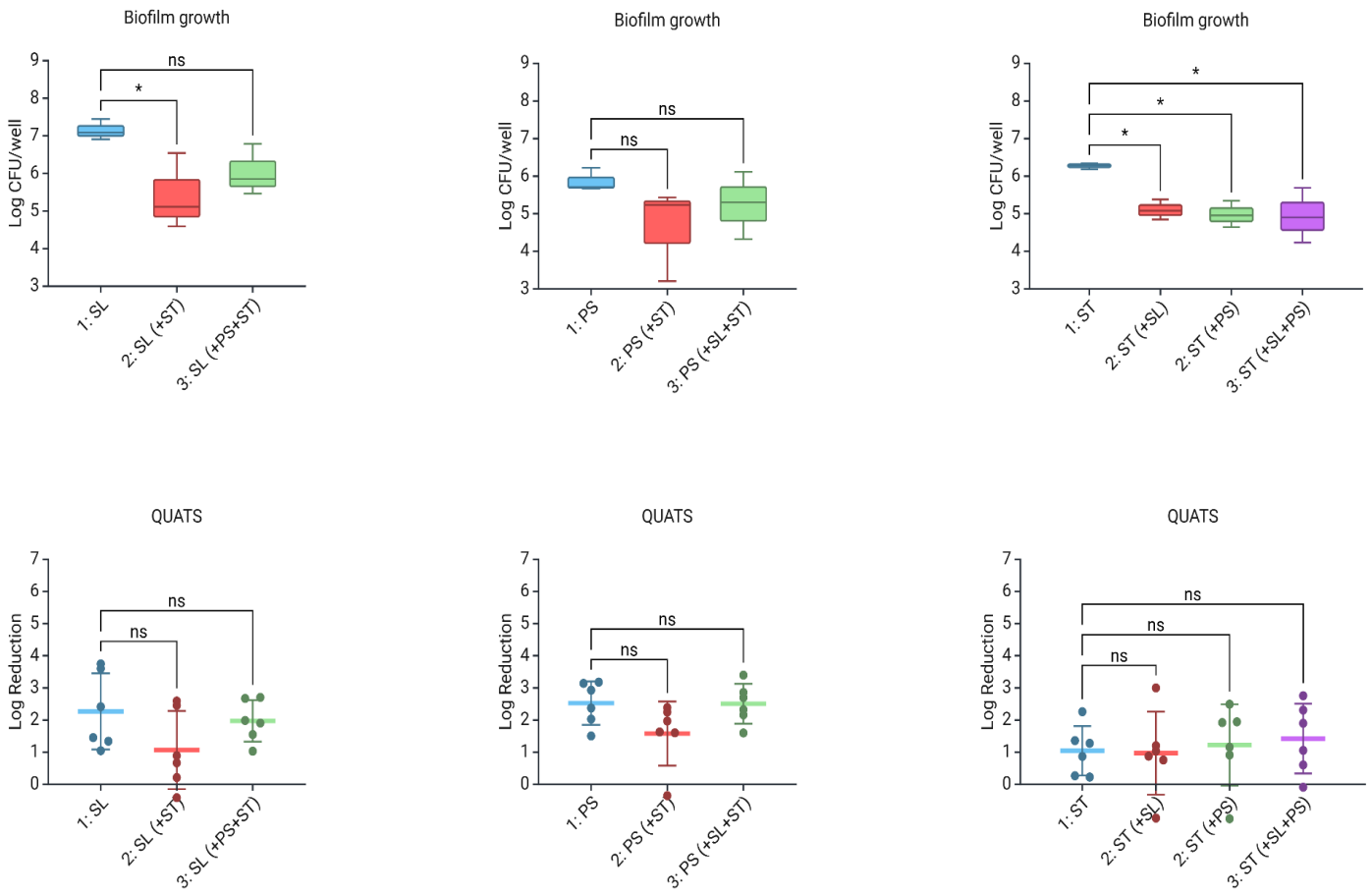

Figure 4.3. Bacterial loads (upper panel) and reductions (lower panel) in biofilms with *Salmonella* Typhimurium as a companion microorganism. Bacterial loads in monomicrobial (1), binary (2), and ternary (3) biofilms were quantified prior treatment with QUATS in polystyrene plates and plotted as min to max values (n = 3). Reductions were quantified following QUATS treatment at concentration of 50–200 ppm, and results are presented as mean ± SD (n = 6). Statistical significance was assessed using one-way ANOVA with Dunnett's multiple comparisons. *: $P < 0.05$ and ns: no significance. SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; ST: *Salmonella* Typhimurium. Graphs were created using www.biorender.com.

