DISSERTATION

APPLICATION OF BIOTECHNOLOGY IN AGRICULTURE: A BIOINFORMATIC AND MARKET ANALYSIS OF NOVEL INTERVENTION METHODS

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ABSTRACT

APPLICATION OF BIOTECHNOLOGY IN AGRICULTURE: A BIOINFORMATIC AND MARKET ANALYSIS OF NOVEL INTERVENTION METHODS

From genetic sequencing to genetic engineering, the use of biotechnology within agriculture has become an essential method for dealing with several alarming issues. One critical application of this technology is the use of these advancements within agriculture. The use of known biological processes to create novel and innovative technologies is called biotechnology. One of those issues is the overuse of antibiotics and antimicrobials within livestock and processing facilities, giving rise to an increase in antimicrobial resistance. Further still, is the ever-growing population and the challenge in feeding this population in a sustainable manner and in such a way that consumers deem acceptable. Therefore, three studies were conducted to highlight the use of biotechnology in agriculture for potential innovative ways in pathogen intervention and diagnosis as well as understanding the market pathways in which brand new biotechnology can be brought into commerce.

The first study was conducted to investigate the establishment and persistence of *Listeria* spp. within a new meat processing facility and to examine the relationship between the core facility microbiome and *Listeria* spp. presence. *Listeria* spp. contamination in meat processing facilities poses threats to both the meat industry and public safety. A study was conducted to identify how *Listeria* spp. in a state-of-the-art meat processing facility might be controlled through its microbial ecology. The study explored potential innovative ways for pathogen control

and intervention. Samples were obtained from drains, door handles, and walls of the facility for microbial analysis and *Listeria* spp. analysis. Real-time PCR and conventional plating methods were used for *Listeria* spp. confirmation. A total of 1,009 environmental samples were sequenced with 59 samples confirmed by rtPCR based detection and conventional plating methods to be *Listeria* spp. Following speciation, 26 samples were *Listeria monocytogenes*. DNA extraction of the microbial samples were subjected to 16S rRNA gene amplicon sequencing. Taxonomic analysis, alpha diversity, beta diversity, and weighted Random Forest predictive techniques were compared for *Listeria* spp. presence or absence and for different species of *Listeria*. Alpha diversity metrics found no significant difference between *Listeria* absence and presence or between differing species of *Listeria.* Beta diversity distances revealed community separation was driven by room function and evaluated statistically using PERMANOVA ($P < 0.05$). Differential abundance analysis determined several genera to be differentially abundant for *Listeria* spp. presence or absence as well as association with *L. monocytogenes.* These genera included *Pseudomonas, Psychrobacter, Acintobacter, Janthinobacterium,* and *Prevotella.* Weighted Random Forest algorithms were found to predict *Listeria* presence/absence with an overall accuracy score of 61% indicating a propensity to accurately predict *Listeria* absence but not predict *Listeria* presence. Larger sample sizes will be needed to accurately predict *Listeria* presence using machine learning techniques. Overall findings from this study lay the ground work for utilizing meat processing facility microbial ecology to better control foodborne pathogens.

The second study evaluated the relationship between the fecal and nasal microbiomes and disease states within very early life dairy calves. The early life microbiome of dairy calves undergoes extensive changes, and diseases such as Bovine Respiratory Disease (BRD) and calf

scours could affect microbiome diversity and composition during this developmentally important timeframe. The objective of the current study was to describe the establishment of the early life (herein defined as the first 21 days) fecal and nasal microbiomes of dairy calves as well as evaluate the correlation between disease states and early life microbial ecology. Nineteen dairy calves were evaluated weekly during the first three weeks of life for fecal and nasal microbial composition. The health status was also assessed for each calf utilizing the Wisconsin Calf Health System, which included assessment of fecal scores and lung health via ultrasound. Samples were collected using sterile cotton-tipped swabs and DNA was extracted with the 16S rRNA gene subsequently sequenced to estimate microbiome composition and diversity. Taxonomy analyses and diversity analyses were used to characterize the establishment of early life nasal and fecal microbiomes and to correlate disease state and early life microbiomes. Across the three-week sampling period, only five calves were identified as having scours and one calf had clinical signs of both BRD and scours. Nasal microbiomes were dominated by the families *Moraxellaceae* (average relative abundance 49.42%), *Mycoplasmataceae* (16.24%) and *Pastuerellaceae* (3.4%) and fecal microbiomes were dominated by *Bacteroidaceae* (40.9%), *Ruminococcaceae* (12.72%) and *Lachnosporaceae* (9.83%). Nasal samples had higher alpha diversity as compared to fecal samples and compositional analysis revealed distinct separation between nasal and fecal samples ($P < 0.05$). Differential abundance analyses revealed two features to be differentially abundant during the first three weeks of life. *Neisseriaceae* was differentially abundant in nasal samples and decreased in relative abundance over the first three weeks. *Bifidobacterium* was differentially abundant in fecal samples and decreased in relative abundance over time ($P < 0.05$). Nasal samples indicated no significant differences in alpha diversity over the three sampling time points, but compositional changes were detected across

sampling points ($P < 0.05$). Alpha diversity increases and compositional changes were detected for fecal samples across the three sampling time points Fecal samples of healthy calves had higher alpha diversity compared to scouring calves ($P < 0.05$) while no significant differences were seen in composition. The current study revealed that fecal microbiomes undergo diversity increases as well as changes in composition, indicating early fecal microbial life is subject to quick change. Nasal microbiomes indicated no diversity shifts however, compositional changes were seen, which could be suggestive of higher rates of environmental contamination of the nasal microbiome. Disease state within fecal microbiomes (i.e., calf scours) revealed that healthy calves had higher alpha diversity compared to scouring calves. This finding provides insight for further research to be conducted to evaluate the potential of diversity changes to be indicative of disease onset.

The third and final project focused on market research to evaluate the commercialization of an innovative use of CRISPR-Cas9 machinery as an antimicrobial for pathogen control. CRISPR biotechnology is a promising option for alternative pre-slaughter intervention methods in foodborne pathogen control for production livestock operations. A customizable CRISPR-Cas9 targeted pathogen killing system has recently been studied and offers potential as a novel antimicrobial product in the production animal market. Questions remain however regarding the commercialization and market acceptability of the new CRISPR-Cas9 product. Our lab designed a study to evaluate the product's value, the target customer, and the regulatory aspects of how the product will move in the target market ecosystem. Our study details the market pathways for commercialization of a novel CRISPR-Cas9 biotechnology. The current study uses a three-pronged integrated methodology involving attendance of a market research workshop to learn the tools necessary to complete market research, an interview process, and a comprehensive

literature review. The study approached product value by assessing its potential to reduce other antimicrobial use, its customizability, and its innovation of pre-slaughter interventions. The study included 20 interviews with industry experts, production livestock operations personnel, and academicians. Surprisingly, interviews revealed that the target customer for our CRISPR-Cas9 product to be the owners of production livestock operations because these individuals make the financial decisions to purchase new products. Adoption of the product by our target customer would also require verification that the product is effective, does not increase cost, and is safe for animal use. Insights from the interviews revealed the regulatory process would be the primary focus of the market ecosystem. These interview insights made it clear that new biotechnology for use in livestock animals such as our product would be regulated by the U.S. Food & Drug Administration (FDA), with the Center for Veterinary Medicine being the main regulatory component of the FDA for new animal food products and drugs. This insight was established on the basis of the definition of the product being one of a new animal drug with intent to reduce foodborne pathogens in the public sector. Overall, our study maintains that our new CRISPR-Cas9 biotechnology offers producers an alternative intervention method that could improve consumer perception and outlines a regulatory pathway for the target market ecosystem.

ACKNOWLEDGEMENTS

For my wife, Lauren Emily Frazier…

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Chapter One

Literature Review: Biotechnology and Agriculture

In recent years, technology has advanced at exponential levels. From the advent of self-driving cars to cellular phones that are more powerful than some computers, technological advances have made modern life more manageable. Of particular interest is the use of biotechnology in food and food animals. Biotechnology is the use of biological processes to develop technological products. Throughout human history, we have used biology in everyday life such as baking (i.e., using yeast to make breads). While this is a simple example, it is one that highlights the practicality of using biotechnology. Following the advancement of genetic engineering, biotechnology rapidly developed and is widespread in its applications.

The demand for food is ever increasing as the world population continues to grow. By the year 2057, it is estimated that the Earth will be home to 10 billion people (United Nations, 2019). While the importance of healthcare and medicine cannot be understated, the need for food might outweigh any problem humanity may face as evidence suggests the hope for eradicating world hunger by 2050 has become questionable (FAO, 2009). Therefore, one of the main purposes of any new technology in agriculture should be to promote sustainable practices for producers. There is a need for developing new ways to meet these challenges. One of those ways is the use of biotechnology in agriculture. In agriculture, the United States Department of Agriculture (USDA) defines the use of biotechnology as a range of tools for developing new products or modifying existing products as well as improving plant and animal growth or using microorganisms for specific benefits. These benefits include the use of biotechnological tools for

increased crop productivity, enhanced crop protection, improvements in food processing, improved nutritional and flavor profiles, and better produce (Wieczorek, 2003).

The use of biotechnology in agriculture is widespread and is used in many applications, such as food production, animal welfare and food safety. While there are many great things that these new technologies are capable of, and there is a dire need for increased production of quality food and protein sources, consumers remain a barrier. Genetically modified organisms (GMOs) appear to be held in a negative light by consumers, particularly with genetically modified animal products (Lin *et al*., 2019). Consumers lean towards buying animal products that were derived from conventional rearing methods (Ufer *et al*., 2019). However, consumers hold fewer negative connotations towards genetically modified plants (Riberio *et al*., 2016). This is mainly due to ethical concerns on using genetic engineering technologies on living organisms as it could be harmful to the animal or is seen as unnatural (Frewer *et al*., 1997). To alleviate the consumer fear of genetically modified organisms, this review highlights the use of biotechnologies in an indirect manner for the betterment of animal health, safe and quality food products, and how these methods are regulated. The hope is to provide producers efficient ways to use new biotechnology tools for their operations and alleviate consumer concern over products derived from genetic manipulation.

1.1. Next Generation Sequencing

Next generation sequencing (NGS) techniques have revolutionized biological science. Also known as massively parallel or deep sequencing, NGS allows for the sequencing of millions of fragments of DNA in parallel, resulting in high-throughput data. Bioinformatic tools

are then used to analyze the fragments and piece together the target genome via a reference genome set. The advancement of NGS technology now provides an extensive amount of data in a timely manner for marginal cost. For example, the Human Genome Project took 15 years and a $3 billion budget to generate approximately 3 gigabytes (GB) of data; today, 3 GB of raw data can be completed for around ~$24 and an entire human genome can be completed for ~$689 (Venter *et al*., 2001; Wetterstrand, 2020). Furthermore, NGS techniques have been applied to crucial studies involving human and animal health (e.g., disease diagnosis in humans, Mall *et al*., (2019); integration into veterinary diagnostics, Harris *et al*., (2021)). The use of NGS in agriculture has played an important role for animal science and food safety. Applications include tracing and understanding antimicrobial resistance (AMR) of foodborne pathogens, animal health and welfare, and livestock production (Crofts *et al.,* 2017; Ganda, 2017; Sharma *et al*., 2017). While not limited to these roles, NGS use may allow for noninvasive ways to produce more quality driven food without the need for harming the animal. Manipulating host microbiomes could allow for these things because several beneficial traits in animals are directly tied to microbiome influences and could increase host fitness (Mueller & Sachs; 2015). Studying the role that microorganisms, proteins, and metabolites play in animal production systems may allow for scientists and industry personnel to produce food that consumers would not oppose as compared to genetically engineered animal products. For instance, studies could elucidate microbiomes or microbiome functions that could serve as candidates for marking altered states (i.e., disease) and identify key microbial taxa for studies in how these bacteria could be targeted for growth/inhibition to promote key features in performance and health (Mueller & Sachs, 2015; Clemmons *et al*., 2019).

1.1.1. Antimicrobial Resistance in Livestock and Next Generation Sequencing

Antimicrobial resistance (AMR) is an important and challenging combatant for public health and animal welfare alike. Antibiotics are necessary for fighting disease and eliminating the pathogens responsible for the onset of illness. However, the overuse of antimicrobials has led to an increase in AMR. The use of antimicrobials has exposed human microbiotas to high levels of these drugs and has subsequently led to promoting the selection of AMR genes in bacterial populations (Blazquez *et al*., 2002). In addition, it is documented that use of antibiotics in animals has led to an increase in drug resistance in foodborne pathogens (Marshall & Levy, 2011; Kimman *et al*., 2010; Gilchrist *et al*., 2007). To further complicate things, consumers of animal products have indicated the desire for products that are labeled as "antibiotic-free". The rise of AMR has caused the animal health sector to come under scrutiny for the use of antimicrobials to treat disease in livestock animals (Singer *et al*., 2019). However, the necessity of using antimicrobial drugs to treat sick livestock is of importance. A recent study evaluated conventional production and antibiotic-free production of pigs when faced with the onset of porcine reproductive and respiratory syndrome virus (PRRSV). The study elucidated that antibiotic-free pigs had a mortality or removal incidence of 57.98% as opposed to the treated groups who saw mortality rates of 20.94% and 24.98% respectively (Dee *et al*., 2018). Interestingly, the study ultimately had to be halted due to concerns regarding the welfare of the animals. This is of importance due to the recent survey conducted by Singer *et al*. (2019) in which it was reported that the perception of consumers was that animals raised without antibiotics would have improved welfare; the resulting information led to the conclusion that antibiotic-free production was favored to conventional production however, it was not driven by prioritizing animal welfare. The survey showed that the respondents felt that animal welfare was

less important, and the antibiotic-free label was the highest priority. The use of biotechnological advances could provide ways to begin innovating upon how to reduce antibiotics. High-throughput sequencing techniques along with metagenomic selection has allowed for an increase in understanding resistomes from many habitats and could provide information that could lead to new NGS therapeutic techniques (Crofts *et al*., 2017).

1.1.2. Antimicrobial Resistance and Livestock Welfare

The dichotomy of antimicrobial use for animal welfare and antibiotic-free product labeling is a major hurdle for livestock producers. The rise in AMR is a driving factor behind this contradiction. However, as previously mentioned, the use of NGS techniques have allowed scientists the unique ability to understand AMR in differing systems. Furthermore, it allows for techniques and intervention protocols to be elucidated without the use of antimicrobials for the betterment of animal welfare and animal product quality and safety. For example, Kim *et al.,* (2017) employed microbiome analyses of before and after antimicrobial treatment on poultry carcasses; the study highlighted that as further processing occurred, Proteobacteria, which are known for being phylogenetically related to foodborne diseases, decreased. The information garnered here could provide food producers with insights into how to process meat products without the need for antibiotics or antimicrobials. NGS methods, such as shotgun metagenomics, have enabled the study of bacterial community composition along with their corresponding antibiotic resistance genes (ARGs). Berglund *et al*., (2019) introduced a novel method, fARGene, for the identification and reconstruction of ARGs from metagenome data. fARGene is a method that uses gene models to identify resistance genes of previously unknown origin including those that have a sequence similarity to known resistance genes. The genomic tool for

ARG identification was shown to demonstrate that >80% of novel ARGs predicted in *Escherichia coli*, a prominent foodborne pathogen, induced a resistance phenotype. Furthermore, the use of NGS techniques has potential to provide the food safety industry vital information on traceability and underlying mechanisms of AMR of foodborne pathogens as well as understanding the transfer of ARGs between bacterial species (Carroll *et al*., 2017; Moran-Gilad, 2017; Karkman *et al*., 2017).

Whole genome sequencing (WGS) based AMR surveillance is already being used in the United States via the National Antimicrobial Resistance Monitoring System, or NARMS (Oniciuc *et al*., 2018). The NARMS surveillance program tracks AMR changes found in humans, retail meat products and food animals utilizing information from the CDC, the Food and Drug Administration (FDA) and the USDA. These WGS-based techniques are based on literature findings highlighting major foodborne pathogens such as *Salmonella*, *Campylobacter*, Shiga-toxin producing *E. coli* (STEC), *Listeria monocytogenes* and *Staphylococcus aureus* and their corresponding ARGs (Zhang *et al*., 2015; Dallman *et al*., 2015; Gordon *et al*., 2014; Chen *et al*., 2013; Allard *et al*., 2012). The knowledge that lies within these WGS-based AMR surveillance programs could allow for insights into how antibiotic usage in livestock production systems affects both the product and public safety as well as the animal's welfare and health. For instance, the use of NGS technologies have allowed for assessment of virulence factors of known foodborne pathogens in cattle grown conventionally or naturally (i.e., feedlot cattle vs. antibiotic free; Weinworth *et al*., 2017). The study indicated that there were no differences observed in pathogen load or virulence factor providing insight that cattle production programs are likely not a major driver for foodborne pathogen virulence or load. Therefore, using this information might provide innovations upon the use of antibiotics within traditional feedlot settings in such that a

reduction or an increase in use will not answer problems associated with foodborne illness within the public.

1.2. The Microbiome and its Application to Livestock and Food Safety

Another exciting use of biotechnology is the study of the microbiome. Microbiomes are communities of microorganisms living and interacting with one another in an environment. Microbiomes are found everywhere in nature and are vital components to the ecosystem they are found. Within the livestock production world, these environments and ecosystems include but are not limited to, the animal gut microbiome, the rumen microbiome (e.g., cattle, sheep, goats), and the built environment of processing plants. 16s rRNA gene amplicon sequencing is the most common NGS method for revealing phylogenetic and taxonomic profiles for microbial community structures within a microbiome (Panek *et al*., 2018). While it is the most common in community profiles, 16s rRNA sequencing is not the only NGS technique used. Other disciplines include the use of shotgun metagenomic sequencing to identify all genes in all organisms present in a sampled microbiome; however, shotgun sequencing has a few limitations including that of the difficulty of deriving what genome a read is associated with (Sharpton, 2014). There are several other "-omics" fields that are currently emerging with the betterment of biotechnology including but not limited to metabolomics (i.e., the study of metabolites and small molecules; (Dettmer *et al*., 2004; Tzoulaki *et al*., 2014; Tran *et al*., 2020)); proteomics (i.e., the study and characterization of proteins expressed within a cell or tissue; (Wilkins *et al*., 1996; Bendixen, 2005); and transcriptomics (i.e., study of transcripts in a given cell or tissue; (Martyniuk, 2020). Understanding the role of the microbiome and its associated metabolites, proteins and transcripts for example, could lead to techniques allowing for the manipulation the microbiome of livestock

animals or their environments, and meat products and the processing environments, which might provide researchers, veterinarians, and producers alternative ways to promote health and productivity as well as sustainable food products.

1.2.1. The Microbiome and Nutrition

One of the major drivers in microbial composition of livestock animals is diet. In ruminant animals, the diet could in fact be more powerful in deriving microbial composition than the host itself (Purushe *et al*., 2010). This could be due to the actual characteristics of the feed make-up itself, both chemical and physical, as the feed make-up can determine the available microbial niches within the rumen (Hooper *et al*., 2012). Studies have also indicated that differing types of diets lead to higher abundances of particular microbes when compared to another diet. For example, Henderson *et al*. (2015) established that forage-based diets resulted in higher abundances of *Bacteroidales* and *Ruminococcaceae* while grain-based diets displayed higher numbers of *Prevotella* and *Succinivibrionaceae*, regardless of the ruminant species. This once more highlights the ability of feed given its composition to drive microbial community composition and this could simply be the amount of food eaten by the ruminant (Hooper *et al.,* 2012). Other studies have shown that manipulation of early-life microbiota in goats by diet led to differences in composition and function (Abecia *et al*., 2014a). Similarly, Abecia *et al*. (2014b) showed that nutrition played a major role in the developing microbiome, especially the make-up of archaeal composition, in kid goats and could therefore be used for intervention techniques. It has also been detailed in a recent review by Stokes (2017) that the microbiome has a major impact on the gut mucosal immune system in pigs. Moreover, it has also been shown in broiler chickens that the order *Clostridiales* is associated with higher residual feed intake leading to

improved productivity (Liu *et al*., 2021). Taken together, the above information could provide methods of manipulating the microbiome of livestock to promote health and production without added growth promoters or genetic engineering of the animal. Research into this is already underway; Foditsch *et al*. (2015) demonstrated that administering *Faecalibacterium prausnitzii* orally to week-old calves significantly reduced the onset of calf scours (i.e., diarrhea) during the first seven weeks of life.

1.2.2. The Microbiome and the Environment

Environmental microbiomes often have significant roles on animal and animal product microbiomes. For example, a recent study indicated the upper respiratory tract microbiota of cattle differed depending on the animal's farm of origin (Nicola *et al*., 2017). Studies have indicated that when exposed to unfavorable environmental conditions, symbiotic bacteria can act as opportunistic pathogens or simply become ineffective at providing the host protection from (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Lokmer & Wegner, 2015; Bahrndorff *et al*., 2016). A study conducted by Chen *et al*. (2018) determined that heat stress resulted in a lower microbial diversity for dairy cows exposed to extreme heat conditions. Other studies have also supported this finding in both dairy cows and in murine models indicating that heat stress can alter the microbial population and diversity (Bailey *et al*., 2011; Yadav *et al*., 2013; Min *et al*., 2016; Chen *et al*., 2018). It is also theorized that environmental stressors such as heat stress and the subsequent changes to microbial composition and diversity are linked to the onset of disease and lower metabolisms in dairy cows (Chen *et al*., 2018). The ability to understand these relationships due to the advances in NGS biotechnology could bring forth improved welfare protocols for livestock production as it is further investigated.

1.2.3. The Microbiome and the Built Environment

The environment for animal product processing exposes the products to a new microbial environment which may affect the quality and safety of the product. For example, the causation of listeriosis, a major foodborne illness caused by *Listeria monocytogenes*, is said to be attributed to foodborne illness at a rate of 99% in the United States (Scallen *et al*., 2011). Ready to eat (RTE) products are of particular concern as they are foods that are generally eaten raw or prepared in a fashion that does not require additional microbial intervention steps. These products can become contaminated via *L. monocytogenes* during storage due to the fact that the pathogen is capable of growth at varying temperatures and other environmental factors (Buchanan *et al*., 2017). Tan *et al*. (2019) also demonstrated that the microbiome of three different processing facilities of fruits had different environmental microbial diversities once again highlighting the importance the built environment has on *Listeria* spp. contamination. The application of this knowledge could come in the form of innovating upon sanitation techniques within the processing environment.

Common control methods and sanitation protocols rely on potent biocides, which can give rise to resistance genes (Lerma *et al*., 2012). More so, the formation of biofilms presents another challenge for processing environments. Fagerlund *et al*. (2017) indicated that biofilms containing *L. monocytogenes* developed an increase in tolerance to sanitation procedures over a three-day period. Therefore, it is crucial to establish alternative methods in controlling foodborne pathogens within processing environments.

1.2.4. Interspecies Relationships

The use of NGS techniques such as 16s rRNA amplicon sequencing can also allow for insight into how bacterial species interact with one another. An understanding of interspecies relationships could provide livestock producers and processing facilities novel methods in pathogen control. Recent studies have indicated that certain interspecies interactions can either inhibit or enhance the growth and persistence of foodborne pathogens. *Pseudomonas* spp. have been shown in various studies to have a positive effect on the attachment of *L. monocytogenes* to stainless steel surfaces (Bokulich *et al*., 2016; Beuchat *et al*., 2004; Hassan *et al*., 2004). However, *Staphylococcus sciuri* was indicated to be a microorganism capable of inhibiting the growth of *L. monocytogenes* biofilms on stainless steel (Leriche & Carpentier, 2000). The underlying mechanisms in which these organisms are capable of inhibiting or promoting growth of pathogenic bacteria in the processing environment are not well established; however, the knowledge gained from the microbiome could be used to implement novel intervention techniques that do not require genetic alteration of the food product and could alleviate consumer stress around food products in general. For example, Tan *et al*. (2019) concluded that *Pseudomonas* dominated a fruit processing facility with a high abundance of *L. monocytogenes*. Therefore, the control of *Pseudomonas* could be of value in preventing the onset of *L. monocytogenes* establishment within processing environments. Future studies should further indicate if this relationship between *Pseudomonas* and *Listeria* could in in fact be exploited for intervention techniques. A year earlier, a study was conducted by Zhang *et al*. (2018) investigating the use of equisetin, a secondary metabolite isolated from the marine fungus *Fusarium* sp. Z10, as an inhibitor for quorum sensing in *Pseudomonas aeruginosa* and concluded that the metabolite was capable of inhibiting quorum sensing virulence phenotypes

within *P. aeruginosa*. It is also known that *Pseudomonas* spp. are common spoilage organisms in food and as mentioned above harbor potential to increase prevalence of pathogenic bacteria. Therefore, the use of secondary metabolites to control *Pseudomonas* spp. could allow for a new intervention method for food processing plants without the need for genetic alterations or harmful biocides. Thereby, the use of the microbiome as a biotechnological tool could enhance the industry's ability to produce food and food products in a manner in which consumers deem suitable. It could also prove as an alternative to antimicrobial use in these facilities furthering the consumer's likeability of food products.