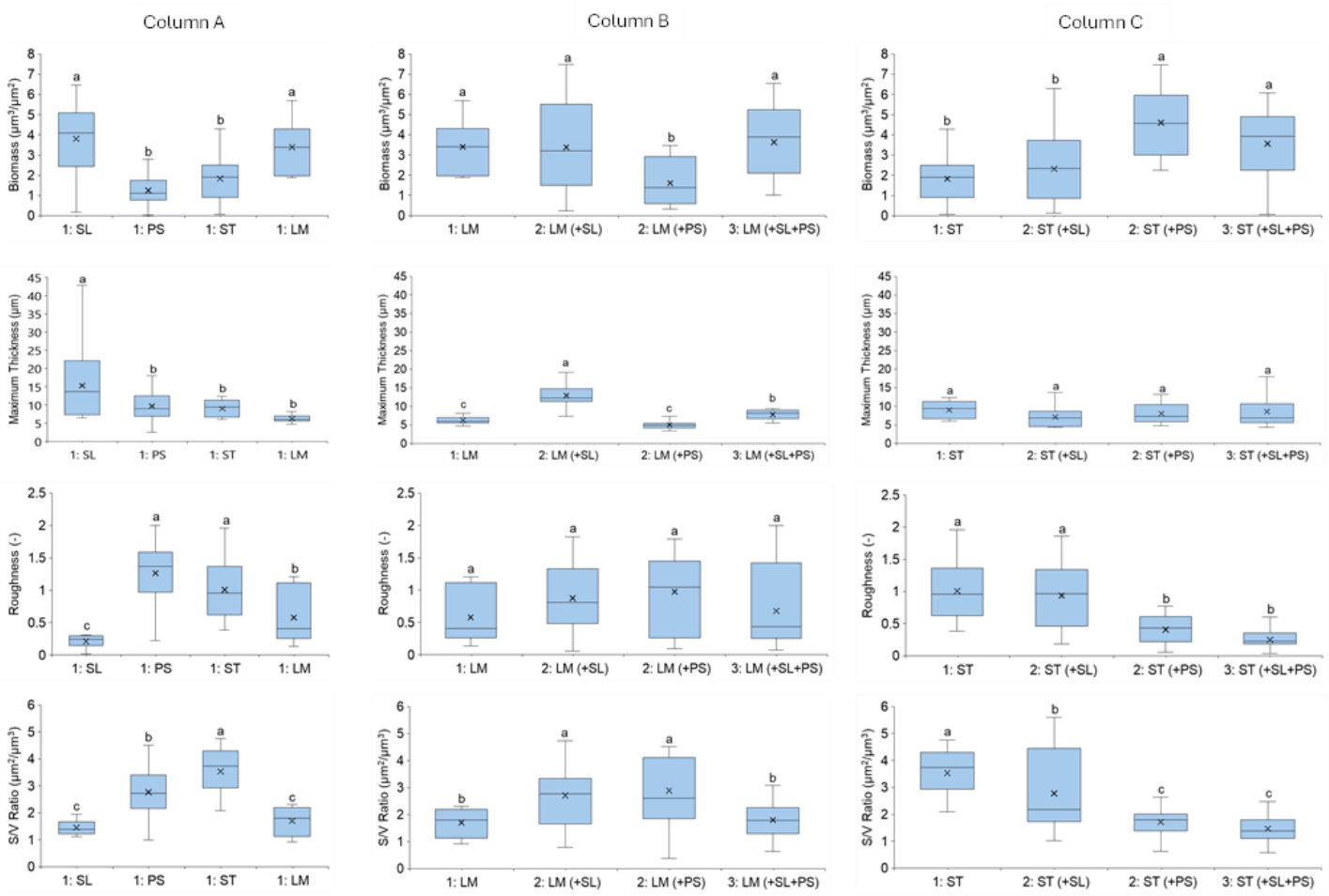

Figure 4.4. Biomass, maximum thickness, roughness coefficient, and surface-to-volume ratio in untreated monomicrobial (1), binary (2), and ternary (3) biofilms based on live-cell populations. Column A represents these parameter values for monomicrobial biofilms. Column B represents these parameters for *Listeria monocytogenes* monomicrobial biofilms, along with its binary and ternary biofilms. Column C represents these parameter values for *Salmonella* Typhimurium monomicrobial biofilms, along with its binary and ternary biofilms. Statistical significance was assessed using one-way ANOVA followed by Duncan's post hoc test, with different letters indicating significant differences in mean values ($P < 0.05$). SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; ST: *Salmonella* Typhimurium; LM: *Listeria monocytogenes*.