1.3. CRISPR-Cas Systems and Agriculture

Another exciting tool for furthering biotechnology in agriculture is the use of CRISPR-Cas systems for genetic editing. The use of these systems allows for genome engineering to provide products that are capable of being resistant against disease and biocides. However, these food products have the undesirable "genetically modified organism" label associated with them and cause controversy among producers and consumers alike. Interestingly, one of the more exciting applications is the use of CRISPR-Cas systems in an indirect fashion to promote health and quality of livestock and food products by utilizing the genetic editing tool against harmful microorganisms. This use of CRISPR-Cas systems could provide a novel way for pathogen intervention without being directly used on the animal or plant genome.

1.3.1 Brief History of CRISPR Systems

Clustered regularly interspaced palindromic repeats, or CRISPRs, were first identified in 1987 by Japanese researchers studying the structure of the *Escherichia coli* genome (Ishino *et al*., 1987). The novel DNA repeat family termed CRISPR is a defense mechanism found in many species of bacteria and archaea that is utilized for protection against foreign invaders. A locus in which CRISPR is found to contain direct repeat sequences that are separated by unique spacer sequences (Jansen *et al.* 2002b). The spacer sequences are derived from the DNA of infectious virus or plasmid and are then used as a memory bank upon reinfection of the corresponding virus or plasmid (Khanzadi & Khan, 2019). Furthermore, it is often that *cas* genes are found adjacent to the repeat and spacer arrangement; these *cas* genes originally thought to be used for DNA repair, are theorized to be analogous to the eukaryotic RNA interference defense system (Makarova *et al*., 2006; Barrangou & Horvath, 2012). Numerous *cas* genes have been identified however, of those *cas* genes, only two, *cas1* and *cas2*, appear to be universal to all CRISPR-Cas systems (Markarova *et al*., 2015; Sternberg *et al*., 2016; Jackson *et al*., 2017).

1.3.2. CRISPR-Cas System Identification and Classification

CRISPR-Cas systems have been placed in two different system classes, comprised of six different system types, which reflect the complex classification of CRISPR systems which are broken up into two classes based on effector module design (Mir *et al*., 2017; Koonin *et al*., 2017; Koonin & Makarova, 2019). This approach of classification takes into account several criteria: i.) the signature *cas* genes ii.) the similarity of sequences shared between Cas proteins iii.) Cas1 protein's phylogeny due to it being the most conserved of the Cas proteins iv.) where the gene lies within the CRISPR-cas locus and v.) the actual structure of the CRISPR mechanism (Makarova *et al*., 2011b; Makarova *et al*., 2015; Koonin *et al*., 2017). Class 1 CRISPR systems

differ from Class 2 systems as Class 1 systems have multisubunit effector complexes that are made up of many Cas proteins; Class 2 systems on the other hand have a large, single effector module and is considered a single, multidomain protein (Koonin & Makarova, 2019). Within each class of CRISPRS, there are subgroups referred to as types that are characterized based the effector module's architecture using unique signature proteins (Koonin & Makarova, 2019). Class 1 systems are made up of Type I, Type III and the rare Type IV (Koonin *et al*., 2017). Type I CRISPR-Cas systems are identified via a universal presence of a large signature gene termed *cas3*. The *cas3* gene is thought to be involved in R-loop-dependent target DNA cleavage (Cady & O'Toole, 2011). The Type III CRISPR-Cas systems are distinguished via the signature *cas10* gene which encodes for a repeat-associated mysterious protein (RAMP) which is involved in processing CRISPR RNAs (crRNA) and target DNA cleavage (Anantharaman *et al*., 2010). Type IV systems are very rare and lack an adaption module in its rudimentary CRISPR-Cas loci (Koonin *et al.,* 2017). Type I systems seem to occur in multiple phylogenetic clades of both bacteria and archaea; however, it is notable that Type II systems are exclusive to bacteria and Type III systems appear more commonly among archaeal species (Makarova *et al*., 2011). Furthermore, Type I and Type III systems have several similarities including the reliance on the signature *cas6* gene to cleave the repeat sequences of crRNA to produce small crRNA's (Barrangou & Marraffini, 2014). Type I and Type III also require the use of a large complex of Cas proteins for crRNA-guided DNA targeting (Doudna & Charpentier, 2014).

Class 2 CRISPR system types include Type II, Type V and Type VI (Koonin *et* al., 2017). Type II CRISPR-Cas systems are the systems that typically contain the signature *cas9* gene. The *cas9* gene encodes for a large protein with multiple functions and also harbors the ability to generate crRNA—which were shown to serve as guides in a concert of Cas proteins to

disrupt virus proliferation in *Escherichia coli*—and are capable of targeting phage and plasmid DNA for degradation (Brouns *et al*., 2008; Garneau *et al*., 2010; Deltcheva *et al*., 2011). The CRISPR model found within *S. thermophilus* is indeed a Class 2, Type-II system (Barrangou *et al*., 2007; Barrangou & Horvath, 2012). In contrast to Class 1Type I and III systems, Class 2 Type II require very little Cas-associated mechanisms for immune responses; these systems utilize a trans-encoded CRISPR RNA (tracrRNA)for crRNA biogenesis (Deltcheva *et al*., 2011, Barrangou & Marraffini, 2014). DNA targeting and cleavage in Type II systems is carried by a large single, multidomain protein – Cas9 (Barrangou & Marraffini, 2014). Type V systems contain the predicted effector protein Cpf1 (Cas12a) that unlike Cas9, does not require the additional tracrRNA for DNA cleavage, making it a potential alternative to genome editing (Zetsche *et al*., 2015). Type VI systems contain the signature *cas13* gene and has only two recognizable features termed HEPN1 and HEPN2 (Koonin *et al*., 2017).

1.3.3. CRISPR-Cas9 Functionality and Mechanisms of Genome Targeting and Editing

First identified as a large multifunctional protein (Makarova *et al*., 2002), the *cas9* protein (formerly COG3513) is the essential Cas protein in Type II CRISPR-Cas systems (Doudna & Charpentier, 2014). The protein has two putative nuclease domains: i.) the HNH motif, which is present in various nucleases including colicin E9 – causes cell death by forming double-stranded breaks into the DNA (Saravanan *et al*., 2004; Bolotin *et al*., 2005); and ii.) RuvC-like, a canonical two-metal dependent catalysis whose mechanisms have been proposed for viral large terminases (Makarova *et al.,* 2006; Doudna & Charpentier, 2004: Xu *et al*., 2017). While studying the Type-II CRISPR-Cas model organism, *S. thermophilus*, it was shown that the Cas9 protein was essential for defense against bacteriophages (Barrangou *et al*., 2007).

Furthermore, it was proposed that Cas9 could be responsible for introducing double-stranded DNA breaks in the targeted bacteriophage or plasmid genome (Garneau *et al*., 2010). It was also determined that Cas9 enabled for *in vivo* phage and plasmid targeting in bacteria and that HNH and RuvC domains were required to disrupt plasmid transformation (Deltcheva *et al*., 2011; Sapranauskas *et al*., 2011). It has further been shown while studying the CRISPR-Cas locus in *Streptococcus pyogenes* that tracrRNA is a critical component to the CRISPR machinery in that it is needed to activate crRNA maturation; this maturation step eventually leads to sequence-specific immunity against foreign invaders (Deltcheva *et al*., 2011).

1.3.3.1. CRISPR-Cas Mechanistics

Briefly, CRISPR systems typically work in three stages to facilitate an immune response in the host species. The first stage is known as the acquisition stage; here, DNA from the foreign bacteriophage or plasmid is taken up and incorporated into the host species' CRISPR locus as spacers between crRNA repeats (Wang *et al*., 2016). This initiates the second stage in which Cas proteins are expressed and the transcription of the spacer genetic material into pre-crRNAs begins. Next, the maturation process of pre-crRNAs into mature crRNA takes place via Cas proteins and host factors such as tracrRNA; the mature crRNA acts as a guide containing the spacer sequence and then targets the invading genetic material (Deltcheva *et al*., 2011; Doudna & Charpentier, 2014; Wang *et al*., 2016). The third stage sees the guide crRNA and Cas proteins recognize the target DNA and initiate cleavage of the foreign genome. Most CRISPR systems, including Cas9, utilize a sequence specific protospacer-adjacent motif (PAM), adjacent to the crRNA target site in the invading genome for target action; host organisms in Type I and Type II

CRISPR-Cas systems lack the PAM sequence, thus protecting its own genome from cleavage (Bolotin *et al*., 2005; Shah *et al*., 2013; Wang *et al*., 2016).

1.3.3.2. CRISPR-Cas9 and Genomic Editing

The CRISPR-Cas9 system is the most widely utilized system for genomic editing. Cas9 and the duplex of its crRNA and tracRNA can be manipulated for genomic editing and regulation. For the most practical and simplified use of this system, Jinek *et al*. (2012) demonstrated that the crRNA-tracrRNA duplex can be fused into a chimeric single guide RNA (sgRNA). Because of this, the Cas9-sgRNA is the most widely used variant for gene editing purposes (Wang *et al*., 2016). The Cas9-sgRNA system works by binding DNA with base pairs that can bind the sgRNA and also have an adjacent PAM, thereby facilitating cleavage of the target DNA base-pair region. A simple manipulation of an approximately 20-nucleotide region of the sgRNA to pair with the target DNA, the Cas9 protein can be "programed" to target any genomic locus containing a PAM sequence. This makes it an easy tool to be utilized for specific genome targeting (Wang *et al*., 2016).

1.3.4. Applications of CRISPR-Cas9 in Agriculture and Food Safety

Since the discovery of CRISPR-Cas systems and specifically the CRISPR-Cas9 system, many industries and multidisciplinary fields of science have sought to harness its genome editing abilities. Application of the CRISPR machinery can be found in many aspects of food and agriculture. The first reported study in agriculture utilizing CRISPR-Cas9 was done by Feng *et al*., (2013) in which genomic engineering of rice was evaluated. The study highlighted the ease

in which the CRISPR-Cas9 system was able to modify the genome of rice and sparked further evaluation of the use of the gene editing tool in agriculture.

1.3.4.1. CRISPR-Cas9 Machinery and Crops

In crop production, the CRISPR-Cas9 machinery has seen use in creating virus-resistant plants (Ali *et al*., 2015; Baltes *et al*., 2015; Ji *et al*., 2015; Tashkandi *et al*., 2018); resistance to fungal diseases (Wang *et al*., 2014; Li *et al*., 2017; Foster *et al*., 2018); resistance to bacterial pathogens (de Thomazella *et al*., 2016); improvements in stress resistance, both physical and chemical (Svitashev *et al*., 2015; Shi *et al*., 2017); and enhancements in nutrition (Andersson *et al*., 2017; Andersson *et al*., 2018; Nakayasu *et al*., 2018). The versatility that CRISPR-Cas9 gives to genomic editing is imperative to new discoveries in food and crop science. It has given researchers and industry personnel the ability to generate crops that are capable of withstanding several stressors and could prove highly beneficial to meeting the global demand for food.

1.3.4.2. CRISPR-Cas9 in Livestock Production and Genetics

The CRISPR-Cas9 machinery has also shown promise for utilization within food animal production and genetics. For example, Ikeda *et al*. (2017) applied the CRISPR-Cas9 machinery to repair a single nucleotide mutation in the *IARS* gene of Japanese Black cattle. The mutated gene is responsible for the recessive genetic disorder in Japanese Black cattle known as Isoleucyl-tRNA synthase (IARS) syndrome, which alters neonatal fitness and causes more prenatal deaths when affected with the disorder. The results of the study showed promise in using CRISPR genome editing to reduce the waste of meat and genetic resources. Additionally,

Bi et al. (2016) demonstrated CRISPR-Cas9's ability to generate elite genetic lines of cloned pigs for a reliable strategy to minimize potential biological risk of environmental contamination of mutated cells.

1.3.4.3. Animal Welfare and CRISPR-Cas9

Additional research has focused on the utility of CRISPR-Cas9 to enhance health in livestock animals. In 2016, Whitworth *et al*. engineered pigs that were resistant to porcine reproductive and respiratory syndrome virus (PRRSV) via the CRISPR-Cas9 machinery. The team effectively knocked out the viral entry receptor on cells known as CD163. Furthermore, a 2017 study demonstrated the use of CRISPR-Cas9 technologies to insert the NRAMP1 gene into the cattle genome to produce resistance against tuberculosis (Gao *et al*., 2017). In an attempt to better animal welfare, a major talking point of public concern surrounding cattle production, Schuster *et al*. (2020) established the CRISPR-Cas12a system as a viable method to insert the Polled Celtic variant of the *polled* locus of Holstein-Friesian cattle into the genome of horned Holsteins to generate offspring of the polled (de-horned) phenotype. The CRISPR-Cas12 system is a Type V system within class 2 CRISPR systems. It is in the same class as the Type II CRISPR-Cas9 system.

1.3.4.4. CRISPR-Cas9 as an Antimicrobial

CRISPR-Cas9 has also been used to target foodborne pathogens found within livestock production systems. A study was designed that demonstrated the ability of two cloned guide RNAs (gRNA) target and kill *Escherichia coli* containing the Shiga-toxin producing genes *stx1*

and *stx2*, found in Shiga-toxin producing *E. coli* – STEC (Jia *et al*., 2018). The CRISPR system was able to target and kill the pathogens while leaving *E. coli* that did not carry the Shiga-toxin producing genes unaffected; the system also did not target other bacterial populations as well. Other studies have indicated the ability of CRISPR-Cas9 systems to disrupt self-repair systems in the *E. coli* genome resulting in cell death (Cui & Bikard, 2016). In 2016, Kim *et al*. exhibited the ability of CRISPR-Cas9 to re-sensitize antibiotic resistant *E. coli*. It was shown that 99% of *E. coli* cells transformed with a plasmid harboring the CRISPR-Cas9 machinery were able to be killed with Ampicillin. Further still, in 2019, a study was shown to deplete populations of antibiotic resistant *Enterococci* species while not disrupting other microbial species (Rodrigues *et al*., 2019). Furthermore, it was demonstrated by Yosef *et al*. (2015) that ARGs in *E. coli* could be targeted via CRISPR-Cas9 phage-delivered machinery and cleaved resulting in cell death; the approach allowed for antibiotic resistant bacteria to be targeted while antibiotic sensitive species were left intact. Citorik *et al*. (2014) also demonstrated the ability of RNA-guided nucleases via a CRISPR-Cas9 system to target and cleave multidrug-resistant genes in *E. coli*. Furthermore, studies have also indicated the ability of CRISPR-Cas9 systems to target and kill methicillin resistant *Staphylococcus aureus* (MRSA) as well as develop more efficient methods of phage-delivered CRISPR-Cas systems (Park *et al*., 2017). Further studies have found to be in support of these findings (Gomaa *et al*., 2014; Jiang *et al*., 2013). The use of CRISPR-Cas9 in this manner provides a potential alternative to the overuse of antibiotics and could alleviate the selective pressure of ARGs in pathogenic bacteria.

1.3.4.5. The Microbiome and CRISPR-Cas9

The use of CRISPR-Cas systems is anticipated to be used to manipulate the microbiome of plant and animal systems within the food chain along with the farm environment to provide competent alternatives in effectively controlling pathogenic outbreaks (Barrangou & Notebaart, 2019). For example, studies have indicated the ability of lytic phages to be used on carcasses in processing for control of pathogens (Hagans & Loessner, 2010). With the knowledge that phage-mediated CRISPR-Cas delivery systems have effectively reduced virulence and antibiotic resistance genes, the use of these systems as biocontrol agents could prove useful in the processing environment. Further research is needed to reveal the effects of CRISPR systems on environments and establish safe measures for use in these systems. The use of CRISPR technologies in conjunction with microbiome science and NGS/WGS provide an exciting new field of research and could provide producers with new methods to combat antimicrobial resistance.

1.3.4.6. Consumer Perception Regarding the Use of CRISPR-Cas9 in Livestock

Moreover, the promising new technology could allow for an improved consumer perception of livestock animal products as it is not being used as a direct genetic editing tool on animals. It could prove to be an ethical and consumer friendly technique in pathogen prevention and control in both livestock systems and processing environment systems. Interestingly enough, studies have found that consumers have positive outlooks regarding genetically modified food and food products given they are informed of the facts (Hoban, 1998; Lusk *et al*., 2004; Delwaide *et al*., 2015; Shew *et al*., 2018). However, these studies focused on plants and associated plant food products. The use of genetically modifying tools in animals is a bit trickier. The negative impacts that could occur in animals resulting from the use of genetic engineering is

one of the main concerns consumers have regarding the use of biotechnology in animals (Miles & Frewer, 2001; Schuppli & Weary, 2010). What is of interest however, is one study found that consumers were willing to accept the use of genetic engineering tools in cattle for dehorning purposes due to the fact that dehorning is an animal welfare issue and is scrutinized by consumers (USDA, 2018; McConnachie *et al*., 2019). Furthermore, Kilders & Caputo (2021) found an increased willingness to pay for gene-edited secondary animal products (i.e., milk, meat) when the use of genetic modification was marketed as an animal welfare benefit strategy. Therefore, gene editing via CRISPR-Cas biotechnologies in livestock animals could be accepted if marketed in a manner that both informs the consumer of how the animal is being treated and also that the technology is intended to have beneficial consequences for the animal.

1.4. Regulatory Hurdles for Biotechnologies

While new biotechnology in agriculture can provide exciting new avenues of research and discovery, the use of these tools is heavily regulated. There are stringent regulations that must be adhered to when discussing biotechnologies such as CRISPR. In the United States, there are three agencies that regulate GMOs: the U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and the Environmental Protection Agency (EPA). The FDA is responsible for regulating genetically altered foods such that if foods differ significantly from components "generally recognized as safe" (GRAS), the product must have FDA approval before commercialization begins (Es *et al*., 2019). It is the responsibility of the FDA to regulate livestock animal genetic modification and cloning (Van der Berg *et al.,* 2020). Furthermore, the FDA uses the guidance of the Federal Food, Drug, and Cosmetic (FD&C) Act and the Public Health Service (PHS) Act to regulate any new animal or human product including

that of biotechnology. The FDA's Center for Veterinary Medicine (CVM) is the oversight agency within the FDA that handles defining a new product's regulatory pathway. The CVM utilizes the FD&C act which in sections 402 and 403 describes the requirements to meet the definition of "food" (i.e., articles used for food or drink in man or animal). The CVM uses this definition to thereby define any additional additives that might go into the food of livestock and pets, including any application of CRISPR or other biotechnologies that could be used as an alternative to current antimicrobials. The second regulatory agency involved in biotechnology product regulation is the USDA, which is responsible for regulating genetically altered plants and crops. It is the responsibility of this agency to regulate planting, transport, import/exports and commercialization of genetically altered crops (Es *et al*., 2019). The third agency involved in CRISPR regulations is the EPA. The EPA is tasked with regulating any microorganism or pesticide developed with genetic engineering machinery (Es *et al*., 2019).

Interestingly, when it comes to regulating the food given to livestock production animals (i.e., cattle, poultry, swine, etc.) or pet animals, there are far more regulations involved. Not only must the product meet regulatory standards set forth by one or more of the above agencies, it must also be regulated by the Association of American Feed Control Offices (AAFCO), which is charged by local or federal law for regulating the sale and distribution of animal feed and animal drugs. Animal feed must meet the uniform definitions set forth by these cooperating agencies in order to enter commerce.

Together, these three agencies (USDA, FDA, EPA) make up an oversight system was established in 1986 termed the Coordinated Framework for Regulation of Biotechnology; this Coordinated Framework is based upon existing laws designed to protect the environment and human and animal health (Bari & Zaman, 2021). Biotechnology tools and their associated

products are assessed using this risk-based system. Each of the agencies have developed their own regulations and guidance documents regarding biotechnology and under the Coordinated Framework, they implement these guidance documents and regulatory protocols under already established laws to both ensure safety and that the biotechnology in question is effective. With the advent of new biotechnologies such as CRISPR-Cas systems, the question of "do these systems fall under these regulatory entities or should there be a separate oversight and regulatory system for these technologies" arises. The Coordinated Framework and its associated regulatory agencies are constantly being updated with new regulations and policies, and therefore, should serve well to regulate new technologies used in agriculture. Understanding how producers and consumers alike view new technologies could provide insight into this debate and allow for further evaluation of oversight regulations. Consumer values surrounding production of their food strongly influence the price they are willing to pay for the product; furthermore, consumer perspectives on how biotechnology is used in production systems of animals can heavily influence the success or failure of the technology (Ufer *et al*., 2019). Therefore, it is important to note the role of the consumer in determining how new technologies are regulated. If a product is regarded as safe by any agency or oversight committee, but is deemed as unacceptable by the consumers, the new technology faces extreme hurdles in getting through the commercialization process and could potentially face demanding proof of scientific research to be regarded as safe.

1.5. Conclusions

Biotechnology in agriculture has been applied to many systems for production of food products. From crops to livestock animals, biotechnology methodologies have proven useful in providing producers and consumers new ways in managing livestock and food production. Two

of the most prominent advances in biotechnological sciences are that of next generation sequencing and the genome editing tool of CRISPR-Cas9. These technologies have provided ways for producers to not only produce crops that are resistant to herbicides and disease, but also alleviate strain of disease in animals. Furthermore, research into manipulating microbiomes and the use of CRISPR-Cas systems as an alternative to antimicrobials has given rise to the use of biotechnology in an indirect manner. While consumers have a preference that lends to food products that are not genetically modified (Ufer *et al*., 2019), the use of these biotechnologies in such a way that does not directly affect a living plant or animal could alleviate the stress of antibiotic usage and consumer perception to current pathogen intervention protocols within production systems. Consumers, industry personnel and regulators alike should keep up with the science of these biotechnologies and come to agreement on how the regulatory framework of these tools can best be used to harness the extreme power they possess to provide the world with safe, quality food and the quantity the world population will demand.

The current review highlights the use of next generation sequencing techniques and CRISPR-Cas systems for potential methods in controlling pathogenic foodborne outbreaks While CRISPR been shown to be useful in genetically modifying living organisms to be resilient to disease, the regulation of these genetic lines along with consumer perception could inhibit the widespread use of these techniques within the livestock and food industries. The use of these technologies must be further researched and be demonstrated to the public that the manner in which they are used can provide products that are not only safe to eat but safe for the plant or animal they are being used in or on. Finally, science must be communicated to the public in a way they can easily interpret and make unbiased decisions on whether or not to buy or use these

products. It is imperative that work be done in this area to improve consumer-producer relations in regards to new biotechnologies.

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Chapter Two

Listeria spp. Establishment and Persistence Within a Newly Constructed Meat Processing Facility and the Role of the Core Microbiome

Summary

Listeria spp. contamination in meat processing facilities poses threats to both the meat industry and public safety. A study was conducted to identify how *Listeria* spp. in a state-of-the-art meat processing facility might be controlled through its microbial ecology. The study explored potential innovative ways for pathogen control and intervention. Samples were obtained from drains, door handles, and walls of the facility for microbial analysis and *Listeria* spp. analysis. Real-time PCR and conventional plating methods were used for *Listeria* spp. confirmation. A total of 1,009 environmental samples were sequenced with 59 samples confirmed by rtPCR based detection and conventional plating methods to be *Listeria* spp. Following speciation, 26 samples were *Listeria monocytogenes*. DNA extraction of the microbial samples were subjected to 16S rRNA gene amplicon sequencing. Taxonomic analysis, alpha diversity, beta diversity, and weighted Random Forest predictive techniques were compared for *Listeria* spp. presence or absence and for different species of *Listeria*. Alpha diversity metrics found no significant difference between *Listeria* absence and presence or between differing species of *Listeria.* Beta diversity distances revealed community separation was driven by room function and evaluated statistically using PERMANOVA ($P < 0.05$). Differential abundance analysis determined several genera to be differentially abundant for *Listeria* spp. presence or absence as well as association with *L. monocytogenes.* These genera included *Pseudomonas, Psychrobacter, Acintobacter,*

Janthinobacterium, and *Prevotella.* Weighted Random Forest algorithms were found to predict *Listeria* presence/absence with an overall accuracy score of 61% indicating a propensity to accurately predict *Listeria* absence but not predict *Listeria* presence. Larger sample sizes will be needed to accurately predict *Listeria* presence using machine learning techniques. Overall findings from this study lay the ground work for utilizing meat processing facility microbial ecology to better control foodborne pathogens.

Introduction

Listeria monocytogenes is a Gram-positive, opportunistic pathogen that is the causative agent of the foodborne illness listeriosis. Scallan *et al*. (2011) estimates that in the United States 99% of all listeriosis infections result from *L. monocytogenes* contamination of food. While most cases of infection are often mild, severe cases of listeriosis have been associated with a variety of foods, including meat and meat products (Scallan *et al*., 2011). Uniquely, this pathogen is genetically heterogeneous and can be grouped into clonal complexes (CC); CC9 and CC121 have been shown to be hypovirulent strains of *L. monocytogenes* and are associated with cattle and beef products at a high rate, especially ready-to-eat (RTE) products (Maury *et al*., 2019).

Contamination from food processing environments is of concern for food safety and public health. Processing environment microbiomes are of concern for food safety because foodborne pathogens from animal sources, such as the hide and fecal material, can result in product contamination (Bell, 1997; Hellstrom *et al*., 2010; Wieczorek *et al*., 2012). A built environment-adapted microbiome may allow for colonization and the persistence of pathogens that can be transferred from the production facility to food sources. Previous studies of built

environments have concluded that the microbial composition of these facilities can lead to facilitation of *L. monocytogenes* establishment (Tan *et al*., 2019). Reconstruction of a meat processing facility also proved to be a vector for the spread of *L. monocytogenes* (Stessl *et al*., 2020). The interactions between species are of critical value when analyzing microbial communities. The relationship between *L. monocytogenes* and other bacterial species is well-documented and has shown contradictory findings; some organisms have been shown to inhibit the establishment of *Listeria* while others can facilitate *Listeria*'s growth (Hassan *et al*., 2004; Leriche & Carpentier, 2000. While processing environment contamination remains a challenge for food safety, a better understanding of the microbial ecology of pathogens may improve intervention techniques.

Metagenomic tools such as 16S rRNA amplicon sequencing have recently been applied to map the microbiomes of food processing facilities, and have discovered that food processing environments have well established microbiomes and that these microbiomes are also found on or in food products (De Filippis *et al*., 2021). Studies have also been conducted to understand the differences in food-associated microbiomes (Jarvis *et al*., 2018). Hultman *et al.* (2015) reported that distinct rooms and materials used in meat processing facilities have unique taxonomic relative abundances. Amplicon sequencing has become a powerful tool for the food industry to try and understand the dynamics occurring between microbiota as well as answering relevant questions regarding food safety. This technology has been utilized to determine that spoilage associated microorganisms can enhance meat processing contamination by *Listeria monocytogenes* (Zwirzitz *et al*., 2021). While the built environment plays a role in food product microbiomes, there is a critical need to enhance understanding how the built environment

microbiome interacts with the establishment of pathogens in food products and production facilities.

While many of these studies have been conducted within facilities that are either in-use or undergoing renovations (Tan *et al.,* 2019; Wagner *et al*., 2020; Stessl *et al*., 2020; Zwirzitz *et al*., 2021), few studies have been conducted in newly built processing facilities. Microbiomes of operational facilities at the time of sampling could be mostly established and critical information could be missed in understanding how those microbial profiles exist. To enhance understanding regarding some of these relationships and challenges, a study was designed to analyze the establishment and persistence of *Listeria* spp. in a newly constructed meat processing facility and, further, determine the role of the microbiome in a new facility in facilitating or inhibiting establishment of *Listeria*. 16S rRNA amplicon sequencing and *Listeria* spp. surveillance of environmental surfaces were applied in simultaneously to investigate the microbial ecology of *Listeria* spp. This study sought to identify potential taxonomic relationships that could elucidate interspecies interactions for innovative intervention techniques. Machine learning techniques were used to explore the ability to predict the presence of *Listeria* spp. based on surface microbial ecology in a meat processing facility.

Methods and Materials

Experimental Design and Sample Collection

A study was designed to analyze the establishment and persistence of *Listeria* spp. in the newly built Global Food Innovation Center in Fort Collins, Colorado. The state-of-the-art

processing and research facility was designed with space allocated for specific room function roles to mimic the operation of a small commercial processing facility (Figure 2.1). These room functions included live animal (where animals were housed); harvest (where animals were slaughtered and converted to carcasses); fabrication and processing (where carcasses are transformed into meat products); product holding (where animal carcasses were stored prior to fabrication and rooms where fresh product was stored or frozen); and non-product (where no production occurred or live animals were not introduced). Sampling occurred after post-construction sanitary protocols were completed and continued monthly from January 2019 through August 2020; sampling ceased due to the COVID-19 global pandemic during the months of February-May 2020. A total of 64 sites were specified for sampling including drains, door handles and walls throughout the processing facility. A total of 14 sampling events were conducted to sample for both microbial analyses and for *Listeria* spp. establishment. A sterile, double-tipped swab (BD; Franklin Lakes, NJ) was used to collect microbiome analysis samples and a buffered sponge (Whirl-Pak; Madison, WI) was used for the collection of samples utilized for *Listeria* spp. analyses. For drains, the top and bottom drain cover was swabbed as well as the opening of the drain itself. Door handles were swabbed at the point of employee hand contact or on the actual doorknob; furthermore, if there were multiple doors (i.e., swinging doors) the right door was chosen for sampling. The smokehouse doors in the production facility were the only doors to have both door handles swabbed for sampling. Wall samples were collected at a designated location approximately five feet high and six square inches in area. It should be noted that wall samples were subsequently excluded from sequencing and *Listeria* spp. confirmation due to the lack of microbial biomass found. Microbiome analysis samples were frozen at -4◦C

immediately following sampling while *Listeria* spp. samples were processed for PCR (see below).

Sample Prep for Listeria spp. rtPCR Using GENE-UP®

Samples were placed into *Listeria* Phage Technology (LPT) Broth (i.e., a *Listeria* enrichment media; Biomerieux: Marcy-I'Etoile, France), hand massaged for 60 s immediately after collection and then placed into an incubator (37º C) for ± 22 h. Samples were prepped for GENE-UP® PCR per the manufacturer's instructions (Biomerieux: Marcy-I'Etoile, France). Briefly, incubated samples were taken, and one mL of each sample was placed into a corresponding tube (Eppendorf, manufacturing location); these tubes were then used for cell lysis. Twenty microliters of each sample were taken and pipetted into individual lysis strips and placed into a 96-well plate holder. The plate was then vortexed for five minutes. Ten microliters of lysed sample were then placed into the GENE-UP® LIS-2 kit (Biomerieux: Marcy-I'Etoile, France) for PCR. Using the GENE-UP® Routine software, a plate map was generated for each PCR run. Two positive and two negative controls were used with the positive controls being a pure *Listeria innocua* culture and a pure *Listeria monocytogenes* culture. The negative controls were uninoculated LPT broth.

GENE-UP® Listeria spp. Confirmation and Cryopreservation

Samples prepared for GENE-UP® PCR were then placed into the GENE-UP® thermocycler and with the corresponding plate map were ran ~ 1 h cycling time. Positive

samples were then subjected to culture confirmation via microbiological plating techniques. Briefly, if a sample was detected as positive, a sterile inoculating loop was utilized to plate each original sample on individual Modified Oxford plates supplemented with yeast extract (MOX/YE). The plates were then incubated at 37◦ C for ~ 48 h. If typical *Listeria* spp. colonies formed, three individual colonies were taken and plated individually on new MOX/YE plates and incubated for 37◦ C for ~48 h. An additional plating/incubation step was done for a purification step. Following the purification step, each isolate was then subjected to GENE-UP® preparation (i.e., lysis and PCR prep) as described above. The isolates were then placed into the GENE-UP® thermocycler for PCR confirmation. Positive isolates were deemed "confirmed-positives" and were frozen in a 10% glycerol stock and stored at -80◦ C for future analyses.

Listeria Speciation

Samples confirmed as a positive *Listeria* spp. sample were speciated utilizing the API® LISTERIA (Biomerieux: Marcy-I'Etoile, France) kit. The samples to be tested were prepared and used per the manufacturer's instructions. Briefly, frozen stock cultures of confirmed *Listeria* spp. were placed onto Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar and incubated for 48 h at 37◦ C; a purification step was then preformed on these cultures. Next, one isolated colony from purified plates were individually plated onto new PALCAM agar plates and incubated at 37◦ C for 24 h. The API® LISTERIA kit was then used for speciation of the samples. An incubation box was prepared by distributing about 3 mL of distilled water into the honeycombed wells. The testing strip was then placed into the incubation box and the sample name was written on the box. Several well isolated colonies from each

sample were individually placed into the API® Suspension Medium and the turbidity was to be equivalent to 1 McFarland. Once the inoculum was prepared, each sample was then pipetted into the wells (100 µL for the |DIM| test – an enzymatic substrate used for differentiation of *L. innocua* and *L. monocytogenes* – and 50 µL for the remainder of the tests in the test strip). All incubation boxes were closed and incubated at 37° C for roughly 24 h. Following incubation of the strips, ZYM B reagent (Biomerieux: Marcy-I'Etoile, France) was then placed into the |DIM| test and reactions from all wells were read and recorded. Results were recorded and a numerical profile was generated. The numerical profile was entered into the APIWEB™ software database (Biomerieux: Marcy-I'Etoile, France) and speciation of the sample was attained.

DNA Extraction and Sequencing

Paired-end 16s rRNA gene sequencing was utilized for analysis of microbial community composition. Qiagen PowerSoil Kits (Qiagen: Hilden, Germany) were used to extract the DNA per manufacturer's instructions across the sampling sites. Extraction was done via 96-well plates each with seven negative controls and one positive control (e.g., a microbiome mock community; Zymo; Irving, CA). Amplification and sequencing of the extracted DNA was done following the Earth Microbiome Project Protocols (EMP: www.earthmicrobiome.org) (Thompson *et al.*, 2017). The 515f forward primer and the 806R reverse primer were each barcoded with an error-correcting golay barcode which allowed for multiplexing. Picogreen Quant-iT (Invitrogen, Life Technologies; Grand Island, NY) was used for PCR product quantification and the quantified PCR products were then pooled at an equimolar concentration used for sequencing. Sequencing occurred via a 500-cycle kit on the Illumina miSeq sequencing platform (Illumina; San Diego,

CA). Samples were randomly placed across four sequencing lanes due to the high number of samples and time between sampling events. Randomization was done to prevent confounding effects due to technical errors.

Statistical Analysis and 16s rRNA Sequencing Analysis of Listeria

A generalized linear model was fit, and the estimated marginal means were established for predictions of the presence of presumptive and confirmed *Listeria* within the various rooms of the facility. 16S rRNA gene amplicon data were demultiplexed and denoised via the DADA2 plugin within the QIIME2 software (Bolyen *et al*. 2019; Callahan *et al*. 2016). Using a pre-trained machine learning classifier and the SILVA reference database, taxonomy of the demultiplexed sequences was obtained (Pedregosa *et al*. 2011). The taxonomy was used for filtering mitochondria and chloroplasts out along with additional filtering steps that removed sequences that appeared in less than 10% of the samples. A phylogenetic insertion tree was constructed following filtering along with rarefaction at a sampling depth of 9,204; using these parameters, phylogenetic analyses were conducted using the core metrics pipeline in QIIME2 (Janssen *et al*., 2018; Matesen *et al*., 2010; Bolyen *et al*., 2019). Alpha diversity was measured for both *Listeria* presence or absence as well as *Listeria* species across room function utilizing Shannon's Index (Shannon & Weaver, 1949) to calculate the richness and diversity of the sample while Faith's phylogenetic diversity (Faith, 1992) was used to measure the biodiversity while incorporating phylogenetic differences between species. Richness was also measured for alpha diversity (McIntosh, 1967). Kruskal-Wallis pairwise testing (Kruskal & Wallis, 1952) was used to analyze alpha diversity metrics with significance set at alpha level 0.05. Beta diversity

analyses used unweighted UniFrac distances (Lozupone *et al*. 2007) to measure the community structure differences in the presence or absence of *Listeria* across room functions as well as the unweighted UniFrac distance for community structure of different *Listeria* species across room functions. Further, Chi-Squared Pearson's Correlation test was used to analyze the relationship between room function and *Listeria* species with alpha level set at 0.05. Differential abundance was investigated using ANCOM (Mandal *et al*., 2015) to determine differentially abundant ASVs (amplicon sequence variants) when *Listeria* was present. Machine learning was utilized to predict *Listeria* presence within a microbiome using the QIIME2 sample-classifier plugin (Bokulich *et al*., 2018). Statistical testing and visualizations were done using RStudio version 3.5.1.

Results

Listeria spp. Presence in the Facility

A total of 70 samples were designated as presumptive for *Listeria* spp., with 59 of these samples confirmed as *Listeria* spp., and a total of 26 samples were speciated as *Listeria monocytogenes* (Figure 2.2). The probabilities of finding *Listeria* spp. within a newly constructed meat facility were evaluated using a generalized linear model. Due to the low number of samples confirmed as *Listeria monocytogenes,* probabilities were not estimated for these samples. Presumptive samples (Figure 2.3a) indicated a probability of finding *Listeria* 50% of the time in livestock holding, with the second highest being fabrication (33%) and third highest being 'dirty harvest' (31%). Confirmed samples (Figure 2.3b) revealed livestock holding

was again the highest probability at 50%, fabrication the second (27%) and carcass holding being the third highest (20%).

Microbiome Diversity Metrics

Alpha diversity metrics resulted in no difference ($P > 0.05$) when *Listeria* was either present or absent for any of the room functions (Figure 2.4). There were no differences found for any *Listeria* species and room function for alpha diversity (Figure 2.5). Community composition was visualized using an unweighted-UniFrac PCoA plot (Figure 2.6) and revealed that room function drove separation regardless of *Listeria* spp. presence or absence. When comparing the species of *Listeria*, certain species also correlated to separation and clustering. *Listeria innocua* samples were more correlated with live animal and harvest rooms and separated from *Listeria monocytogenes*, which was more correlated with fabrication and processing as well as non-product and product-holding (Figure 2.7). PERMANOVA analysis of room function when *Listeria* spp. was present indicated compositional changes between the microbiomes (p-value < 0.001).

Microbiome Differential Abundance

Differential abundance was tested using ANCOM for the presence and species of *Listeria*. Several features were indicated as being more abundant when *Listeria* spp. were present. These included the species *Chryseobacterium piscium* and *Corynebacterium marinum,* the genera *Brevundimonas, Flavobacterium, Acinetobacter*, and the family *Planococcaceae*.

Interestingly, there were far more abundant features when *Listeria* spp. was not present. These included the genus' *Psychrobacter, Staphylococcus, Pseudomonas*, *Alkanindiges* and *Chryseobacterium* as well as the families *Comamonadaceae* and *Planococcaceae* (Figure 2.8).

Differential abundance of *Listeria* spp. presence by room function reported three features to be more abundant when *Listeria* spp. were present within the live animal space (Table 2.1). The genera *Chryseobacterium* and *Flavobacterium* were found to be more abundant in the presence of *Listeria*. The differentially abundant features in the presence of *Listeria* spp. for harvest rooms can be found in Table 2.2. These included the genera *Clostridia* UCG-014, *Muribaculaceae, Eubacterium* and *Prevotellaceae*. Fabrication and processing revealed genera *Chryseobacterium, Acinetobacter* and *Psychrobacter* as more abundant in the presence of *Listeria*; the genera *Acinetobacter* and *Chryseobacterium* were associated with features more abundant in the absence of *Listeria* spp. (Table 2.3).

Pseudomonas was differentially abundant with the presence of *Listeria monocytogenes* (Figure 2.9). The genus *Janthinobacterium* was shown to be differentially abundant in fabrication and processing and associated with *Listeria innocua*. *Acinetobacter* was found to be differentially abundant in live animal, and was associated with all species of *Listeria.* The genus *Prevotella* was also found to be differentially abundant in harvest and live animal and the genus *Pedobacter* was also found to be differentially abundant in the presence of *L. monocytogenes* in product holding and fabrication and processing (Figure 2.9).

Microbiome-based prediction of Listeria spp.

A machine learning model was used to predict the presence of *Listeria* spp. from microbiome samples across different room functions. Using a Random Forest classifier, models were fit across all rooms, and then individually for room functions live animal, harvest, and fabrication and processing. The resulting confusion matrices can be found in Figure 2.10. For live animal, an overall accuracy score of 84% was achieved with area under the curve (AUC) scores being 0.96 for both "no" and "yes" for *Listeria* spp. presence. For both harvest and fabrication and processing room functions, a limited sample size could have skewed the results. The machine learning model for harvest, an overall accuracy score of 95% was achieved with AUC scores for "no" and "yes" being both 0.97. The overall accuracy score for the fabrication and processing model was 96% and AUC scores for "no" and "yes" were 0.85 respectively. To correct for imbalanced class size within this dataset, a Weighted Random Forest model was constructed to analyze predictability of the facility microbiome and the presence or absence of *Listeria* spp (Figure 2.11). However, the overall accuracy score of the model was 61% as it could only accurately predict the absence of *Listeria* spp. at an accurate level.

Discussion

To the best of our knowledge, the current study is the first to detail the environmental differences in facility microbial ecology leading to the establishment of *Listeria* spp. in a newly constructed meat processing facility. Our findings indicate a relationship between the core microbiomes of individual rooms and rooms of different function were associated with differing species of *Listeria*. Similar to previous findings, our results displayed a higher incidence of confirmed *Listeria* spp. within rooms housing live animals due to the hide of livestock animals

being a major contributor to carcass contamination at slaughter (Bell, 1997; Hellstrom *et al*., 2010; Wieczorek *et al*., 2012).

While it has been previously established that *L. innocua* is considered a surrogate for *L. monocytogenes* due to their close genetic relationship, the impact of differing environmental conditions is distinct between the two species (Glaser *et al*., 2001; Meylheuc, *et al*., 2002; McLaughlin *et al.,* 2011; Milillo, *et al*., 2012). The findings within the current study indicate that the presence of *L. monocytogenes* or *L. innocua* could be linked to the environmental conditions within a particular room function. For example, *L. monocytogenes* is considered a psychrotolerant bacterium due to its capability of growth at temperatures below freezing due to changes within the membrane, production of cold shock proteins, and the ability to acquire cryoprotective compounds (Phadtare *et al*., 1999; Neunlist *et al*., 2005; Chan & Wiedmann, 2009; Cordero *et al*., 2016). The meat processing facility within the current study adhered to strict temperature regulations and studies have indicated the ability of the hypovirulent clones of *L. monocytogenes* clonal complexes (CCs) 9 and 121 to be highly associated with meat and meat products particularly due to genetic alterations enabling them to be highly adapted to the meat processing environment (Maury *et al.,* 2019; Stessl *et al*., 2020). These findings validate our results of *L. monocytogenes* confirmation in the fabrication and processing rooms because the CCs of *L. monocytogenes* could explain the higher occurrence of this species within the colder meat storage or processing environments as opposed to live animal rooms. While the microbiome within the individual sample sites (i.e., drain, door handle) could in fact play a role in the establishment of the two different *Listeria* species, the findings within this study, coupled with previous findings, indicate a potential link between the spatial environments. Further studies would be needed to address these hypotheses.

Sanitation and cleaning of the facility could also play a role in *Listeria* spp. establishment. Indeed, all rooms are subjected to the same sanitation protocols, and cleaning of the facility occurs regularly. However, live animal holding, and slaughter rooms are subjected to higher traffic of potentially contaminated sources such as animal hide and fecal material. Carcasses may be contaminated during the slaughter process although sanitation methods of the carcass have been studied for the effective removal of pathogens from the carcass such as the use of steam and high-pressure water treatments (Dixon *et al*., 2019). Because carcasses are sanitized prior to fabrication, fabrication and processing areas were less likely to encounter severely contaminated meat and meat products. Not surprisingly, the diversity of further processing rooms would be lower due to adequate sanitation. The current study provides support that individual room environments influence microbial diversity and therefore, possibly the establishment of *Listeria* spp.

While diversity differences were not seen within each room in association with the presence or absence of *Listeria* spp., taxonomic associations were observed. Similar to previous findings, live animal rooms were shown to have associations between *Listeria* spp. presence and the genera *Flavobacterium* and *Chryseobacterium* (Bernardet & Bowman, 2006; Oosthuizen *et al*., 2019). *Chryseobacterium* has previously been isolated from *L. monocytogenes* positive drains and has been linked to biofilm formation (Dzieciol *et al*., 2016; Wagner *et al*., 2020). These previous findings are consistent with the findings in the current study. Because *Prevotella* is known to be a dominant member of the ruminant microflora, the differential abundance of the family *Prevotellaceae* in harvest rooms could be linked to the removal of the rumen and viscera on the harvest floors (Stevenson & Weimer, 2007). However, there is not a well understood relationship between *Listeria* spp. and *Prevotellaceae*. Fox *et al*. (2014) demonstrated that there

was an association between *Listeria* spp. negative drains and the presence of *Prevotella* although the causation for this relationship remains unknown. One theory for this association could be due to the ability of *Prevotellaceae* to produce propionate (Polansky *et al*., 2016). Propionate has been recognized as a safe antimicrobial food additive that has shown an ability to reduce the growth of *L. monocytogenes* in ready-to-eat meats (Glass *et al*., 2013; Dussault *et al*., 2016). Research has also shown that in solid food types such as meat and surfaces where *L. monocytogenes* has greater adherence, the combination of propionate and the lack of oxygen could be extremely effective in preventing *L. monocytogenes* growth (Rinehart *et al*., 2018). A negative association between *Prevotellaceae* and *Listeria* spp. could be due to the antimicrobial effects of secondary metabolites produced by certain genera of *Prevotellaceae*. This hypothesis requires further investigation.

An interesting finding of the current study was the differential abundance of the genera *Pseudomonas*, *Psychrobacter*, and *Acinetobacter* in the presence/absence of *Listeria* spp. due to the ability of these genera to form biofilms (Puga *et al*., 2016; Wagner *et al*., 2020). The major component of biofilm formation is that of the extracellular matrix (ECM) and is a primary driver for biocide resistance (Flemming *et al*., 2016). The ability of certain species, primarily that of *Pseudomonas*, to produce EPSs (extracellular polymeric substrates) allows for *L. monocytogenes* to take shelter within the matrix (Huis in't Veld, 1996; Liao, 2006; Puga *et al*., 2018). The current study demonstrated an association between *Pseudomonas* and *Listeria* spp. absence; however, one feature, determined to be *Pseudomonas*, was differentially abundant in the presence of *L. monocytogenes.* This finding is validated by the literature as contradicting findings have determined the ability of certain species of *Pseudomonas*, particularly that of *P. fluorescens,* has been shown to either inhibit the growth of *L. monocytogenes* or facilitate the

pathogen's growth and survival due to protective effects of biofilm formation (Buchanan & Bagi, 1999; Carpentier & Chassaing, 2004; Puga *et al*., 2016). While *Pseudomonas* spp. are considered a common member of the meat processing microbiome and have been shown in higher proportions when associated with raw meat product, the environment in which *Pseudomonas* is isolated could be the key in understanding how *Listeria* spp. and *Pseudomonas* will interact (Hultman *et al*., 2015; Roder *et* al., 2015; Dziecol *et al*., 2016; Maes *et al*., 2019; Wagner *et al*., 2020).