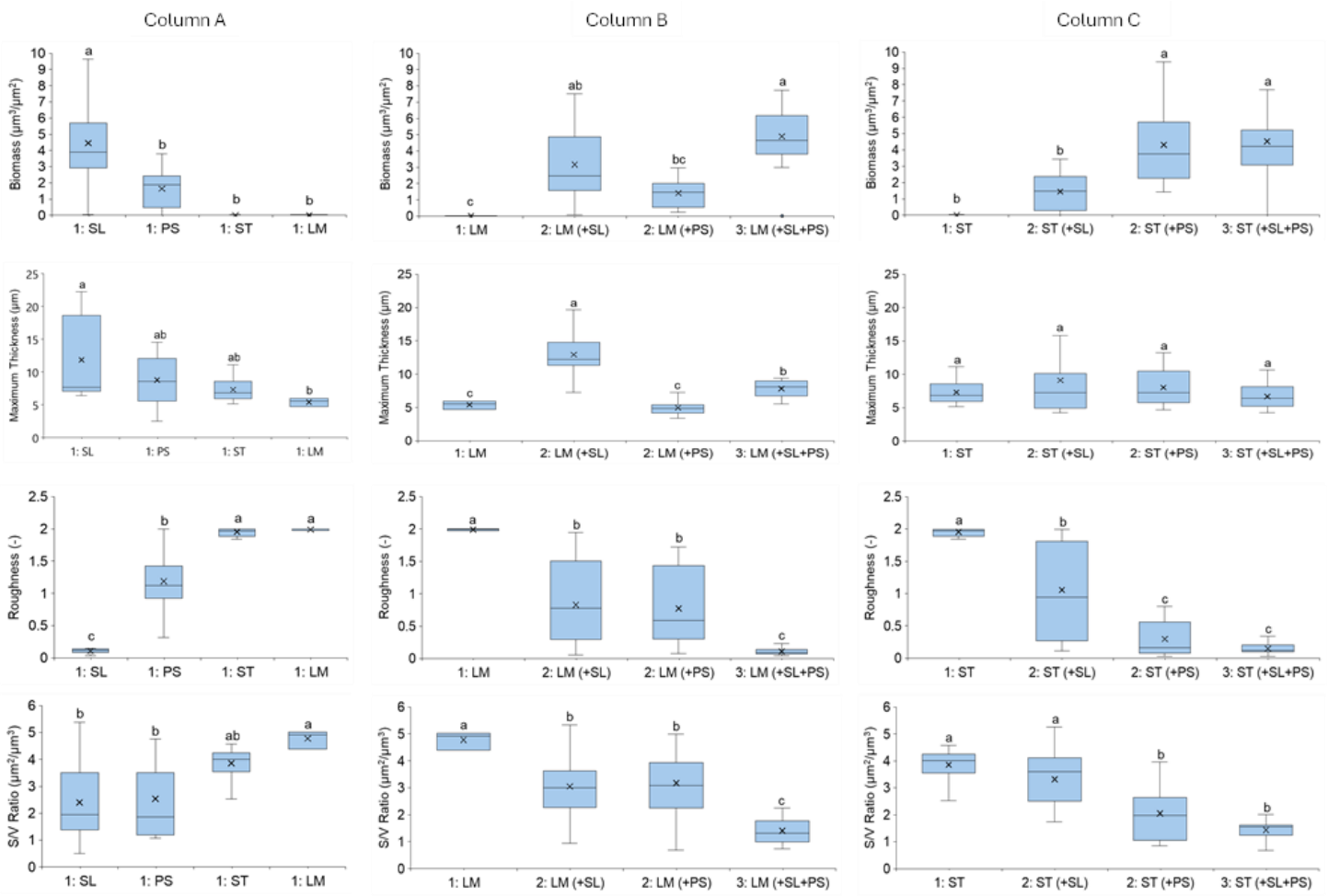

Figure 4.5. Biomass, maximum thickness, roughness coefficient, and surface-to-volume ratio in untreated monomicrobial (1), binary (2), and ternary (3) biofilms based on dead-cell populations. Column A represents these parameter values for monomicrobial biofilms. Column B represents these parameters for *Listeria monocytogenes* monomicrobial biofilms, along with its binary and ternary biofilms. Column C represents these parameter values for *Salmonella* Typhimurium monomicrobial biofilms, along with its binary and ternary biofilms. Statistical significance was assessed using one-way ANOVA followed by Duncan's post hoc test, with different letters indicating significant differences in mean values ($P < 0.05$). SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; ST: *Salmonella* Typhimurium; LM: *Listeria monocytogenes*.