For example, psychrotrophic *Pseudomonas* are known for their dominance within meat processing environments. Studies have indicated that *Pseudomonas* can enhance *L. monocytogenes* growth on stainless steel surfaces; and in the presence of higher salt concentrations or colder temperatures (i.e., 10 ◦C) *L. monocytogenes* has an enhanced ability to adhere to biofilms (Hassan *et al*., 2004; Lee *et al.,* 2017; Lee *et al.,* 2019). Salt is a common preservative of meat and meat products and its application to meat usually occurs within the colder temperature processing rooms. Additionally, it has been reported that *P. fluorescens* can inhibit the growth of *L, monocytogenes* in low salt conditions (Buchanan & Bagi, 1999; Carpentier & Chassaing, 2004), and associations between *Pseudomonas* and *Listeria* spp. could be driven by the environmental conditions such as salt concentration found within individual rooms within a processing facility. Nutrient competition or the lack thereof could also influence how *L. monocytogenes* responds in meat processing biofilms. The association between *L. monocytogenes* and *Pseudomonas* within the current study occurred in the more diverse live animal room. A potential theory for this finding is that possible adherence to *Pseudomonas*-produced biofilms could protect *L. monocytogenes* and allow it to grow and establish because normal background microflora or more diverse environments could reduce the growth of *L.*

monocytogenes (Jia *et al*., 2020). Additional studies of *Listeria*-biofilm interactions will be needed to validate this claim.

The current study revealed that *Janthinbacterium* had features associated with both *L. innocua* and *L. monocytogenes*, which challenges previous findings. For instance, Fox *et al*. (2014) found that *Janthinobacterium* was associated with *Listeria*-negative drains in association with a multi-species biofilm however, the mechanistic features of this interaction within a meat processing environment are still unknown. Environmental conditions within each room of the meat processing facility in the current study are likely drivers for microbial community composition and diversity. The relationship between *Janthinobacterium* and *Listeria* spp. in the current study provides further evidence that environmental factors (i.e., temperature, pH, salt concentration) influences the ecology of *Listeria* spp. in a meat processing environment. The relationship between *L. monocytogenes* and *Janthinobacterium* in the current study could also be due to the ability of certain species of *Janthinobacterium* to produce antimicrobial compounds that have an inhibitory effect on Gram-positive bacteria (O'Sullivan *et al*., 1990). Secondary metabolites produced by certain biofilm-forming genera such as *Prevotella* and *Janthinobacterium,* could allow for the use of innovative processing intervention techniques by using live microorganisms for the control of pathogenic bacteria such as *L. monocytogenes*. The true nature of the *Listeria*-biofilm relationship was undetermined as a major limitation of the current study was that biofilms were not directly assessed. Differences in product type (i.e., poultry vs. beef vs. dairy) could also alter how inter-species relationships unfold in biofilms and were also not assessed during this study. Future studies of biofilm microbial communities, product type, *Listeria* spp. interaction, and environmental factors will need to be analyzed to better understand these findings and inferences.

One of the goals of the study was to analyze the microbiome of the meat processing facility for a potential role in predicting the presence of *Listeria* spp. Random Forest machine learning algorithms were applied for this purpose. Due to the low rate of confirmed *Listeria* spp. samples, the model was trained on imbalanced data. Because of this, the model favors majority class prediction, leading to accurate predictions about the majority class, but far less accurate predictions on the minority class (Kubat *et al*., 1998). Our models were predicting accurately for microbiomes absent of *Listeria* but were unable to accurately predict *Listeria*-positive microbiomes. Studies have used methods such as the synthetic minority over-sampling (SMOTE) technique to correct for models predicting *Listeria* spp. in poultry samples (Chawla *et al*., 2002; Golden *et al*., 2019). It has been reported that training imbalanced data sets using Weighted Random Forest models also corrects for class imbalance by applying a penalty for over-sampling of the majority class improving model accuracy (Chen *et al.,* 2004); unfortunately, no improvements were seen in the current study. Overall, models of imbalanced class dataset are difficult to predict by and the current study suffered from these dataset imbalances due to the low number of positive *Listeria* spp. samples obtained. Future studies should maintain a more even dataset to establish the true predictive power of machine learning in assessing *Listeria* spp. presence or absence within a meat processing microbiome.

Conclusion

The aim of this study was to assess the establishment and persistence of *Listeria* spp. in the context of surface microbial ecology in a newly constructed meat processing facility. The overall diversity of the facility indicated that there was no difference between the microbiomes

containing *Listeria* spp. and those without. This could be due to sanitation techniques employed by the facility or sample size being too small. Further studies could reveal the role diversity plays on the establishment of *Listeria* spp. Several genus-level features were established as differentially abundant when either associated with *Listeria* spp. or when *Listeria* spp. was absent. These findings could potentially lead to pathogen control techniques using the microbial community to reduce or inhibit the establishment of pathogenic bacteria. Use of machine learning to predict pathogenic bacteria within a built-environment could prove useful in being able to assess hazardous microbiomes for meat and meat-product production. The current study's models were imbalanced and future studies should control for imbalance to give a more accurate depiction of what predictive strategies could be applied. Future studies should consider looking at biofilm formation and the microbial communities that make-up biofilms. Additional "omics" techniques could be used to establish which organisms are producing metabolites that either inhibit or enhance the growth of *Listeria monocytogenes* for potential use as food safety intervention techniques.

Table 2.1. The differential abundance of features in room function "live animal" in the presence of *Listeria* spp. was measured using ANCOM. The W statistic indicates the ratio of a feature's relative abundance to the relative abundance of other features detected to be significantly different at FDR-adjusted $p < 0.05$. Groups were labeled as "no" (i.e., no *Listeria* spp. were present) and "yes" (i.e., *Listeria* spp. was found).

Feature/Taxonomy	W	Percentile	0	25	50	75	100	0	25	50	75	100
		Group	no	no	no	no	no	yes	yes	yes	yes	yes
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae; g__Chryseobacterium	1261		1	1	1	1	12	1	1	40	140.25	887
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium	1233		1	1	1	3.5	226	1	12.75	98.5	206.25	956
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium	1144		1	1	1	1	424	1	6.5	53	113.5	2313

Table 2.2. The differential abundance of features in room function "harvest" in the presence of *Listeria* spp. was measured using ANCOM. The W statistic indicates the ratio of a feature's relative abundance to the relative abundance of other features detected to be significantly different at FDR-adjusted $p < 0.05$. Groups were labeled as "no" (i.e., no *Listeria* spp. were present) and "yes" (i.e., *Listeria* spp. was found). Features with the same name but different W scores could indicate differing species within the same genus.

Feature/Taxonomy	W	Percentile	0	25	50	75	100	0	25	50	75	100
		Group	no	no	no	no	no	yes	yes	yes	yes	yes
d__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__Clostridia_UCG-014	2013		1	1	1	1	43	1	1	13	38.5	116
d__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__Clostridia_UCG-014	1976		1	1	1	1	29	1	1	6.5	26.5	79
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Muribaculaceae; g__Muribaculaceae; s__uncultured_bacterium	1931		1	1	1	1	3	1	1	3	13.75	113
d__Bacteria; p__Firmicutes; c__Clostridia; o__Oscillospirales; f__coprostanoligenes_group; g__coprostanoligenes_group	1912		1	1	1	1	59	1	1	7	22.75	128
d__Bacteria; p__Spirochaetota; c__Spirochaetia; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__Treponema_porcinum	1843		1	1	1	1	628	1	1	5.5	220.25	653
d__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridia_UCG-014;	1798		1	1	1	1	70	1	1	10	50.75	308

f__Clostridia_UCG-014; g__Clostridia_UCG-014												
d__Bacteria; p__Firmicutes; c__Clostridia; o__Christensenellales; f__Christensenellaceae; g__Christensenellaceae_R-7_group; s__uncultured_prokaryote	1768		1	1	1	1	29	1	1	8.5	20.75	33
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotellaceae_NK3B31_group	1762		1	1	1	1	219	1	1	6	81.50	717

Table 2.3. The differential abundance of features in room function "fabrication and processing" in the presence of *Listeria* spp. was measured using ANCOM. The W statistic indicates the ratio of a feature's relative abundance to the relative abundance of other features detected to be significantly different at FDR-adjusted $p < 0.05$. Groups were labeled as "no" (i.e., no *Listeria* spp. were present) and "yes" (i.e., *Listeria* spp. was found). Features with the same name but different W scores could indicate differing species within the same genus.

Feature/Taxonomy	W	Percentile	0	25	50	75	100	0	25	50	75	100
		Group	no	no	no	no	no	yes	yes	yes	yes	yes
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae; g__Chryseobacterium	769		1	1	1	10	1425	1	83	186	373	10434
d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter	768		1	1	1	1	1170	1	80	160	248	774
d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter	746		1	1	1	1	678	1	10.5	99	244	1884
d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Psychrobacter	743		1	1	1	12	579	1	29.5	79	132	726
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae; g__Chryseobacterium; s__Chryseobacterium_piscium	724		1	1	1	1	715	1	15	35	63	300

d__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae	718		1	1	1	1	326	1	1	1	111.5	228
d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae; g__Chryseobacterium	700		1	1	1	1	1016	1	5	18	100	518

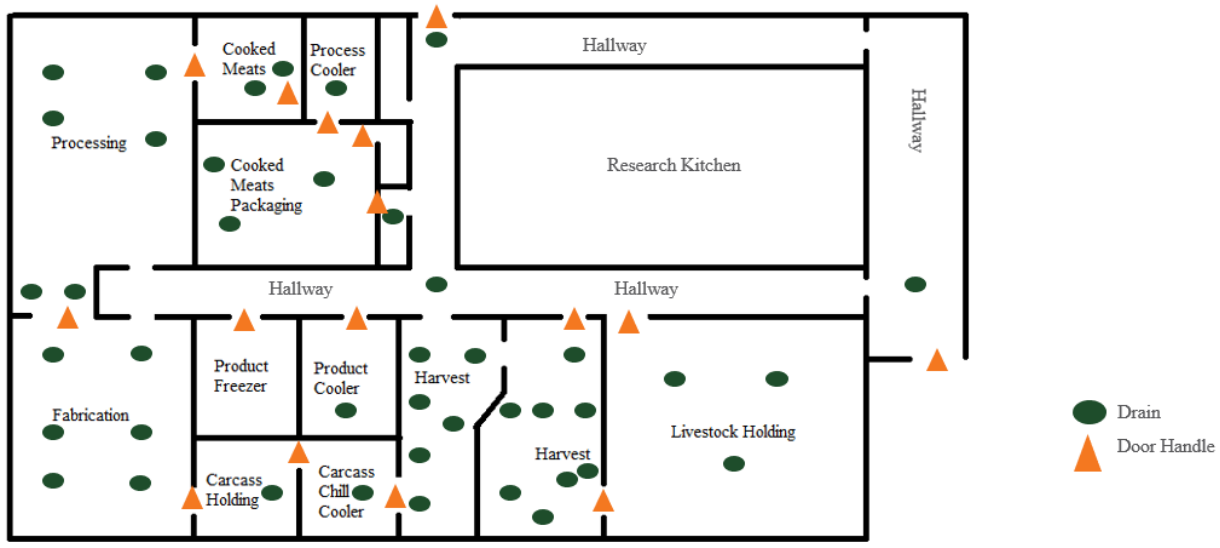

Figure 2.1. The Global Food Innovation Center facility map details sample sites (i.e., drains represented as green circles and door handles represented as orange triangles) and rooms within the facility. The rooms were divided in groups based on room function. Live Animal refers to livestock holding where animals were housed in the facility prior to slaughter. Harvest rooms describe both harvest rooms where animals were slaughtered and turned into carcasses. Product holding rooms are indicative of the rooms where carcasses were held (i.e., carcass holding, carcass chill cooler) or where meat or meat product was stored (i.e., product freezer, product cooler, process cooler). Fabrication and Processing encompass rooms where carcasses are fabricated and processed into meat product (i.e., fabrication, processing, cooked meats, cooked meats packaging). Non-Product rooms indicate the areas where no product or live animals were introduced (i.e., hallways). The research kitchen was not sampled.

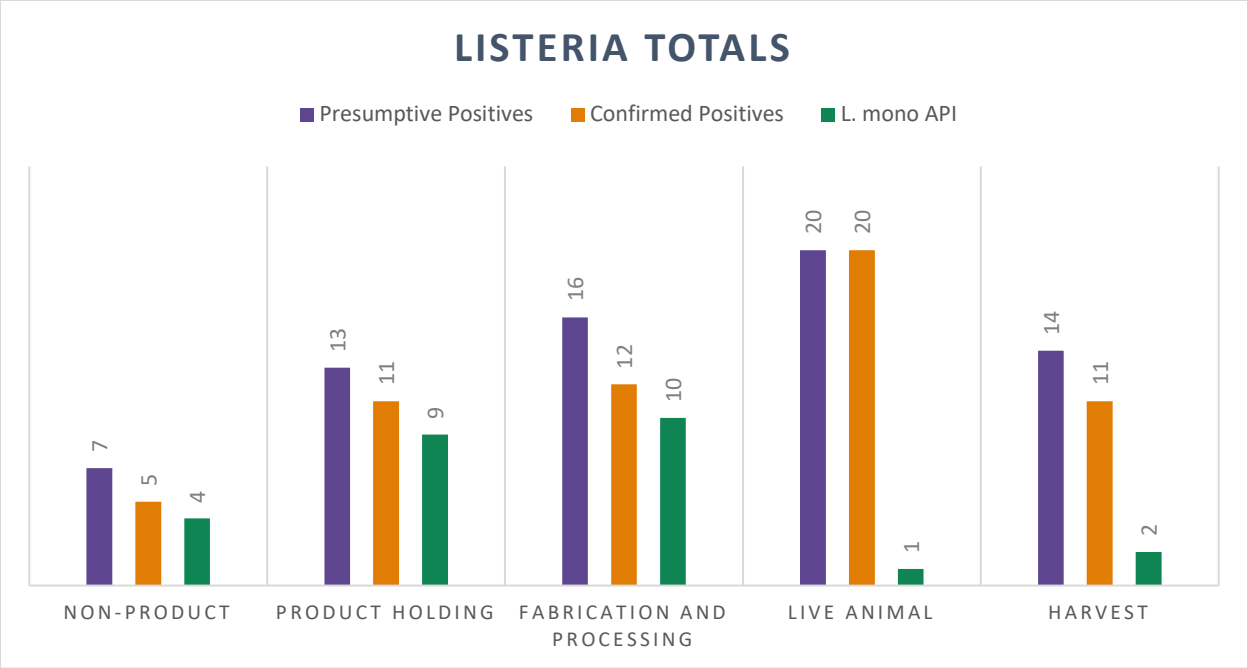

Figure 2.2. The total number of *Listeria* spp. presumptive, confirmed and *L. monocytogenes* as determined by GENE-UP® real time PCR and API® speciation were counted based on room function. Non-product rooms refer to rooms were no live animals or meat/meat products were stored or processed. Product holding rooms are rooms where carcasses or meat/meat products were stored in temperature regulated coolers or freezers. Fabrication and processing rooms are temperature regulated rooms within the facility where carcasses are fabricated and processed into prospective meat products. Live animal refers to the livestock holding area of the facility. Harvest rooms are the slaughter rooms of the facility. Purple represents presumptive samples; orange indicates confirmed samples; and grey represents samples that were confirmed as *L. monocytogenes*.

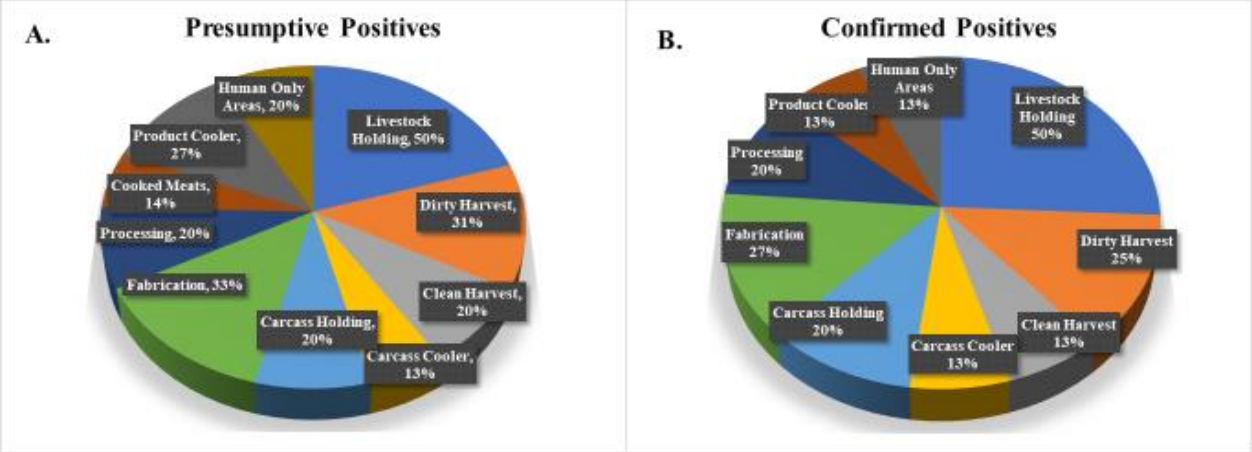

Figure 2.3. Estimated margin of means probabilities were calculated via a generalized linear model for both presumptive and confirmed positive *Listeria* spp. samples in the individual rooms of the facility. Human only areas refer to areas where neither live animals nor meat products were introduced. Livestock holding is the area of the facility where animals are housed prior to slaughter. Dirty and clean harvest rooms refer to the slaughter floor rooms of the facility. The carcass cooler is where carcasses are placed immediately following slaughter and are moved to the carcass holding area prior to fabrication. Fabrication and processing rooms are the areas of the facility in which the carcasses are fabricated into meat and meat products. Cooked meats refers to the ready-to-eat portion of the facility. The probabilities of finding the presence of *Listeria* spp. for presumptive samples are shown in a panel (a). Likewise, the probabilities of the occurrence of confirmed *Listeria* spp. samples are shown in a separate panel (b).

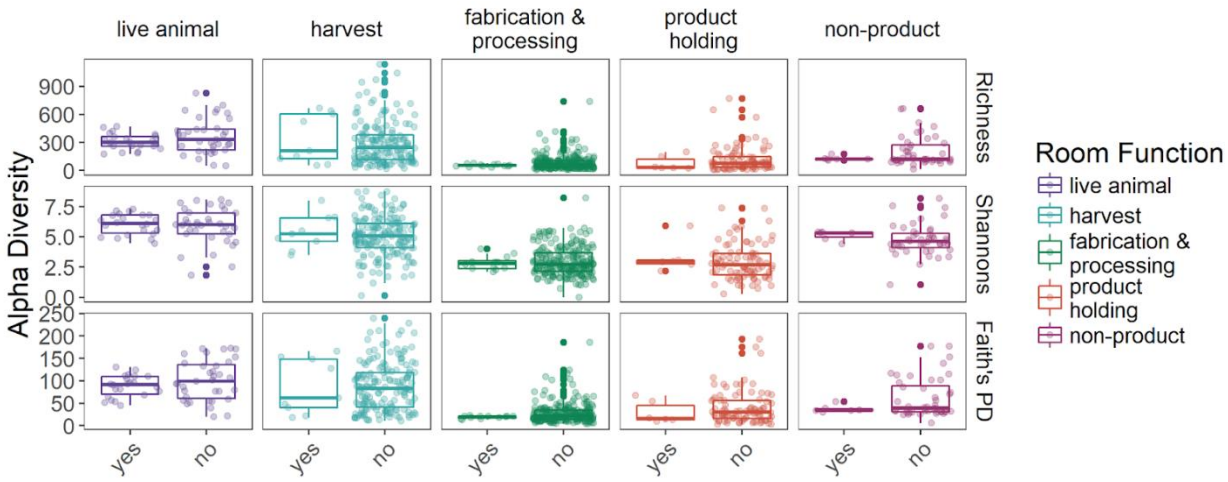

Figure 2.4. The alpha diversity of *Listeria* presence or non-presence was analyzed based on room function. Richness, Shannon's Index and Faith's Phylogenetic Diversity metrics were used along with statistical comparisons being analyzed via pairwise Wilcox test with alpha level set at 0.05. No significant differences were seen for any metric across the different room functions when comparing the alpha diversity of *Listeria* presence or non-presence.

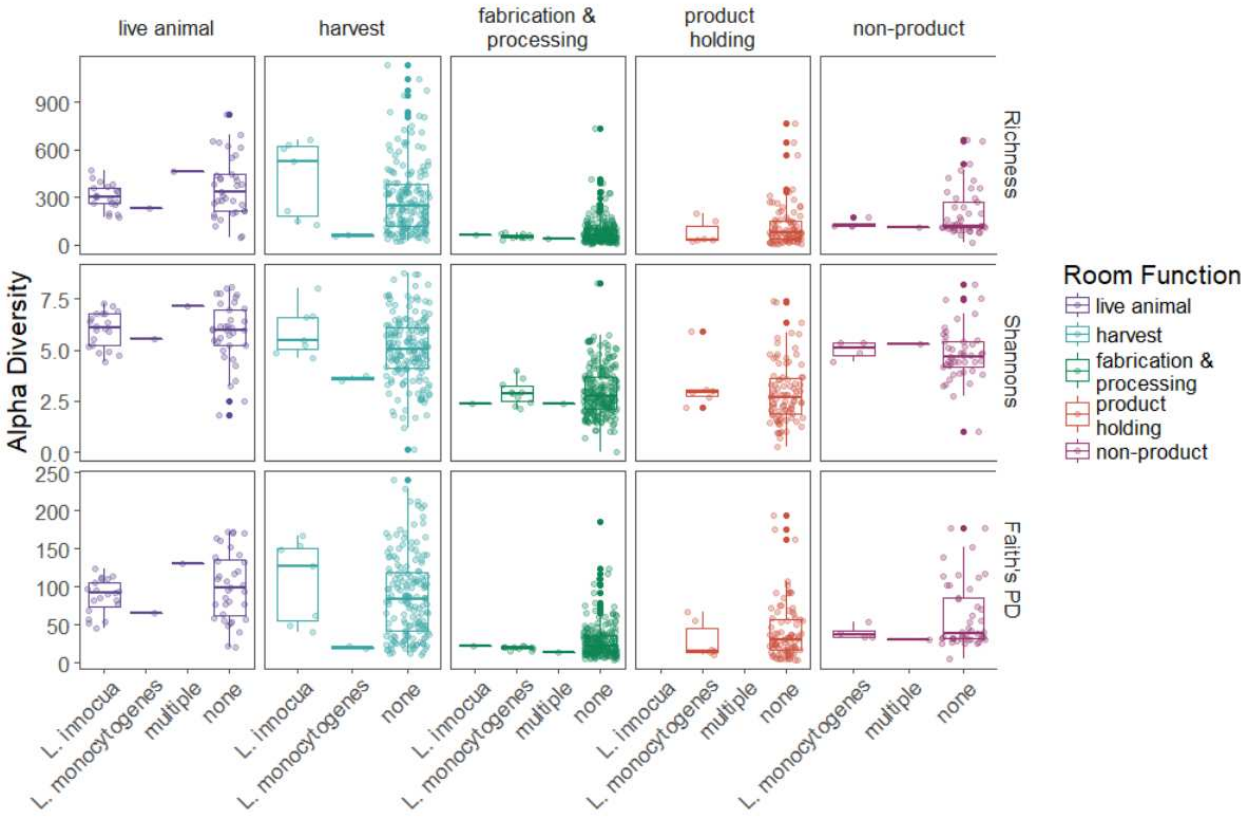

Figure 2.5. The alpha diversity of different *Listeria* species across rooms of different functions. Richness, Shannon's Index and Faith's Phylogenetic Diversity metrics were used along with statistical comparisons being analyzed via pairwise Wilcox test with alpha level set at 0.05. No significant differences were seen for any metric across the different room functions when comparing the alpha diversity of *Listeria* species.

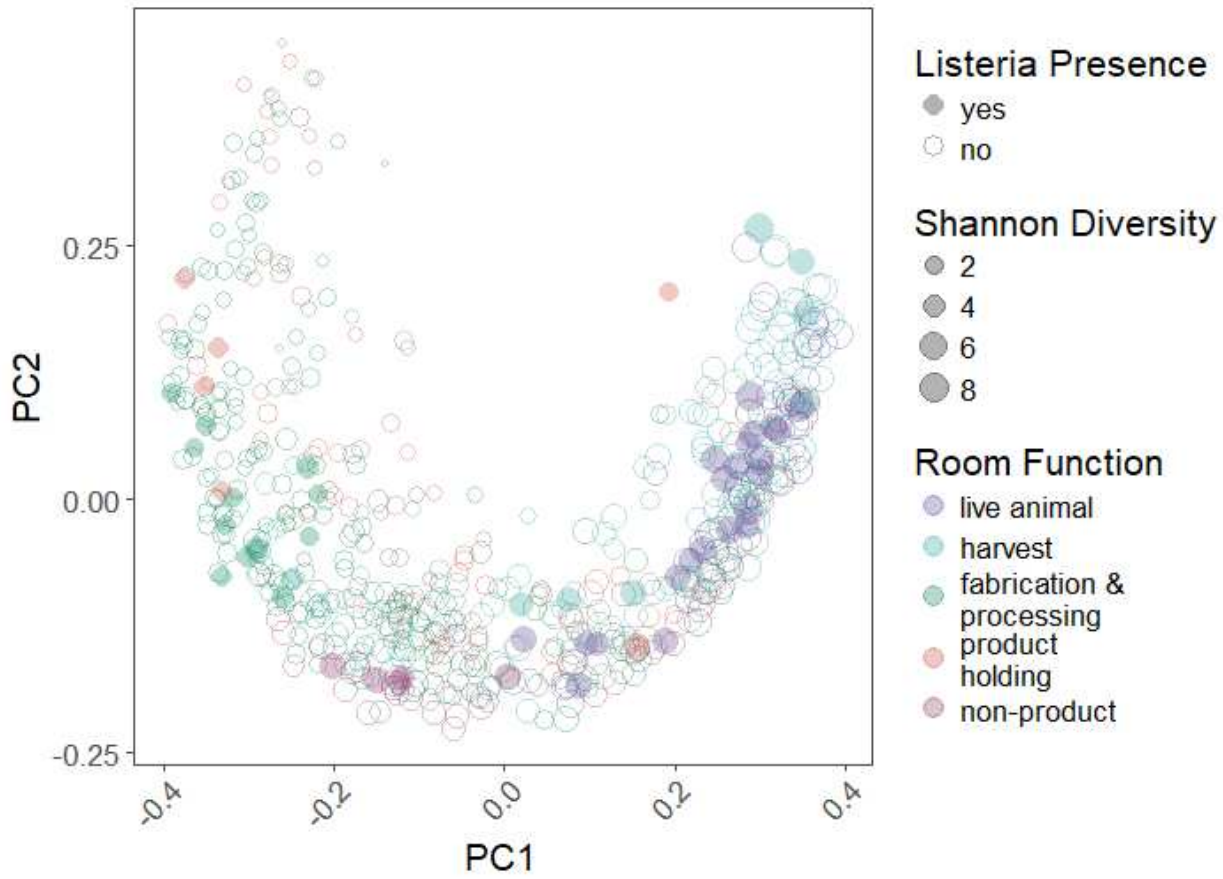

Figure 2.6. Community structure in the presence or absence was analyzed across different room functions using unweighted UniFrac distance. Furthermore, the alpha diversity (Shannon's Index) was also taken into account. Community structure separation was driven by rooms of different function; no statistical differences were seen in Shannon's Index were seen ($\alpha < 0.05$). However, there does appear to be a trend where alpha diversity is higher in rooms where live animals are housed (live animal) or slaughtered (harvest) when compared to fabrication and processing rooms.

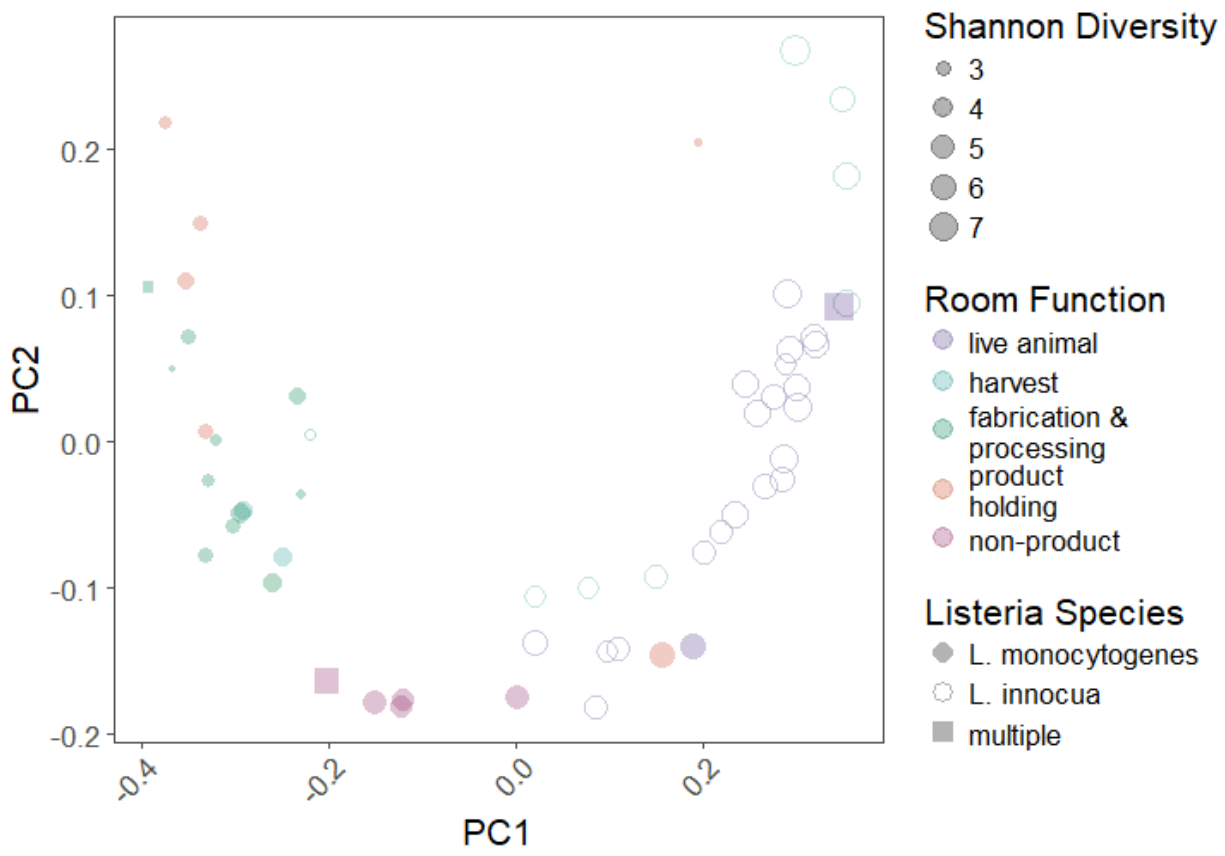

Figure 2.7. The community structure of different *Listeria* species across room function was analyzed via unweighted UniFrac distance. Further, alpha diversity (Shannon's Index) was also measured here. *L. innocua* and *L. monocytogenes* appear to be separated with *L. innocua* being more prevalent in live animal and harvest rooms and *L. monocytogenes* being found more in fabrication and processing areas. A Chi-Square Pearson's Correlation Test was run to analyze this pattern and it was determined that there were indeed significant relationships between room function and *Listeria* species (p-value = 5.884^{-6}). PERMANOVA results also indicated significant impact on the composition of room function beta diversity when *Listeria* spp. where present (p-value < 0.001). Further, no statistical differences were seen in alpha diversity although as mentioned before, there does appear to be higher alpha diversity for live animal and harvest rooms as compared to fabrication and processing areas.

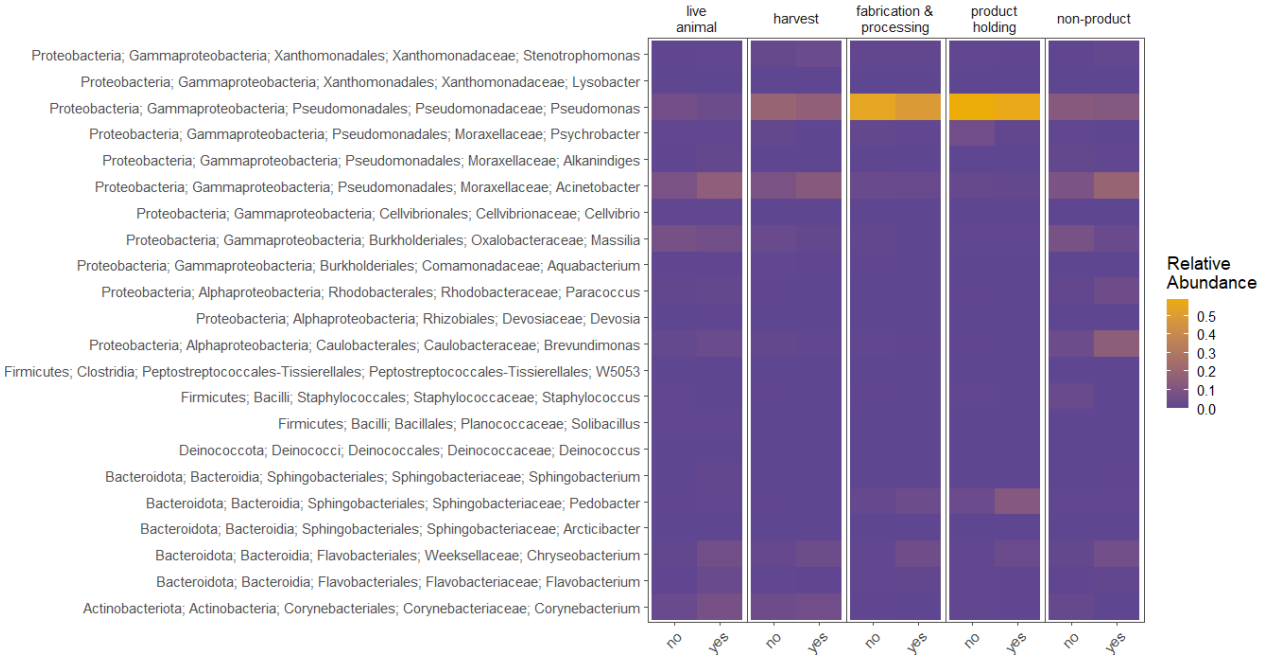

Figure 2.8. The differential abundance of features associated with *Listeria* presence or non-presence across rooms with different functions was measured via ANCOM. The relative abundance of those differentially abundant features was then visualized via a heatmap across rooms of different functions to better demonstrate where certain microorganisms are associated in *Listeria* present or non-present microbiomes.

Figure 2.9. The differential abundance of features associated with different *Listeria* species across rooms with different functions was measured via ANCOM. The relative abundance of those differentially abundant features was then visualized via a heatmap across rooms of different functions to better demonstrate where certain microorganisms are associated with microbiomes containing different species of *Listeria*. 'Multiple' here indicates instances where speciation indicated that the sample could be more than one species of *Listeria*.

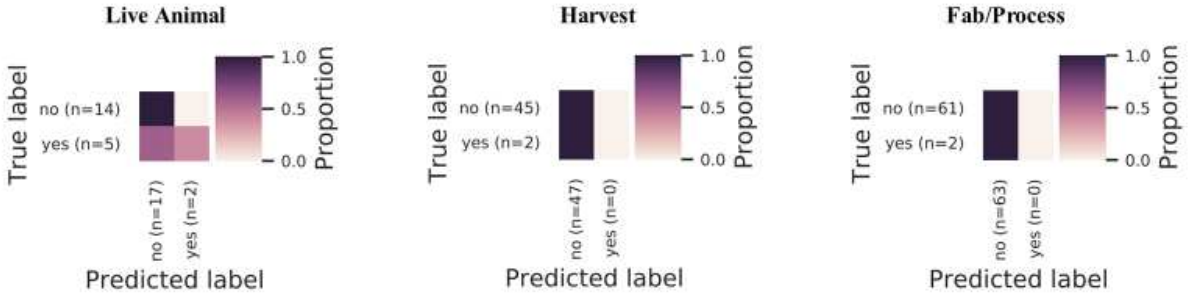

Figure 2.10. Machine learning confusion matrices were obtained via a Random Forest classifier for prediction of *Listeria* spp. in the microbiome in differing room functions. The overall accuracy score for live animal was 84%. Harvest had an accuracy score of 95%. Fabrication and processing had an accuracy score of 96%. Sample size in both harvest rooms and fabrication and processing rooms could have potentially skewed their respective models.

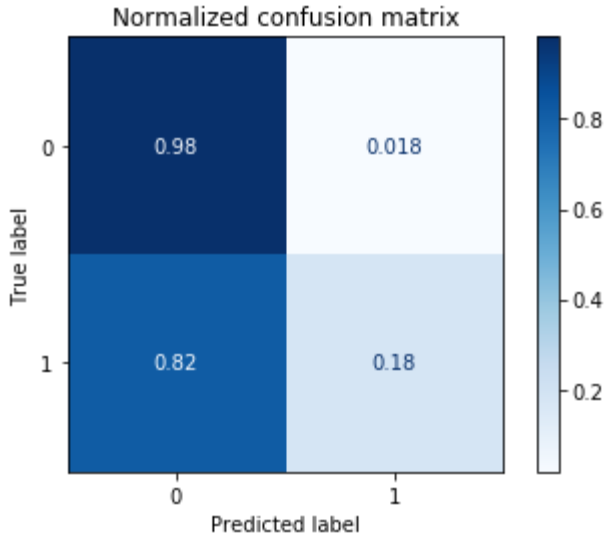

Figure 2.11. A Weighted Random Forest machine learning model was assessed for the use of facility microbial data to predict the presence of *Listeria* spp. The model indicated an overall accuracy of 62.1% in which the model was able to predict the absence of *Listeria* spp. but could not appropriately predict *Listeria* spp. presence.

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Chapter Three

Early Life Microbiomes in Dairy Calves and their Associations with Disease State

Summary

The early life microbiome of dairy calves undergoes extensive changes, and diseases such as Bovine Respiratory Disease (BRD) and calf scours could affect microbiome diversity and composition during this developmentally important timeframe. The objective of the current study was to describe the establishment of the early life (herein defined as the first 21 days) fecal and nasal microbiomes of dairy calves as well as evaluate the correlation between disease states and early life microbial ecology. Nineteen dairy calves were evaluated weekly during the first three weeks of life for fecal and nasal microbial composition. The health status was also assessed for each calf utilizing the Wisconsin Calf Health System, which included assessment of fecal scores and lung health via ultrasound. Samples were collected using sterile cotton-tipped swabs and DNA was extracted with the 16S rRNA gene subsequently sequenced to estimate microbiome composition and diversity. Taxonomy analyses and diversity analyses were used to characterize the establishment of early life nasal and fecal microbiomes and to correlate disease state and early life microbiomes. Across the three-week sampling period, only five calves were identified as having scours and one calf had clinical signs of both BRD and scours. Nasal microbiomes were dominated by the families *Moraxellaceae* (average relative abundance 49.42%), *Mycoplasmataceae* (16.24%) and *Pastuerellaceae* (3.4%) and fecal microbiomes were dominated by *Bacteroidaceae* (40.9%), *Ruminococcaceae* (12.72%) and *Lachnosporaceae* (9.83%). Nasal samples had higher alpha diversity as compared to fecal samples and

compositional analysis revealed distinct separation between nasal and fecal samples ($P < 0.05$). Differential abundance analyses revealed two features to be differentially abundant during the first three weeks of life. *Neisseriaceae* was differentially abundant in nasal samples and decreased in relative abundance over the first three weeks. *Bifidobacterium* was differentially abundant in fecal samples and decreased in relative abundance over time ($P < 0.05$). Nasal samples indicated no significant differences in alpha diversity over the three sampling time points, but compositional changes were detected across sampling points ($P < 0.05$). Alpha diversity increases and compositional changes were detected for fecal samples across the three sampling time points Fecal samples of healthy calves had higher alpha diversity compared to scouring calves ($P < 0.05$) while no significant differences were seen in composition. The current study revealed that fecal microbiomes undergo diversity increases as well as changes in composition, indicating early fecal microbial life is subject to quick change. Nasal microbiomes indicated no diversity shifts however, compositional changes were seen, which could be suggestive of higher rates of environmental contamination of the nasal microbiome. Disease state within fecal microbiomes (i.e., calf scours) revealed that healthy calves had higher alpha diversity compared to scouring calves. This finding provides insight for further research to be conducted to evaluate the potential of diversity changes to be indicative of disease onset.

Introduction

Respiratory disease and gastrointestinal disease in dairy calves carry a large economic burden for dairy cattle operations (USDA, 2007). The multifactorial respiratory disorder known as Bovine Respiratory Disease (BRD) is responsible for extreme economic loss in cattle operations due to the high morbidity and mortality rates of the disease; previous studies found

that BRD is responsible for 22.5% of all documented mortality in pre-weaned calves (USDA, 2007; Gershwin *et al*., 2015; USDA, 2013). Gastrointestinal disease such as calf scouring is another economically important condition for cattle producers as it accounts for 57% of the mortality rate among pre-weaned calves (USDA, 2007). Scours can either be caused by infectious pathogens (e.g., viruses, bacteria, protozoa) or can occur due to non-infectious factors (e.g., diet); the patterns of disease between both non-infectious and infectious scours are markedly similar, and knowing the casual pathogens or factors associated with a scours outbreak is crucial for assessing intervention or management protocols on-farm (Cho & Yoon, 2014). A common on-farm treatment practice of disease in livestock is the use of antimicrobials (Mathew *et al*., 2007; Smith, 2015). Antimicrobial use in livestock animals, including dairy cattle, is highly controversial for various reasons including evidence of a low effectivity rate of antimicrobials in preventing infection and the growing public concern regarding antimicrobial resistance (Thames *et al*., 2012; Smith, 2015; McEachran *et al*., 2015). Therefore, new alternatives to disease control and prevention are needed.

One method for approaching resolution to new methods of disease control and prevention in the dairy industry is the use of Next-Generation sequencing (NGS). NGS techniques have been applied for studying bacterial composition and diversity in livestock. These techniques have increased over the last decade (Tablerlet *et al*., 2012; Sharma *et al*., 2017) and have allowed for a better understanding of the microbiomes of livestock and the relationship between the microbiome and diet (De Menezes *et al*., 2011); methane emissions (Difford *et al*., 2018); and stress and physiology (Chen *et al*., 2018). An additional use of NGS is the application of 16S rRNA amplicon gene sequencing to assess nasal and fecal microbial ecology to understand relationships between the microbiomes and disease state (Oikonomou *et al*., 2013; Holman *et al*.,

2015a; Lima *et al*., 2016; Nicola *et al*., 2017; Nakamura *et al*., 2017; Ma *et al*., 2020; McMullen *et al*., 2020a; Kim *et al*., 2021). While the importance of both the nasal and fecal microbiomes has been well recognized (Malmuthuge & Guan, 2017; McMullen *et al*. 2020b), there are discrepancies among the literature regarding correlations between disease state and the microbiome. For example, Lima *et al*., (2016) reported calves 35 days of age had no significant differences in microbiota structure or diversity between healthy calves and those presenting clinical signs of pneumonia or otitis media. Nakamura *et al*. (2017) also reported while scouring calves had lower diversity compared to healthy calves (at ten days of age), there were no significant compositional differences between the two groups. McMullen *et al*., (2020a) also reported that post-weaned heifers in feedlots demonstrated no significant results or common trends in composition or diversity between cattle that developed BRD or remained healthy. While most previous studies assess the establishment of the microbiome over several weeks or at a static age (i.e., Nakamura *et al*., 2017), very early life (< 3 weeks of life) microbiome establishment and disease state influences remains widely unknown. This understanding provides new avenues for research in analyzing early life microbiome establishment and disease state impacts in dairy calves.

To address this matter, we designed a study to analyze the development of dairy calf nasal and fecal microbiomes during the first three weeks of life and to understand the correlations, if any, early life microbiomes have with respiratory illness and/or gastrointestinal (GI) disease. Our study sought to answer two primary questions: i.) how does the microbiome of dairy calves develop during the first three weeks of life? and ii.) how does very early life microbiomes correlate to health status within the very early life of dairy calves?

Materials and Methods

Experimental Design and Sampling

A longitudinal, repeated measures study was designed to evaluate the establishment of the dairy calf microbiome over the first three weeks of life as well as to evaluate if and how these early life microbiomes correlate with respiratory disease and gastrointestinal disease. Sampling took place at a commercial dairy farm located in Northern Colorado from March 2021 through April 2021. Calves used for sampling in the study were handled following the guidance of the Institutional Animal Care and Use Committee (IACUC #1421). Calves were individually placed into the calf hutches by the dairy. Within seven days after birth, calves were sampled for microbial composition (initial sample). Following the initial sample collection, calves were sampled weekly for two weeks, totaling three sampling time points. A total of 22 calves were sampled for sequencing and subsequent analysis.

Sampling of the calves consisted of a fecal swab and a nasal swab using double-headed sterile cotton tip swabs (BD; Franklin Lakes, NJ) in conjunction with health scoring via the Wisconsin Calf Health System (Calf Health Scorer – Food Animal Medicine, University of Wisconsin, Madison; McGuirk & Peek, 2014). All sampling was done by trained laboratory personnel. The nasal swab was inserted into the calf's left nasal cavity, approximately 2 to 3 inches inside, and nasal contents were gathered; fecal swabs occurred via placing a sterile swab approximately 2 to 3 inches into the anus and rectum of the calf and removing fecal content. Following sampling, the swabs were immediately placed on ice and transported to a -20 °C freezer located at the microbiome laboratory at Colorado State University (Fort Collins, CO) and stored until DNA extraction and sequencing following all sampling time points. Health

scoring via the Wisconsin Calf Health System consisted of scoring the calf's clinical signs of disease such as eye, nasal, joints, ears, cough, fecal score, navel, and temperature (Calf Health Scorer – Food Animal Medicine, University of Wisconsin, Madison; McGuirk & Peek, 2014). Health scoring was done weekly during the calves' first ~56 days of life. The scoring system works based on a scoring range from 0-3, where 3 indicates severe clinical signs of disease. Furthermore, ultrasounds of the lungs were also performed and scored. Ultrasound scores ranged from 0-5, where 0-1 indicates healthy or normal lungs. According to the Wisconsin Calf Health System, a score of 3 or greater would indicate the presence of pneumonia and subsequent respiratory illness. All health scoring was completed by trained personnel.

DNA Extraction and Sequencing

At the conclusion of the sampling time frame, DNA was extracted from all nasal samples (n = 66) and fecal samples (n = 66) was performed using the Qiagen PowerSoil Kit (Qiagen: Hilden, Germany) per the manufacturer's instructions. DNA was extracted from all fecal and nasal swabs using 96-well plates with nine negative control samples and three positive mock microbial community controls (Zymo; Irving, CA) per plate. Once DNA was extracted, microbial communities were then characterized via paired-end 16S rRNA gene sequencing following the Earth Microbiome Project Protocols amplification and sequencing with the forward primer 515f and the reverse primer 806r (EMP; www.earthmicrobiome.org) (Thompson *et al*., 2017). Multiplexing of the sequences occurred using error-correcting Golay barcodes within the PCR primers and the subsequent PCR products were quantified using Picogreen Quant-iT (Invitrogen, Life Technologies; Grand Island, NY). The quantified PCR products were

pooled at equimolar concentrations for sequencing. Pools were then sequenced using a 500-cycle kit on the Illumina miSeq sequencing platform (Illumina; San Diego, CA). Samples were placed into the sequencing wells at random as to avoid confounding due to technical artifacts.