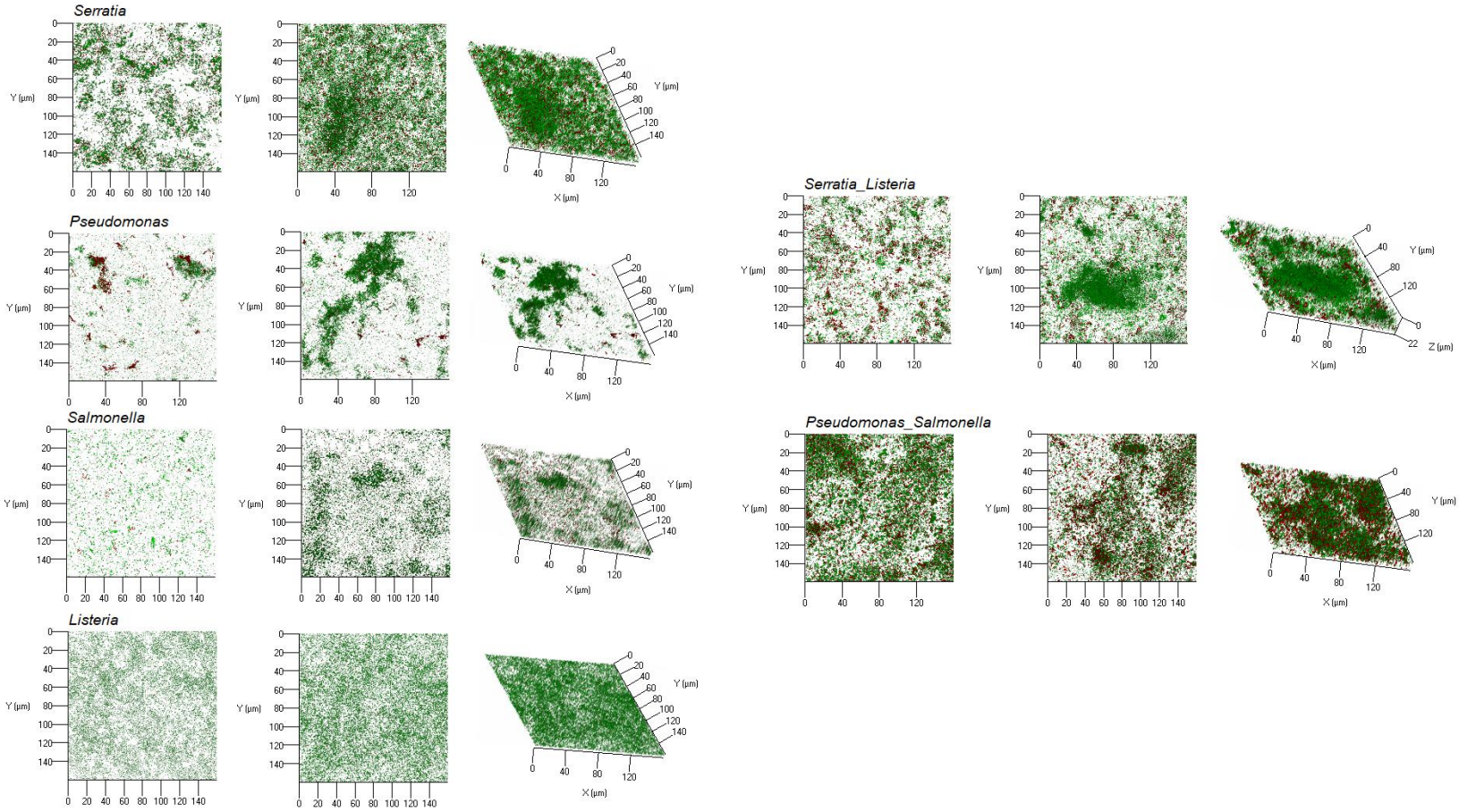

Figure 4.6. Selected confocal images of biofilms, chosen based on their structural parameters. For each biofilm type, the first two images depict the top surface, while the third image presents a rotated view of the second image to highlight the three-dimensional architecture. The biofilm images include monomicrobial biofilms of *Serratia liquefaciens*, *Pseudomonas simiae*, *Salmonella* Typhimurium, and *Listeria monocytogenes*, as well as binary biofilms such as *Serratia liquefaciens* × *Listeria monocytogenes* and *Pseudomonas simiae* × *Salmonella* Typhimurium.