Analysis of Fecal and Nasal Microbiomes

Following sequencing, data processing and analyses were performed using QIIME2 version 2020.8 (Bolyen *et al*., 2019). Multiplexed sequence data was demultiplexed, quality filtered and denoised via DADA2 using the QIIME2-demux and QIIME2-dada2 plugins (Boylen *et al*., 2019; Callahan *et al*., 2016). Taxonomic classification was achieved using the SILVA 138 99% database via the QIIME2 feature-classifier plugin and subsequent filtering of taxa assigning to mitochondria and chloroplasts was performed (Quast *et al.,* 2012; Bokulich *et al*., 2018a). Filtering steps were also conducted to remove sequences that appeared in less than 10% of all samples. Prior to diversity analyses, samples were split into two groups using the QIIME2 filter-samples plugin: fecal and nasal. This was based on the sample type at sampling and was done to analyze the fecal and nasal microbiomes individually and their associations with disease state. Furthermore, based on health scoring, calves were placed into one of four groups per sampling time point for analysis: i.) healthy (i.e., calves presenting no signs of clinical disease ii.) BRD (i.e., calves having clinical signs consistent with respiratory illness iii.) Scours (i.e., calves with clinical signs consistent with gastrointestinal disorder) and iv.) BRD plus Scours (i.e., calves having both respiratory and gastrointestinal disorders at the same time). Calves were also grouped based on fecal severity scores during the three-week sampling time frame.

Phylogenetic diversity analyses were conducted by first creating a phylogenetic insertion tree via the QIIME2 fragment-insertion plugin which utilizes the SEPP program with the SILVA 128 tree used as reference tree backbone; rarefaction was then performed to a sampling depth of 9,060 and phylogenetic diversity analysis was ran using the core metrics pipeline within QIIME2 (Matesen *et al*., 2010; Jansen *et al*., 2018; Bolyen *et al*., 2019). Alpha diversity was measured using Shannon's Index, Faith's Phylogenetic Diversity, and richness metrics (Shannon & Weaver, 1949; McIntosh, 1967; Faith, 1992) in QIIME2 and then tested statistically using a Kruskal-Wallis test with a Benjamini-Hockberg multiple testing correction (Kruskal & Wallis, 1952). Beta diversity was analyzed using unweighted UniFrac distance with statistical comparisons done using PERMANOVA testing with multiple testing correction (Lozupone *et al*., 2005; Lozupone *et al*., 2007). Community diversity changes over time were analyzed using the QIIME2-logitudinal plugin (Bokulich *et al*., 2018b). All diversity testing was done with significance set at $\alpha < 0.05$. Differential abundance was assessed via ANCOM (Mandal *et al*., 2015) to investigate whether individual taxa were differentially abundant in each health group and fecal severity score groups across sampling time points. Visualizations were performed via ggplot2 using RStudio version 4.0.5 (Wickham, 2009).

Results

Calf Health During Sampling

Overall, 22 calves were swabbed for nasal and fecal microbiome samples. Table 3.1 details how each calf was scored using the health scoring system through each sampling time point. Three calves were removed from the study due to one calf being lame, another calf being

moved by the farm and subsequently lost, and the last calf being health scored twice as two different calves. Given this issue, only 19 calves were utilized for microbiome analyses. At microbiome sampling time point one, all 19 remaining calves were identified as being healthy. At sampling time point two, 16 calves were considered healthy and three were determined to have scours. At sampling time point three, 16 were healthy, two had scours, and one calf had both BRD and scours. Demultiplexed sequences resulted in a total of 7,063,380 paired-end reads with an average read length of 46,469.9 base-pairs. Denoising using DADA2 version 2021.8.0 yielded 152 samples, 3,457 features and a frequency of 3,204,242 with a mean feature frequency per sample of 21,080.54. The sequences were then rarified to a depth of 9,060 for diversity analysis. The plates were sequenced with nine negative controls and three positive controls alongside the study samples. Negative quality control checks indicated that the individual controls had low sequencing numbers (0-285 feature count) returned and contamination of the plate was not likely. Positive controls were assessed individually as well. Based on the taxonomic mock community representation, the sequencing run was determined to be valid. Quality control checks of the positive and negative sample controls indicated it was reasonable to remove control samples and utilize the data to answer the three proposed questions.

Development of the Early Life Microbiome

The composition of the nasal and fecal microbiomes revealed little change over the first three weeks of life (Figure 3.1). The family *Moraxellaceae* dominated in nasal samples (49.42% relative abundance) with the families *Mycoplasmataceae* (16.24%) and *Pasteurellaceae* (3.4%) included in the top three most relatively abundant families represented in nasal samples across all

three time points (Figure 3.1). Fecal samples were dominated by the families *Bacteroidaceae* (40.09%), *Ruminococcaceae* (12.72%) and *Lachnospiraceae* (9.83%) throughout the sampling time points (Figure 3.1). Diversity changes were seen during the first three weeks of life. Nasal samples had higher alpha diversity compared to fecal samples for alpha diversity metrics Faith's PD and Richness ($p < 0.05$). Figure 3.2A illustrates the diversity differences found within fecal and nasal samples between different sampling time points. Fecal samples were different for Faith's PD and Richness between sampling time points 1 and 3 ($p = 0.003$ and $p = 0.002$, respectively), and between sampling time points 2 and 3 ($p = 0.016$ and $p = 0.014$, respectively) with diversity increasing over time. No differences were observed for nasal samples for any of the alpha diversity metrics ($p > 0.05$) over the first three weeks of life.

A Principal Coordinate Analysis (PCoA) plot shows distinct separation between each sample type (Figure 3.2B; PERMANOVA $p = 0.001$). Pairwise PERMANOVA analysis within fecal samples also revealed a difference in microbial composition between sampling time points 1 and 2 ($p = 0.002$), 1 and 3 ($p = 0.001$), and 2 and 3 ($p = 0.007$). Relative abundance of the genus *Bifidobacterium* was differentially abundant over time and decreased for sampling time points 2 and 3. Nasal sample microbial composition differed between sampling time points 1 and 2 (PERMANOVA, $p = 0.03$) and time points 1 and 3 ($p = 0.012$) with one feature, in the family *Neisseriaceae*, differentially abundant in nasal samples, showing a decrease over time.

Early Life Microbiomes and Disease Within the First Three Weeks of Life

The number of calves with BRD and scours are listed in Table 3.1. Due to the low number of calves with BRD during the first three weeks of life, early life microbiome association

with disease state during the sampling timeframe was only assessed for scours? in fecal samples. Table 3.2 details the number of calves associated with fecal severity scores. The taxonomic mean relative abundances for fecal severity scores revealed *Bacteroidaceae*, *Ruminococcaceae*, and *Lachnospiraceae* were again the dominant families. Kruskal-Wallis pairwise testing revealed differences for Richness between healthy calves and, moderate ($p = 0.012$) and severe ($p = 0.033$) fecal scores as well as differences for Faith's PD between healthy and moderate scores ($p = 0.008$), and mild and moderate scores ($p = 0.008$; Figure 3.4A) with diversity being higher in calves that were healthy or had lower fecal severity scores, but no compositional differences were detected (Figure 3.4B; PERMANOVA pairwise analysis $p > 0.05$). Not surprisingly then, no features were found to be differentially abundant for fecal severity at any sampling time point.

Discussion

Early life nasal and fecal microbiomes are developing and changing during the first three weeks of life. To the best of our knowledge, this is the first study to characterize early life (< 3 weeks old) fecal and nasal microbiomes in dairy calves and evaluate correlations of disease on early life microbial ecology. The current study is corroborated by previous research as the most dominant families in fecal samples were *Bacteroidaceae*, *Ruminococcaceae*, and *Lachnospiraceae* (Rey *et al*., 2014; Ma *et al*., 2020; Kim *et al*., 2021). Similar to this study previous research also found that *Bacteroidaceae* dominates fecal microbiomes during the first two weeks of early life and decreases in abundance with an increase in age (Rey *et al*., 2014; Kim *et al*., 2021). The ability of Bacteroidetes to maintain gut health and potential use as a healthy gut biomarker has been well studied (Bry *et al*., 1996; Mazmanian *et al*., 2005;

Mazmanian *et al*., 2008; Round *et al*., 2010; Lee *et al*., 2013; Kim *et al*., 2021). Additionally, the families *Ruminococcaceae* and *Lachnospiraceae,* have been linked to gut health for their ability to produce butyrate (Ma *et al*., 2020). Butyrate is a short-chain fatty acid that is considered the primary source of energy for rumen epithelial cells which are mainstays in nutrient absorption as well as water absorption; it has further been shown to have beneficial side effects when supplemented in the diet of early age livestock animals (Gorka *et al*., 2009; Guilloteau *et al*., 2009; Gorka *et al.,* 2011; Xu *et al.,* 2016; Bedford & Gong, 2018; O'Hara *et al*., 2018). Therefore, the dominance of these families should be considered routine in healthy gut maintenance of young calves.

Interestingly, albeit not surprisingly, the current study revealed a decreasing relative abundance of the genus *Bifidobacterium* in fecal samples and supports previous findings as *Bifidobacterium* is associated with milk digestion during a time when their rumens are under-developed metabolically and physically (Pacheco *et al*., 2015; Khan *et al*., 2016; Kelly *et al*., 2016). *Bifidobacterium* has a well-established role in gut health as it provides mechanisms in gut mucosal barrier maintenance by production of beneficial metabolic substrates as well as immunological properties that prevent the attachment of pathogenic organisms (Leahy, *et al*., 2005; Fukuda, *et al*., 2011; O'Connell Motherway *et al*., 2011). While it is common on-farm practice to remove the newborn calves from their dams, the use of *Bifidobacterium* as a direct-fed microbial in milk replacer has been well-studied (Abe *et al*., 1995; Vlkova *et al*., 2006; Kelly *et al*., 2016). The make-up of the milk replacer used at the dairy in the current study is unknown; however, the restrictive milk-feeding process used at commercial dairy operations to promote weaning and quicker transition to solid feed could explain the decrease in *Bifidobacterium* over time as verified in earlier studies (Khan *et al*., 2016). Taken together, *Bifidobacterium*'s presence

is suggestive of its role in maintaining gut health for early life calves. Future studies are needed to determine if these microorganisms could have use as biomarkers for establishing calf health.

Early life microbial diversity and composition in calves is characterized by increasing diversity with age and change in diet, especially in the gut microbiome (Uyeno *et al*., 2010; Mayer *et al*., 2012; Oikonomou *et al*., 2013; Klein-Jobstl *et al*., 2014; Tomassini, 2015; Dill-McFarland *et al*., 2017; Malmuthuge & Guan, 2017). The current study revealed significant increases in diversity for fecal microbiomes over time. Further, microbial composition also displayed significant differences between all sampling time points, suggesting that the diversity and composition of dairy calf microbiomes is indeed undergoing dramatic shifts in the first three weeks of life. While previous studies analyzing diversity shifts were confounded by diet (Klein-Jobstl *et al*., 2014; Dill-McFarland *et al*., 2017), our study demonstrated diversity and composition shifts utilizing calves only fed milk replacer. These results suggest that age and development of the dairy calf can be major drivers for diversity and composition changes regardless of the influence of diet. This inference is supported by Ma *et al*., (2020) in which dairy calves were also studied with a regulated diet of milk replacer and diversity shifts were seen during the first eight weeks of life. Because the decrease of *Bifidobacterium* across the three sampling time points is indicative of the calf rumen transitioning to be more adept for a more complex diet, the increase in diversity and compositional changes over time supports the idea that early life ruminants encounter significant microbial changes as the host animal ages and grows. Previous studies validate this claim of significant microbial changes made in the current study (Uyeno *et al*., 2010).

Past studies corroborate our findings that the nasal microbiomes were dominated by the families *Moraxellaceae*, *Mycoplasmataceae* and *Pasteurellaceae* (Holman *et al*., 2015; Nicola *et*

al., 2017; McMullen *et al*., 2020). However, their physiological role in maintaining health is contentious as these dominant families have been highly associated with both healthy nasal microbiomes and those in a diseased state (Corbeil *et al*., 1985; Allen *et al*., 1991; Catry *et al*., 2007; Angen *et al*., 2009; Pardon *et al*., 2011; Jamali *et al*., 2014; Holman *et al*., 2015a; Lima *et al*., 2016; Gaeta *et al*., 2017; McMullen *et al*., 2020a; McMullen *et al*., 2020b). While studies have concluded that potential pathogenic bacteria could be natural inhabitants of the nasal microbiome (Holman *et al*., 2015a; Gaeta *et al*., 2017; Lima *et al*., 2016), different species within these families associate with health in different manners. For example, the family *Mycoplasmataceae*, known for *Mycoplasma* spp., has been isolated from both sick and healthy calves and is one of the dominant families in the current study (Allen *et al*., 1991; Angen *et al*., 2009; Nicola *et al*., 2017). At the species level however, *M. bovis*, an important etiological agent in the development of BRD, has been identified in calves and cattle with respiratory disease while the lesser studied *M. dispar* was recently identified as being differentially abundant in beef cattle with healthy nasal microbiomes (Aebi *et al*., 2015; Holman *et al*., 2015a; Gaeta *et al*., 2017; McMullen *et al*., 2020a). While not directly tested in this study due to low numbers of calves positive for BRD, our findings coupled with the literature could indicate that nasal microbiome diversity is not indicative of health status. This inference could be due to the capability of pathogenic species to inhabit the normal microflora of the dairy calf respiratory tract.

It is possible that the environment in which calves reside can affect their microbial ecology. Nicola *et al*., (2017) determined that the nasal microbiomes differed between calves from different farms indicating the living or housing environment can affect the dairy calf upper respiratory tract (URT) microbiome. Raabis *et al.* (2021) theorized that nasal samples from dairy

calves were dominated by *Mycoplasma* spp. due to a high prevalence of *Mycoplasma* spp. found on the farm within the study. While the current study did not assess environmental samples, it could be theorized that the nasal microbiomes of early life calves are influenced by the environment and the health status of dairy calves is linked to on-farm environments. Previous studies indicated environmental contamination could lead to higher diversity in nasal microbiomes compared to that of fecal microbiomes and offers support for results within the current study (Klindworth *et al*., 2013; Holman *et al*., 2015a; Holman *et al*., 2015b; Timsit *et al*., 2016; Lima *et al*., 2016; Gaeta *et al*., 2017; Timsit *et al*., 2017; Holman *et al*., 2017; Nicola *et al.,* 2017).

Contrary to Barden *et al*. (2020) in which *Neisseriaceae* was reported to increase in abundance in oral microbiomes., the current study identified the family *Neisseriaceae* within the nasal microbiomes decreasing in abundance over time. This finding is of interest as our study could be the first in identifying *Neisseriaceae* within nasal microbiomes of young dairy calves. A possible explanation for our findings could be due to overlap between the nasal and oral microbiomes or due to contamination of nasal microbiomes by oral microorganisms as the calf licks or eats. Previous research efforts have reported that the oral microbial composition is known to influence the composition of the lung microbiomes due to saliva intake in humans (Bassis *et al*., 2015). It could be expected then that environmental contamination of the nasal microbiome could increase diversity and allow for more composition changes as the calf comes in to contact with microorganisms from differing sources. Further still, our study provides evidence for microbial overlap between oral and nasal microbiomes. Additional research will be needed to elucidate any significant role of *Neisseriaceae* within young dairy calf nasal microbial ecology.

Diversity differences between healthy and disease animals may act as a potential biomarker for identifying the onset of calf scours. Several studies have revealed that changes in the early life microbiota of calves could be of value in identifying diarrhea onsets (Bartels *et al*., 2010; Ma *et al*., 2020; Kim *et al*., 2021). Similarly, Nakamura *et al*. (2017) indicated that scouring calves had lower diversity levels than healthy calves when assessing Japanese Black calves during the onset of non-infectious scours, although no statistical differences were seen. Similar to these past findings, our results revealed differences in diversity between healthy and scouring calves with calves displaying clinical diarrhea having lower diversities compared to those with mild clinical signs of disease or those that were healthy. The use of diversity shifts as a biomarker has been previously reported within the literature (Kim *et al*., 2021) and therefore, validates the findings within the current study and further facilitates the theory that diversity changes could be used for identifying scours onsets in young calves. Along with diversity changes, certain microorganisms have been studied for potential use as identifiers of disease. While no differentially abundant features or taxa were observed in the current study, previous studies have reported that a decrease in Bacteroidetes and *Bacteroidaceae* may be associated with diarrhea onset in calves and other animals (Larson *et al*., 1977; Oikonomou *et al*., 2013; Guard *et al*., 2015; Suchodolski *et al*., 2015; Zhu *et al*., 2018; Kim *et al*., 2021). Future research will be needed to assess the use of bacteria such as *Bacteroidaceae* as a novel method in identifying disease occurrence in dairy calves.

While there were several strengths to the current study, one of the limitations was that antibiotic usage for severely scouring calves was not assessed. Ma *et al*. (2020) reported calves with diarrhea onsets had a higher Shannon's Index than healthy calves; however, the study attained that this could have been caused by fluctuations in microbial diversity development due

to antibiotic treatment. While on-farm protocols for the diary used in the study treated calves with antibiotics for respiratory disease, it is unclear what consequences arose for fecal diversity in calves that received treatment. The loss of diversity in severely scouring calves could be due to antibiotic treatment although this claim is speculatory. Future studies should investigate the use of treatment protocols on diversity changes within young dairy calves for assessment of how diversity shifts could be used for predictive purposes.

Conclusion

Early life microbiomes in calves are important for the development and maintenance of health. The current study highlights the establishment of fecal and nasal microbiomes during the first three weeks of life of dairy calves and assesses the impact of disease on early life microbial ecology. While taxonomic changes are sparse, microbial diversity within these microbiomes increases over time indicating the fast changes the young ruminant microbiomes go through. The environment in which young dairy calves are subjected to can also influence the changes seen in microbiome establishment and makeup with the nasal microbiome more likely to be shaped by environmental sources. Finally, disease within the GI tract impacts the diversity of microbes and lower microbial diversity could be associated with the onset of calf scours in pre-weaned dairy calves. Diversity shifts within the gut microbiome of young dairy calves could be utilized as potential biomarkers for the onset of calf scours; however, further investigation is required to identify these relationships to causative agents of scours. The findings should be further evaluated in future studies to elucidate potential manipulations of the microbiome to better calf health and identify novel pathogen control interventions.

Table 3.1. Total number of calves in each health category at each sampling time point

Health Category*	Sampling Time Point 1¥	Sampling Time Point 2	Sampling Time Point 3
Healthy	19	16	16
Scours	0	3	2
BRD	0	0	0
BRD/Scours	0	0	1

* Health categories were assessed based on clinical scores using the Wisconsin Calf Health System (McGuirk & Peek, 2014)
¥ Sampling time points refer to initial sampling at < 7 days of age and the two subsequent sampling time points which occurred weekly for an additional two weeks

Table 3.2. Total number of calves associated with fecal severity scores at the sampling time points

Fecal Severity Score*	Sampling Time Point 1	Sampling Time Point 2	Sampling Time Point 3
Healthy (0)	6	4	7
Mild (1)	11	6	7
Moderate (2)	2	5	3
Severe (3)	1	4	2

*Fecal severity scores were assessed using the Wisconsin Calf Health System (McGuirk & Peek, 2014)

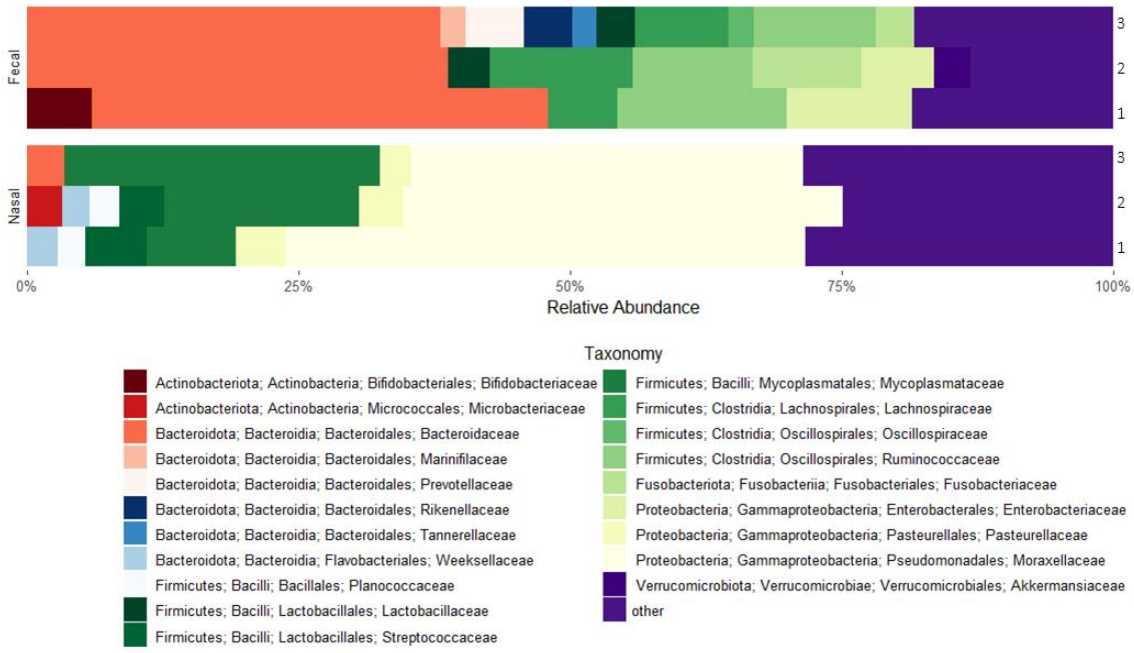

Figure 3.1. Taxonomic mean relative abundance at the family level for both nasal and fecal samples were assessed across the three sampling time points. Sampling time point 1 was the initial sampling time point at calf age < 7 days old. Time points 2 and 3 refer to the subsequent weekly sampling time points.

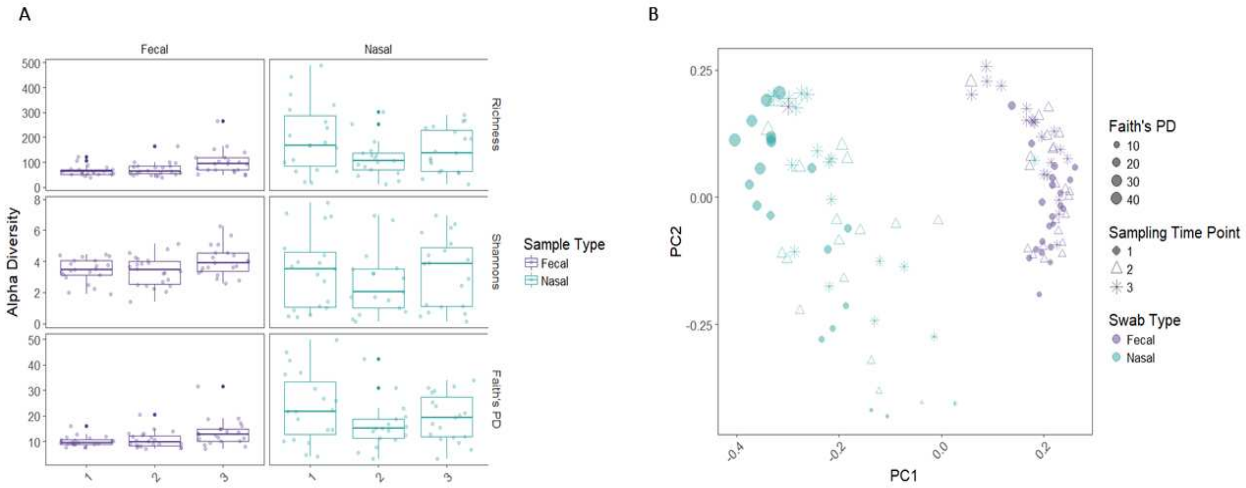

Figure 3.2. Diversity was analyzed for both alpha and beta diversities for each sample type across the three sampling time points. Alpha diversity metrics (Panel A) of each sample type across the three sampling time points were measured using Faith's PD, Shannon's Index and Richness with statistical significance analyzed using Kruskal-Wallis pairwise testing. No differences ($p > 0.05$) were observed for nasal alpha diversity metrics. Fecal samples indicated statistical differences for Faith's PD between sampling time points 1 and 3 ($p = 0.003$), and 2 and 3 ($p = 0.016$) with diversity trending higher as sampling events occurred. Fecal samples also saw statistical differences for Richness between sampling time points 1 and 3 ($p = 0.002$), and time points 2 and 3 ($p = 0.014$) with an increase in diversity over the sampling time points. Beta diversity (Panel B) distance was measured using unweighted UniFrac distance and revealed separation was driven between sample type. PERMANOVA analysis revealed statistical difference in unweighted UniFrac distance for each sample type ($p = 0.001$). PERMANOVA pairwise analysis revealed statistical differences in beta diversity for fecal samples between all sampling time points ($p < 0.05$). Nasal samples showed statistical differences in beta diversity between time points 1 and 3 ($p = 0.03$) and time points 2 and 3 ($p = 0.012$).

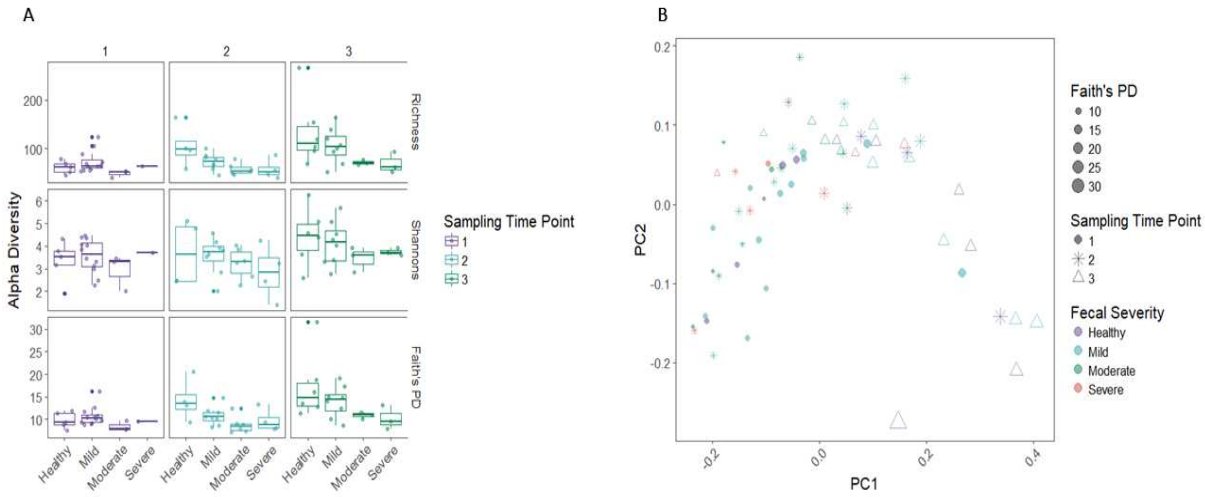

Figure 3.3. Diversity analyses of fecal samples and their association with fecal severity scores across sampling time points. Panel A details alpha diversity metrics (i.e., Faith's PD, Richness, Shannon's Index) for fecal samples and fecal severity at each sampling time point. Kruskal-Wallis pairwise testing revealed statistical differences for Richness between healthy calves and, moderate and severe fecal scores ($p = 0.012$ and $p = 0.033$, respectively); and between mild scores and, moderate and severe fecal scores ($p = 0.006$ and 0.039, respectively). Statistical difference was also observed for Faith's PD between healthy and moderate scores ($p = 0.008$) and mild and moderate scores ($p = 0.008$). Panel B details the distance between sampling time points and fecal severity scores for fecal samples only. PERMANOVA pairwise analysis revealed statistical differences in beta diversity for fecal samples between all sampling time points ($p < 0.05$) but differences within fecal severity were not observed.

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Chapter Four

Utilizing a CRISPR-Cas9 Targeted Foodborne Pathogen Antimicrobial Within Livestock Feed: A Market Research Perspective

Summary

CRISPR biotechnology is a promising option for alternative pre-slaughter intervention methods in foodborne pathogen control for production livestock operations. A customizable CRISPR-Cas9 targeted pathogen killing system has recently been studied and offers potential as a novel antimicrobial product in the production animal market. Questions remain however regarding the commercialization and market acceptability of the new CRISPR-Cas9 product. Our lab designed a study to evaluate the product's value, the target customer, and the regulatory aspects of how the product will move in the target market ecosystem. Our study details the market pathways for commercialization of a novel CRISPR-Cas9 biotechnology. The current study uses a three-pronged integrated methodology involving attendance of a market research workshop to learn the tools necessary to complete market research, an interview process, and a comprehensive literature review. The study approached product value by assessing its potential to reduce other antimicrobial use, its customizability, and its innovation of pre-slaughter interventions. The study included 20 interviews with industry experts, production livestock operations personnel, and academicians. Surprisingly, interviews revealed that the target customer for our CRISPR-Cas9 product to be the owners of production livestock operations because these individuals make the financial decisions to purchase new products. Adoption of the product by our target customer would also require verification that the product is effective,

does not increase cost, and is safe for animal use. Insights from the interviews revealed the regulatory process would be the primary focus of the market ecosystem. These interview insights made it clear that new biotechnology for use in livestock animals such as our product would be regulated by the U.S. Food & Drug Administration (FDA), with the Center for Veterinary Medicine being the main regulatory component of the FDA for new animal food products and drugs. This insight was established on the basis of the definition of the product being one of a new animal drug with intent to reduce foodborne pathogens in the public sector. Overall, our study maintains that our new CRISPR-Cas9 biotechnology offers producers an alternative intervention method that could improve consumer perception and outlines a regulatory pathway for the target market ecosystem.

Introduction

By the year 2050, the United Nations (2019) estimates the world population is expected to reach nearly 10 billion people. An increase in human population could be expected to result in an increase in the consumption of meat. The increase of meat and meat product consumption could also increase in foodborne pathogen outbreaks and due to this issue, there is cause for concern.

The United States Department of Agriculture (USDA) is responsible for mandating and reporting foodborne pathogens in the United States. More specifically, the USDA's Food Safety and Inspection Service (FSIS) is responsible for collecting information and determining if there is association between regulated products and human disease (FSIS, 2019). In 2019, there were 16 foodborne outbreaks in the United States with 15 of those outbreaks causing illness in more

than one state. These outbreaks resulted in approximately 1,000 hospitalizations and more than 175 hospitalizations. The most prevalent food products involved were meat products with beef products (37.5%) being the most common food source for pathogenic contamination. *Salmonella* (43.8%) and Shiga toxin-producing *Escherichia coli* (STEC; 37.5%) were the two most common pathogens among all product types (FSIS, 2019).

Cattle, along with other ruminants, are the primary reservoir of harboring and shedding STEC, mainly *E. coli* O157:H7 (Ferens & Hovde, 2011; Munns *et al*., 2015; Stein & Katz, 2017). Previous studies have attributed approximately 75% of all human *E. coli* O157 outbreaks to food products coming from cattle (Callaway *et al*., 2009). While cattle are typically unaffected by the pathogen, because the pathogen resides in the gastrointestinal tract, specifically at the recto-anal junction at the distal colon, their hide can become contaminated due to shedding the bacteria in fecal matter (Savageau, 1983; Naylor *et al*., 2003; Arthur *et al*., 2010). Hide contamination is considered the primary source of beef carcass contamination at slaughter (Barkocy-Gallagher *et al*., 2003; Nou *et al*., 2003; Bosilevac *et al*., 2004; Arthur *et al*., 2007). Because of contamination factors, processing facilities have procedures in place to reduce the contamination of carcasses and subsequent products. Current guidelines for processing intervention facilities exist to control for the entry and contamination of foodborne pathogens that outline procedures for sanitation, control of cross contamination, and carcass treatment for visible fecal contamination (FSIS, 2019).

While processing facility intervention methods are mostly effective, if pathogen contamination of the hide is large enough, current facility intervention methods could be less than adequate in pathogen removal from the carcass. New pre-harvest intervention methods for the control of *E. coli* O157:H7 have been investigated for better foodborne pathogen control.

These intervention methods consist of direct-fed microbials, vaccine technology, and the application of chemicals such as sodium chlorate and neomycin sulfate (Loneragan & Brashears, 2005). For example, Younts-Dahl *et al*. (2005) established that *Lactobacillus* NP51 used as a direct-fed microbial was able to reduce the recovery of *E. coli* O157:H7 in cattle and is currently available on the market as a pre-harvest intervention. Even though there are commercialized products on the market such as NP51 (Loneragan & Brashears, 2005), new biotechnology continues to be investigated.

Recently, the use of the gene-editing tool CRISPR-Cas9 has been investigated for use in livestock. The CRISPR-Cas9 machinery is an RNA-guided nuclease (i.e., Cas9) that works through helical double-stranded DNA base pairing and makes for a highly specific and efficient tool for gene editing in various cell types and organisms (Garneau *et al*., 2012; Jinek *et al*., 2012; Gasiunas *et al*., 2012; Ran *et al*., 2013). The use of this technology in agriculture is well documented in crops for creating plants that are resistant to viruses, fungal disease, and bacterial pathogens (Wang *et al*., 2014; Ali *et al*., 2015; Baltes *et al*., 2015; Ji *et al*., 2015; de Thomazella *et al*., 2016; Li *et al*., 2017; Foster *et al*., 2018; Tashkandi *et al*., 2018). CRISPR technology has also been applied to create genetic lineages in livestock to be resistant to certain diseases, to repair genetic mutations that can cause disease, and uses in improving animal welfare and health qualities (Bi *et al*., 2016; Whitworth *et al.,* 2016; Ikeda *et al*., 2017; Gao *et al*., 2017; Schuster *et al*., 2020).

The success of CRISPR-Cas9 in plants and crops has given way for research to be conducted for use in livestock production systems. CRISPR-Cas9 systems have ultimately been studied as a method to target foodborne pathogens in livestock, albeit to differing degrees. In 2016, a study was done to demonstrate the ability of antibiotic resistant *E. coli* cells to be re-

sensitized to Ampicillin via a CRISPR-Cas9 plasmid at an effect rate of 99% (Kim *et al*., 2016). A similar study was conducted in which the use of the CRISPR-Cas9 machinery was able to target and deplete antibiotic resistant *Enterococci* species while leaving other microbial species within the system undisturbed (Rodrigues *et al*., 2020). Both studies highlight the power and novelty in using CRISPR systems to be used as intervention methods for food safety and public health protocols. The detail in manner of which CRISPR-oriented protocols would be used or how is still not well understood although new research could provide critical evidence to implement these procedures. In a novel strategy, this gene-editing machinery has now been applied in such a manner that it could be used as an antimicrobial. In 2018, Jia *et al*. successfully demonstrated the ability of a CRISPR-Cas9-mediated plasmid packaged into bacteriophages to target and cleave *Escherichia coli* O157:H7 cells harboring the Shiga-toxin producing genes *stx1* and *stx2* within rumen fluid. The system was able to target and kill pathogenic *E. coli* O157 cells while leaving all other microflora intact, including that of other strains of *Escherichia coli*. This technology works by inserting the genes to be targeted within the machinery of CRISPR and could prove as a highly efficient and viable alternative to current pre-harvest intervention methods currently available today on the market. Furthermore, the target genes can be completely customizable; meaning, this technology could theoretically target any pathogenic bacterial species.

Many pathogenic bacterial species can cause illness within both humans and animals. A critical component to food safety involving livestock animals is the use of in-feed antibiotics for the treatment of illness in the animal. The prophylactic use of antibiotics in livestock production practices is a common misconception among consumers (Curtis, 1987; Wolf *et al*., 2016; Redding *et al*., 2021). While antibiotics are useful for animal welfare and production when used

in the appropriate manner, many consumers today perceive food products labeled “antibiotic-free” or “organic” to be superior and safer compared to traditionally produced food products (Hughner *et al*., 2007; Van Loo *et al*., 2010; Van Loo *et al*., 2013; Redding *et al*., 2021). Due to consumer perception, many producers may feel pressure to avoid management practices that involve antibiotic usage, which could lead to a void in treatment options for sick animals (Flaten *et al*., 2006; Habing *et al*., 2016; Ekakoro *et al*., 2018; Wemette *et al*., 2020; Redding *et al*., 2021). The customizable nature of a CRISPR-Cas9-mediated antimicrobial could provide a new approach in addressing both antibiotic use and consumer perception of livestock products. While antibiotics are not used for the treatment of foodborne pathogens within the host animal, the potential to reduce the need of antibiotics using a customizable, target-specific CRISPR-Cas9 antimicrobial could enhance consumer perception of both animal welfare and public safety. The use of CRISPR biotechnology in livestock production systems offers value although consumer studies have reported a negative outlook on the use of genetic engineering tools due to the negative impacts that could arise in the animal (Miles & Frewer, 2001; Schuppli & Weary, 2010). However, research has also found that consumers are willing to accept the use of biotechnological products in animals if it is deemed to better the welfare of the animal (Miles & Frewer, 2001; Schuppli & Weary, 2010; USDA, 2018; McConnachie *et al*., 2019; Kilders & Caputo, 2021). Marketing a novel CRISPR-Cas9 antimicrobial product to reduce the use of antibiotics in livestock animals could allow consumer perception to increase because of the current negative views surrounding animal welfare and antibiotic usage. Research has yet to reveal if this approach to CRISPR-Cas9 use and marketing will be valuable.

While the research behind the novel technology is well-known for our lab, the commercialization routes for this technology is less understood. The use of these novel tools and

products in the United States are regulated by three federal agencies: the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA) and the Environmental Protection Agency (EPA) (Es *et al*., 2019; Van der Berg *et al.,* 2020). All three agencies play major roles in the regulation of any product used in humans and animals. The major regulatory agency for animal foods and food products is the FDA's Center for Veterinary Medicine (CVM). The agency uses the guidance of the Federal Food, Drug, and Cosmetic Act (FD&C Act) to define food for consumption by humans or animals and more specifically, the agency uses Guide 1240.3605 to classify new animal foods or ingredients for regulation by the FD&C Act. However, there are new pathways introduced when discussing the use of biotechnology within the food of animals. As of date, there are no precedents set forth for the use of CRISPR-Cas9 biotechnology in livestock animals.

To address these challenges, our lab designed a market research study to evaluate the market acceptability of a new biotechnology such as a CRISPR-Cas9 antimicrobial, to determine who a potential customer would be, producer acceptability, and to determine the route in which a product of this nature will be regulated. Our hypothesis for the study is that the target customer will be the farmers, ranchers and feeders of livestock as they will be the ones to use the product in their feed for livestock; moreover, we hypothesize given the current definitions defined by the FDA, CVM, the FD&C Act and Guide 1240.3605 that this product will be regulated as a new animal drug feed additive with the intended use of downstream public health and food safety.

Methodology

A primary issue when conducting market research is bias in questioning and a tendency to "sell" the technology instead of asking questions regarding the movement of materials and data. Our methodology consisted of an integrated three step structure to combat question bias that could result from asking yes or no questions. The first step was the attendance of the Research to Market program presented by Colorado State University to obtain the proper tools and techniques for market research. The second step used the interview process learned during the Research to Market program to gather data on the market for our product. Finally, the third step was a literature search which was integrated into the interview process in order to clarify previous insights or to access new points of questioning for the interviewees.

Research to Market

The tools and techniques utilized for this study were obtained through attendance of the Research to Market (R2M) Cohort Five through Colorado State University's Ventures Program. The purpose of this multi-week program took place during the months of January 2020 and February 2020 with the primary goal of helping scientists learn market research techniques for bringing new technologies from research laboratories to commercialization. This program established the baseline and etiquette for interview conduction and provided instruction on how to purposefully use the information obtained during the interviews.

At the beginning of the R2M program and prior to interviews being performed, our lab assessed our product for the product's values and strengths as well as identified the benefits it could bring to a target customer. The product value would be used for further evaluation of who our target customer could be. With this value in mind, an initial target customer was identified

for the beginning of the interview process. Additionally, a preliminary market ecosystem map was created (Figure 4.1). A market ecosystem map is designed to help organize economic activity in a target market with a specific target customer in mind. The interview process started after the preliminary ecosystem map, product value, and initial target customer were established.

Interviews

Interviews were conducted in-person, over the phone, and through email to determine the following: the target customer; the willingness to adopt the technology; and the regulatory process of bringing a new agricultural biotechnological product to market. For this study, interviews focused on the beef industry, primarily the feedlot industry, because of the status of the laboratory findings of the technology at the time these interviews took place. The current research of our lab focuses on reducing *E. coli* O157:H7, a common foodborne pathogen that contaminates beef and beef products, through the use of our product. Potential interviewees were evaluated based on credentials identified through our lab and consultation from the Research to Market Cohort 5 experts. The interview composition is summarized in Table 4.1 and included cattle feedlot owners, feedlot mill managers, industry experts, state and federal government agency employees and members of academia. The interview process occurred in two stages. The first set of interviews took place during and after the R2M Cohort 5 workshop (Jan. 2020 – Feb. 2020). The primary goal of these interviews was to identify a target customer and understand the pathways in which a new product would travel from idea conception to commercialization of the new product. The second set of interviews took place from March 2021 – May 2021 with the primary goal of these interviews being that of product regulation. The target interview goal was set at 30 participants.

To not bias the results and because of tools learned at the Research to Market Cohort 5 at Colorado State University, a questionnaire style interview was not performed. Instead, we addressed each individual as a storyteller where we would need to decipher and interpret their answers (Colorado State University's Research to Market Cohort 5, 2020). For example, we would ask questions that were open-ended and did not allow for "yes" or "no" answers. With this style of interview, we were able to harbor information without extreme bias. For this study, bias would result from an attempt to sell the product, making the interview more about the technology itself and not about the interviewee's role or job. It was the job of the interviewer to not attempt a sale of the potential technology. Questions were asked to the interviewee to gain insight about the individual's specific job or knowledge about how products move through the market ecosystem and not specifically about their interest in our proposed product to control the conversation. An additional aspect of this interview approach was to obtain additional interview recommendations which could lead to answering the proposed research questions. All in-person interviews were recorded via detailed note taking and the use of a recording device for transcription. Phone interviews consisted of detailed note taking and email correspondence was archived for later follow up.

The Value Proposition Canvas and Ecosystem Map

The Value Proposition Canvas (VPC) was designed using the Value Proposition Design as described by Osterwalder *et al.* (2014). Briefly, the VPC is a tool to detail the benefits provided to the target customer through our products services. The VPC is divided into two visual sections: the Value Proposition Map (i.e., value or product benefits) and the Customer Profile. The Value Proposition Map consisted of the products' services and benefits, the gains

created for the customer, and the pains alleviated for the customer. The Customer Profile described the job description of the customer, the gains the customer would like to achieve, and the pains the customer is trying to reduce or eliminate. Our VPC was established prior to interviews with an initial target customer in mind and adjusted throughout the interview process until customer fit was obtained. “Fit”, the process in which our product’s value matches the customer profile, was achieved once the proper target customer was identified through the interview process.

Once the VPC obtained the correct fit, the market ecosystem map was assessed. An ecosystem map is a physical map created with the intent to help organize the economic activity within our target market with a specific target customer in mind. The ecosystem map allows us to understand the steps in commercialization, those who are participating, who potential partners are going to be and who will gain value, and how the product will eventually arrive to our target customer. The interview process at this point was catered to industry leaders for an understanding in how our product would move through the target market to the target customer for product use. This effort was done by analyzing three primary questions: how materials flow through the ecosystem, how money exchanges hands, and how data is exchanged between partners. This process also involved answering questions to address how new biotechnology would be regulated and how the regulatory process would affect the market ecosystem flow. An additional approach to the ecosystem map was the identification of influencers, competitors, and saboteurs. Briefly, influencers are those individuals or companies that can influence the target customer into adopting the technology; competitors are individuals and companies that have the potential to produce and market similar products that would rival our product on the market; and saboteurs are those individuals and companies that could effectively kill the product and any

others like it before making it on the market to maintain their own product(s) market viability. Once the following was established, a working ecosystem map was generated. It should be noted that a market ecosystem map is not static and could change as further market research is conducted.

Results

Interviews

Because of the global COVID-19 Pandemic of 2020, the target interview goal of 30 was not reached. A total of 20 interviews were completed and conducted either in-person, the use of voice or video call, or through email correspondence. Thirteen interviews were conducted in-person; four were conducted via phone or video conference; and three were completed in email correspondence. Fourteen of the total interviews were conducted with individuals within industry positions (i.e., companies or individuals responsible for creating and marketing products for use in agriculture or those in livestock production); four were within the regulatory sector; and two were within academia (Table 4.1). Three of the individuals interviewed played roles in both the industry and regulatory sectors.

Interviews originated with our proposed target customer identified during the preliminary phases of R2M Cohort 5 and progressed as new individuals were identified through the interview process. Table 4.1 highlights the key takeaways from the interviewees. Additionally, regulatory agencies and pathways were determined as well as product manufacturing insights. From here, the value proposition, VPC, and ecosystem map could be analyzed.

Value Proposition, the Target Customer, and the Value Proposition Canvas

The value proposition for our product is detailed in Table 4.2. Key components of the product's value included being a targeted pathogen reduction technology due to the CRISPR-Cas9 design, a customizable technology capable of targeting many pathogens, and it could also be designed to target multiple pathogens at once. Other services and features of our product's value also included the use of a bacteriophage delivery system and being an affordable alternative intervention. Gains created for the customer by use of our technology were identified as being an effective treatment as shown by previous research within our lab, affordability, profit loss reduction, and time saved. Pains alleviated by use of our product were determined to be the price at which the product could be sold would be competitive in the market making it easier for consumers to purchase the product, a potential replacement for traditional therapies of animal illness, and the possibility of improving public concern surrounding antibiotic usage in livestock animals.

The starting target customer was considered to be feed mill managers or those responsible for making/mixing the feed at the livestock operation. A secondary target customer was nutritionists as these individuals formulate and ration feed for the animals. The primary result from the interviews was the target customer was identified as the owners and managers of livestock production operations. While feed mill managers and nutritionists were originally thought to be a target customer group, the interview process revealed that livestock production operation owners/managers make the financial decisions and ultimately decide which products to buy or not to buy. Interviews concluded that the target customer would be the owners and managers of production livestock operations. The interviews concluded that feed-mill managers do not in fact formulate or ration feed themselves. Instead, they are given the feeding formulas

and rationing information from the feedlot nutritionist(s) and make and ration feed based on the nutritionist requirements. It was then determined the nutritionists were only able to formulate feed based on the ingredients that the owners of the operations were willing to purchase and use for their cattle. The Customer Profile is detailed in Table 4.3 for our target customer. The job description of feedlot owners and managers was determined to be buying cattle appropriately and properly, feeding appropriately to maximize efficiency, minimize the cost of treatment as much as possible, and selling cattle at the highest profit value. The gains needed to be created to warrant adoption of the technology were revealed as being able to fix an issue without increasing cost, maximize cattle performance, increase profit, and make economic sense to the owners. Customer pains of our target customer revealed the need to improve public perception regarding animal illness treatment procedures, market risk, the cost of implementing new interventions, and the time that goes into running a production livestock operation. The VPC was determined to have a good "fit" between our product's value proposition and our customer profile (Figure 4.2).

The Ecosystem Map and Regulatory Process

The ecosystem map (Figure 4.3) was adjusted over the interview process and consisted of two primary pathways. One pathway identified was a route in which the product's commercialization and delivery to the target customer would all be handled in-house. A second pathway involved the use of partnerships and outsourcing aspects of the commercialization process. The ecosystem map details the movement of the product through each pathway and the routes the product would take for commercialization including manufacturing, validation, regulation, storage, shipping, and delivery to the target customer. Partnerships were identified as individuals or companies responsible for animal drug manufacturing and development as well as

individuals or companies involved in animal feed production. Influencers, competitors, and saboteurs were remarkably revealed as having potential to be the same companies as those who could become partners.