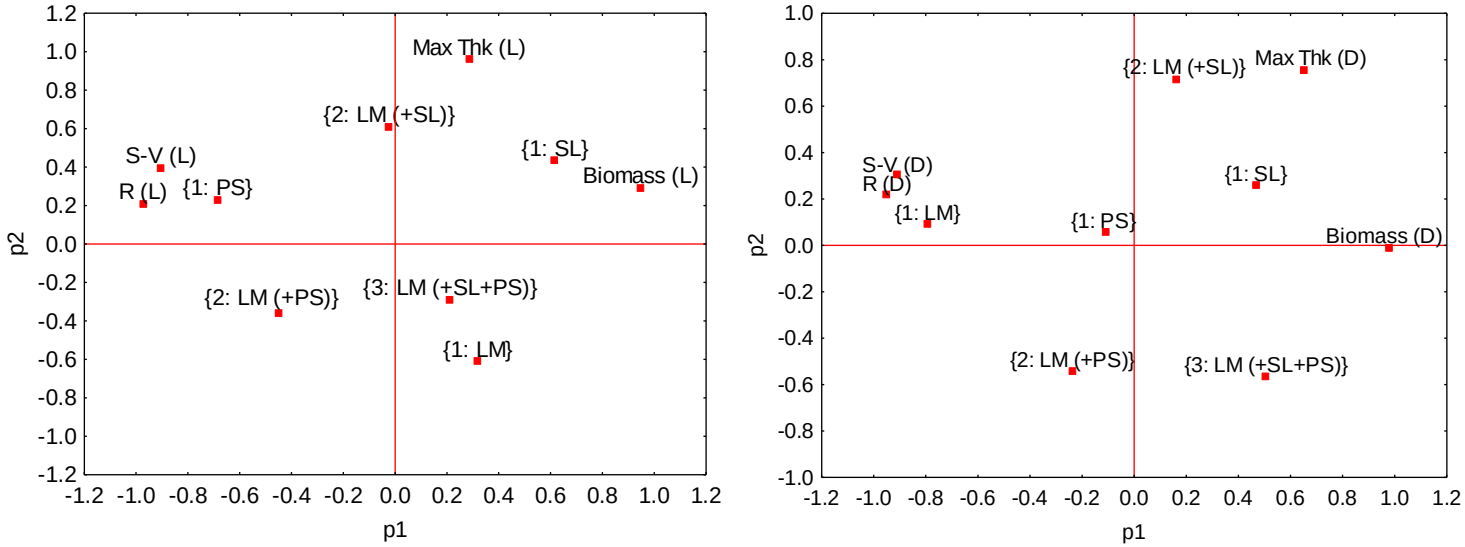

Figure 4.7. The loading scatterplots (p1 vs. p2) show clustering among the structural parameters as extracted from green and red channels, representing live (L) and dead (D) cells. Variables placed close to each other influence the PCA model in a similar way, indicating an association between them. The further a variable is from the origin, the more influential it is in determining the PCA model. The following continuous variables were analyzed: biomass, maximum thickness (Max Thk), roughness (R), and surface to volume ratio (S-V). Biofilms having *Listeria monocytogenes* as a companion microorganism were selected as categorical variables. SL: *Serratia liquefaciens*; PS: *Pseudomonas simiae*; LM: *Listeria monocytogenes*.

CHAPTER 5

OVERALL CONCLUSION AND FUTURE RESEARCH DIRECTION

Overall, our studies found that microbial counts in edible insects can vary based on the product type, and these products may be contaminated with bacterial spores, including *Bacillus cereus* biovar Emeticus. Microbiome analyses of retail food products, such as fresh produce, deli meats, and cheese, revealed that fresh produce tends to have higher alpha diversity compared to other product types. Beta diversity analyses showed distinct microbial community compositions across these retail product categories. Moreover, we observed that cheese and deli meats were dominated by fermented-associated bacterial genera whereas fresh produce had a high relative abundance of bacterial genera linked to food spoilage or plant diseases. Additionally, our research demonstrated that the microbiome of food environments can contribute to the survival of foodborne pathogens against sanitizer exposure. For instance, we observed that *Pseudomonas* and *Serratia* increased the resistance of *Listeria monocytogenes* to sanitizer when *Listeria* was present in binary biofilms alongside these bacteria. Based on our findings, the following research directions could be pursued to further advance understanding in these research areas.

1. The demand for alternative proteins is increasing, and edible insect protein presents a significant market opportunity. However, the food safety aspects associated with edible insect protein need further research. A comprehensive research approach should focus on microbiome analyses and the investigation of potential foodborne pathogens throughout the production and processing cycle. This will help optimize process controls to tackle food safety challenges associated with these novel products. Additionally, since edible insects

undergo processing steps such as roasting or drying, it is important to conduct research to validate these technologies in effectively eliminating or minimizing foodborne pathogens of concern, such as emetic toxin-producing *Bacillus cereus*, in these products.

2. Based on my research into ready-to-eat food products, such as fresh produce, deli meats, and cheese, the presence of microbial genera related to food spoilage was confirmed in various products. Future studies could concentrate on analyzing the microbiome of these items throughout their shelf-life to determine which spoilage-related microbial taxa become more dominant over time. This understanding could enable the food industry to implement targeted strategies aimed at reducing these microbes during food production and processing.
3. Lastly, studies on biofilms have demonstrated that the microbes residing in food environments can protect foodborne pathogens from antimicrobial agents. Future research should aim to understand the genetic mechanisms that allow pathogens to survive antimicrobial stress in biofilm settings. Therefore, to gain deeper insights, studies on biofilms may include metatranscriptomic approaches, which will provide a more comprehensive understanding of pathogen's gene expression during the formation of biofilms and antimicrobial exposure.