Interviews concluded that feedlot operation owners prefer buying products from a trusted brand name, indicating that the second pathway involving partnerships would be a critical step in getting the product to the target customer. Partnerships would require transfer/sale of intellectual property (IP) from our lab regarding our product to the partner. This IP transfer would first require the validation and regulation of the product as determined by interviews with individuals representing animal drug manufacturing companies. IP transfer or sale would also require scrutiny of these companies as many potential partners could be competitors or saboteurs as described by our ecosystem map. Through the interview process, it was determined that research would need to be done in order to show the efficacy of the product in order for industry partners to adopt the technology. An interview with an expert within the industry claimed that before a partnership would arise, the product would have to be approved by AAFCO and further regulation would more than likely be needed before a partnership or IP sale would be complete. The efficacy of the product and AAFCO approval, plus any additional regulatory requirements, would need to be verified prior to moving the product through the ecosystem map via a partnership route.

The common theme between both pathways was determined to be the regulatory process our product would need to go through in order to make it to the customer. Interviews with industry experts, regulatory officials and academicians confirmed that there would be several regulatory hurdles in getting the product to market. The FDA was identified as the primary regulatory agency for new biotechnology for use in livestock animals. One of the primary

hurdles for regulation of the product was determined to be the definition of the product (i.e., what exactly the product is in terms of application). The product would need to be defined as either a feed additive or a medicated feed additive. Additionally, the FDA's Center for Veterinary Medicine (CVM) was determined to be the primary regulating agency within the FDA for new biotechnology for use as an animal feed additive or medicated feed additive. The FDA and CVM ultimately revealed that the CRISPR-Cas9 mediated product would be defined as a drug according to the FD&C Act Section 201(g)(1)(c) it would be ultimately be intended to affect the structure or the function of the host animal by altering the host microbiome by removing pathogenic bacteria due to cleavage of the virulence genes resulting in cell death; the product would also meet the definition of a drug under Section 201(g)(1)(B) because the overarching goal is to reduce pathogenic bacteria in beef products intended for human consumption and thereby implicitly intended to reduce disease outbreak in humans. Moreover, if the product were to be considered a "food additive", it would have to act on pathogenic bacteria within the host animal *feed* to ensure food safety of the animal food. This sentiment was further backed up by the Food Safety and Inspection Service (FSIS). The USDA's FSIS does not regulate CRISPR-Cas9 technology and would not regulate it for intended use as an antimicrobial. The information garnered through this interview however did allow for further backing that the FDA would be the primary driver in determining the safety of this product and its GRAS status.

Determining the product would be defined as a new animal drug led to an insight from an interview that the next hurdle would be the categorization of the drug. The interviewee detailed that the category in which a new animal drug falls can affect a customer's willingness to adopt and use the product. A category I drug requires no withdrawal times and poses no additional burden on the feed-yard, making it more attractive based on simple economics. A category II

drug however, does require withdrawal times leading to additional feeding and economic concerns for the feeding operation. A category II drug would need to scientifically show excellent efficacy and returns for the feedlot before adoption of the product would be readily used.

Regulations would also have to be passed regarding environmental safety. The EPA would be the leading regulatory agency for new biotechnology in regards to assuring the environment would not be damaged or altered due to product use. Interviews concluded that several important questions would need to be addressed prior to product commercialization and sale. These questions included answering how the product would affect the environment in the short and long term once it has been introduced into the wild and understanding how the EPA could impact the movement of the product in and to the market. Interviews with nutritionists and regulatory agency experts revealed scientific evidence would be needed for EPA approval of the new product.

Discussion

The current study is the first study to detail the market pathways for potential commercialization of a novel CRISPR-Cas9 biotechnology in cattle production. While many CRISPR-Cas9 studies have been done to attempt and harness the gene-editing tool for use as an antimicrobial, the challenge for these studies is to be able to apply the new technology within real world scenarios and address the many hurdles a new biotechnology may face. The current study approached these challenges and attempted to provide insight into the marketplace by forming a road map from idea conception to the sale of a novel product. We focused on the beef

cattle feeding sector for our technology, but it should be noted that the customizable nature of the technology allows for use in many livestock production systems given a target gene is known.

Our target customer, feedlot operations owners/managers, is responsible for many duties which include providing a product that is safe for consumers. Product contamination of beef and beef products generally occurs at the beef packing plants as muscle tissue of beef carcasses is effectively free of bacteria until exposed to the environment and hide during skinning and carcass processing (Nottingham, 1982; Bell, 1997; Yang *et al*., 2017). While packing plants bear the burden of foodborne pathogen intervention, feedlot operations are likely to be called upon to implement pre-slaughter strategies for foodborne pathogen control (Galyean *et al*., 2011). Adequate buying and selling of cattle are primary jobs for feedlot operations and previous studies have shown that product recalls due to pathogen contamination cause a ripple effect in the price of cattle resulting in a negative impact on the live cattle futures prices within the market (McKenzie & Thomsen, 2001; Lusk & Schroeder, 2002; Moghadam *et al*., 2013). Because of the market risk in cattle buying, there is financial incentive for feedlots to consider alternative, pre-slaughter techniques in reducing foodborne pathogens such as the proposed technology in our study.

Consumer misconception regarding antibiotics or antimicrobial usage in a production livestock system pose significant hurdles for our target customer. Similar to our findings and interview insights, a primary source of external pressure in cattle feeding operations results from the consumer interest and demand for “organic” beef (Hughner *et al*., 2007; Van Loo *et al*., 2010; Van Loo *et al*., 2013; Redding *et al*., 2021). This demand for organic beef and beef products impacts consumer perception regarding the therapeutic use of antibiotics for the treatment of illness; while the USDA has acknowledged the value in antibiotic use for treating

illness in livestock, the consumer pressure of antibiotic free products has led to the agency wanting to limit the use of antimicrobials (Sharfstein, 2009). The shift of consumer preference to organic products presents a problematic approach for livestock operations owners in typical treatment protocols (Flaten *et al*., 2006; Having *et al*., 2016; Ekakoro *et al*., 2018; Wemette *et al*., 2020; Redding *et al*., 2021). While the product in its current state is not designed to treat animal illness, the customizable feature of the technology provides avenues for preventative measures (Jia *et al*., 2018). An appropriate marketing scheme could also improve consumer acceptance of beef products that have been exposed to a new technology such as a CRISPR-Cas9 antimicrobial as research has found increased willingness to pay for gene-edited secondary animal products (i.e., milk, meat) when the use of genetic modification was marketed as an animal welfare benefit strategy (Kilders & Caputo, 2021). Product marketing that allows consumers to easily understand the safety of our product, foodborne pathogen control capabilities, and adaptations to improve animal welfare issues, could make the movement of our product through the market ecosystem easier.

While there are safety concerns by the target customer for use of the product in live animals, previous research supports CRISPR technology as a safe tool for use in live animals for pathogen control and reduction (Kim *et al*., 2016; Jia *et al*., 2018; Rodrigues *et al*., 2020). Additionally, studies provide evidence for use of CRISPR technology for targeting specific bacterial strains to improve animal health (Stout *et al*., 2017) which could lead to better willingness to accept our technology. Currently, our product is not in a live animal testing phase however, Song *et al*. (2019) demonstrated that CRISPR-Cas technology applied in microbiome systems protect beneficial bacterial species by transferring genetic agents making them more resilient in their environment while removing particular strains that are destructive to the health

of the microbiome and the animal host. Future studies involving live animals will be needed to address live animal safety more adequately although the current status of research proves promising.

The largest hurdle for new biotechnology is the regulatory process. Similar to information gained from the interview process and literature search, the FDA is responsible for regulation of genetic modification to animals and livestock (Van der Berg *et al*., 2020). The FDA works in tandem with the USDA and EPA in regulating new biotechnology as established by the Coordinated Framework for Regulation of Biotechnology in 1986 (Bari & Zaman, 2001). Our original hypothesis was that the technology would be considered a new feed additive and be regulated as a feed additive drug as detailed in the CVM's Guide1240.3605. Contrary to our hypothesis, interview insights revealed that the new product would not be considered an additive even though it would be added to the feed of the animals. An interview with the CVM detailed that the product would be regulated as a new animal drug and highlighted section 201(g) of the Federal Food, Drug, and Cosmetic Act (FD&C Act) in which it defines the term "drug" to include articles intended for use in the "diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals, and the articles intend to affect the structure or function of the body of man or animals" (FDA, 2018). Our initial thought process considered the product to not act on the host body or function, but interview information indicated elimination/reduction of a pathogenic species such as *E. coli* O157:H7 will in fact alter the microbiome of the animal.

This interview insight is consistent with previous studies in which differential abundance analysis indicated significant differences between the microbiotas of cattle with normal shedding of *E. coli* O157:H7 and those considered to be super shedders (Wang *et al*., 2018). Given this information, our product would not meet the requirements of a food additive and because the

intended use is for food safety and public health safety of humans, under sections 201(g)(1)(C) and 201(g)(1)(B) of the FD&C Act the product would in fact meet the definitions of a new animal drug with intended use for mitigation of disease in humans.

Several important questions arise with knowledge that our product will be considered and regulated as a drug. The first and foremost of these questions that would have to be answered is defining the category of drug in which this product would be classified. As previously mentioned, a Category I drug is one that can be administered to the animal with no withdrawal period required (FDA, 2016). This category of drug is more likely to be accepted by producers simply because using it does not require any additional resources or feeding times due to a withdrawal period. Category II drugs however require a withdrawal period as the drug could leave behind harmful residues if ingested by humans and must be void within the animal prior to slaughter (FDA, 2016). The Federal Register also detailed that approved medicated feeds are considered Type A feeds (FDA, 2016). In this docket, Category I, Type A medicated feeds can be handled by both licensed and unlicensed feed mills while Category II, Type A medicated feeds can only be handled and used by licensed feed mills. Therefore, defining the new proposed product will be crucial in determining how and where it can be used. These questions must be met with experimental data that answer if CRISPR-Cas9 technology used as a pathogen control technique via direct-fed microbials in livestock will require a withdrawal period and if so, how long, and what measures must be taken for the product to not affect humans who consume livestock products subjected to the new technology. Experimental data will be needed to answer if the product, now considered a new animal drug, would have adverse effects on the environment, the host animal and the consumer of meat products.

The concerns over the use of CRISPR-Cas9 with regards to environmental and host safety need to be answered. The environmental concerns regarding the use of CRISPR has been readily studied in the use of gene drives in insects for insect control and has revealed many concerns such as the potential for the new technology to alter insect populations and even ecosystems (Beumer *et al*., 2013; Esvelt *et al*., 2014; Oye *et al*., 2014; Champer *et al*., 2016; Taning *et al*., 2017). This environmental concern will extend to the product in question within the current study and will need to be addressed. According to the FDA, the intended use of the product as an antimicrobial will alter the host microbiome, there is reason to speculate the product could alter environmental microbiomes once released from the animal. Off-target mutations within the target genome have potential to be very common if the guideRNA (gRNA) is not of proper design within the CRISPR machinery (Sander & Joung, 2014). Potential risk of modified gene sequences being transferred to other organisms when genetic technology is being used is another concern for this biotechnology (Taning *et al*., 2017). Because the intended nature of the current product is to act on pathogenic bacteria species and reduce/kill them (Jia *et al*., 2018), the more demanding answer needed is the microbiome consequences that could arise by elimination of a particular species of bacteria, both within the host microbiome and within environmental microbiomes. While theories such as Chesson's coexistence theory (Chesson, 2000) describe how microbes exist with one another, and how antibiotic resistant bacterial strains and susceptible strains of the same bacterial species are able to coexist (Letten *et al*., 2021), removal of the resistant species could rewrite these theories in some respects. The use of the CRISPR machinery to remove or kill pathogenic bacteria could therefore open new microbial niches or coexistence mechanisms that change the population dynamics of differing

microbiomes. The effects of these changes will need to be understood prior to use of CRISPR-Cas9 antimicrobials in either live animal models or environmental models.

While our study provided valuable insight into the commercialization and regulatory process for a new biotechnology, the study is not without flaws. One of the major limitations of the study is the number of interviews conducted. The major complication for interviews was, of course, the global COVID-19 pandemic of 2020. This viral outbreak disrupted many lines of communication necessary for proper interviews. Nevertheless, many studies involved in market research of food science technology surpass hundreds of interviews (Vigani *et al*., 2015). Alongside the number of interviews, a limitation of the study could be the manner of the interviews. Without a structured interview/questionnaire process, such as that of the Delphi survey method described by Linstone & Turoff (1975). The primary purpose of the interview structure used in our study was to mitigate bias and induce answers that are controlled by the interviewer and not limit the interviewee in answers. The issue with this approach is there are not standardized questions as each interviewee is asked questions based on their job description. When using methods such as those dictated by the R2M Cohort 5, interviews are less scientific and more opinionated and pertinent information learned from detailed interviews should be verified through more data driven research and the literature.

Conclusion

In summary, the target customer for our product would ultimately be the owners of production livestock operations due to their ability to dictate which products are bought and used at the feedlot or site of operation. While feed mill managers and nutritionists can be influencers

for the target market economy, these individuals can only formulate and mix feed based on the owner's product preferences. Any new product must meet safety and efficacy standards to be considered for adoption into practice. These standards are set by regulatory frameworks which are primarily dictated by the FDA and CVM. These agencies will make the ultimate decision as to what category drug our product will be. Defining the product's drug category will have major implications in determining the market acceptability of the product. Through the Research to Market and interview process, our product was evaluated to be a promising pre-slaughter intervention method for pathogen control and can have profit incentive for feedlots to consider. The use of our CRISPR-Cas9 technology could also prove a viable alternative to antibiotic and antimicrobial therapies currently in use at traditional production livestock operations. By using alternative therapeutic methods such as our product, public perception of the livestock industry could be enhanced if marketed in a proper fashion. Prior to commercialization, proper validation will be needed to determine the safety of the product on the host animal, the environment, and the consumer. Future research efforts should consider obtaining data to provide the efficacy of the product and potential safety risks or the lack thereof. New market research should focus on consumer acceptance of products that use CRISPR-Cas9 technology for foodborne pathogen control.

Table 4.1. The total number and category of interviewees

Interviewees*	Category	Number of Participants	Key Highlights from Interviews
Feedlot Owners	Industry	1	Market risk of new product use and public misconceptions of animal welfare practices; prefer partnerships with trusted name-brand
Feedlot Mill Managers	Industry	1	Mix feed based on nutritionist feed formulations and rations
Government Agency Experts	Regulatory	4	FDA and CVM are primary regulatory agencies; product will be regulated as new animal drug based on definition of a new animal drug
Livestock Product Companies or Experts	Industry	12	Will not partner with companies until product is regulated and tested for validation, efficacy and safety
Academia	Academic	2	Nutritionists formulate feed based on what products feedlot producers are willing to purchase; environmental concerns will need scientific evidence

* Individuals identified either at the beginning of the Research to Market Cohort 5 or through previous interview recommendations

Table 4.2. The value proposition for the CRISPR-Cas9 antimicrobial technology*

Product services and Features	Creating Customer Gains	Alleviation of Customer Pains
Customizable to fit customers need	Effective treatment option	Potential replacement of standard livestock operations therapy protocols
Targeted pathogen killing system	Improve performance	Improving consumer perspectives of traditional livestock operations
Multiple pathogen killing capabilities	Reduction of profit loss due to affordability	Sale of new products at no added costs; can product be sold at cost similar to antibiotics and antimicrobials currently in use
Use of bacteriophage delivery system	Time saved through use of product	
Affordable alternative to antibiotics	Less work required for managing healthy animals	

* Value proposition followed guidelines set forth by Osterwalder *et al.* (2014)

Table 4.3. The customer profile of our target customer (feedlot owners and managers)*

Job Description and Responsibilities	Gains Needed from Product	Customer Pains
Accessing cattle and buying cattle properly	Fixes problem or illness without increasing cost	Public disconnect between consumers and livestock operation practices
Feeding adequately and efficiently	Maximizes performance and efficiency	Market risk
Minimizing cost of treatment as much as possible	Increases profit value	Antibiotics in use because they work as intended
Selling cattle at highest profit value	Make sense for use of product to be adopted	No funding for extra expenditures
Running a business from top to bottom		Time invested into running the operation

*Customer profile dictated by guidelines set forth by Osterwalder *et al.* (2014)

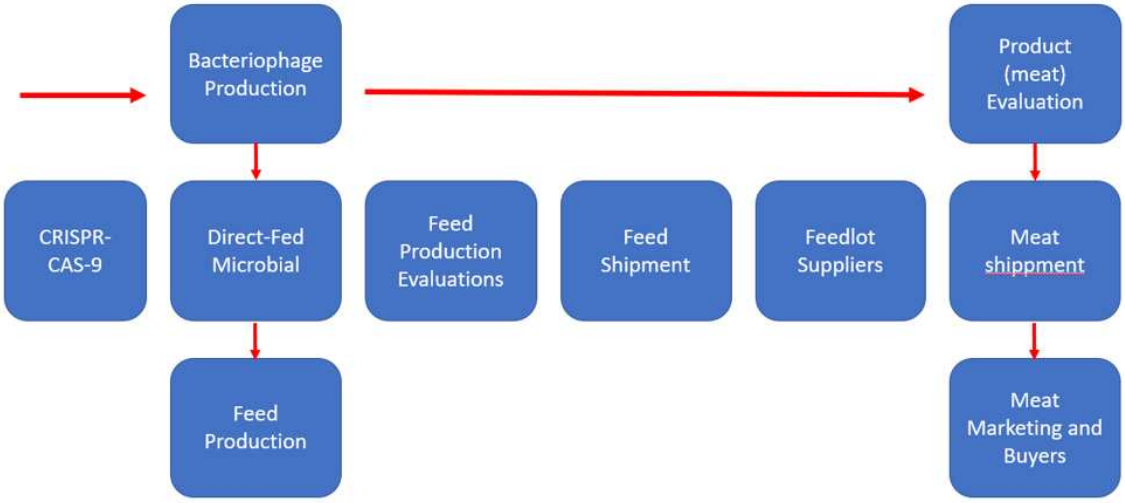

Figure 4.1. The preliminary ecosystem map established during the Research to Market Cohort 5. This map was established without prior knowledge of the product's target market or target customer with a purpose to highlight how proper market research influences understanding of market ecosystems. Red lines indicate the possible flow of materials and data to the target customer.

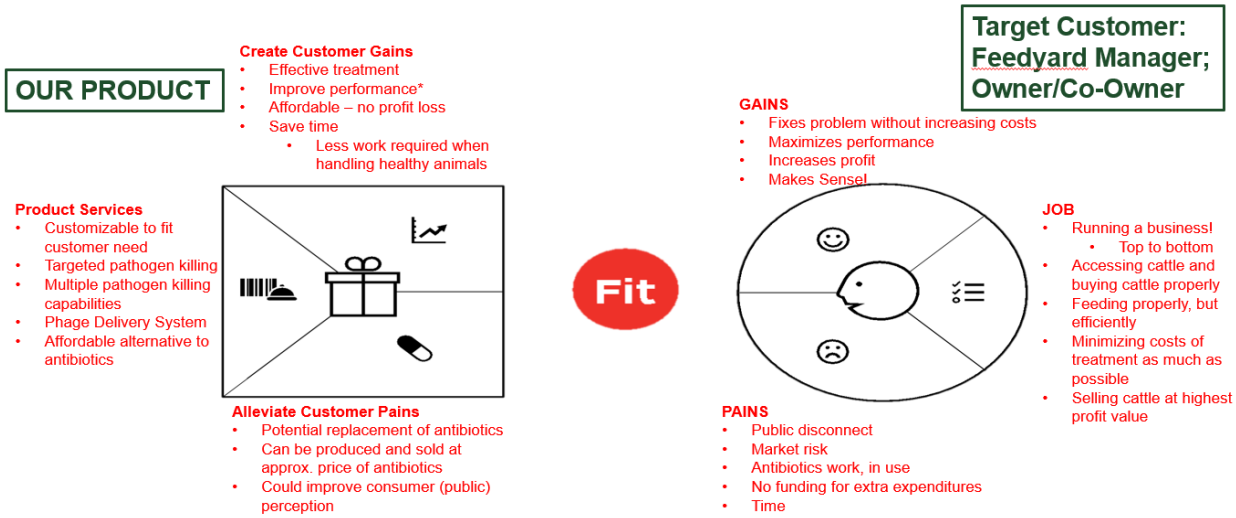

Figure 4.2. The Value Proposition Canvas details the product services and features along with the profile of the targeted customer. The product services indicate both the pains that the product hopes to alleviate for the customer and the gains the product could create for the end-user. Furthermore, the customer profile details the everyday pains and gains that are essential to understand for the value of our product to be correctly placed with the customer.

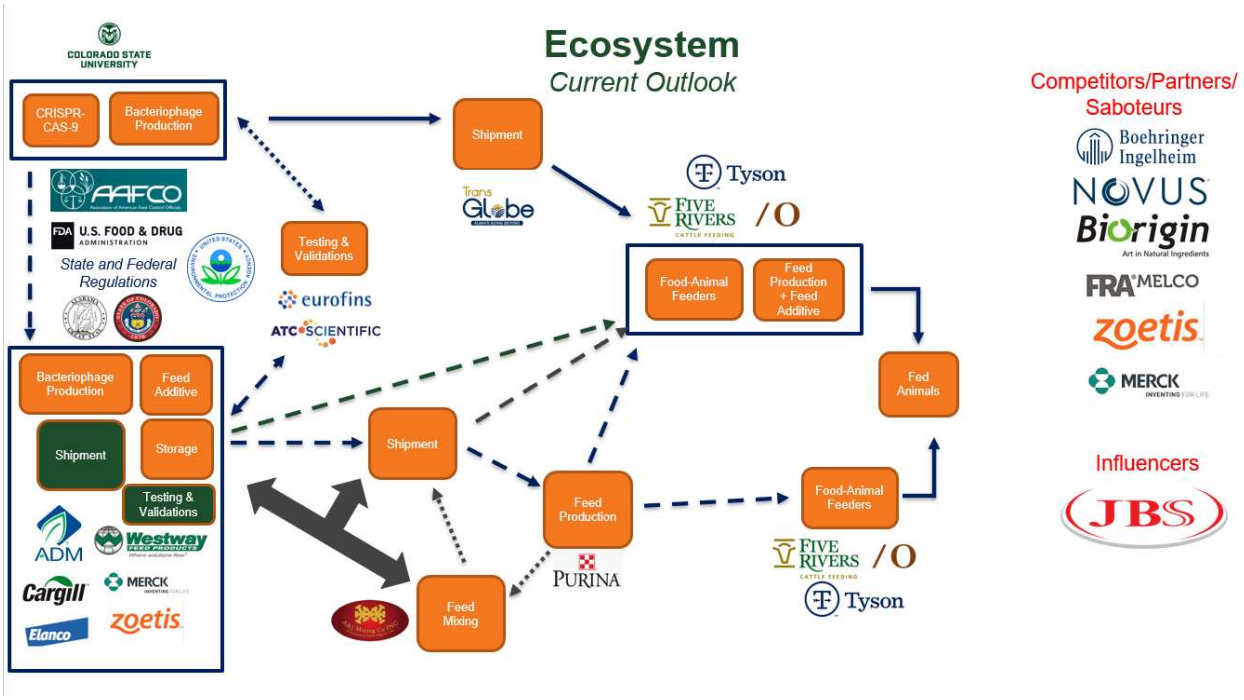

Figure 4.3. The Ecosystem map that was established during the interview process. The ecosystem map allows for visualization of the movement of our product through the commercialization process. Solid lines indicate direct modes of movement while dotted lines indicated the use of partners or third-party movement. The map also allows for us to understand the potential partners, competitors, saboteurs, and influencers that our product could encounter in the ecosystem.

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